Genotoxicity of 1,2,4-Benzenetriol:

Roles of Oxygen-Derived Active Species and Quinones

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Genotoxicity of 1,2,4-Benzenetriol:

Roles of Oxygen-Derived Active Species and Quinones

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ABSTRACT

Benzene causes leukemia in humans. It also induces structural and numerical chromosomal aberrations in lymphocytes and bone marrow of exposed workers. Recent evidence indicates that such chromosomal changes are important in the progression of many cancers, including leukemia. Studying the mechanisms whereby benzene induces chromosomal alterations will provide information regarding the mechanism of its carcinogenicity. Benzene, however, is unlikely as the immediate toxic species since its metabolism is required for toxicity. 1,2,4-Benzenetriol is one of the most reactive metabolites of benzene. This research characterizes the genotoxicity exhibited by benzenetriol and investigates the mechanisms involved.

Benzenetriol produced both structural and numerical chromosomal changes, as measured by a modified micronucleus assay. Micronuclei containing either whole chromosomes or acentric chromosome fragments can be distinguished by using an antikinetochore antibody. Benzenetriol increased the frequency of micronucleus formation eight-fold in HL60 cells. Addition of copper ions (Cu^{2+}) enhanced the frequency of benzenetriol-induced micronuclei a further three-fold and altered the pattern of micronuclei from predominantly kinetochore-positive to kinetochore-negative.

The cell-free system confirms the influence of Cu^{2+} in changing the mechanism of benzenetriol-induced micronucleus formation from aneuploidy to clastogenicity. In this system, Cu^{2+} changed the free radical propagated chain reactions from one-electron transfer to two-electron transfer. Reactive oxygen species are implicated in the Cu^{2+}-mediated chromosome breakage through their direct or metal-mediated interactions with DNA. 8-Hydroxy-2'-deoxyguanosine (8-OH-dG) is a marker of oxidative DNA damage

and ultimately leads to point mutations. Benzenetriol increased the level of 8-OH-dG, and Cu^{2+} again enhanced this effect.

Benzenetriol also induced numerical aneuploidy in the form of hyperdiploidy of chromosomes 7 and 9, which was determined by fluorescence *in situ* hybridization (FISH). The hyperdiploidy frequencies were increased approximately three-fold over the controls. Microtubule integrity is required for proper chromosomal segregation. Benzenetriol disrupted normal microtubular organization, as shown by immuno-cytochemical staining with anti-tubulin antibodies. The quinonoid oxidation products of benzenetriol and reactive oxygen species are the most likely cause of this disruption and the mitotic abnormalities which result in aneuploidy. These data provide further insight into the mechanisms involved in benzene-induced genotoxicity and leukemia.

DEDICATION

To my mother whose spirit has been encouraging me to succeed in my studies and scientific career, and to my country where I grew up and received my early education.

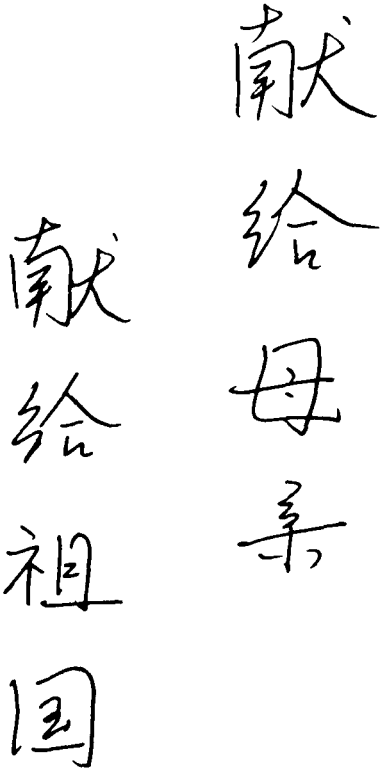

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TABLE OF CONTENTS

CHAPTERS

APPENDICES

LIST OF FIGURES

I. General Introduction

II. Benzene Metabolite, 1,2,4-Benzenetriol Induces Micronuclei and Oxidative DNA Damage in Human Lymphocytes and HL60 Cells

III. Detection of 1,2,4-Benzenetriol Induced Aneuploidy and Microtubule Disruption by Fluorescence *in situ* Hybridization and Immunocytochemistry

IV. Effect of Metals, Ligands and Antioxidants on the Reaction of Oxygen with 1,2,4-Benzenetriol

LIST OF TABLES

Chapter I

Chapter II

Chapter III

Chapter IV

PREFACE

This dissertation comprises five chapters including a general introduction, three papers written for publication, and a final conclusion.

The general introduction (Chapter I) outlines the context and goals of the research. It summarizes the mechanisms of toxic quinones and reactive oxygen species, the effects of 1,2,4-benzenetriol, and chromosomal aberrations. This study investigates the role of oxygen-derived active species, metal ions and quinones in the genotoxicity of benzenetriol as well as the possible mechanisms involved.

Chapter II has been recently published in the journal of *Environmental Molecular Mutagenesis* (21:339-348, 1993). In this section, we demonstrate that benzenetriol induces micronuclei and oxidative DNA damage in human cells. We also present the data as evidence that copper ions alter benzenetriol's genotoxicity from aneuploidy to clastogenicity.

Chapter III has been accepted for publication in the journal of *Mutation Research.* Here, we provide further evidence that benzenetriol can induce aneuploidy and microtubular disruption in human HL60 cells.

Chapter IV has been submitted to the journal of *Free Radical Biology & Medicine* for publication. This final study consists of observations of the stimulatory or inhibitory effect of metals, ligands and antioxidants on the autoxidation of benzenetriol. It describes the free radical propagated chain reactions during the oxidation.

The last section (Chapter V) is an overview of the findings in this research. It concludes that benzenetriol induces numerical and structural chromosomal alterations and point mutations by two distinct mechanisms. The disruption of cytoskeletal microtubules and the damage of DNA by oxygen-derived active species and toxic quinones are involved in the benzenetriol induced genotoxicity in human cells.

The respective contributions of the author and each co-author in this research are listed in Appendix 7.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 BACKGROUND

Annual production of benzene in the United States was reportedly 11.8 billion pounds in 1988 (CEN 1989). The high volume production and the volatility has contributed to benzene being a ubiquitous environmental pollutant and a public health concern (USEPA, 1980; Mehlman, 1991). High levels of human exposure occur during the manufacturing of benzene or its use as an organic solvent or as a starting material for the synthesis of other chemicals in the pharmaceutical and chemical industries (Brief et al., 1980). In addition, benzene from other sources such as gasoline emission, automobile exhaust and cigarette smoke further contribute to exposure. Epidemiological studies have established that benzene is a human carcinogen and occupational exposure to benzene can lead to the development of various blood disorders including aplastic anemia, pancytopenia, and acute myelogenous leukemia (IARC, 1982). The mechanism by which benzene causes genetic damage remains unclear. However, it has been postulated that the genotoxicity of primary metabolites of benzene such as hydroquinone, catechol and 1,2,4-benzenetriol are associated with benzene-induced carcinogenicity. Benzenetriol is a highly reactive metabolite and its oxidation leads to the formation of genotoxic oxygen-derived active species. The goal of this research is to characterize genetic alterations induced by benzenetriol and to assess whether it may potentially play a role in benzene-induced carcinogenicity.

1.1.1 Human Exposure to Benzene

Human contact with benzene occurs primarily from occupational and environmental exposure. Occupational exposure to benzene numbers approximately 0.2 to 2 million workers in North America (NIOSH, 1977; OSHA, 1985). A air concentration of benzene in the workplace has been reported at 1600 mg/m^3 (500 ppm) and at times in excess of 3200 mg/m^3 (1000 ppm) (IARC, 1982). The Occupational Safety and Health Administration (OSHA) has set the standard limit for occupational exposure to benzene at 10 ppm time-weight average (TWA) per 8-hour (OSHA, 1985). In compliance with the OSHA standard, benzene exposure levels have decreased dramatically (Runion and Scott, 1985). In 38,000 air samples from 7 factories using or producing benzene in the U.S., the concentrations of benzene in air were below 1 ppm in 87% of samples, between 1 and 10 ppm in 11.4% , and greater than 10 ppm in only 1.6% (Runion and Scott, 1985).

The recommended benzene exposure level of 10 ppm TWA per day was designed to prevent developing aplastic anaemia and/or other perturbances which cause depression of cellular elements in blood (Yardley-Jones et al., 1991). Subsequently, epidemiologic studies indicated an excess risk for the development of benzene-induced leukemia at 10 ppm (Rinsky et al., 1987). OSHA (1987), therefore, has recently promulgated a new standard limiting worker exposed to 1 ppm 8-hour TWA in the U. S. Occupational exposure in many Third World countries frequently exceeds 10 ppm in industrial settings (Holmberg and Lundberg, 1985). And in extreme cases such as Chinese workers, the exposure levels are still up to 100 ppm benzene.

Although occupational exposure levels in the U. S. have been declining, benzene is still present above recommended levels in ambient air (Holmberg and Lundberg, 1985). Thus, environmental exposure spreads to the general population. An estimated 75% of the U.S. population has been environmentally exposed to benzene, usually 2-3 orders of magnitude lower than occupational exposure (Van Raalte, 1982). Environmental sources of benzene exposure include industrial processes, gasoline engine emissions, gasoline re-fueling, solvents in consumer products, and cigarette smoke.

Cigarette smoke is a major component of environmental exposure with levels in smokers reported at 15 $\mu g/m^3$ compared to 1.5 - 2 $\mu g/m^3$ in non-smokers (Wallace, 1989). Cigarette smokers are exposed to an estimated 2 mg of benzene per day (approximately 60 µg / cigarette). Non-smokers are exposed to benzene from 'sidestream' smoke since a single cigarette emits between 12 to 480 µg of benzene into the air (IARC, 1986; Wallace et al., 1987).

Based on linear extrapolation of leukemia risk at higher levels of exposure, Wallace (1989) estimated that 1000 cases of leukemia per year in the U. S. can be attributed to benzene exposure, nearly half of them are associated with cigarette smoking. Indeed, cigarette smokers have a 50% higher risk of developing leukemia than non-smokers (Sandler, 1985; McLaughlin et al., 1989; Severson et al., 1990). In summary, smokers and those occupationally exposed face the greatest risk of developing benzene-induced leukemia.

1.1.2 Toxic Effects of Benzene

Toxic responses to benzene in humans include central nervous system (CNS) anesthesia from short-term, intensive exposures, hematopoietic toxicity from relatively long-term, chronic exposure, and leukemia from much longer-term exposure to much lower concentrations of benzene.

Acute exposure to 20,000 ppm benzene in human was fatal within 5-10 min (Flury, 1928). Workers exposed to acute, non-lethal levels (<20,000 ppm) of benzene developed CNS depression, loss of consciousness, irregular heart-beat, dizziness, headache, and nausea (Deutsche F., 1974). In a cohort of more than 500,000 occupationally exposed persons, benzene poisoning, defined as peripheral leukocyte counts less than 4000 cells/mm^3 with an occupational history of benzene exposure and CNS symptoms, was detected with average exposures as low as 12.5 ppm (Yin et al., 1987). Since benzene is regulated at concentrations far below those required to induce CNS toxicity, its neurotoxic effect is infrequently studied (Snyder, 1988).

Humans are susceptible to benzene myelotoxicity (hematopoietic toxicity) as shown by the high incidence of blood dyscrasias among workers with significant exposure (Goldstein, 1989). Ever since Santesson (1897) and Selling (1916, both referenced in IARC, 1982) reported that chronic exposure to benzene could cause death from aplastic anemia, the toxic effects of benzene to the blood of man were recognized. In addition to aplastic anemia, benzene exposure induces thrombocytopenia, granulocytopenia, lymphocytopenia, pancytopenia, leukopenia, and bone marrow hypoplasia or aplasia (Laskin and Goldstein, 1977; IARC, 1982; OSHA, 1987).

Pluriopotent bone marrow stem cells divide and differentiate into all the major blood cell types (Figure 1). Damage of the stem cells could potentially lead to disorders of various mature blood cells (Golde, 1991). The decrease or absence of erythrocytes, leukocytes, platelets and their precursors within the bone marrow can result from alterations in processes governing stem cell self-renewal (aplastic anemia). Another major disease that can ensur from stem cell toxicity is leukemia, in which the stem cell acquires a heritable alteration in processes controlling replication and differentiation, and populates the blood system with immature, non-functional leukocytes (Dexter et al., 1985). It remains to be determined why benzene is toxic to bone marrow stem cells, and how it acts at the molecular level to alter self-renewal and differentiation. However, the correlation of benzene exposure levels to specific hematopoietic effects in humans has been well documented and is summarized in Table 1.

An association between occupational exposure to benzene and the development of leukemia was noted by Delore and Borgomano as long ago as 1928. Since then, numerous case reports and more than 100 occurrences of leukemia associated with benzene exposure have been published (Vigliani, 1976). In the 1970's, Aksoy showed that the incidence of leukemia among 28,500 Turkish shoe workers exposed chronically to benzene was 13.6 per 100,000, significantly above the incidence of 6 per 100,000 in general population (Aksoy, 1972, 1976 and 1977). Rinsky et al. (1987) examined a cohort of 1165 men working in a rubber industry in Ohio, and demonstrated an overall standardized mortality ratio (SMR; the ratio of observed over expected deaths x 100) of 337 for leukemia and 409 for multiple myeloma. And a clear dose-response relationship was discerned between the cumulative benzene exposure and development of leukemia in this thorough retrospective study.

The largest investigation to date involved 233 benzene factories and 83 control factories in 12 Chinese cities (Yin et al., 1987; 1989). The benzene-exposed cohort includes 28,460 workers and the control cohort contains 28,257. The overall SMR for exposed cohort was 574 (containing 76.6% of the acute leukemia cases). Most benzene-induced malignancies are particularly acute myelogenous leukemias (AML). The International Agency for Research on Cancer (IARC, 1982) states:

"*The relationship between benzene exposure and the development of acute myelogenous leukemia has been established in epidemiologic studies*".

Based on these epidemiologic data, benzene has been classified as a human carcinogen. In addition to AML, other leukemias, such as multiple myeloma, chronic myelogenous leukemia, chronic lymphocytic leukemia, Hodgkin's lymphoma, non-Hodgkin's lymphoma, and acute lymphocytic leukemia, are also associated with benzene exposure (reviewed by Goldstein, 1977; Kipen et al., 1988; Austin, 1988). Benzene also induces an excess incidence of cancer at sites other than the hematopoietic system, including the urinary bladder (Steineck et al., 1990) and lung (Aksoy, 1976; Yin et al., 1989).

1.1.3 Metabolism of Benzene

Benzene, like some other carcinogens, apparently lacks direct-acting carcinogenicity and mutagenicity. It must first be metabolized to the "proximate" carcinogens (Andrews et al., 1977). Thus, benzene by itself is weakly mutagenic or non-mutagenic in standard bacterial and mammalian cell mutation assays (Dean, 1978; Huff et al., 1989). The inability to detect

benzene-induced mutation with these assays may be explained by the lack of enzymes needed to convert benzene to its metabolites (IARC, 1982; Glatt et al., 1989). The liver is the primary site for bioactivation of benzene because it contains the requisite cytochrome P450 enzymes. The cytochrome P450 2E1 in the P450 family has been established to specifically metabolize benzene (Koop et al., 1989).

The metabolism of benzene is complex and not completely understood. The major pathways of benzene metabolism and excretion are shown in Figure 2. Benzene is metabolized primarily in the liver by cytochrome P450 2E1 to benzene epoxide which rearranges to yield phenol (PH) or conjugates with glutathione to yield phenyl mercapturic acid (Tunek, 1978; Koop et al., 1989). Benzene epoxide can also be catalyzed by epoxide hydrolase to benzene-1,2-*trans*-dihydrodiol which is further converted by cytosolic dehydrogenation (Jerina, 1968) to catechol (CAT). This is the major pathway from benzene to catechol (Sata et al., 1963; Tomaszewski et al., 1975). Phenol can be hydroxylated mainly to *p*-hydroquinone (HQ) and perhaps to *o*-catechol (Sawahata and Neal, 1983). CAT can be further hydroxylated to form the triphenolic metabolite of benzene, 1,2,4-benzenetriol (BT) (Tunek et al., 1980). BT can also arise from the hydroxylation of HQ (Inoue et al., 1989a). The ring-opened metabolite, *trans,trans*-muconaldehyde can also be formed from benzene dihydrodiol (Latriano et al., 1986) or benzene oxide oxepin (Gad-El-Karim et al., 1986) by various pathways (Figure 2).

Most above benzene metabolites have been detected in rodent blood, bone marrow and urine (Rickert et al., 1979), and in human urine (Inoue et al., 1989a, b). Parke and Williams (1953a, b) administered ^{14}C-benzene (0.34 - 0.5

ml/kg) orally to rabbits, and 84-89% of the dose was recovered as radioactivity in expired air (43%), urine (34.5%), feces and body tissues (5-10%). The 34.5% radioactivity recovered in urine consisted of PH (23.5%), HQ (4.8%), CAT (2.2%), BT (0.3%), *trans,trans*-muconic acid (1.3%) and L-phenyl mercapturic acid (0.5%). The more stable metabolites are found at higher concentrations in blood, bone marrow, lymphoid tissue, and fat (Bergman, 1979; Greenlee et al., 1981). Surprisingly, no one has measured the BT level in blood and bone marrow yet because the BT is too unstable.

The relationship among benzene, its metabolites, and bone marrow toxicity is complex. Although metabolism of benzene is necessary for bone marrow toxicity, the reactive metabolites are formed in the liver and transported to bone marrow. The currently accepted mechanism of the transport from liver to bone marrow is shown in Figure 3. Once the metabolites are formed in the liver, they enter the blood and preferentially accumulate in the bone marrow where they exert their toxicity (Andrews et al., 1979; Rickert et al., 1979). The most potent toxicants may be, not the primary benzene metabolites, but the secondary metabolites arising from the further action of myeloperoxidase (MPO) in bone marrow (Smith et al., 1989). Thus, bone marrow provides another site for further metabolism, in which yet more toxic quinone metabolites of benzene are derived and more reactive oxygen species are generated (Greenlee et al., 1981; Irons, 1985). Both quinones and reactive oxygen species, therefore, are candidates for the acute toxicants which contribute to benzene-induced hemotoxicity and carcinogenicity (Smith et al., 1989; Subrahmanyam et al., 1991a).

1.2 TOXIC QUINONES AND REACTIVE OXYGEN SPECIES

Quinones are aromatic diketones, widely distributed in nature, and found in plants, fungi, bacteria, and animals. They function as components of the electron transport chains involved in cellular respiration and photosynthesis. Quinones have been used as anticancer chemotherapeutic drugs and as pigments for dyes and cosmetics. Quinones are also prevalent as environmental pollutants many of which are toxic in human. The chemistry and toxicity of quinones have been recently reviewed in detail by Monks et al. (1992a). Quinones have reduction potentials appropriate to redox-cycling, and therefore they create oxidative stress by generating reactive oxygen species.

1.2.1 Toxicity of Benzene-Derived Quinones

Polyhydroxylated metabolites of benzene , CAT, HQ, and BT each oxidize (either enzymatically or spontaneously) to their respective semi-quinones and quinones (Greenlee et al., 1981; Subrahmanyam et al., 1991b). Benzene-derived quinones include 1,2-benzoquinone (*o*-BQ), 1,4-benzoquinone (*p*-BQ), and 2-hydroxy-benzoquinone (2-OH-BQ) as well as their corresponding semiquinones (Figure 2). The semiquinones (at physiological pH) exist as anion radicals, each containing one unpaired electron (Mukherjee et al., 1989). Benzene-derived semiquinone radicals and quinones exert various toxicities both *in vitro* and *in vivo*.

Quinone-mediated toxicities include neurotoxicity, cytotoxicity, genotoxicity and perhaps carcinogenicity (Smith et al., 1989; Monks et al., 1992a). As mentioned above, acute exposure to benzene results in a central

nervous system (CNS) depression (Haley, 1977) probably caused by quinone metabolites of benzene (Snyder, 1988). A well known neurotoxicant, 6-hydroxydopamine, however, provides a good example for quinone-induced neurotoxicity. It undergoes a rapid autoxidation to its quinone form which is cytotoxic through genesis of the products of partial reduction of oxygen (Gee and Davison, 1989).

BQ, HQ, CAT and BT are cytotoxic in both human lymphocytes and HL60 cells (Yager et al., 1990; Levay and Bodell, 1992; Zhang et al., 1993). To be toxic in bone marrow, benzene's phenolic metabolites require further bioactivation. Bone marrow stroma consists predominantly of two cell types, macrophages and fibroblastoid stromal cells. HQ is selectively toxic at the level of the macrophage in bone marrow stroma cells, whereas the fibroblastoid stromal cell is relatively resistant (Thomas et al., 1989). Thus selective toxicity toward macrophage stroma cells in the bone marrow is because they contain considerably greater peroxidase activity and less DT-diaphorase (NAD(P)H : quinone oxidoreductase) than the fibroblastoid cells. Peroxidases catalyze the further oxidation of the phenolic metabolites to their quinones, and DT-diaphorase is an obligate two-electron reductase, which converts quinones to their parent hydroquinones (Ross et al., 1990). Consequently, HQ is activated to *p*-BQ in the bone marrow macrophage stroma cells rather than in the fibroblastoid cells (Thomas et al., 1990).

The resultant quinones or semiquinone radicals can react directly with DNA, RNA, and proteins. Benzene and its metabolites bind to DNA in rabbit bone marrow *in vitro* (Rushmore et al., 1984), and to RNA in rat and mouse organs *in vivo* (Arfellini et al., 1985). The DNA adducts in HL60 cells treated

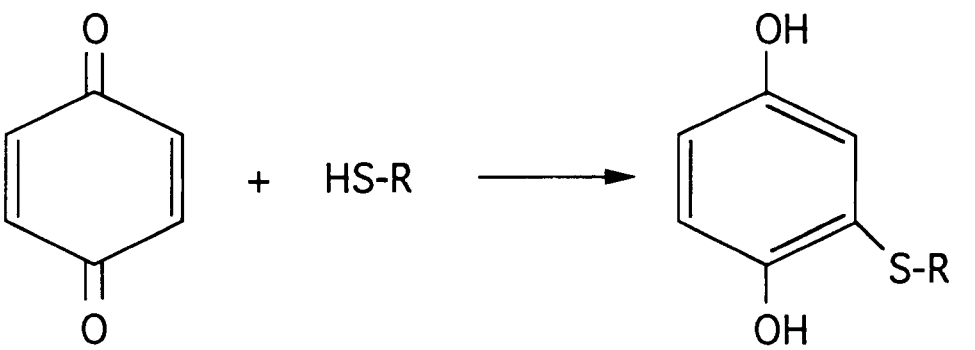
with CAT, HQ and BT have been identified by ^{32}P postlabeling (Levay and Bodell, 1992). In addition, these benzene-derived quinones form adducts with glutathione (GSH) (Tunek et al., 1980) and proteins such as hemoglobin (Adams and Biemann, 1990), cytoskeletal protein (Irons, 1985), and enzymes including prostaglandin H synthase (Schlosser et al., 1990). Interestingly, all of these adducts have been identified as the same structural product of the "Michael addition", namely, the formation of quinone-thioethers.

Benzene-derived quinones, then, are cytotoxic and genotoxic, and in addition mutagenic and carcinogenic. For instance, BQ produced a 100-fold increase in the frequency of expression of 6-thioguanine resistance in Chinese hamster V79 cells (Glatt et al., 1989). HQ, BT and CAT also induced this gene mutation but PH did not, suggesting that formation of quinones is responsible. However, none of them induced substantial mutations in *S. typhimurium* (Glatt et al., 1989). The mechanisms underlying benzene-induced bone marrow toxicity and leukemogenesis are therefore presumed to result from the toxic effects of benzene-derived quinones.

1.2.2 Mechanisms of Quinone-Mediated Toxicities

The toxicological activity of benzene-derived quinones resides in their ability to react directly with cellular nucleophiles and also their ability to undergo redox cycling inducing oxidative stress (Figure 4). In the context of toxicology, or more generally in their interactions with macromolecules in living cells, quinones possess two paramount chemical properties. They are electrophiles and oxidants.

Quinones, as electrophiles, react directly with cellular nucleophiles such as protein and nonprotein sulfhydryls. GSH is the major intracellular nonprotein sulfhydryl reagents (Reed, 1985). The conjugation of potentially toxic electrophiles with GSH usually results in detoxification and excretion (Figure 4). Quinones conjugated with GSH are excreted in urine as their corresponding sulfate conjugates and/or as mercapturic acids. The multiple conjugates have been recently shown to have their own toxic properties (Monks and Lau, 1992b). Interaction of quinones with nucleophiles may lead to toxicity by inhibition of critical thiol groups in enzymes, or by alteration of the thiol balance in the cell, either of which can interfere with cellular regulatory processes (Sies, 1985). Reactions of quinones with thioles by Michael addition (Finley, 1974) result in formation of hydroquinone thioethers.



p-Benzoquinone

2-Alkylthiohydroquinone

Quinones, as oxidants, are enzymatically reduced by both one-electron (NADPH-cytochrome P450 reductase, etc.) and two-electron (DT-diaphorase) reductions, resulting in the formation of semiquinones or hydroquinones. The reduction of 2-OH-BQ, as an example of a benzene-derived quinone, is depicted in Figure 5. Two-electron reduction of quinones catalyzed by

DT-diaphorase has generally been considered to be a detoxification pathway since the resulting hydroquinones can be further conjugated and excreted (Figure 4). In some cases, such as with certain bioreductive alkylating agents, the reduction by DT-diaphorase leads to activation of the compound. For example, the reduction of 2-OH-BQ, by DT-diaphorase, may lead to redox cycling dependent on the rate of reduction to BT followed by its autoxidation back to the parent quinone (Brunmark and Cadenas, 1988). Formation of semiquinones therefore occurs during either the reduction of quinones or the oxidation of hydroquinones (Figure 5).

Semiquinones disproportionate to their quinone and hydroquinone forms with great facility (Mukherjee et al., 1989), enabling semiquinones and quinones to be "redox facilitators". Thus, if their redox potential is sufficiently low, semiquinones can transfer their single unpaired electron to other oxidants such as molecular oxygen (O_2). If there is an efficient mechanism for re-reduction of the quinone product to the semiquinone, a redox cycle facilitated by the quinone/semiquinone couple ensues (Figure 4). Semiquinones can react with O_2 forming superoxide anion radical ($O_2^{\bullet -}$) and regenerating the parent quinone, which is then available for re-reduction in a redox cycling. The net result of this redox cycling is oxidative stress resulting from disproportionate consumption of cellular reducing equivalents and the generation of active oxygen species. Ultimately, the flux of $O_2^{\bullet -}$ and other oxygen-derived active species destroys unsaturated lipids, DNA, proteins, and other essential cellular molecules, with lethal results. Much recent research has emphasized the role of oxidative stress and redox cycling in quinone and polyphenol toxicity (Smith et al., 1985).

1.2.3 Toxicity of Reactive Oxygen Species

During the oxidation of benzene's polyhydroxy metabolites and their semiquinones, molecular oxygen (O_2) is primarily reduced to $O_2^{\bullet -}$, and a number of oxygen radicals and active species are generated (Figure 5). Superoxide dismutase can further convert $O_2^{\bullet -}$ to hydrogen peroxide (H_2O_2), which may generate hydroxyl radicals ($HO^{\bullet}$) in the presence of metal ions and reducing equivalents. Singlet oxygen ($^1O_2$) is perhaps produced during the formation of benzene-derived quinones based on the chemiluminescence seen in the oxidation of at least one triphenol (Fatur and Davison, 1987). Since quinones are colored and absorb ultraviolet and visible light energy, photo-excited quinones can be quite reactive to hydrogen and electron abstractions, and/or energy transfer to another acceptor molecule, such as O_2 (i.e., chemical photosensitization) (Bruce, 1974).

All of these reactive oxygen species cause oxidative stress and cellular injury. Subjecting cells to oxidative stress can result in severe dysfunctions, including peroxidation of membrane lipids, depletion of nicotinamide nucleotides, rises in intracellular free Ca^{2+} ions, cytoskeletal disruption and DNA damage. The latter is often measured as formation of single-strand breaks, double strand breaks or DNA adducts etc.. Indeed, DNA damage has been almost invariably observed in a wide range of mammalian cell types exposed to oxidative stress (Halliwell and Aruoma, 1991). However, neither $O_2^{\bullet -}$ nor H_2O_2 induce scission of DNA, as measured by strand breakage (Rowley and Halliwell, 1983) or by chemical changes in the deoxyribose, purines or pyrimidines (Aruoma et al., 1989a and b). The ultimate damaging species is putatively the $HO^{\bullet}$ radical.

The $HO^{\bullet}$ radical induces oxidative changes in DNA bases (Figure 6), resulting, for example, in 8-hydroxy-2'-deoxyguanosine (8-OH-dG), as a end-product of the attack of $HO^{\bullet}$ on guanine at the C-8 site (Figure 7). 8-OH-dG thus represents the covalent addition of $HO^{\bullet}$ (or $^1O_2$) to guanine in DNA (Floyd et al., 1986; Devasagayam et al., 1991) and has become a useful and sensitive marker of oxidative DNA damage both *in vitro* and *in vivo* (Roy et al., 1991). This is a potentially mutagenic change since the hydroxylated DNA base can be misread at the modified and adjacent base residues in the DNA-polymerase reaction *in vitro* (Kuchino et al., 1987). Thus, 8-OH-dG causes the DNA point mutations from G —> T substitution (Cheng et al., 1992).

Abundant evidence indicates the involvement of reactive oxygen species in mutagenesis and carcinogenesis. Thus the rate of spontaneous mutation is enhanced in *E coli* that lacks the activity of superoxide dismutase (Touati, 1989). Similar mutagenic effects have been shown in a range of mammalian cell types subjected to oxidative stress. Single base changes and deletions were the main patterns of mutation in cells exposed to H_2O_2 (Moraes et al., 1990). The majority of base changes were at GC base pairs, the GC to AT base transition being predominant. The base deletions cause frameshift leading to loss of the information caved in the affected gene. Moreover, mutations caused by active oxygen species in certain coding regions of tumor-related genes might be responsible for subsequent activation of oncogenes and/or inactivation of tumor suppressor genes (Wei, 1992). Such changes can cause neoplastic transformation of normal cells through an increase in expression of certain proto-oncogenes such as c-*fos*, c-*jun*, and c-*myc* (Sherman et al., 1990; Yin et al., 1992). Furthermore, reactive oxygen species are involved in initiation (irreversible change in DNA), promotion (further changes in gene expression),

and perhaps progression (production of malignant tumor) in the multistage model of carcinogenesis (Sun, 1990; Halliwell and Aruoma, 1991).

Taken together, reactive oxygen species cause cellular injury, DNA damage, gene mutation, and probably cancer. Most studies have focused on oxidative DNA damage, but this can not involve direct attack of $O_2^{\bullet -}$ or of H_2O_2 on the DNA, unless transition metal ions are present to allow $HO^{\bullet}$ formation. The formation of $HO^{\bullet}$ catalyzed by transition metal ions is crucial in oxidative DNA damage. DNA damage induced by oxidative stress may involve the direct reaction of $HO^{\bullet}$ with DNA or the oxidative activation of nucleases which cleave the DNA backbone (Halliwell and Aruoma, 1991). Metal ions play a essential role in both mechanisms, as summarized in Figure 8.

1.2.4 DNA Damage by Oxidative Stress

Hydroxyl radicals damage DNA. A major source of $HO^{\bullet}$ radicals is the one-electron reduction of H_2O_2 catalyzed by transition metal ions. The most efficient intracellular metal ions in physiological systems are iron and copper (Miller et al., 1990), which play essential roles in many enzymes and proteins. If they become "decompartmentalized", traces of their soluble ions can convert H_2O_2 to $HO^{\bullet}$ via Fenton and Haber-Weiss type reactions:

$$(1) \qquad H_2O_2 \xrightarrow{Fe/Cu} HO^{\bullet} + HO^{-} \qquad \text{(Fenton)}$$

$$(2) \qquad O_2^{\bullet -} + H_2O_2 \xrightarrow{Fe/Cu} HO^{\bullet} + HO^{-} + O_2 \qquad \text{(Haber-Weiss)}$$

Redox cycling of the metal ions amplifies their ability to transfer electrons to H_2O_2 (Chevion, 1988). Thus, the above reaction (2) involves two electron-transfer steps as below:

(3) $O_2^{\bullet -} + Fe^{3+} / Cu^{2+} \longrightarrow Fe^{2+} / Cu^{+} + O_2$

(4) $H_2O_2 + Fe^{2+} / Cu^{+} \longrightarrow Fe^{3+} / Cu^{2+} + HO^{\bullet} + HO^{-}$

Such mechanisms can mediate DNA damage only if the $HO^{\bullet}$ is generated upon or very close to the DNA. Otherwise, the high reactivity of $HO^{\bullet}$ precludes its diffusing significant distances within the cell. Since H_2O_2 crosses biological membranes easily, it can penetrate to the nucleus and react with DNA-bound iron or copper to form 'site-specific' hydroxyl radicals. The metal ions might be those originally bound to the DNA *in vivo*, or those released within the cell as a result of oxidative stress, and then bound to the DNA (Dizdaroglu et al., 1991; Halliwell, 1987).

1.2.5 DNA Damage by Activation of Endonucleases

Oxidative stress initiates a series of events within the cell, which leads to activation of Ca^{2+}-dependent endonucleases that can fragment DNA (Birnboim and Kanabus-Kaminska, 1985; Birnboim, 1988). Thus, oxidative stress increases intracellular free Ca^{2+} by: (1) concomitant inactivation of Ca^{2+}-binding by the endoplasmic reticulum, (2) inhibition of plasma membrane Ca^{2+}-extrusion systems, and (3) release of Ca^{2+} from mitochondria (Halliwell and Aruoma, 1991).

The two mechanisms are not mutually exclusive. Changes in the availability of Ca^{2+} may depend upon, or give rise to, changes in the availability of iron or copper ions. DNA damage could be inhibited by preventing the rise either of Cu^{2+} / Fe^{3+} or of Ca^{2+}, using their appropriate chelators (Goldstein and Czapski, 1990; Dypbukt et al., 1990) or by scavenging the reactive oxygen species using endogenous defense systems (Sun, 1990).

1.2.6 Defenses against Reactive Oxygen Species

Aerobic organisms have evolved multiple defense systems to protect themselves against the reactive oxygen species generated *in vivo.* The enzymatic defenses remove $O_2^{\bullet -}$ by superoxide dismutase, and H_2O_2 by catalase and glutathione peroxidase which works in coordination with glutathione reductase and glucose-6-phosphate dehydrogenase (Sun, 1990). These enzymes function in cooperation to diminish oxidative damage (Figure 9). Moreover, DNA repair enzymes can correct certain oxidative DNA defects such as single-strand breaks (Friedberg, 1985), but may not double-strand breakage (Kohen et al., 1986). Since the $HO^{\bullet}$ radical oxidizes non-specifically almost all biomolecules, including superoxide dismutase and catalase, no specific enzymes can selectively destroy it. Non-enzymatic defenses protect against uncontrolled oxidative reactions initiated by $HO^{\bullet}$.

The non-enzymatic defenses comprise metal-binding and chain breaking antioxidants. Low molecular mass antioxidants for chain breaking include lipid-soluble vitamins, such as α-tocopherol, carotenoids and retinoids which prevent lipid peroxidation and peroxidative damage by scavenging or quenching oxygen-derived active species. Ascorbic acid and GSH, as water-soluble

antioxidants, may consume HO• formed in the cytoplasm. Other scavengers, including urate, formate and polyalcohols (e. g. sugars) also protect the system against radical mediated damage both *in vitro* and *in vivo* (Goldstein and Czapski, 1990). In addition, the administration of synthetic scavengers may diminish the damage in mammals subjected to experimental oxidative stress.

Metal chelators which remove a metal ion from access to redox cycling can block the oxidant action of the metal. For example, desferrioxamine, a synthesized chelator, binds specifically iron and copper as well as other metal ions into redox resistant chelates (Halliwell, 1989). Since desferrioxamine does not readily cross cell membranes, its effects *in vivo* are variable (Halliwell and Aruoma, 1991). As a copper chelator, bathocuproine inhibits copper-initiated human DNA damage in cultured human cells (Kawanishi et al., 1989), and D-penicillamine extends the lifespan of patients with copper overload diseases (Goldstein and Czapski, 1990). Proteins can also bind metal ions in forms unable to accelerate free radical reactions (Halliwell and Aruoma, 1991). Ferritin and metallothionein can store Fe^{3+} and Cu^{2+}, respectively (Miller et al., 1990). Even relatively weak ligands which do not defer redox cycling or HO• production can prevent 'site specific' oxidative damage. They do so by displacing the metal from more sensitive target so that the damage occurs at less vital cellular components.

In summary, oxidative DNA damage results when reactive oxygen species and/or metal ions are not adequately removed, for example, when antioxidants are depleted and/or if the formation of reactive oxygen species is increased beyond the capacity of the defense systems (Sies, 1991).

1.2.7 Roles of Reactive Oxygen Species in Benzene Toxicity

Although the benzene-derived quinones (discussed in 1.2.1) most likely mediate benzene-induced toxic effect, little attention has been paid to oxygen radicals and active species as further mediators of toxicity. The myelotoxic effects of benzene have been attributed to free radical formation, as metabolites of benzene, oxygen radicals, or lipid peroxidation products. The potential role of oxygen free radical in benzene-induced myelotoxicity and leukemia has been carefully reviewed by Subrahmanyam et al. (1991a).

The variety of reactions generating reactive oxygen species during benzene metabolisms is summarized in Figure 10. Those specific to bone marrow may occur (1) during peroxidative metabolism catalyzed by myeloperoxidase (MPO) (or epoxidase and perhaps prostaglandin H synthase), (2) during autoxidation of benzene's phenolic metabolites, or (3) by the membrane-bound NADPH-oxidase activity of phagocytic bone marrow cells.

However, the role of active oxygen species playing in benzene-induced myelotoxicity and carcinogenesis remains unknown. To further understand the roles and mechanisms involved in the toxicities of benzene-derived quinones and reactive oxygen species, this research has focused on studies of the genotoxicity of 1,2,4-benzenetriol (BT). BT, a triphenolic metabolite of benzene, has a strong ability to activate oxygen and to be oxidized to its corresponding quinones (Figure 5). Relatively few studies have been conducted on toxicity of BT and little is known about the mechanisms involved. BT's toxic effects are reviewed in next section.

1.3 TOXICITY OF 1,2,4-BENZENETRIOL

1.3.1 BT is one of the most reactive metabolites of benzene.

BT has been identified as a human urinary metabolite of benzene. In the urine of benzene-exposed workers, the concentration of BT is related linearly to the intensity of benzene exposure (Inoue et al., 1989a, b). BT is presumably also formed in bone marrow via the activation of primary benzene metabolites by myeloperoxidase (MPO) (Subrahmanyam et al., 1991b) or hydrolysis of *o*-BQ (Mason, 1949). The accumulation of BT in blood and bone marrow remains unknown because it is unstable. Although BT is a relatively minor product in the liver during the metabolism of benzene in quantitative terms (Rusch et al., 1977), the low steady state levels may be biologically significant because of its high reactivity.

1.3.2 BT activates molecular oxygen to reactive oxygen species.

BT is more unstable in molecular oxygen than the other metabolites due to its high potency to donate electrons (Greenlee et al., 1981). BT's two *o*-hydroxy substitutes are capable of binding transition metal ions that catalyze its autoxidation (Bandy et al., 1990). Thus, it readily autoxidizes to its corresponding quinones and activates molecular oxygen to a mixture of reactive species, including superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$) as well as perhaps singlet oxygen ($^1O_2$) (Figure 5). These species are known to damage DNA and other cellular macromolecules (Halliwell and Aruoma, 1991; Stadtman and Oliver, 1991).

1.3.3 BT causes oxidative DNA damage both *in vitro* and *in vivo*.

BT induces: (1) single- and double-stranded DNA breaks in the mouse lymphoma cell line, L5178YS (Pellack-Walker and Blumer, 1986), (2) DNA cleavage in isolated human DNA fragments (Kawanishi et al., 1989), and (3) supercoiled DNA breakage in isolated *Escherichia coli* plasmids (Li, 1992). The BT-induced DNA damage is elevated by transition metal ions, such as Cu^{2+} and Fe^{3+}, but inhibited by superoxide dismutase, catalase and hydroxyl radical scavengers (Lewis et al., 1988; Kawanishi et al., 1989; Li, 1992). However, all of these findings were *in vitro*. In the only *in vivo* study (Kolachana et al., 1993), the administration of BT to mice induces a significant increases in the level of 8-hydroxy-2'-deoxyguanosine (8-OH-dG) in bone marrow DNA. Here BT has much higher potency than the other metabolites of benzene tested (PH, CAT and HQ). In another study, the repeated administration of BT to rats for six weeks induced myelotoxicity (Rao et al., 1988).

1.3.4 BT binds to DNA and inhibits DNA synthesis.

In addition, BT or its oxidative products can bind to DNA directly with uncertain consequences. The DNA adducts have been identified by ^{32}P postlabelling in HL60 cells treated with BT (Levay and Bodell, 1992). Moreover, BT inhibits nuclear DNA and/or RNA synthesis in L5178YS cells (Pellack-Walker et al., 1985) and in mouse bone marrow cells (Lee et al., 1989). BT also decreases the mitotic index and slows the cell cycle in human lymphocytes (Erexson et at., 1985).

1.3.5 BT causes sister chromatid exchanges and chromosomal alterations.

BT induces dose-related increases in the sister chromatid exchanges (SCE) in human peripheral blood lymphocytes (Erexson et at., 1985) and Chinese hamster V79 cells (Glatt et al., 1989). Tice and co-workers (1982) further demonstrated that a 4 h exposure to 28 ppm benzene in DBA/2 and C57B1/6 mice significantly increased SCE. However, increased frequencies of SCE were not seen in workers exposed to benzene, on the contrary, decreased frequencies have been reported (Watanabe et al., 1980). BT induces both HPRT (Hypoxanthine-guanine Phosphoribosyl Transferase) gene mutation (6-thioguanine resistance) and micronuclei in Chinese hamster V79 cells (Glatt et al., 1989). Little is known about the mechanisms involved in BT-induced toxicity. It therefore seemed to us timely to investigate the formation of micronuclei and other chromosomal alterations caused by BT, which has not been determined in human cells yet.

1.4 CHROMOSOMAL ABERRATIONS

There are two principal categories of chromosome abnormalities: (1) structural chromosome alterations (deletion, inversion, and translocations etc.), and (2) numerical chromosome alterations (the gain or loss of entire chromosomes). Inductions of such non-random chromosomal changes are thought to be primary events crucial to the onset of neoplasia. They are associated with a variety of cancers, including leukemia (Yunis, 1983; Rowley, 1984).

1.4.1 Common Chromosomal Changes in Leukemia

Most human leukemias and lymphomas have at least one type of nonrandom clonal abnormality. Chromosome gains and losses are often seen in both myeloid and lymphoid neoplasms. An extra chromosome 8 or 21 is the most common change, and a loss of chromosome 5 or 7 also occurs in acute myelogenous leukemias (AML) (LeBeau and Rowley, 1984). Trisomy of chromosome 12 is often found in chronic lymphocytic leukemia (CLL) (Han et al., 1983; Losada et al., 1991). In addition to these numerical chromosomal aberrations, the remarkably specific structural changes are seen in certain hematological leukemias. Among the most dramatic are the structural rearrangements uniquely associated with a particular leukemia. Thus, translocations of chromosomes 8 and 21 [t(8; 21) (q22, q22)], and chromosomes 15 and 17 [t(15; 17) (q22, q21)] are observed in AML, whereas a translocation of chromosome 9 and 22 [t(9; 22) (q34, q11)] is seen in most (>90%) cases of chronic myelogenous leukemia (CML) and in some patients with AML and ALL. The chromosomal alterations in human cancers and leukemias have been extensively reviewed by Sandberg (1990).

These specific chromosomal abnormalities are reportedly involved in tumor development. The products of genes adjacent to the breakpoints indicate how chromosome translocations facilitate tumor initiation and progression (Rabbitts, 1991). The Philadelphia chromosome (Ph^1) represents a reciprocal translocation, t(9; 22) (q34, q11). The Abelson proto-oncogene, c-*abl*, normally located on chromosome 9, resides on the Ph^1 (De Klein et al., 1982). The translocation fuses the c-*abl* gene with *bcr*, a transcription unit on chromosome 22. The *bcr* sequence stimulates the transcription of c-*abl*, activates the tyrosine

kinase activity of c-Abl (Konepka et al., 1984) and converts Abl to a transforming protein for hematopoietic cells (McLaughlin et al., 1987). The chimeric protein of the *bcr-abl* fusion gene (p210) has growth promoting tyrosine kinase activity (Sawyers et al., 1991) and can induce CML in transgenic mice (Heisterkamp et al., 1990). Thus, the c-*abl* proto-oncogene is converted to an oncogene by this chromosomal rearrangement.

In addition, the loss of the long arm portions of chromosome 5 and 7 predicts a high risk of leukemic transformation and particularly occurs in AML. Deletions of 5/5q and/or 7/7q were found in patients who developed myelodysplastic syndrome (MDS) or AML arising secondary to cytotoxic therapy for a previous malignant disease (Rowley et al., 1981). A specific gene involved in the genesis of MDS has not been identified, but the 5q chromosomal region is the gene location of several hematopoietic growth factors (IL-3, IL-4 IL-5, M-CSF, GM-CSF, IL-9) and growth factor receptors (M-CSF receptor, platelet-derived growth factor) (Groopman et al., 1989).

1.4.2 Benzene Induced Chromosomal Aberrations

Benzene consistently induces chromosome alterations in mammalian *in vivo* and *in vitro* test systems (reviewed by Dean, 1985). Frequencies of altered structural chromosome changes and of abnormal chromosome numbers are increased in lymphocytes and bone marrow cells from workers exposed to benzene. Sasiadek (1992) recently reported that benzene-exposed workers had breakpoints primarily in chromosomes 2, 4 and 7. The chromosome 5 anomaly was observed in lymphocytes of workers exposed to benzene or other organic solvents (Van der Berghe et al., 1985). Chromosomal abnormalities in

the chromosome C-group (through chromosomes 6 to 12 and X), such as trisomy of chromosomes 6, 8, and 9, monosomy of chromosome 7, or deletion of the long arm of chromosome 7 (7q-) were detected in patients with benzene-induced pancytopenia and leukemia (Erdogan and Aksoy, 1973). Interestingly, the C-group chromosomes are also frequently abnormal in hematological disorders and leukemias (Chen et al., 1992; Poddighe et al., 1991; Anastasi et al., 1990). Study of these non-random chromosome alterations provides an opportunity to investigate potential genetic regulation of hematopoiesis, and may provide clues to the molecular mechanisms of benzene-induced leukemia.

1.4.3 Micronucleus Formation and Detection

Micronuclei are novel markers of genetic damage, which are visible in the cytoplasm as small nuclear bodies. They contain either chromosome fragments or entire chromosomes that fail to incorporate into the daughter nuclei during cell division (Schmid, 1975). Benzene unequivocally induces micronuclei in bone marrow cells of mice and rats (Tice et al., 1980; 1982). Lymphocytes isolated from benzene-exposed workers also contain excess micronuclei (Dean, 1978; IARC, 1982). Micronuclei arise following exposure to chemicals that cause structural (chromosome breakage) or numerical (disruption of chromosome segregation in mitosis) chromosome aberrations. In assessing their action, the micronucleus assay compares well with chromosome aberration assays (Fench, 1993).

The micronucleus assay was originally developed by Evans and co-workers (1957) to detect genotoxicity in irradiated plant root tips. The assay was subsequently modified to detect cytogenetic damage in human peripheral

lymphocytes (Fenech and Morley, 1985), red blood cells (Schlegel et al., 1986), bone marrow cells (Heddle, 1973), and exfoliated cells (Stich and Rosin, 1984). In the modified micronucleus assay, cytochalasin b is used to disrupt actin filaments, thus preventing the cell from undergoing cytokinesis. Therefore, cells that have initiated cell division are distinguished by their multinucleated form, and only cells having undergone one cell division (containing binuclei) are scored for the presence of micronuclei. However, the cytokinesis-blocked micronucleus assay fails to determine by which mechanisms micronuclei are formed.

Discovery of antibodies that recognize the kinetochore proteins (Moroi et al., 1980; Brenner et al., 1981) extended the cytokinesis-blocked micronucleus assay (Vig and Swearngin, 1986; Degrassi and Tanzarella, 1988). This new micronucleus assay distinguishes micronuclei containing chromosome fragments (kinetochore-negative) from those containing entire chromosomes (kinetochore-positive). It differentiates chemicals that cause chromosome breakage (clastogens, e. g. radiation and sodium arsenite) from those which interfere with chromosome separation during mitosis (aneuploidogens, e. g. diethylstilbestrol or colchicine) (Eastmond and Tucker, 1989). The mechanisms whereby these agents induce micronucleus formation are fairly well understood. Chromosome breakage occurs most often by scission of the DNA and sometimes by interference with DNA-associated proteins such as DNA polymerases (Gualden, 1987). In contrast, chromosome lagging reflects chromosomes improper segregation into the daughter nuclei during cell division. The principle of the modified cytokinesis-blocked micronucleus assay system and these two mechanisms of micronucleus formation are summarized in Figure 11.

Using the anti-kinetochore antibody adapted micronucleus assay, Yager et al. (1990) investigated induction of micronuclei in human lymphocytes by individual metabolite of benzene, such as PH, CAT, HQ and BQ but not BT. Their ability to elevate the frequency of micronucleated cells is: HQ >> BQ > CAT > PH. In combination, HQ and CAT (at equimolar concentrations) acted synergistically in increasing both micronucleated cells and kinetochore-positive micronucleated cells (Robertson et al., 1991). We, therefore, selected the modified micronucleus assay to investigate the ability of benzene's metabolite BT to cause genotoxicity in this study.

1.4.4 Aneuploidy and Fluorescence *in situ* Hybridization

Aneuploidy is the loss or gain of whole chromosome(s) in the cell karyotype. It is common in leukemia and plays an important role in cancer progression and metastasis (Barrett et al., 1987; Fearon and Vogelstein, 1990). Aneuploidy is detected by traditional karyotyping and augmented by the modified micronucleus assay (kinetochore-positive micronucleus). However, karyotyping can be applied only to cells that can be stimulated into mitosis and reliably banded. This limits cytogenetic studies in solid tumors. In addition, karyotypic analyses are time consuming and labor intensive. Moreover, some chemicals such as mitomycin C cleave the kinetochore proteins from chromosomal centromeres (Brinkley and Shaw, 1969). Thus, use of this modified micronucleus assay to detect aneuploidy may lead to false positive results. To avoid the limitations and possible errors by these two assays, a better cytogenetic technique is needed for detecting aneuploidy.

In recent years, a new method, fluorescence *in situ* hybridization (FISH), has become available for detecting numerical and structural chromosomal changes in interphase nuclei and metaphase spreads (Gray and Pinkel, 1992; Zahed and Vekemans, 1991). This method (Figure 12) utilizes *in situ* hybridization with DNA probes specific to blocks of repetitive DNA sequences on defined regions of specific chromosomes (Willard and Waye, 1987; Pinkel et al., 1986; 1988). The visualization of chemically-modified probes involves the use of non-radioactive fluorescent antibodies. The determination of aneuploidy is performed by simply counting the number of label regions representing a particular chromosome of interest within the isolated nucleus. This method has been widely used for the molecular cytogenetic analysis of cancer cells (Cremer et al., 1988a, b; Hopman et al., 1991), but has also been applied to cultured human lymphocytes (Raimondi et al., 1989; Eastmond and Pinkel, 1990), bone marrow (Poddighe et al., 1991; Jenkins et al., 1992) and exfoliated human cells (Moore et al., 1993).

Many carcinogens are also aneuploidogens (Oshimura and Barrett, 1986). Benzene is a well known human leukemogen, and its primary metabolite HQ is an inducer of aneuploidy (Miller and Adler, 1992). It seemed timely to determine whether BT induces aneuploidy using the FISH technique and which mechanism is involved.

1.4.5 Roles of Microtubules in Aneuploidy

There are several potential targets for aneuploidy induction in mitotic cells (Liang and Brinkley, 1985). For example, colchicine and diethylstilbestrol interfere with microtubule assembly by inhibiting polymerization of the tubulin

subunits. Ethdium bromide affects the centrioles / centrosomes, mitomycin C removes kinetochores from chromosomes, and chloral hydrate blocks pole-to-pole microtubule elongation.

Since the mitotic apparatus consists predominantly of microtubules, any compound that affects microtubules might cause lagging chromosomes so as to be a potential aneuploidy-inducer. One function of microtubules is to attach to the chromosomes at the kinetochore and to guide them to their respective poles during cell division (Figure 13). The disruption of microtubules can lead to the formation of micronucleus and/or the induction of aneuploidy.

Microtubules are composed primarily of polymers of alpha and beta tubulin, which are rich in nucleophilic sulfhydryl groups important for microtubule assembly. The polyhydroxy benzene metabolites, BT and HQ, inhibit tubulin polymerization *in vitro* , with BT being approximately twice as potent as HQ (Irons et al., 1981; Irons, 1985; Pfieffer and Irons, 1983). Another triphenolic compound similar to BT, the neurotoxin 6-hydroxydopamine (6-OHDA) inhibits microtubule assembly *in vitro* (Davison et al., 1986). All these hydroquinones are readily oxidized to their electrophilic quinone and semiquinone intermediates, which can interact with nucleophilic sulfhydryls of tubulin and cause mitotic abnormalities resulting in aneuploidy. In the latter study, however, the quinonoid oxidation products were relatively ineffective.

In conclusion, both structural and numerical chromosomal aberrations have been observed in benzene-exposed workers and in laboratory animals treated with benzene. These aberrations may therefore be important in benzene-induced leukemia. Thus, studying the mechanisms whereby benzene

or its metabolites produce chromosomal damage may reveal details of the mechanisms of benzene-induced leukemia.

1.5 OBJECTIVES

1.5.1 Specific Aims

The purpose of this research was to study the role of oxygen-derived active species and toxic quinones in benzenetriol-induced genotoxicity and to understand the possible mechanisms involved. We therefore decided to investigate benzenetriol-induced genetic damage, including structural and numerical chromosomal alterations, oxidative DNA damage, and microtubule disruption by the modified micronucleus assay, the FISH technique, HPLC-EC analysis, and immunocytochemical methods. The human myeloblastic leukemia (HL60) cell line was chosen as a surrogate of bone marrow cells. HL60 cells are from human origin, at an early stage in hematopoietic development, with high myeloperoxidase content, and with a rapid growth rate (Gallagher et al., 1979). In addition, a cell-free chemical system was used to study role of radicals and transition metal ions in the mechanisms of free radical propagated chain reactions of benzenetriol with oxygen. The specific aims were as follows.

(1) To characterize the genotoxicity of 1,2,4-benzenetriol and determine the extent to which produces micronuclei and aneuploidy in HL60 cells.

(2) To characterize benzenetriol-induced numerical (kinetochore-positive micronuclei) and structural (kinetochore-negative micronuclei) chromosomal alterations, and to determine whether the pattern of micronucleus formation is contingent upon the presence or absence of metal ions (Cu^{2+}, Fe^{3+}).

(3) To study the mechanisms involved and to determine the mechanisms of induction of kinetochore positive and negative micronuclei, aneuploidy, and oxidative DNA damage.

(4) To examine the effects of metals, chelators and antioxidants on the oxidation of benzenetriol, and to determine how metal ions change the pathways of free radical chain reactions.

1.5.2 Hypotheses

This dissertation addresses three main sets of hypotheses as follow:

Hypothesis I:

1,2,4-Benzenetriol increases the frequency of micronuclei and levels of 8-OH-dG in human cells. Cu^{2+} may enhance the benzenetriol-induced genotoxicity and alter the mechanisms involved.

Hypothesis II:

1,2,4-Benzenetriol induces aneuploidy and microtubule disruption in HL60 cells. The benzenetriol-induced microtubule disruption may be a predominant mechanism of the induction of aneuploidy.

Hypothesis III:

Metal ions stimulate and antioxidant enzymes inhibit the autoxidation of 1,2,4-benzenetriol. Cu^{2+} may change the free radical propagated chain reactions from the sequential one-electron transfer to a concerted two-electron transfer.

This research is intended to provide a better understanding of the mechanisms involved in benzene-induced genotoxicity and will ultimately aid understanding of how and why benzene causes human leukemia.

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LEGENDS FOR FIGURES

Figure 1. Blood Cell Development. The schematic process from stem cells in bone marrow to matured cells in blood.

Figure 2. Pathways of Benzene Metabolism and Excretion. Values in parentheses are percentages of metabolic products detected in urine of animals (rabbits, rats, mice, dogs) or humans. Asterisks denote putative or demonstrated alkylating activity toward intracellular nucleophiles, AHH, aryl hydrocarbon hydroxylase; UDPG, uridine diphosphate glucuronyltransferase; PAPS, 3'-phosphoadenosine-5'-phosphosulfate. Dashed lines indicate putative pathways.

Figure 3. Proposed Mechanism of Benzene-Induced Myelotoxicity. The abbreviations are: P450, cytochrome P450 2E1; PH, phenol; CT, catechol; HQ, *p*-hydroquinone; BT, 1,2,4-benzenetriol; and MPO, myeloperoxidase.

Figure 4. Redox Cycling and Biological Fates of Quinones. Q, quinones; QH_2, hydroquinones; $Q^{\bullet -}$, semiquinones; and GSH, glutathione. Multiple arrows mean multiple steps. Dashed line indicates putative pathway.

Figure 5. Redox Cycling Pathways for 1,2,4-Benzenetriol and Its Quinones. The reaction scheme demonstrates the concomitant production of reactive oxygen species and prevention of antioxidant enzymes. SOD, superoxide dismutase; and CAT, catalase.

Figure 6. DNA Base Adducts of Hydroxyl Radicals. Some of the end-products that result from attack of hydroxyl radicals upon the bases of DNA.

Figure 7. Formation of 8-Hydroxy-2'-Deoxyguanosine.

Figure 8. Hypothetical Mechanisms of DNA Damage Resulting from Exposing Cells to Oxidative Stress.

Figure 9. Antioxidant Enzymes in the Cellular Defense Systems. SOD, superoxide dismutase; and CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; G6PD, glucose-6-phosphate dehydrogenase; GSH, glutathione; and GSSG, oxidative glutathione.

Figure 10. Hypothetical Scheme for the Possible Pathways of Free Radical Formation during Benzene Metabolism. The induction of oxygen activation, lipid peroxidation, glutathione oxidation and DNA/protein damage. The abbreviations are: BZ, benzene; PH, phenol; CT, catechol; HQ, *p*-hydroquinone; BT, 1,2,4-benzenetriol; *t,t*-MA, *trans, trans*-muconaldehyde; SQ, semiquinones; HSQ, 2-hydroxy-*p*-semiquinone; BQ, benzoquinones; HBQ, 2-hydroxy-*p*-benzoquinone; P450, cytochrome P450 2E1; and MPO, myeloperoxidase.

Figure 11. Anti-Kinetochore Antibody Modification of the Micronucleus Assay.

Figure 12. Schematic Representation of a Basic Fluorescence *in situ* Hybridization (FISH) Technique.

Figure 13. Involvement of Microtubules in Mitotic Chromosomal Segregation.

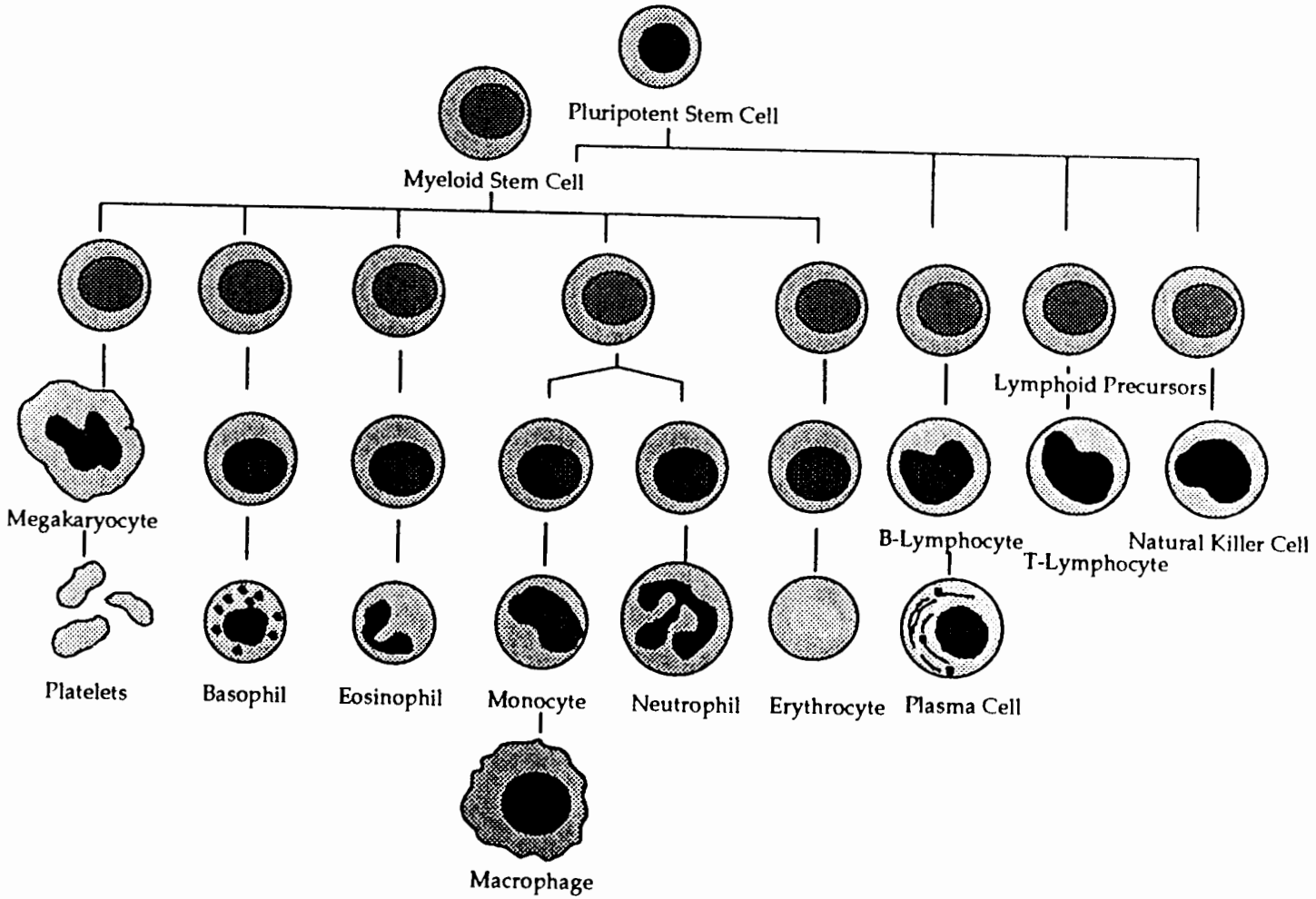


FIGURE 1. Blood Cell Development. (Adapted from Golde, 1991; Courtesy of Dr. Kathleen Meyer)

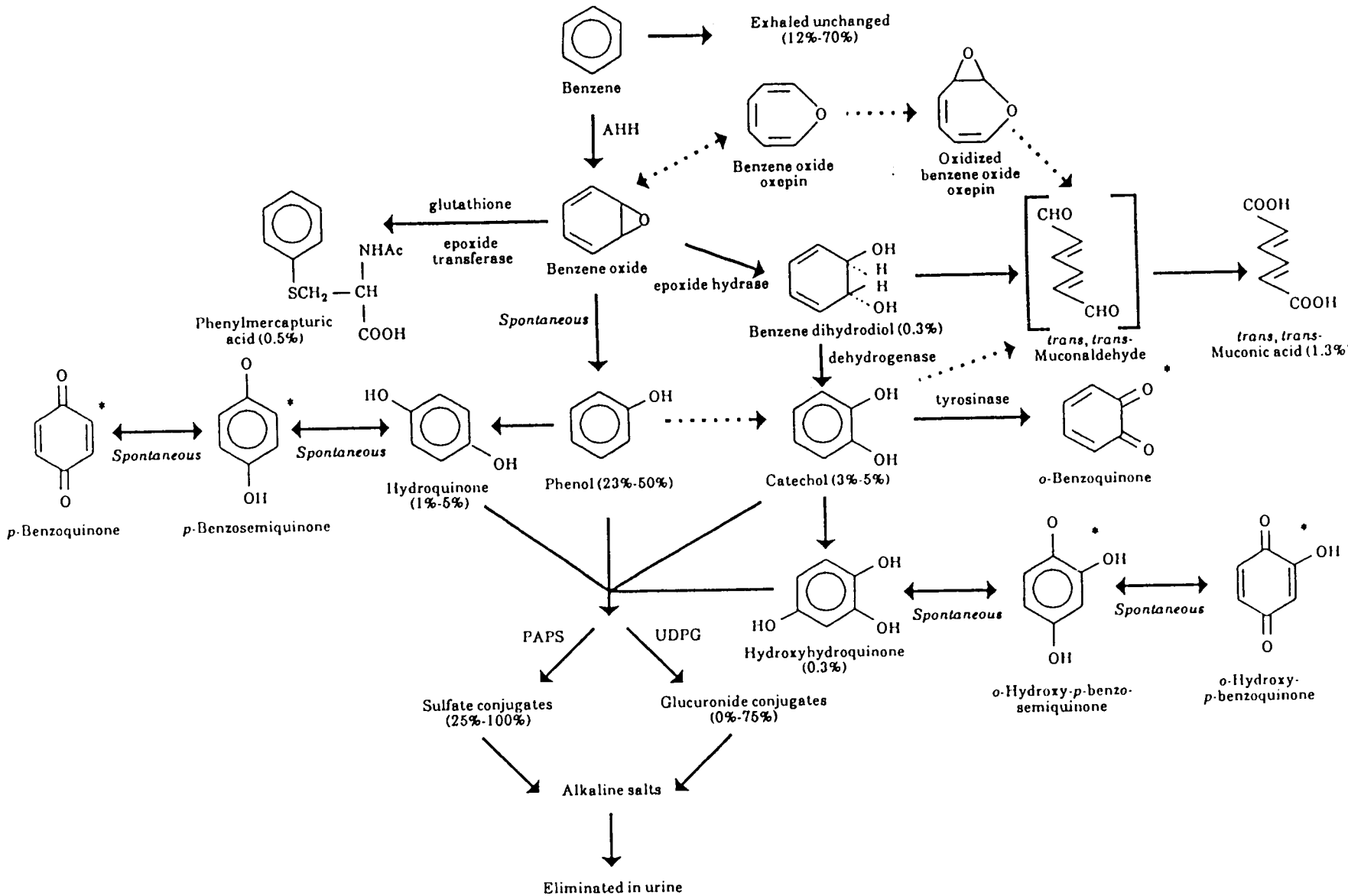


FIGURE 2. Pathways of Benzene Metabolism and Excretion. (from Huff et al., 1989)

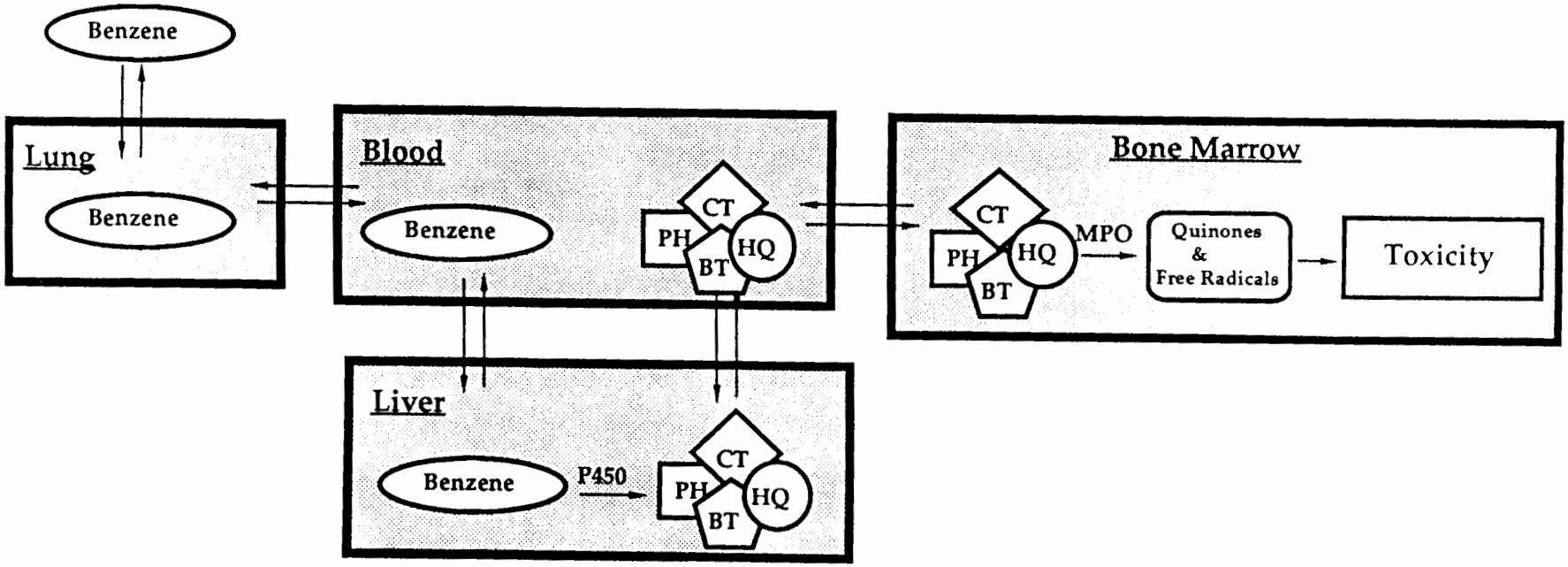


FIGURE 3. Proposed Mechanism of Benzene-Induced Myelotoxicity.

(Modified from Smith et al., 1989; Courtesy of Dr. Kathleen Meyer)

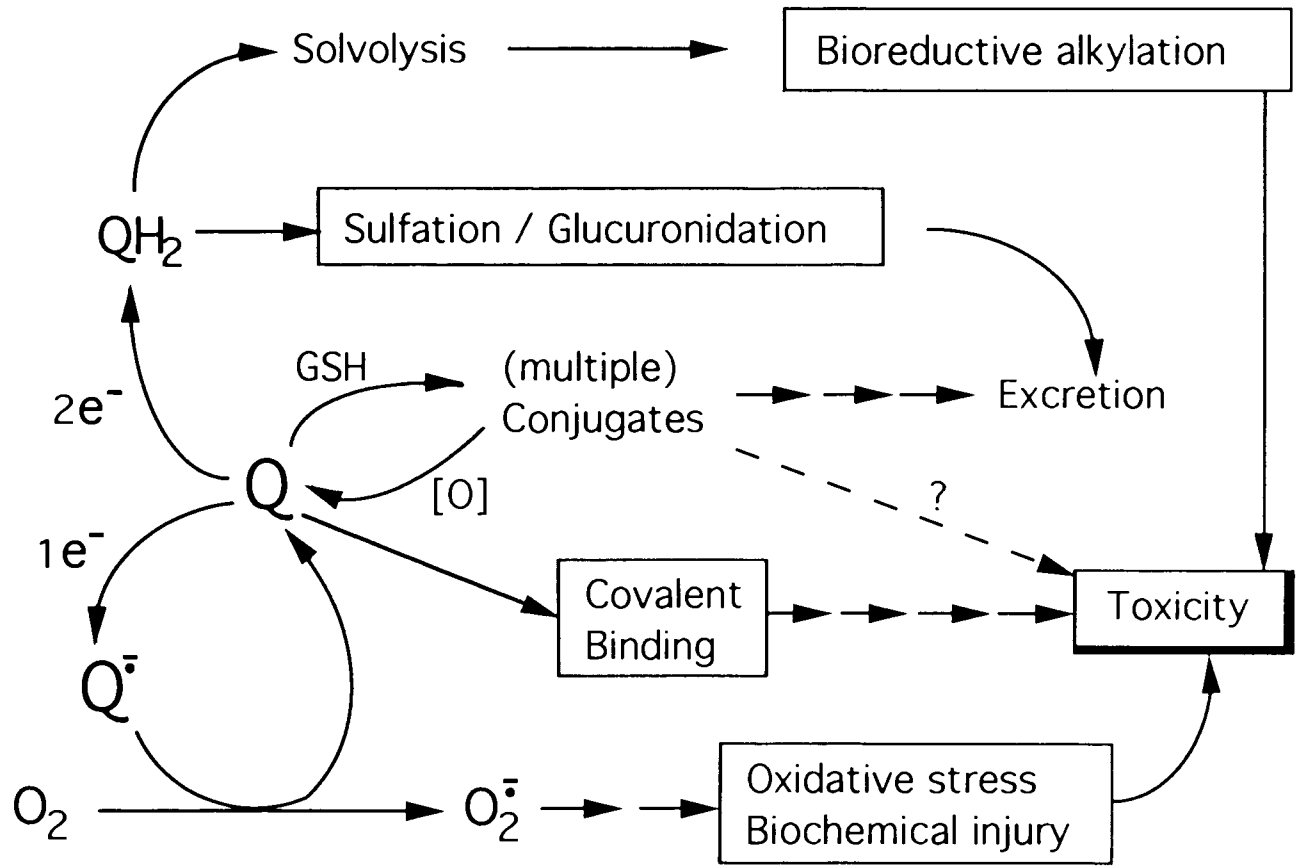


FIGURE 4. Redox Cycling and Biological Fates of Quinones. (from Monks et al., 1992a)

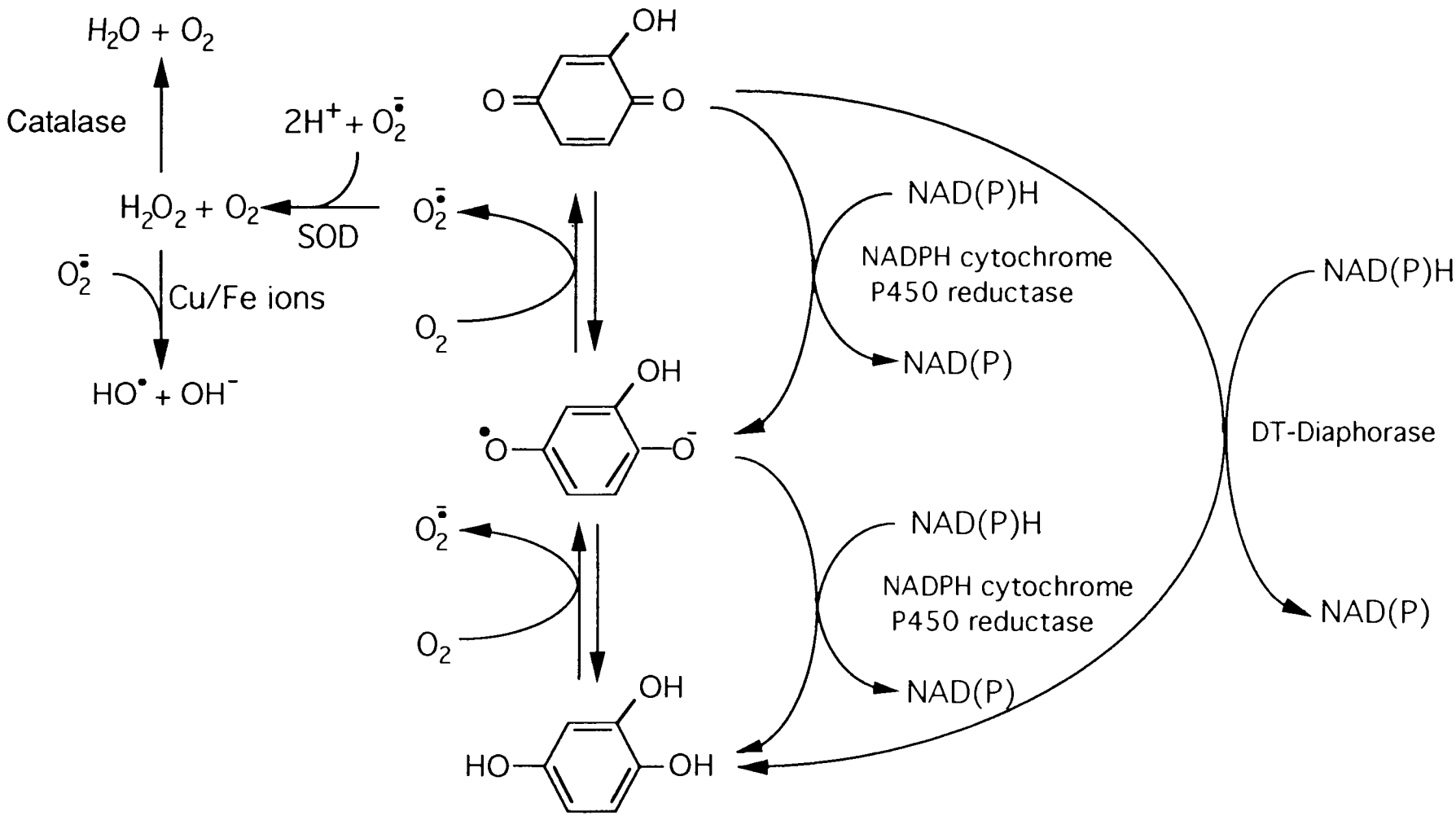
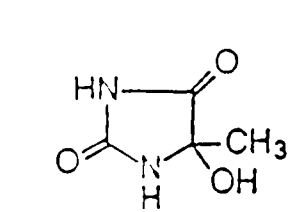
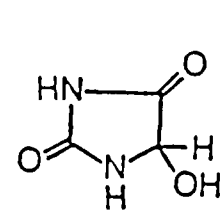


FIGURE 5. Redox Cycling Pathways for 1,2,4-Benzenetriol, Its Quinones, and Reactive Oxygen Species.

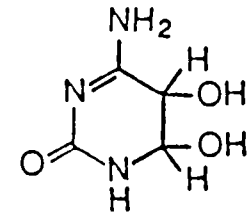
(Adapted from Pellack-Walker and Blumer, 1986)



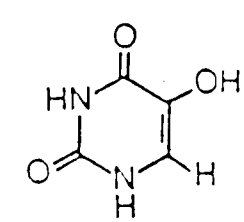
5-hydroxy-5-methyl-hydantoin



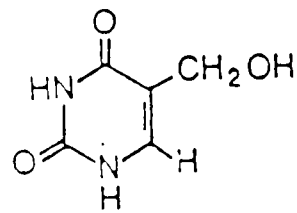
5-hydroxyhydantoin



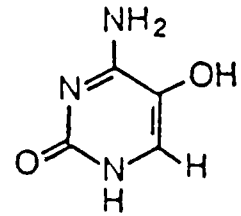
cytosine glycol



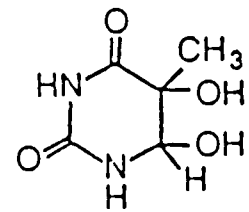
5-hydroxyuracil



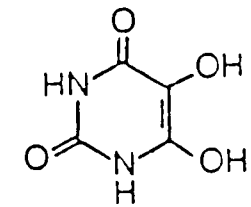
5-hydroxymethyl-uracil



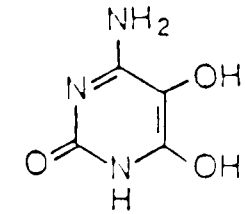
5-hydroxycytosine



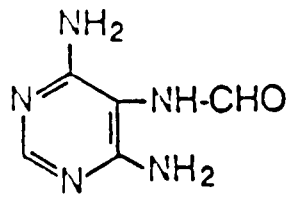
thymine glycol



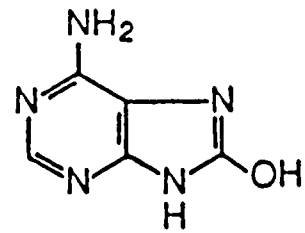
5,6-dihydroxy-uracil



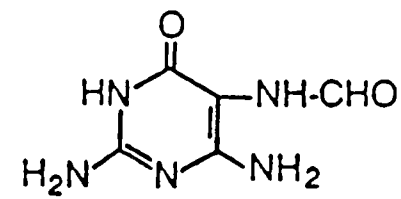
5,6-dihydroxy-cytosine



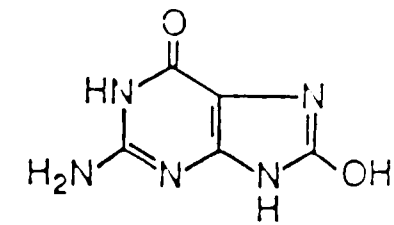
4,6-diamino-5-formamidopyrimidine



8-hydroxyadenine



2,6-diamino-4-hydroxy-5-formamidopyrimidine



8-hydroxyguanine

FIGURE 6. Some of the end-products that result from attack of hydroxyl radicals upon the bases of DNA.

(from Halliwell and Aruoma, 1991)

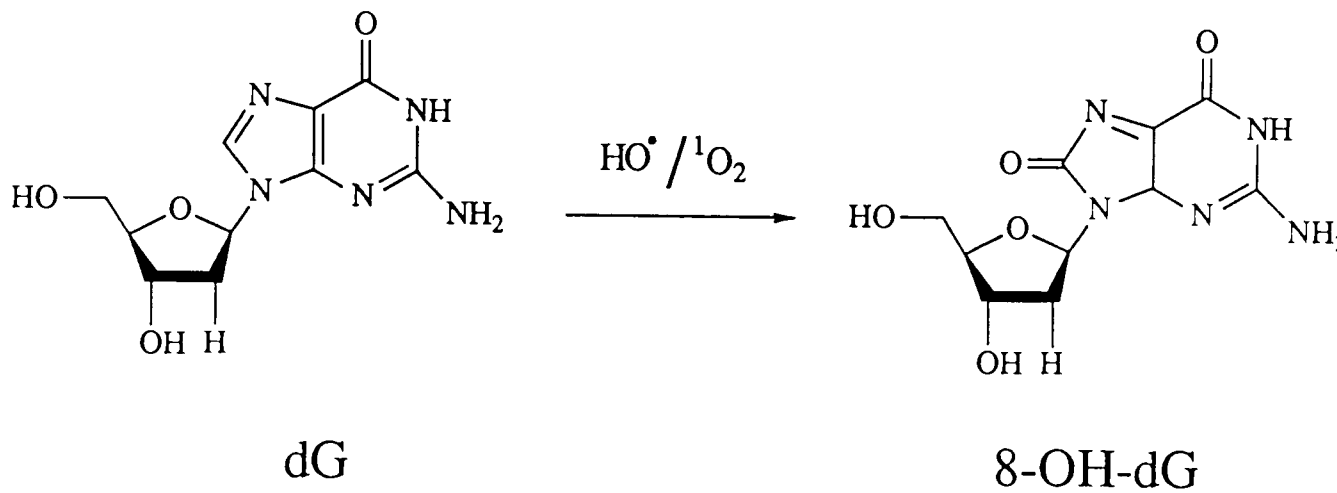


FIGURE 7. Formation of 8-Hydroxy-2'-Deoxyguanosine.

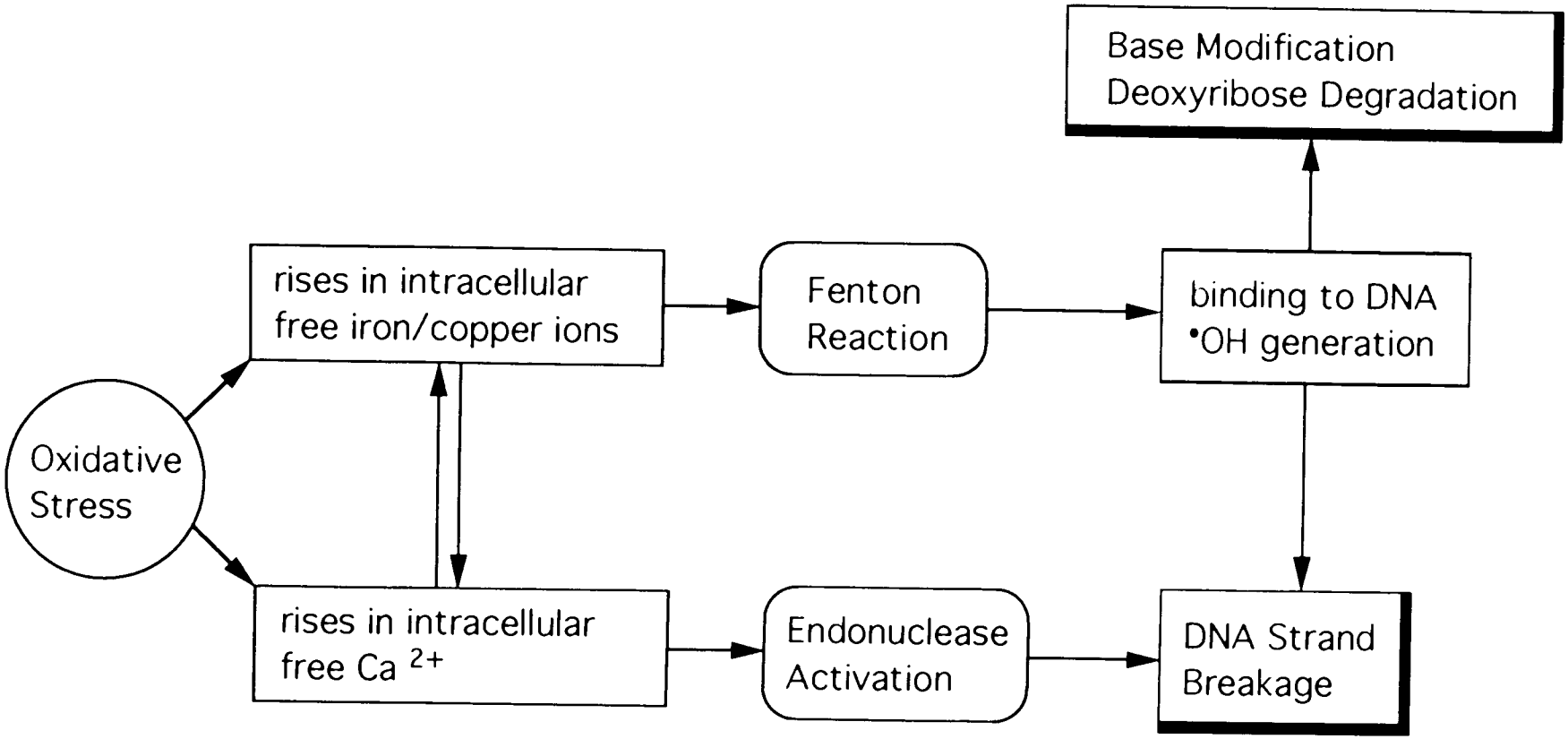


FIGURE 8. Hypothetical Mechanisms of DNA Damage Resulting from Exposing Cells to Oxidative Stress.

(Adapted from Halliwell and Aruoma, 1991)

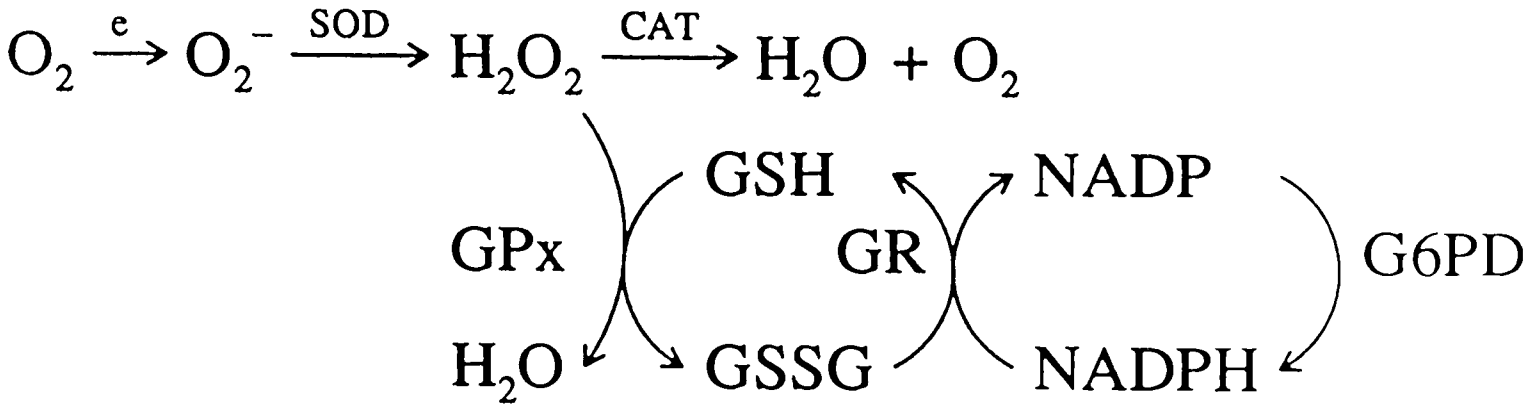


FIGURE 9. Antioxidant Enzymes in the Cellular Defense Systems. (from Sun, 1990)

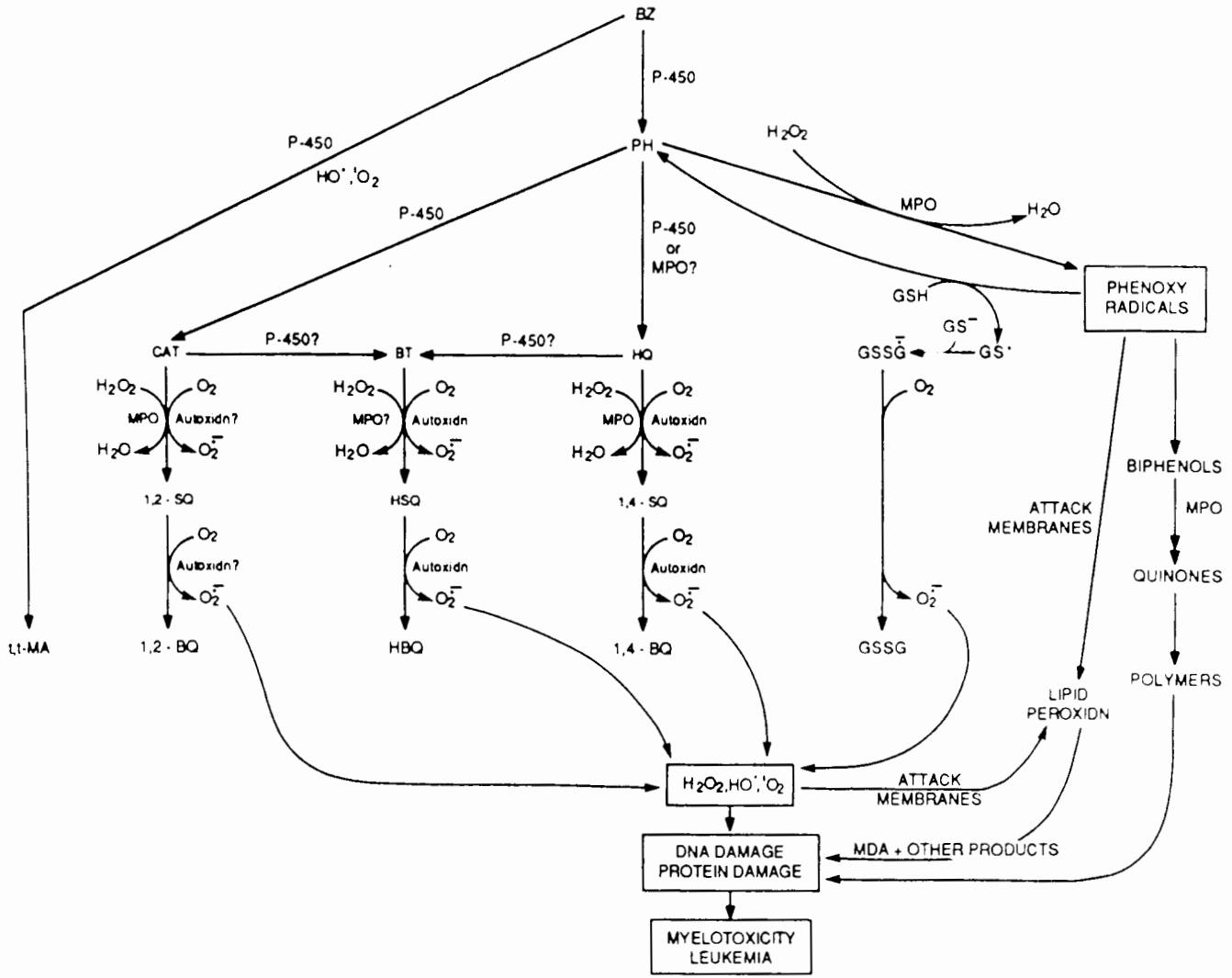
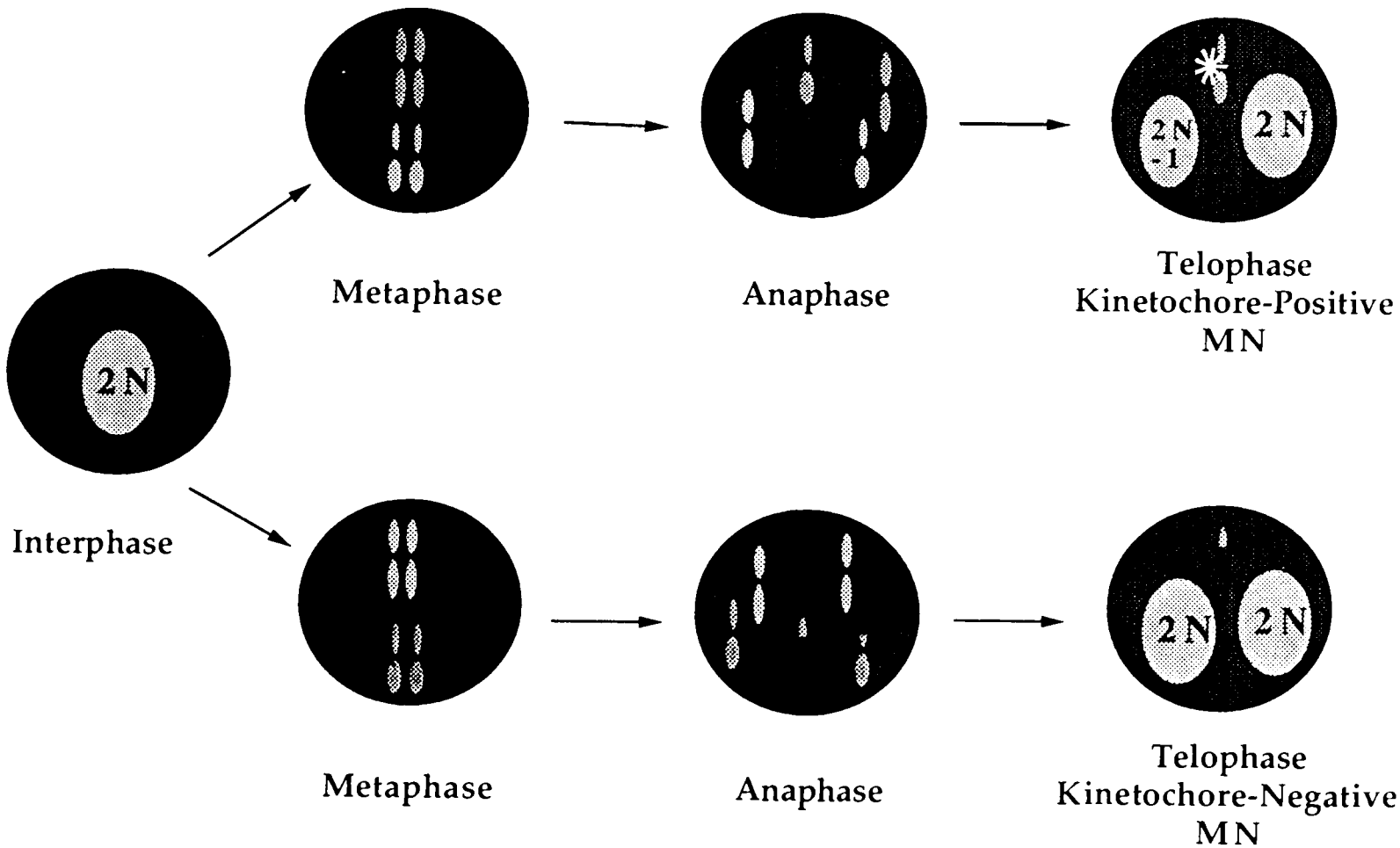


FIGURE 10. Hypothetical Scheme for the Possible Pathways of Free Radical Formation during Benzene Metabolism. (from Subrahmanyam et al., 1991)

Figure 11. Micronucleus Assay with Anti-Kinetochore Antibody Staining



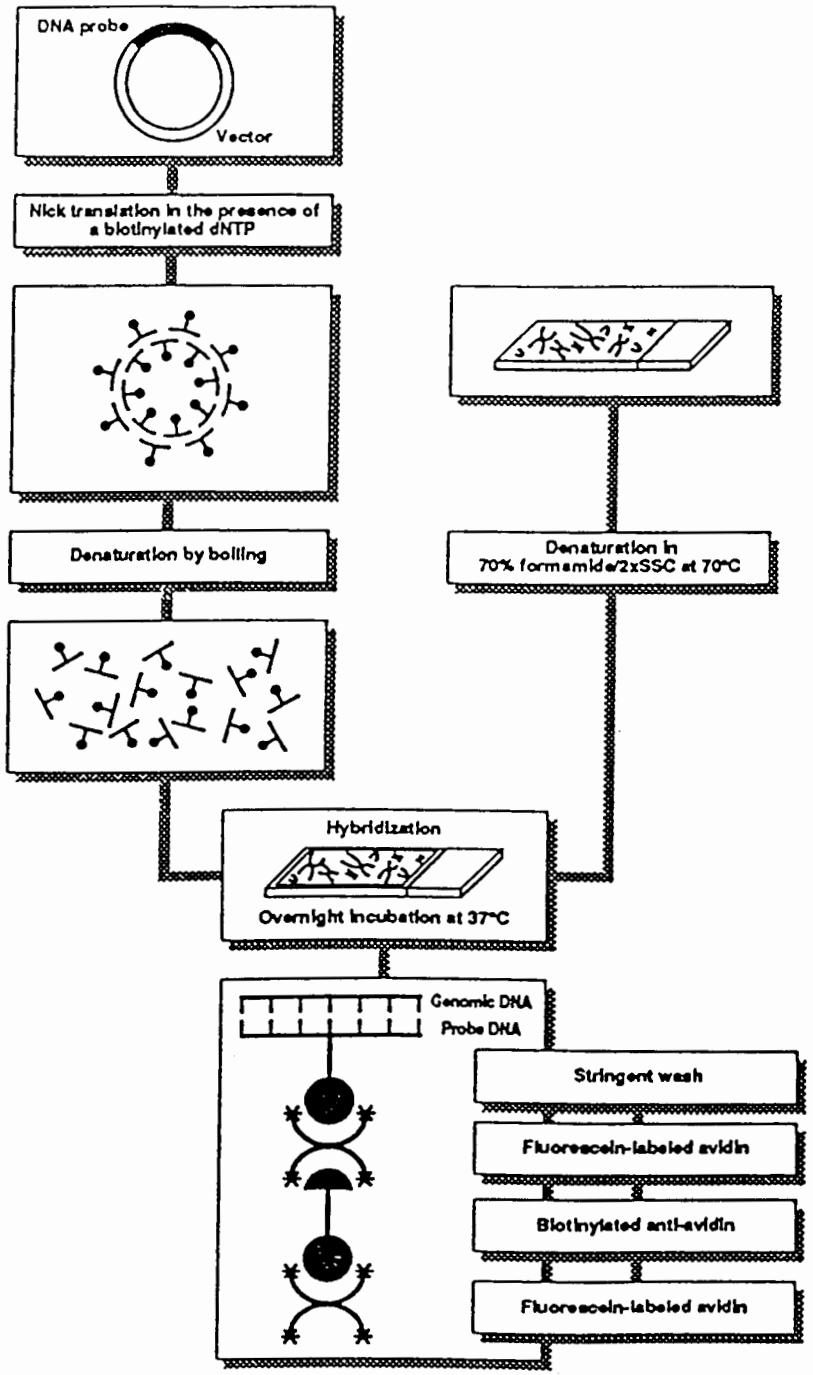


FIGURE 12. Schematic Representation of a Basic Fluorescence *in situ* Hybridization Technique. (from Zahed and Vekemans, 1991)

FIGURE 13. Involvement of Microtubules in Mitotic Chromosomal Segregation.*
(from *Scientific American*, October 1989)

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Table 1 **Effects of Benzene Exposure in Humans.**

(Adapted from Holmberg and Lundberg, 1985)

Concentration (ppm)	Effects
10,000	Death after a few hours
4,000	Narcosis
200-400	Risk from very severe pancytopenia Abnormal blood values in 50-80% of cases
125-200	Considerable risk for leukemia Severe pancytopenia
65-125	Milder forms of pancytopenias and other cytopenias With acute exposure headache and fatigue after a few hours
40-65	Hemocytopenia
25-40	Red blood cells affected Reduced blood hemoglobin
10-20	Probably the critical level for leukemia risk
1-10	Chromosomal damage
1	Odor threshold

CHAPTER TWO

Benzene Metabolite, 1,2,4-Benzenetriol Induces Micronuclei and Oxidative DNA Damage in Human Lymphocytes and HL60 Cells

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Running Title: Genotoxicity of Benzenetriol in Lymphocytes and HL60 Cells

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Abbreviation: BN, binucleated cells; BT, 1,2,4-benzenetriol; dG, deoxyguanosine; K^-, kinetochore-negative; K^+, kinetochore-positive; MN, micronuclei; MPO, myeloperoxidase; 8-OH-dG, 8-hydroxy-2'-deoxyguanosine.

ABSTRACT

The triphenolic metabolite of benzene, 1,2,4-benzenetriol (BT) ,is readily oxidized to its corresponding quinone via a semiquinone radical. During this process, active oxygen species are formed that may damage DNA and other cellular macromolecules. The ability of BT to induce micronuclei (MN) and oxidative DNA damage has been investigated in both human lymphocytes and HL60 cells. An antikinetochore antibody based micronucleus assay was used to distinguish MN containing kinetochores and potentially entire chromosomes (kinetochore-positive, K^+) from those containing acentric chromosome fragments (kinetochore-negative, K^-). BT increased the frequency of MN formation twofold in lymphocytes and eightfold in HL60 cells with the MN being 62% and 82% K^+, respectively. A linear dose-related increase in total MN, mainly in K^+-MN, was observed in both HL60 cells and lymphocytes. Addition of copper ions (Cu^{2+}) potentiated the effect of BT on MN induction threefold in HL60 cells and altered the pattern of MN formation from predominantly K^+ to K^-. BT also increased the level of 8-hydroxy-2'-deoxyguanosine (8-OH-dG), a marker of active oxygen-induced DNA damage. Cu^{2+} again enhanced this effect. Thus, BT has the potential to cause both numerical and structural chromosomal changes in human cells. Further, it may cause point mutations indirectly by generating oxygen radicals. BT may therefore play an important role in benzene-induced leukemia.

Key Words: **1,2,4-benzenetriol, micronuclei, oxygen radicals, 8-hydroxy-2'-deoxyguanosine, human leukemia**

INTRODUCTION

Benzene is an important industrial chemical and an ubiquitous environmental pollutant. It causes myelotoxicity and myelocytic leukemia in humans [Goldstein, 1977; IARC, 1988] and multiple forms of cancer in rodents [Huff et al., 1989]. Benzene itself is unlikely to be the ultimate carcinogen. It is metabolized in the liver to its primary metabolite phenol (PH), which is further hydroxylated to catechol (CAT), hydroquinone (HQ), and 1,2,4-benzenetriol (BT) [Inoue et al., 1989a, b]. *trans,trans*-Muconic acid, a ring-opened benzene metabolite, is also formed via a different pathway [Inoue et al., 1989c]. Each of these metabolites and intermediary species may play a role in benzene-induced carcinogenesis.

Even though BT is a relatively minor benzene metabolite, its chemical properties suggest a high biological potency. BT is more unstable in molecular oxygen than the other metabolites, has a strong ability to bind transition metal ions and rapidly autoxidizes to its corresponding quinone via semiquinone radical intermediates [Greenlee et al., 1981; Bandy et al., 1990]. During the autoxidation of BT, oxygen is converted to active oxygen-derived species, including superoxide anion radical, hydrogen peroxide, hydroxyl radical, and perhaps singlet oxygen [Greenlee et al., 1981; Pellack and Blumer, 1986; Bandy et al., 1990; Zhang and Davison, 1990]. These reactive oxygen species can damage DNA and other cellular macromolecules [Halliwell and Aruoma, 1991; Stadtman and Oliver, 1991]. BT has been reported to induce DNA strand breaks, which are elevated by Cu^{2+} and Fe^{3+} but inhibited by superoxide

dismutase, catalase and hydroxyl radical scavengers [Lewis et al., 1988; Kawanishi et al., 1989]. BT also induces sister chromatid exchanges (SCEs) in human lymphocytes [Erexson et al., 1985] and gene mutations (6-thioguanine resistance) in V79 cells [Glatt et al., 1989]. Further, BT suppresses mitogenic responses and interferes with microtubule assembly in lymphocytes presumably via formation of quinones and active oxygen species [Irons and Neptun, 1980; Irons et al., 1981; Irons, 1985]. These data suggest that BT may produce both chromosomal or DNA breakage (clastogenicity) via oxidative mechanisms and chromosomal lag (aneuploidy) by disrupting microtubules.

Here, we have investigated the ability of BT to produce numerical and structural types of chromosomal changes using a modified micronucleus assay [Fenech and Morley, 1985; Eastmond and Tucker, 1989]. Using cells that have been blocked from cytokinesis by cytochalasin B, the presence or absence of micronuclei (MN) can easily be scored in cells having completed exactly one nuclear division. Use of an antikinetochore antibody allows differentiation of MN containing entire chromosomes from those containing chromosome fragments. Kinetochore-positive micronuclei (K^+-MN) have a high probability of containing entire chromosomes, whereas kinetochore-negative micronuclei (K^--MN) contain only chromosomal fragments [Vig and Swearngin, 1986; Degrassi and Tanzarella, 1988; Gudi et al., 1990]. We have successfully used this technique previously to show that benzene's primary phenolic metabolites PH, CAT and HQ are each capable of producing both K^+-MN and K^--MN in human lymphocytes [Yager et al., 1990]. We have also discovered that HQ and CAT act in concert to produce a synergistic induction of primarily K^+-MN in human lymphocytes [Robertson et al., 1991].

Whereas lymphocytes have been used in this and previous studies, the human HL60 myeloid leukemia cell line is a particularly suitable model to characterize the effects of benzene's metabolites and to study the mechanisms involved [Meyer et al., 1991]. HL60 cells are representative of early lineage myelocytic bone marrow cells and contain high levels of myeloperoxidase (MPO), which facilitates the bioactivation of many benzene metabolites, including BT [Gallagher et al., 1979; Smith et al., 1989; Subrahmanyam et al., 1992]. In this report, we have extended our studies to BT and shown that BT induces both K^+-MN and K^--MN in human lymphocytes and HL60 cells. In combination with copper (Cu^{2+}), the pattern of MN formation was altered from primarily K^+ to K^- accompanied by an increase in total MN. This suggests that active oxygen species, whose production is catalyzed by Cu^{2+}, are involved in K^--MN formation. To further investigate their role, we used an assay for the hydroxylated DNA base, 8-hydroxy-2'-deoxyguanosine (8-OH-dG) and show that BT increases levels of this modified base in HL60 cells, an effect also potentiated by Cu^{2+}. These data indicate the involvement of active oxygen species in BT-induced DNA damage and implicate the potential importance of BT-mediated genetic damage in benzene-induced human leukemia.

MATERIALS AND METHODS

Cell Culture Human lymphocytes were isolated from peripheral blood of a healthy adult female using Ficoll-Paque (Pharmacia, Piscataway, NJ) density gradients and cultured as described previously [Yager et al., 1990; Robertson et al., 1991]. Briefly, 1 x 10^6 lymphocytes were seeded into 2 ml of culture medium consisting of RPMI 1640 supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin (Gibco, Grand Island, NY), and 1.5% phytohemagglutinin (PHA) (HA15, Burroughs-Wellcome, Greenville, NC). The lymphocytes were grown in a humidified incubator with 5% CO_2 at 37 °C for 72 hr. HL60 cells, a human myeloid cell line originally derived from the peripheral blood leukocytes of a patient with acute myeloid leukemia [Gallagher et al., 1979], were obtained from American Type Culture Collection (Rockville, MD). HL60 cells were also cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 50 μg/ml gentamicin sulfate (UCSF, San Francisco, CA) at 37 °C in a 5% CO_2 moist atmosphere and passaged twice weekly to maintain a density between 1 x 10^5 to 1 x 10^6 cells/ml. The cells in the plateau phase of growth were then seeded in 15 ml sterile centrifuge tubes and diluted with fresh complete medium to a density of 0.5 x 10^6 cells/ml (2 ml per each culture).

Treatment Conditions 1,2,4-benzenetriol (99%) (Aldrich, Milwaukee, WI) was dissolved in phosphate-buffered saline (PBS, Ca^{2+} and Mg^{2+} free, pH 7.4) immediately prior to treatment, and a solution of copper (II) chloride (Aldrich, Milwaukee, WI) was made fresh in distilled-deionized water. At 24 hr after culture initiation, lymphocytes were treated with BT in complete medium continually until 72 hr, and HL60 cells were treated in PBS with BT only or in

combination with Cu^{2+} for 1 hr then washed and resuspended in complete medium. All treatments were performed in duplicate for each dose and repeated at least three times in HL60 cells.

Cytochalasin B (Sigma, St. Louis, MO) was added at 44 hr for lymphocytes and at 28 hr for HL60 cells to block the cells in cytokinesis (3 μg/ml, final concentration). Both treated lymphocytes and HL60 cells were harvested onto glass slides at 72 hr and 48 hr, respectively, using a cytocentrifuge (Cytospin-II, Shandon, Sewickley, PA) at 600 rpm for 10 min.

Staining Procedures Detailed procedures for performing the micronucleus assay with the antikinetochore antibody have been described previously [Eastmond and Tucker, 1989; Yager et al., 1990]. Briefly, following fixation of cells in methanol for 15 min, slides were dried completely in air, rinsed in PBS containing 0.1% Tween 20 (Fisher, Fair Lawn, NJ) for 5 min, and stained with an antikinetochore antibody (Chemicon, Los Angeles, CA) diluted 1:1 with 0.2% Tween 20 in PBS. The slides were then coverslipped and incubated for 1 hr, washed twice in 0.1% Tween 20 in PBS and incubated with fluoresceinated rabbit or goat antihuman IgG (Chemicon, Los Angeles, CA) diluted 1:120 with PBS containing 0.5% Tween 20 for another hour. Again the slides were washed twice and then stained with the DNA-specific stain 4,6-diamino-2-phenylindole (DAPI) (Sigma, St. Louis, MO) prepared in an antifade solution [Johnson and Nogueira Araujo, 1981].

Scoring The stained slides were randomized and coded prior to scoring; 1,000 binucleated cells per dose (500 per duplicate) were then scored for the presence of MN using a Nikon microscope equipped with epifluorescent

illumination, a 100x oil immersion lens and the appropriate filters for fluorescein and DAPI [Robertson et al., 1991]. The presence or absence of a kinetochore spot in each micronucleus was determined by switching from the DAPI filter to the fluorescein filter. Both the total number of MN, including K^+-MN and K^--MN, and the number of cells containing at least one micronucleus were recorded.

Cell Viability and Cell Division Kinetics Cell viabilities were determined at 72 hr (lymphocytes) or 48 hr (HL60 cells) using trypan blue (0.16%). Replicative index (R. I.), a measure of cell division kinetics, was calculated by scoring 400 cells per dose (200 per duplicate) (see Table I legend).

Extraction, Purification, and Enzymatic Hydrolysis of DNA from HL60 Cells DNA was isolated from HL60 cells according to a phenol extraction procedure [Gupta, 1984]. High purity double-distilled phenol (International Biotechnologies, New Haven, CT) was used for extraction to avoid additional oxidative DNA damage by peroxide or quinone contaminants in phenol. Following the DNA isolation, 200 - 400 μg of DNA from HL60 cells was resuspended in 200 μl of 20 mM sodium acetate (pH 4.8) and digested to nucleotides with 20 μg of nuclease P1 (Sigma, St. Louis, MO) at 70 °C for 15 min. Then, 20 μl of 1 M Tris-HCl (pH 7.4) was added to the nucleotide mixture to adjust the pH, and the mixture was subsequently treated with 1.3 units of *E.Coli* alkaline phosphatase (Sigma, St. Louis, MO) at 37 °C for 60 min.

Synthesis of 8-OH-dG Standard Standards of 8-OH-dG were synthesized by Udenfriend's hydroxylating system [Kasai and Nishimura. 1984]. Deoxyguanosine (dG) was hydroxylated at the C-8 position by sequentially

adding 25 µl of 0.1 M dG, 14 µl of 1 M ascorbic acid, 6.5 µl of 1 M ethylene diamine tetra acetic acid (EDTA), and 13 µl of 0.13 M $FeSO_4$ to 0.942 ml of 0.1 M sodium phosphate buffer (pH 6.8). The mixture was incubated for 15 min at 37 °C in the dark with vigorous shaking. Following incubation, aliquots of the reaction mixture were analyzed by high pressure liquid chromatography (HPLC). Fractions containing 8-OH-dG were collected from HPLC eluates, lyophilized and stored at 4 °C.

Determination of 8-OH-dG in DNA The hydrolyzed DNA solution was filtered using an "ultrafree" millipore filtration system (10,000 dalton cutoff). The amount of 8-OH-dG in DNA was measured by HPLC equipped with electrochemical detection using an ESA Coulochem detector (model 6010). The applied potential was 0.05 V at the first detector and 0.35 V at the second detector. Levels of 8-OH-dG were expressed relative to the content of DNA detected by absorbance at 260 nm. Standard solutions of 8-OH-dG (1 - 5 pmol) and dG (0.5 mM) were simultaneously analyzed to calibrate levels of 8-OH-dG and DNA, respectively. Data were digitized by a Nelson 760 (Cupertino, CA) analytical interface and processed by Nelson analytical series 4400 data acquisition software on a Hewlett-Packard 9816 computer. Results are expressed in pmol 8-OH-dG/µg DNA.

Statistical Analysis The statistical comparisons of multiple means were assessed by analysis of variance (ANOVA). Individual means were compared using a one-tailed Fisher exact test. The minimum level of significance chosen was $p < 0.05$. Slopes of dose response and Pearson's correlation coefficient (r-value) were determined, where appropriate, by regression analysis.

RESULTS

Effect of BT on Micronucleus Induction in Human Lymphocytes

Figure 1 shows the induction of MN in human lymphocytes treated with a range of BT concentrations in a complete medium. BT increased the total number of MN per 1000 binucleated cells in a dose-dependent manner (Fig. 1). The mechanism of MN formation was examined using an antikinetochore antibody to distinguish between K^+-MN and K^--MN. More K^+-MN were formed in BT treated lymphocytes than K^--MN at each concentration (Fig. 1). Although BT produced a minimal response between 10 and 50 μM, at 100 μM (the maximum concentration tested) it caused a 2.3-fold increase ($p < 0.01$) of total MN above background, with 62% being K^+. However, notable cytotoxicity as indicated by trypan blue dye exclusion (65%) and R. I. (1.47) was also observed at this concentration (Table I). BT is therefore only a moderate inducer of MN in human lymphocytes.

Effect of BT on Micronucleus Induction in HL60 Cells

To avoid potential confounding effects of antioxidants and other substances present in complete medium, HL60 cells were treated with BT for 1 h in PBS rather than in medium. A dose-dependent increase ($p < 0.01$) in the total number of MN was observed in HL60 cells treated with BT between 5 and 50 μM (Fig. 2). The proportion of K^+-MN was consistently increased ($p < 0.001$) over the BT concentration range tested. The minimum concentration of BT tested (5 μM) induced a twofold increase in MN with 73% being K^+. The maximum effective concentration of BT used (50 μM) induced an eightfold increase in MN with 82% being K^+. In contrast, 68% of the total MN were K^--MN in untreated HL60 cells. At 100 μM BT, MN frequency and the R. I. were

unscorable and cell viability was decreased to 15% (Table I). Regression analysis showed that the dose-related increase in both total MN and K^+-MN was linear (correlation coefficient $r > 0.99$). The slopes of the dose response curves were MN: 0.947 > K^+-MN: 0.825 >> K^--MN: 0.122. The increase in K^--MN was not statistically significant. Consequently, the increase in total number of MN is almost entirely due to an increase in K^+-MN. BT therefore induces predominantly K^+-MN in HL60 cells.

Effect of Cu^{2+} on the Induction of Micronuclei in HL60 Cells by BT

Since transition metal ions (Cu^{2+} and Fe^{3+}) stimulate BT-induced DNA strand breaks and accelerate the autoxidation of BT [Lewis et al., 1988; Kawanishi et al., 1989; Zhang and Davison, 1990], the effect of Cu^{2+} on the level and type of MN induced by BT was investigated. Cu^{2+} was chosen because it is more efficient than Fe^{3+} [Kawanishi et al., 1989; Zhang and Davison, 1990]. When HL60 cells were incubated with varying concentrations of BT (0 - 50 μM) while maintaining Cu^{2+} constant at 5 μM, the total number of MN induced reached a maximum at 10 μM BT (Fig. 3A). When compared with 10 μM BT alone (Fig. 2), the presence of 5 μM Cu^{2+} increased the incidence of total MN twofold ($p = 0.05$) and the formation of K^--MN fivefold ($p < 0.001$) but decreased the proportion of K^+-MN from 75% to 30%. Treatment with 5 μM Cu^{2+} by itself did not significantly effect MN or K^--MN formation. Thus the addition of Cu^{2+} not only potentiated the MN formation but also altered the nature of BT-induced MN from K^+ to K^-. In contrast to the linear dose response seen in Figure 2, the total number of MN decreased at concentrations above 20 μM BT in the presence of Cu^{2+} (Fig. 3A). This may be attributed to the combined toxicity of BT and Cu^{2+}. However, cell viability and the R. I. were not measurably reduced at these doses (Table I). A possible explanation for this is

that the measurements of cytotoxicity may not estimate the true toxicity because they were assessed 24 h after the treatment and damaged cells might have been lost to analysis.

Similarly, BT (5 μM) with varying concentrations of Cu^{2+} (1 - 10 μM) induced primarily K^--MN in HL60 cells, with at least twice as many K^--MN as K^+-MN (Fig. 3B). At the maximum response, the simultaneous administration of 5 μM BT and 2 μM Cu^{2+} produced a threefold increase ($p < 0.001$) in the total number of MN with 85% being K^--MN in comparison with 5 μM BT alone, which produced only 27% K^--MN. Although the combination of BT (5 μM) with 5 or 10 μM Cu^{2+} again decreased the total number of MN, these concentrations were essentially nontoxic as measured by cell viability and R. I. (data not shown). Cu^{2+} (1 - 10 μM), by itself, had no statistically significant effect on the induction of MN (data not shown). In summary, co-incubation with Cu^{2+} increases the total number of MN induced by BT and alters the pattern of MN formation from K^+-MN to K^--MN.

Effect of BT on 8-OH-dG Levels in DNA of HL60 Cells

8-OH-dG is a DNA-hydroxyl radical or DNA-singlet oxygen adduct [Kasai and Nishimura, 1984; Floyd et al., 1986, 1989; Devasagayam et al., 1991]. It has become a useful and sensitive marker of oxidative DNA damage [Floyd et al., 1986]. To test BT's ability to cause oxidative DNA damage, 8-OH-dG formation in the DNA of HL60 cells treated with different concentrations of BT (5, 10 and 50 μM) in PBS was measured (Fig. 4A). BT, at all concentrations tested, increased the 8-OH-dG content in DNA to 0.10 - 0.30 pmol/μg DNA above the background level of 0.06 pmol/μg DNA. Treatments with 5 and 10 μM BT for 30 min caused a twofold and fivefold increase over the control, respectively. These

values returned to steady-state levels by 60 min suggesting an efficient DNA repair mechanism. Cells treated with 50 μM BT took twice as long (60 min) to show a fourfold increase in the 8-OH-dG level, which returned back to the original level after further incubation for 24 hr at 37 °C (data not shown). Though the reason for this is unclear, it may be due to excess BT and/or its semiquinone acting as an antioxidant and reacting with active oxygen species, thereby delaying the rise in 8-OH-dG. In PBS controls, the 8-OH-dG level remained almost constant around 0.06 pmol/μg DNA (Fig. 4A).

Effect of Cu^{2+} on BT-Induced 8-OH-dG Formation

Because simultaneous treatment with 5 μM BT and 2 μM Cu^{2+} induced the maximum response in K^--MN formation (Fig. 3B), the same combination was chosen to examine the formation of 8-OH-dG in HL60 cells (Fig. 4B). As mentioned earlier, 5 μM BT alone increases the 8-OH-dG level twofold after 30 min. Levels of 8-OH-dG were almost unchanged when cells were treated with 2 μM Cu^{2+} alone for 15, 30 and 60 min. However, at 15 and 30 min, the combination of 5 μM BT and 2 μM Cu^{2+} increased 8-OH-dG levels by 0.04 pmol/μg DNA above the 5 μM BT treatment (Fig 4B). Thus the presence of Cu^{2+} potentiated BT-induced oxidative DNA damage. Moreover, the level of 8-OH-dG failed to return to steady-state by 60 min. This may be explained by Cu^{2+} catalyzing the continuous production of hydroxyl radicals, singlet oxygen, and other active oxygen species that damage DNA. The presence of Cu^{2+} would therefore be expected to enhance BT-induced chromosome breakage, which appears to be the case since Cu^{2+} also potentiates BT-induced K^--MN formation.

DISCUSSION

Our laboratory continues to investigate the role of different benzene metabolites in benzene-induced human leukemia. Each metabolite tested so far, including PH, CAT, HQ, and 1,4-benzoquinone, induces genetic damage in human lymphocytes as measured by their ability to increase the incidence of micronuclei (MN) [Yager et al., 1990; Robertson et al., 1991]. In this report, we have extended our investigations to BT another benzene metabolite, which is relatively unknown. BT has been detected in the urine of workers exposed to benzene [Inoue et al., 1989b]. Even though it is a minor metabolite in quantitative terms [Rusch et al., 1977], BT may be important due to its instability in air and its strong ability to bind metals [Greenlee et al., 1981; Pellack and Blumer, 1986; Bandy et al., 1990; Zhang and Davison, 1990]. Thus it readily autoxidizes and activates molecular oxygen to a number of reactive species including superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) as well as perhaps singlet oxygen ($^1O_2$). These reactive species are known to damage DNA and other cellular macromolecules [Halliwell and Aruoma, 1991; Stadtman and Oliver, 1991]. The products of BT oxidation, mainly, 2-hydroxy-1,4-benzoquinone (2-OH-BQ) and its corresponding semiquinone radical (s-$Q^{\bullet -}$), could also produce genetic damage by, e.g., adduction to DNA or by disrupting the mitotic spindle leading to chromosome loss. Different pathways for the induction of genetic damage by BT and its oxidative products in cellular systems are proposed in Figure 5.

Here, we have shown that BT increases the incidence of MN in human lymphocytes (Fig.1). MN arise when replicating cell populations are subjected to chromosomal breakage by clastogens or to chromosome lag by mitotic

spindle dysfunction. In the antikinetochore antibody modification of the MN assay [Fenech and Morley, 1985; Eastmond and Tucker, 1989], K^+-MN represent the misincorporation of whole chromosomes into the daughter nuclei or aneuploidy and K^--MN indicate the formation of acentric chromosome fragments or clastogenicity [Vig and Swearngin, 1986; Degrassi and Tanzarella, 1988; Eastmond and Tucker, 1989; Gudi et al., 1990]. Using this assay, we have shown that BT can increase both chromosomal lagging and chromosomal breakage. These events are significant genetically since they represent a high probability of a net loss of genetic material.

Relatively few studies have been conducted on the genotoxicity of BT and data regarding BT's potency relative to other benzene metabolites are mixed. Our results show that in lymphocytes BT behaves similarly to HQ, with the dose required to produce the maximal response being in the 100 μM range [Yager et al., 1990]. HQ, however, produces a much higher number of MN than BT. Glatt et al. [1989] found HQ and 1,4-benzoquinone (*p*-BQ) to be more efficient than BT at producing MN in Chinese hamster V79 cells with BT being more comparable to CAT. In the same study, SCEs were analyzed and BT and CAT were found to be more efficient than the other metabolites tested. This is in contrast to results obtained in human lymphocytes, where BT was the least efficient at producing SCEs except for PH [Erexson et al., 1985]. Overall, BT appears to consistently test positive as a genotoxic agent, leaving little doubt that it may contribute to the genotoxicity observed following benzene exposure.

Our investigations were continued using HL60 cells, a human myeloid leukemia cell line, representative of early lineage myelocytic bone marrow-derived cells [Gallagher et al., 1979]. Similar to bone marrow, HL60 cells

contain an appreciable amount of MPO, which facilitates the bioactivation of BT enzymatically [Subrahmanyam et al., 1992]. In addition, they can be readily cultured *in vitro*. In HL60 cells, BT not only increased the frequency of MN but also induced a high proportion of K^+-MN, which indicates that chromosomes are not being properly segregated at mitosis. This may result from the disruption of the mitotic spindle microtubules by the oxidative products of BT, 2-OH-BQ and/or s-$Q^{\cdot -}$. BT inhibits rat brain tubulin polymerization *in vitro*, accelerates the decay of tubulin-colchicine binding activity, and suppresses mitogenic responses in lymphocytes [Irons and Neptun, 1980; Irons et al., 1981; Pfeifer and Irons, 1981]. Recently, using an immunocytochemical staining assay, we have shown that BT also disrupts microtubule assembly in HL60 cells (Zhang et al., 1993).

The oxidation of BT generates reactive oxygen species, which is stimulated by transition metal ions such as copper (Cu^+, Cu^{2+}) and iron (Fe^{2+}, Fe^{3+}). Thus, BT in combination with metal ions provides a model to investigate the role of these active oxygen species in BT-induced DNA damage. Previous studies have shown that BT induces DNA strand breaks, which are increased in the presence of Cu^{2+} and inhibited by the metal chelating agent, bathocuproine [Kawanishi et al., 1989]. In addition, Cu^{2+} was more efficient than Fe^{3+} at stimulating BT-induced DNA strand breaks [Kawanishi et al., 1989], catalyzing the autoxidation of BT [Zhang and Davison, 1990], and causing DNA base damage in the presence of H_2O_2 [Aruoma et al., 1991]. In this study, we therefore tested the effect of Cu^{2+} on BT-induced DNA damage in two ways: first, by measuring its effect on the clastogenic potential of BT using K^--MN as the marker, and second, by measuring the formation of the hydroxylated DNA base, 8-OH-dG.

We found that BT alone produced only a small proportion of K^--MN but when Cu^{2+} was present, not only were more MN formed but also the proportion of K^--MN increased from 27% to 85%. The fact that Cu^{2+} changes the pattern of MN formation from K^+-MN to K^--MN in HL60 cells is consistent with our previous finding in a chemical system where Cu^{2+} changes BT-initiated free radical chain reactions from superoxide-propagated to Cu^{2+}-mediated [Zhang and Davison, 1990]. In this cell-free system, superoxide dismutase normally inhibited the autoxidation of BT, but when Cu^{2+} was present, it did not do so. Thus, Cu^{2+} will stimulate the oxidation of BT in cells containing superoxide dismutase and produce more active oxygen species, which is probably the primary cause of K^--MN formation. In particular, Cu^{2+} catalyzes continual formation of $\cdot OH$ via the Fenton reaction ($H_2O_2 \longrightarrow \cdot OH + OH^-$), which would cause DNA base modification, strand breaks, and DNA or deoxyribose fragmentation [Halliwell and Aruoma, 1991].

The ability of BT to produce oxidative DNA damage was evaluated using a sensitive HPLC technique to determine 8-OH-dG in DNA. 8-OH-dG represents the covalent addition of $\cdot OH$ or $^1O_2$ to guanine in DNA [Kasai and Nishimura, 1984; Floyd et al., 1986, 1989; Devasagayam et al., 1991] and has become a useful marker of oxidative DNA damage both *in vitro* and *in vivo* [Floyd et al., 1986; Roy et al., 1991]. 8-OH-dG can be misread at the modified and adjacent base residues in a DNA-polymerase reaction *in vitro*, which is potentially mutagenic [Kuchino et al., 1987]. Recently, Loeb and his coworkers [Cheng et al., 1992] have demonstrated that 8-OH-dG causes DNA base substitutions leading to point mutations. We found that exposure of HL60 cells to BT increased the level of 8-OH-dG. This increase was transient, however,

and a decrease was observed during prolonged cell incubation. A possible explanation is that HL60 cells have an efficient repair mechanism, which may function to maintain a low 8-OH-dG level in the cells. In this regard, Kasai et al. [1986] found that 8-OH-dG formation in DNA isolated from liver of γ-irradiated mice rapidly decreased with time and speculated the presence of repair enzymes for 8-OH-dG removal. The presence of Cu^{2+} potentiated the BT-induced increase in 8-OH-dG, which did not return to background levels at 60 min. This most likely reflects the fact that Cu^{2+}-binding sites on macromolecules serve as centers for repeated production of ·OH radicals that are generated via the Fenton reaction.

In conclusion, our data demonstrate that BT is genotoxic in both human lymphocytes and HL60 cells by increasing the frequency of MN and levels of 8-OH-dG. BT-induced MN most likely result either from the disruption of microtubules by its corresponding quinones or from oxidative DNA damage produced by reactive oxygen species generated during its oxidation. BT, by itself, causes mainly aneuploidy as indicated by the predominant induction of K^+-MN. However, in combination with Cu^{2+} or other transition metal ions, BT causes mainly clastogenicity and point mutations as shown by the formation of K^--MN and 8-OH-dG. Thus, BT may cause both numerical and structural chromosome aberrations as well as point mutations by two different mechanisms. BT's high potency suggests that it may play a role in benzene-induced genetic damage and perhaps human leukemogenesis.

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Legends for Figures:

Figure 1. Induction of micronuclei in cytokinesis-blocked human lymphocytes following treatment with 10 - 100 μM 1,2,4-benzenetriol in complete medium. Details of the procedures are described in Materials and Methods.

Figure 2. Effect of 1,2,4-benzenetriol on micronucleus induction in HL60 cells. The data represent the mean ± SE of at least three different experiments.

Figure 3. Effect of Cu^{2+} on 1,2,4-benzenetriol-induced micronuclei in HL60 cells. **A:** HL60 cells treated with different concentrations of 1,2,4-benzenetriol in combination with 5 μM Cu^{2+}. **B:** HL60 cells treated with different concentrations of Cu^{2+} in combination with 5 μM 1,2,4-benzenetriol. Control indicates cells treated with neither BT nor Cu^{2+}. Each bar represents the mean ± SE of at least three different experiments.

Figure 4. Effect of 1,2,4-Benzenetriol on the 8-OH-dG level in the DNA of HL60 cells. **A:** Dose-response for 1,2,4-benzenetriol-induced 8-OH-dG formation. Cells were treated with 5 μM BT, 10 μM BT, and 50 μM BT in PBS for 0, 15, 30 and 60 min. Control cells were incubated in PBS and sampled at the same time points. **B:** Effect of 1,2,4-benzenetriol and Cu^{2+} in combination on 8-OH-dG formation in DNA of HL60 cells. Cells were treated with 5 μM BT, 2 μM Cu^{2+}, and 5 μM BT + 2 μM Cu^{2+} in PBS for 0, 15, 30, and 60 min.

Figure 5. Different mechanisms for the induction of genetic damage by 1,2,4-benzenetriol and its oxidative products.

Figure 1

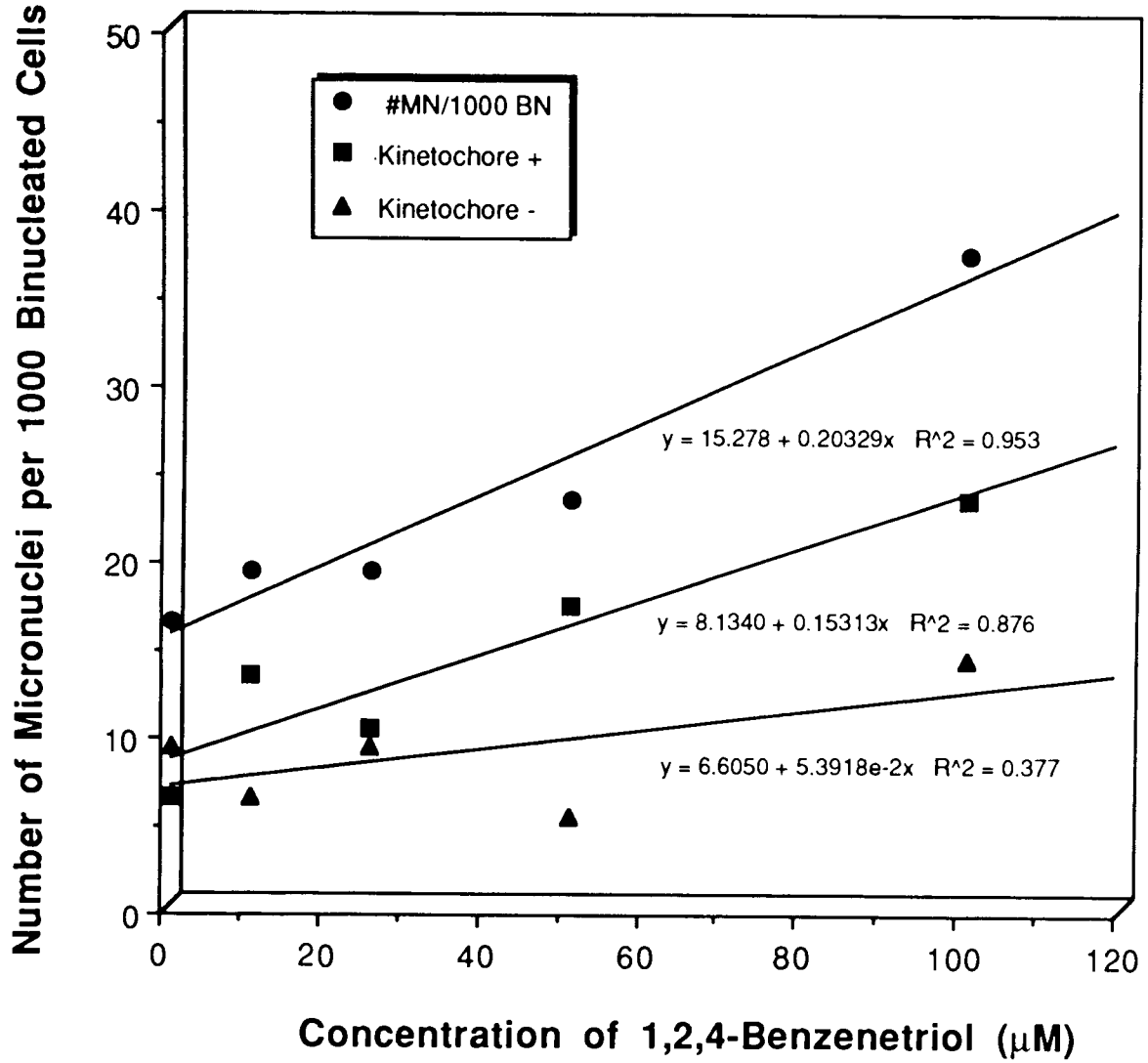

Number of Micronuclei per 1000 Binucleated Cells
Concentration of 1,2,4-Benzenetriol (μM)
#MN/1000 BN
Kinetochore +
Kinetochore -
y = 15.278 + 0.20329x R^2 = 0.953
y = 8.1340 + 0.15313x R^2 = 0.876
y = 6.6050 + 5.3918e-2x R^2 = 0.377
0
10
20
30
40
50
0
20
40
60
80
100
120

Figure 2

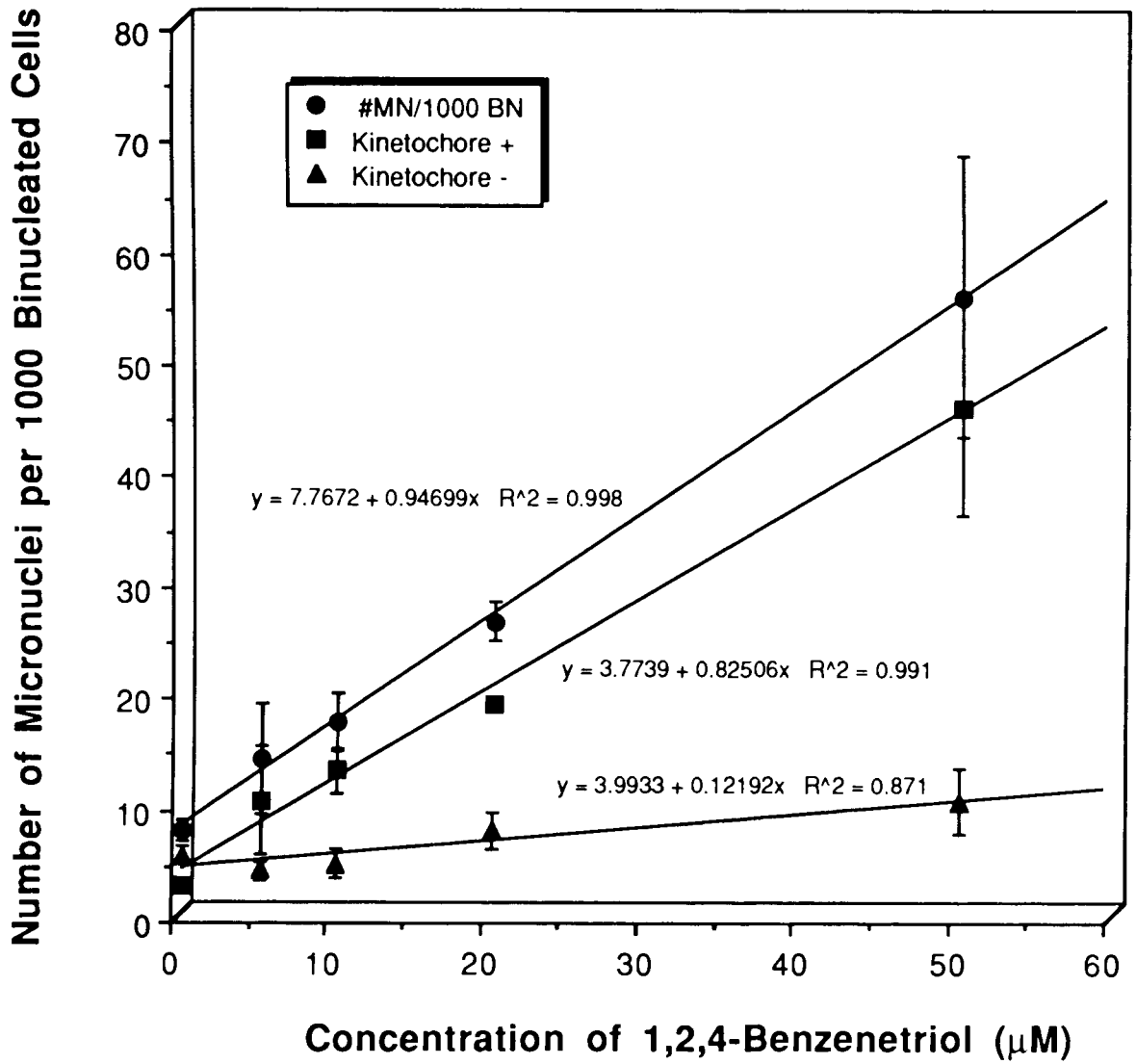

Number of Micronuclei per 1000 Binucleated Cells
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
#MN/1000 BN
Kinetochore +
Kinetochore -
y = 7.7672 + 0.94699x R^2 = 0.998
y = 3.7739 + 0.82506x R^2 = 0.991
y = 3.9933 + 0.12192x R^2 = 0.871
Concentration of 1,2,4-Benzenetriol (μM)

Figure 3A

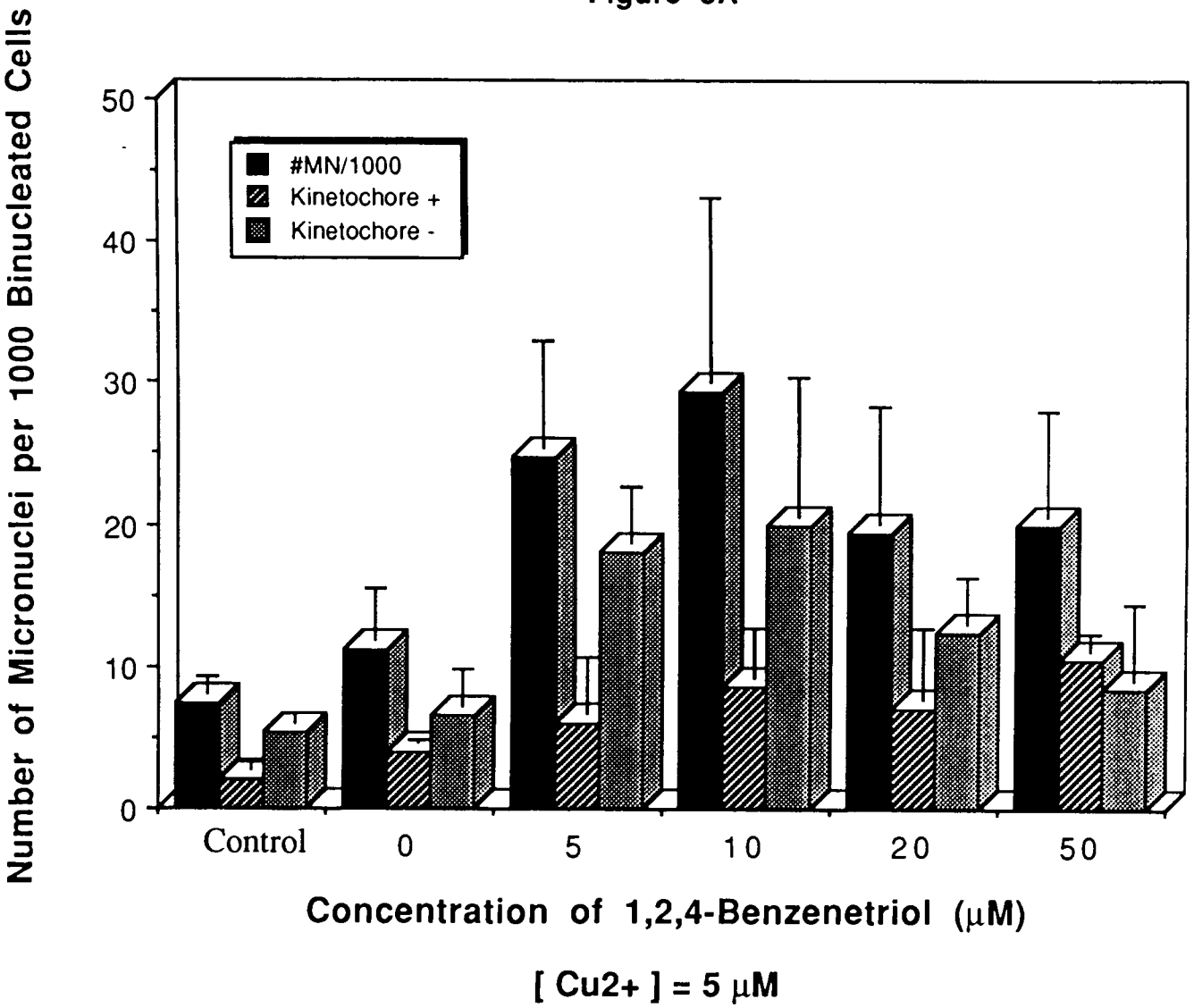

Number of Micronuclei per 1000 Binucleated Cells
50
40
30
20
10
0
#MN/1000
Kinetochore +
Kinetochore -
Control
0
5
10
20
50
Concentration of 1,2,4-Benzenetriol (μM)
[Cu2+] = 5 μM

Figure 3B

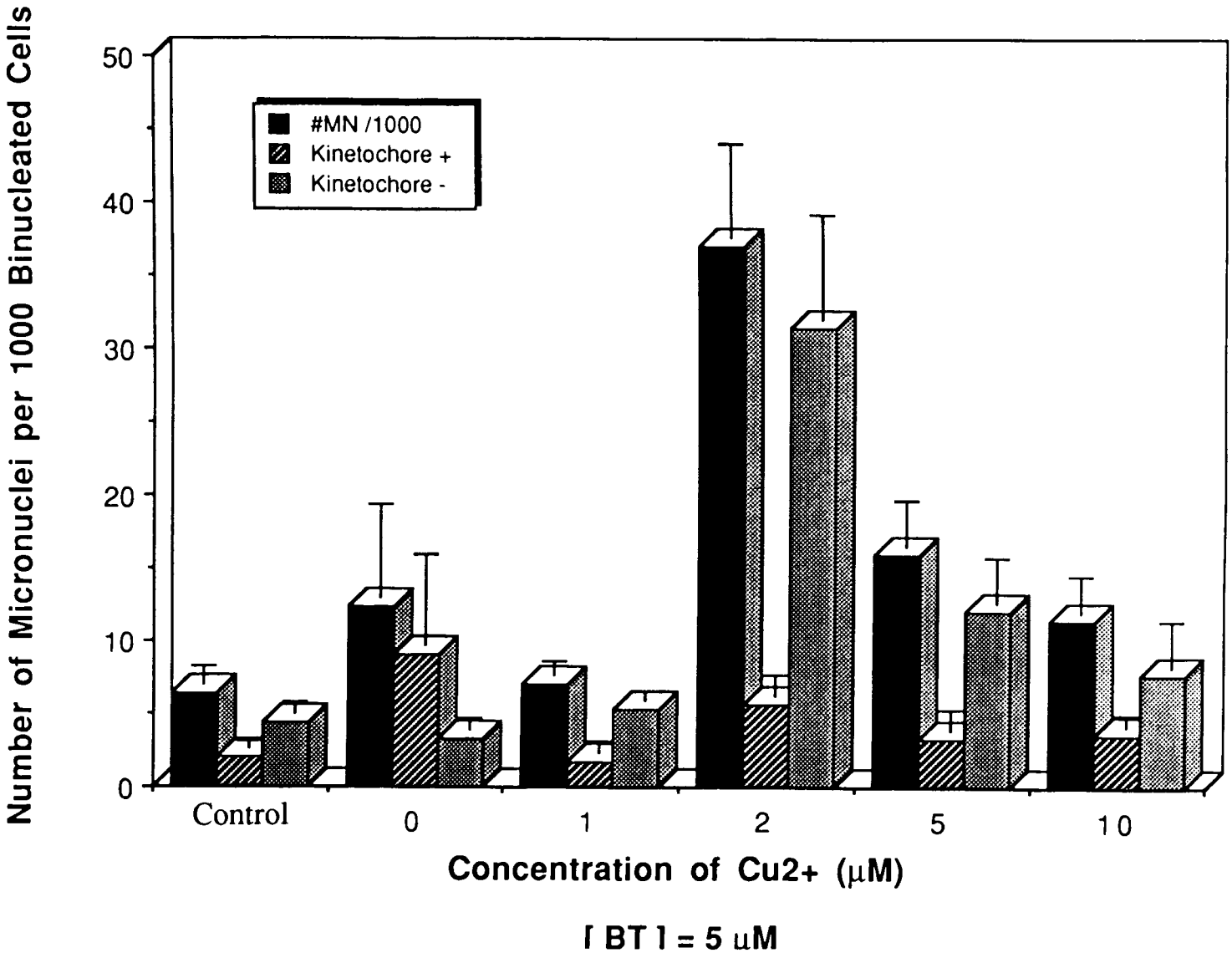

Number of Micronuclei per 1000 Binucleated Cells
50
40
30
20
10
0
#MN /1000
Kinetochore +
Kinetochore -
Control
0
1
2
5
10
Concentration of Cu2+ (μM)
[BT] = 5 uM

Figure 4A

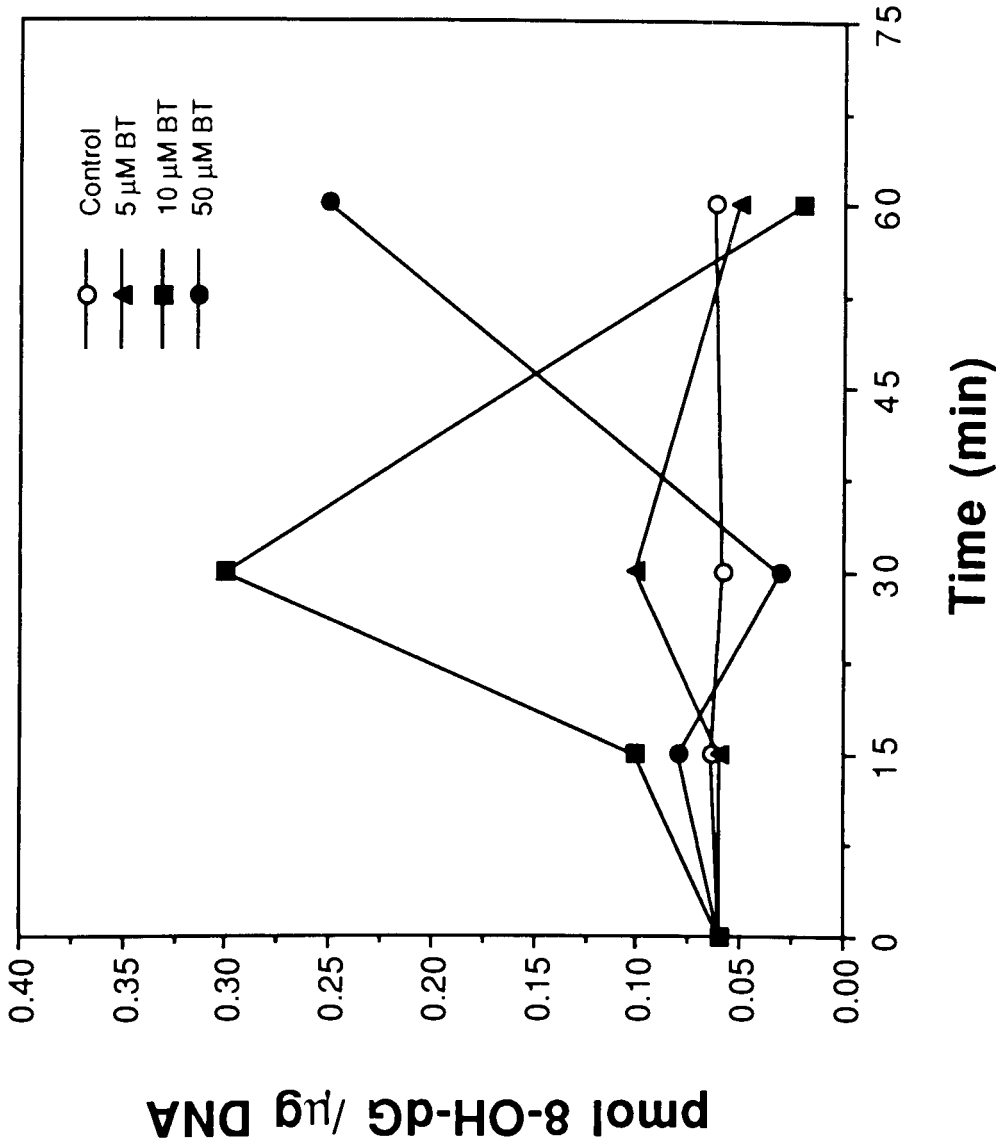
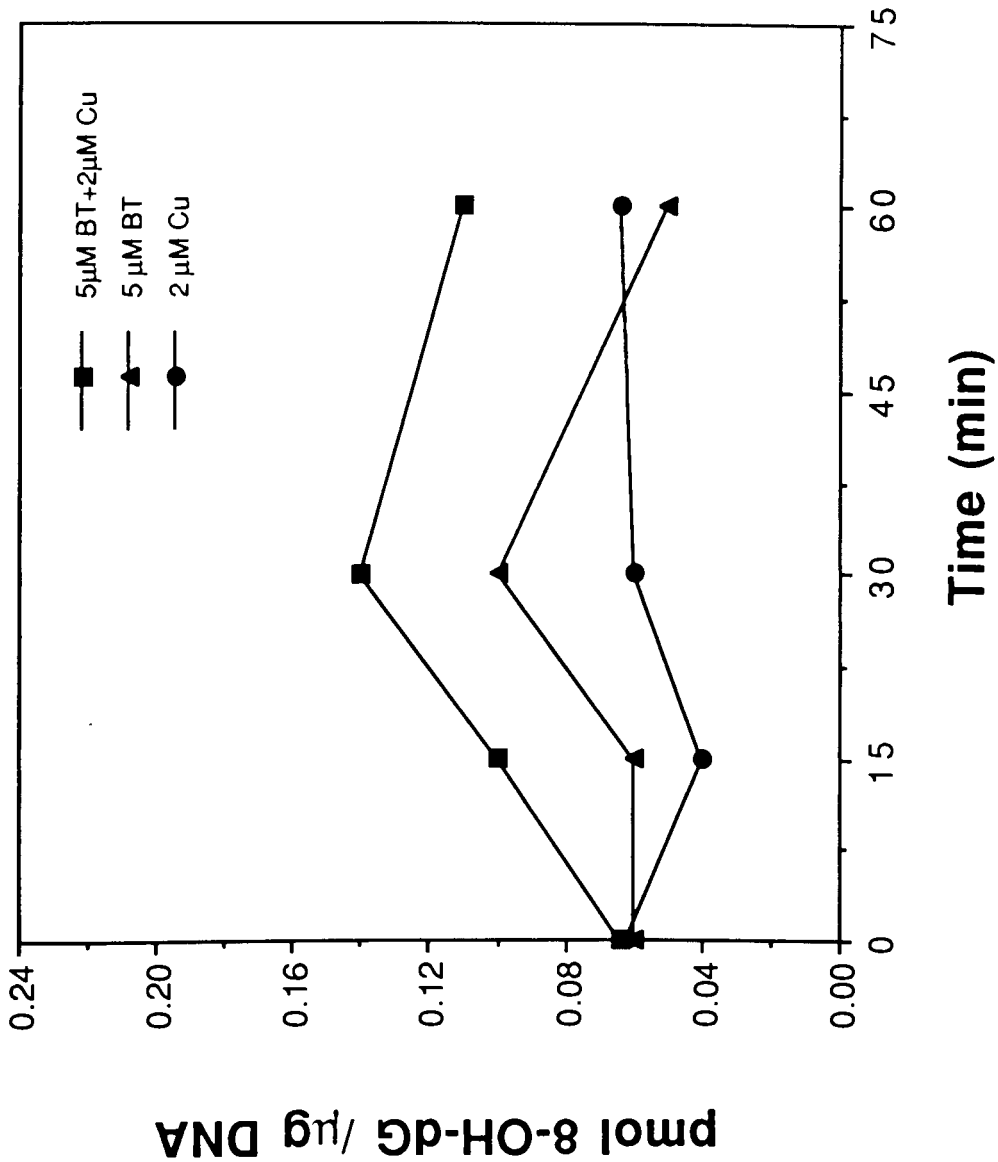

pmol 8-OH-dG /µg DNA
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
15
30
45
60
75
Time (min)
Control
5 µM BT
10 µM BT
50 µM BT

Figure 4B


5μM BT+2μM Cu
5 μM BT
2 μM Cu
0.24
0.20
0.16
0.12
0.08
0.04
0.00
0
15
30
45
60
75
Time (min)
pmol 8-OH-dG /μg DNA

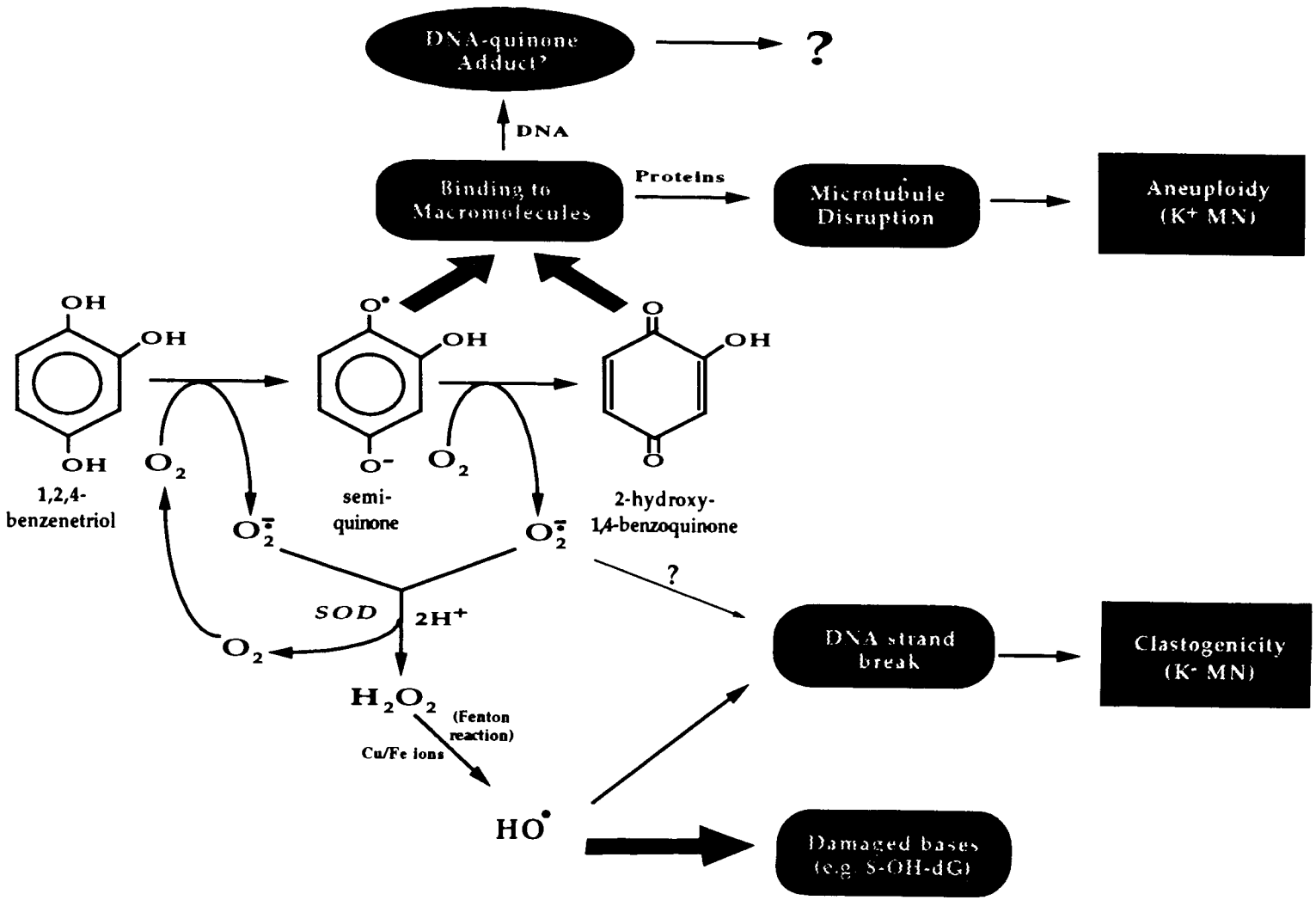


Figure 5. Proposed Mechanisms of Benzenetriol-induced Genotoxicity

Table I. Viability and Replicative Index (R. I.) of Lymphocytes and HL60 Cells Treated with 1,2,4-Benzenetriol (BT) and Copper Ions (Cu^{2+})*

Cell Treatment	Lymphocytes[a] BT / Medium		HL60 Cells[b] BT / PBS		HL60 Cells[b] [BT] + 5 μM Cu^{2+}	
[BT] (μM)	Viability (%)	R. I.	Viability (%)	R. I.	Viability (%)	R. I.
0	95	2.05	94	1.56	94	1.52
1	91	1.85	–	–	–	–
5	–	–	91	1.62	91	1.57
10	86	1.88	91	1.61	95	1.48
20	–	–	90	1.60	92	1.50
25	89	1.86	–	–	–	–
50	87	1.93	84	1.50	86[c]	1.50[c]
100	65	1.47	15[c]	N/A[d]	–	–

* **Cell Viability (%)** = (number of counted living cells / 400) x 100%;

R. I. = [(% mononuclear cells) + (2 x % binuclear cells) + (3 x % tri- or > trinuclear cells)] /100.

a Lymphocytes were treated with BT in complete medium.

b HL60 cells were treated with BT in PBS.

c Results were the average from only two experiments.

d R. I. was unscorable because of the toxicity of 100 μM BT to HL60 cells.

CHAPTER THREE

Detection of 1,2,4-Benzenetriol Induced Aneuploidy and Microtubule Disruption by Fluorescence *in situ* Hybridization and Immunocytochemistry

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Summary

Fluorescence *in situ* hybridization (FISH) is becoming increasingly used to detect chromosomal changes in cancer cytogenetics. Here, we report its use in human HL60 cells to detect aneuploidy induced by the benzene metabolite, 1,2,4-benzenetriol (BT). Human centromeric probes specific for chromosomes 9 and 7 were used. Untreated HL60 cells were 0.72 ± 0.29% hyperdiploid for chromosome 9. Treatment with 5 μM BT increased this level three-fold to 2.20 ± 0.87% and 50 μM increased it four-fold to 2.96 ± 0.74%. Similar results were obtained with the chromosome 7 probe. The induction of aneuploidy by BT is therefore not chromosome-specific nor is it artifactual. Immunocytochemical staining with anti-tubulin antibodies also showed that BT disrupted microtubule organization at these concentrations. Thus, mitotic spindle disruption probably plays an important role in BT-induced aneuploidy. Trisomy and not tetrasomy accounted for the majority of the hyperdiploidy induced by BT in the two C-group chromosomes 7 and 9. Since trisomy of C-group chromosomes is commonly observed in leukemia, BT-induced aneuploidy may be involved in benzene-induced leukemia.

INTRODUCTION

Benzene is an important industrial chemical and environmental pollutant that produces leukemia and other bone marrow disorders in humans (Aksoy, 1977; Yardley-Jones, et al., 1991). It is metabolized primarily to phenol, which is subsequently metabolized to polyhydroxylated metabolites, namely hydroquinone (HQ), catechol (CAT) and 1,2,4-benzenetriol (BT) (Inoue et al., 1989a, b). All of these metabolites are capable of inducing various forms of genetic damage (Erexson et al., 1985; Glatt et al., 1989) including micronucleus formation (Yager et al., 1990; Robertson et al., 1991). Recently, we showed that BT, a minor metabolite of benzene with highly active chemical properties, significantly increased micronucleus formation in both human lymphocytes and HL60 cells (Zhang et al., 1993). Further, by using an anti-kinetochore antibody to delineate the mechanism of micronucleus formation, we observed that the majority of micronuclei formed were kinetochore positive and therefore mainly resulted from chromosome lagging (Zhang et al., 1993). This suggested that BT could be a potent inducer of aneuploidy. In order to test this hypothesis, we have used a novel rapid method of detecting aneuploidy, namely fluorescence *in situ* hybridization (FISH) with chromosome-specific DNA probes (Pinkel et al, 1986; Gray and Pinkel, 1992). This method has been widely used in cancer cytogenetics (Cremer et al., 1988a, b; Hopman et al., 1991) and in cultured lymphocytes (Eastmond and Pinkel, 1990) to detect changes in both chromosome structure and chromosome number.

Aneuploidy is the loss or gain of whole chromosome(s) in the cell karyotype. Recent evidence suggests that it plays an important role in cancer progression and metastasis (Barrett et al., 1987; Fearon and Vogelstein, 1990)

and many carcinogens are also aneuploidogens (Oshimura and Barrett, 1986). Aneuploidy is commonly detected in leukemia and involves changes in specific chromosomes. For example, trisomy of chromosome 8 is often found in acute myeloid leukemia (Le Beau, 1990) and trisomy of chromosome 12 is common in chronic lymphocytic leukemia (Han et al., 1983; Losada et al., 1991). Occupational exposure to benzene has been also shown to cause changes in specific chromosomes especially of the C-group (Erdogan and Aksoy, 1973; Sasiadek, 1992). Here, we have used FISH with centromeric probes specific to chromosomes 7 and 9 to determine whether BT can induce aneuploidy of these chromosomes in the human myeloid HL60 cell line. Further, in an effort to investigate the mechanism of aneuploidy induction, we have also used immunocytochemistry with anti-tubulin antibodies to determine if BT is capable of interfering with mitotic spindle assembly. In this study, we demonstrate that BT induces hyperdiploidy of chromosomes 7 and 9 and disrupts microtubules in HL60 cells. This suggests that BT-induced aneuploidy could play a role in benzene-induced leukemia.

MATERIALS AND METHODS

Cell Culture

HL60 cells, a human myeloid cell line originally derived from the peripheral blood leukocytes of a patient with acute myelocytic leukemia (Gallagher et al., 1979; Dalton et al., 1988), were obtained from American Type Culture Collection (Rockville, MD). HL60 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 50 μg/ml gentamycin sulfate (UCSF, Cell Culture Facility, San Francisco, CA) at 37 °C in a 5% CO_2 moist atmosphere and passaged twice weekly to maintain a density between 10^5 and 10^6 cells/ml. The cells in the plateau phase of growth were then seeded in 15 ml sterile centrifuge tubes and diluted with fresh complete medium to a density of $0.5x10^6$ cells/ml (2 ml per each culture). At this starting concentration of cells, the doubling time of HL60 cells was approximately 36-40 h.

Chemical Treatment

1,2,4-benzenetriol (99%) (Aldrich, Milwaukee, WI) and colchicine (Sigma, St. Louis, MO) were dissolved in phosphate-buffered saline (PBS, Ca^{2+} and Mg^{2+} free, pH 7.4) immediately prior to treatment. At 24 h after culture initiation, HL60 cells were treated with BT in complete medium for 24 h or 1 h and in PBS for 1 h (FISH assay). Colchicine, as a positive control, was added to HL60 cells for 24 h in the media. All treatments were performed in duplicate for each dose. The treated cells were then washed in PBS and harvested following 48 h of culture initiation. The washed cells were incubated with hypotonic solution (0.075 M KCl) for 15 min at 37 °C and fixed three times with Carnoy's solution (methanol : glacial acetic acid = 3:1). The fixed cells were

dropped onto glass slides, allowed to air dry, and stored at -20 °C under a nitrogen atmosphere.

In Situ Hybridization

Biotinylated human centromeric probe specific for chromosome 9 (classical-satellite) and centromeric cocktail probe for chromosome 7 (α-satellite) were purchased from Oncor Inc. (Gaithersburg, MD). Detailed procedures for performing fluorescent *in situ* hybridization with repetitive DNA probes have been described previously (Pinkel et al., 1986; Eastmond and Pinkel, 1990). Briefly, the best spots on each slide were located and marked. Slides were immersed in 70% formamide (Fluka, Buchs, Switzerland) in 2XSSC (0.3 M sodium chloride and 0.03 M sodium citrate, pH 7.0) at 72 °C for 2 min to denature the cell DNA, and then quickly removed to ice-cold 70%, 85% and 100% ethanol series to dehydrate and air dried. The chromosome probes (0.1 ng/μl) was mixed with sonicated salmon sperm carrier DNA (50 ng/μl) in Master Mix 2.1 solution (55% formamide/1xSSC/10% dextran sulfate). The probe mixture was heated at 70 °C for 5 min to denature the probe DNA. After rapid removal to ice, the denatured probe was applied to prewarmed slides (10-15 μl / spot) at 37 °C on a slide warmer. The slides were coverslipped, air bubbles carefully removed, and incubated overnight at 37 °C in a humidified chamber.

Detection and Amplification

The first post-wash was performed 3 times in 50% formamide/2XSSC and once in 2XSSC at 45 °C for 5 min each followed by a second wash in 2XSSC at room temperature for 5 min. The biotinylated probe was detected using FITC conjugated avidin (Vector, Burlingame, CA) 5 μg/ml in pre-block

solution (4XSSC, 0.1% Triton X-100, 0.02% sodium azide, and 5% Carnation non-fat dry milk). After 3 washes in 0.1M phosphate buffer (pH 8.0) of 3 min each with intermittent agitation at room temperature, the hybridization signal was amplified using a biotinylated goat anti-avidin antibody (Vector, Burlingame, CA) 5 μg/ml in pre-block solution followed by a second layer of FITC-avidin. The red fluorescent dye propidium iodide (0.5 μg/ml) in a mounting medium (Vector, Burlingame, CA) was used as counterstain DNA.

Scoring Procedures and Criteria

The stained slides were randomized and coded prior to scoring. 1000 cells per dose from each treatment were then scored by two observers for the presence of fluorescent probe spots in each nucleus. A Nikon microscope equipped with epifluorescent illumination, a 100x oil immersion lens and a filter for fluorescein (excitation at 450-490 nm, dichroic at 510 nm, emission at 520 nm) was used. The nuclei appeared red with bright green-yellow spots indicating the hybridization regions. Both the number of interphase nuclei with 0, 1, 2, 3, 4 and ≥5 spots, and the total number of scored cells were recorded. Each observer scored exactly half of the cells (500 nuclei per dose) from each replicate. Both were trained in the same manner according to the standard scoring criteria described briefly as follows: 1) Scorable interphase nuclei were intact and separated from each other; 2) hybridization regions appeared bright and had the same homogeneous fluorescence intensity; 3) two regions were scored as two spots only if they were clearly separated, otherwise two overlapping spots or split spots were scored as one if they could not be separated by changing focus; 4) if more than 2 spots presented in a single nucleus, they appeared the same in spot size and staining intensity even with a change of focus; and 5) if a nucleus contained no hybridization regions, it was

scored as a zero only when its neighboring cells were clearly stained with fluorescent spots. When cells did not follow these five criteria, they were recorded as unscorable. However, very few, less than 1 per 1000 nuclei in our HL60 cell preparations were unscorable. In general, over 99.8% of the interphase nuclei of HL60 cells showed one, two, three or more *in situ* hybridization regions in all preparations.

Microtubule Staining

The organization of microtubules was assessed in HL60 cells by a new immunocytochemical method (Leung et al., 1992). HL60 cells were treated with BT or colchicine in media for 1 h, and washed twice with PBS (Ca^{2+}/Mg^{2+} free, pH 7.4). They were then resuspended in a microtubule stabilizing buffer (MTSB) consisting of 0.1 M piperazine-N,N'-bis-2-ethane-sulfonic acid (PIPES), 1mM $MgSO_4$, 2 mM ethylene glycol-bis(beta-amino-ethyl ether) (EGTA) and 2 M glycerol, pH 6.9 (all from Sigma, St. Louis, MO). Washed cells were collected directly onto glass slides using a Cytospin-II cytocentrifuge (Shandon, Sewickley, PA) for 10 min at 600 rpm. Slides were fixed in freshly prepared 3.7% para-formaldehyde (Sigma, St. Louis, MO) in MTSB (pH 6.9) for 30 min at room temperature. Fixed cells were incubated with PBS and 0.1 M glycine (Sigma, St. Louis, MO) in PBS for 5 min each, then extracted with 0.3% Nonidet P-40 (Sigma, St. Louis, MO) in PBS for 10 min, and washed in PBS twice. Cells were then stained with a mouse monoclonal anti-beta tubulin antibody (Sigma, St. Louis, MO) diluted 1:200 with PBS followed by a 30 min incubation at 37 °C in a humidified incubator. Following two rinses in PBS, slides were then incubated with a 1:64 dilution with PBS of fluoresceinated goat anti-mouse IgG (Sigma, St. Louis, MO) under the same conditions. The stained slides were washed twice in PBS, mounted using a fresh antifade solution and then stored

at -5 °C in the dark. Microtubule integrity was qualitatively assessed by immunofluorescence microscopy using the following criteria: 1) presence or absence of microtubule staining; 2) fluorescence intensity; and 3) abnormal mitotic figures e.g. lagging chromosomes, micronuclei, monopolar or multipolar mitoses etc..

Photography

Photography was conducted using a Nikon 35-mm camera attachment with either a 5x or 2.5x projection lens coupled with a 100x oil immersion lens. Since the fluorescein label rapidly fades upon long exposure of the slide to light, high ASA rating films were, therefore, used such as Kodak Ektachrome color slide film (ASA 400) or Fujicolor (ASA 1600) color print film. The black and white prints displayed in Figures 2 and 3 were made by laser-imaging from the color slides.

Statistical Analysis

To test for consistency of BT treatment replicates, one-way ANOVA (analysis of variance) was performed on transformed data. In order to stabilize the variance, the arcsine of the square root of the proportion of hyperdiploid cells was used as the transformation. To examine for trend of elevated frequency of hyperdiploidy with increasing concentrations of BT, the Chi-square Test for Trend (Binomial Trend Test) was performed on the raw data. The potential difference in frequency of hyperdiploidy at various doses versus the control was analyzed by using Fisher's Exact Test with the allowance for Type I error adjusted appropriately. Critical values were determined using a 0.05 probability of Type I error unless otherwise stated. The "Stata" program for statistical analyses was used on a MacIntosh IIsi computer.

RESULTS

Fluorescence in situ hybridization in HL60 cells

Scoring cells stained with chromosome-specific fluorescent probes enumerates the number of cell nuclei with 0, 1, 2, 3, 4 and ≥5 hybridization regions (fluorescent spots) corresponding to numbers of a specific chromosome present (Eastmond and Pinkel, 1990). To determine the background frequencies of hybridization regions observed in untreated HL60 cells, a total of 10,000 interphase nuclei were examined after hybridization with a centromeric probe specific for chromosome 9. The frequencies of nuclei containing 0, 1, 2, 3, 4 and ≥5 hybridization regions for this chromosome were 0.02, 9.92, 89.34, 0.63, 0.09 and 0%, respectively (Table 1). A normal diploid cell should contain two copies of chromosome 9, indicated as two bright yellow-green hybridization spots in the cell nucleus. The frequency of apparent hypodiploidy (9.94%) was considerably higher than the hyperdiploid frequency (0.72%). The same phenomenon has been found in untreated human lymphocytes, in which the frequencies of hypodiploidy and hyperdiploidy have been reported to be 9.04% and 0.39%, respectively (Eastmond and Pinkel, 1990). This is most likely due to overlap and visual fusion of the hybridization regions from both copies of the chromosome, which leads to an artifactually-high rate of monosomy. The baseline level of hyperdiploidy in HL60 cells was significantly higher than that of lymphocytes ($p < 0.01$). The frequency of hypodiploidy observed in both untreated HL60 cells and lymphocytes was also statistically different. These differences are probably due to gene instability in the long term transformed cell line.

Colchicine as a positive control for aneuploidy induction

Colchicine is a well-known inducer of aneuploidy. It interacts with microtubules by binding to tubulin proteins and inhibits microtubule assembly leading to mitotic arrest (Wallin et al., 1988). Colchicine was chosen as a positive control to determine whether FISH could be used as a sensitive assay in the HL60 cell line. HL60 cells were treated with 0.1 μM colchicine for 24 h in media. A total of 5000 interphase nuclei of treated HL60 cells were examined with 1000 cells from each treatment being scored. The number of hybridization regions per 1000 cells is presented as mean and standard deviation (S.D. in parentheses) in Table 1. The hyperdiploid frequency (3.04 ± 1.26 %) was significantly elevated above control values ($p < 0.0001$), whereas the frequency of hypodiploid cells (10.26 ± 1.46 %) was approximately the same as in controls (Table 1). Similar results for colchicine-induced aneuploidy, hypodiploidy (12.4%) and hyperdiploidy (5.0%), were obtained in lymphocytes by Eastmond and Pinkel (1990). Thus, colchicine induces similar levels of aneuploidy in both HL60 cells and lymphocytes. The human myeloid HL60 cell line, therefore, is a suitable model system for using FISH to detect aneuploidy-inducing agents.

BT-induced hyperdiploidy of chromosome 9

HL60 cells were treated with BT for 24 h in media and hybridized with DNA probe specific for chromosome 9. Identical experiments with BT treatment were repeated five times for statistical precision, and no significant difference in these replicates were detected by ANOVA. 5000 interphase cells were scored (1000 for each experiment) at each non-toxic dose of BT (0, 5, 10, 20 and 50 μM). Notable cytotoxicity as indicated by trypan blue dye exclusion was

observed only after treatment with 80 and 100 μM BT in HL60 cells, when the cell viability was decreased to 32% and 15%, respectively. Only one to two thousand cells were scorable at these toxic doses. The number of hybridization domains per 1000 nuclei are shown in Table 2. BT treatment at all concentrations ranging from 5 to 100 μM resulted in a significant increase in the frequency (2.20 - 3.40 %) of hyperdiploid cells over the control ($p < 0.001$). To determine if there was a trend in frequency of hyperdiploidy as the dose of BT was increased, we categorized scored cells as either hyperdiploid or non-hyperdiploid and assessed the trend using the Binomial Trend Test. A significant trend ($p < 0.001$) was observed indicating that elevated hyperdiploid frequency is associated with increasing doses of BT (Fig. 1A). However, the frequency of hypodiploidy in BT treated cells was not significantly different from that found in untreated HL60 cells.

To avoid potential confounding effects of thiol-containing compounds and antioxidants, such as glutathione, ascorbate and serum, present in complete medium, HL60 cells were also treated with BT at 5 to 50 μM for only 1 h in PBS and harvested at 48 h after culture initiation. Comparable increases in hyperdiploid frequency were observed (Table 3). The increase (at 50 μM of BT) is statistically significant in comparison with the PBS control ($p < 0.001$). Surprisingly, exposure of HL60 cells to PBS, instead of media, significantly increased the background frequency of hyperdiploidy to 1.70%, which is twice that of cells in media. Thus, PBS may act as an inducer of hyperdiploidy in HL60 cells. However, treatment with BT for 1 h in medium produced only a minimal increase in hyperdiploidy, and a plateau phase was observed at increasing doses (Fig. 1B). This shows the protective effect of complete medium. In addition, the hyperdiploid frequency observed at 50 μM x 1 h in

medium (Fig. 1B) is in agreement with the observation at this same dose (CxT) in Figure 1A. This indicates that 24 h exposure in medium delivered a much higher dose than 1 h exposure even though the exposure level was the same. Overall, BT induced a dose-related increase in hyperdiploidy of chromosome 9 in HL60 cells independent of whether the treatment was performed in media or in PBS.

BT-induced hyperdiploidy of chromosome 7

In addition to the chromosome 9 classical satellite probe, the smaller sized α-satellite probe (Cocktail) specific for chromosome 7 was used. Table 4 shows the background frequencies of nuclei containing 0, 1, 2, 3, and 4 hybridization regions in untreated HL60 cells stained with the chromosome 7 probe. These were 0, 6.40, 92.90, 0.50 and 0.20 %, respectively. Similar background levels (0.30, 6.60, 92.70, 0.40 and 0 %) have been found in human lymphocytes using the same probe (Eastmond and Pinkel, 1990). However, the frequency of monosomy (6.40 %) of chromosome 7 in control cells was significantly lower ($p < 0.01$) than the frequency (9.32 %) of chromosome 9 (Tables 2 and 4). Similar results were also found in treated cells. This suggests that the use of the smaller sized hybridization probe decreases overlapping events which artificially increase the frequency of monosomy. Nuclear spot frequencies in HL60 cells treated with BT at non-toxic doses (5 - 50 μM) for 24 h in media were obtained using the α-satellite probe of chromosome 7 (Table 4). BT, again, significantly increased hyperdiploid frequencies of chromosome 7 at levels similar to that found with chromosome 9. Moreover, the increase in hyperdiploidy was mostly dependent on the increase in the frequency of 3 hybridization domains per nucleus (Table 4).

BT-induced trisomy in hyperdiploidy

BT increased the frequency of hyperdiploidy of chromosomes 7 and 9 in HL60 cells treated with BT both in media (Tables 2 and 4) and in PBS (Table 3). The proportion of nuclei with 3 spots (trisomy) in total hyperdiploidy was consistently two- to four-fold higher than the proportion of cells with 4 hybridization regions (tetrasomy) in both control and treated cells (Table 5). Treatment with BT in PBS tended to increase the proportion of hyperdiploidy in chromosome 9 caused by trisomy. A similar pattern was also seen for treatment with BT in media, which was observed for chromosome 7 but not for chromosome 9 (Table 5). Thus, trisomy accounts for majority of the background and BT-induced hyperdiploidy observed in HL60 cells.

BT-induced microtubule disruption

Disruption of the mitotic spindle leads to improper chromosome segregation during mitosis, resulting in chromosome lag and eventually leading to aneuploidy. To investigate mechanisms of BT-induced aneuploidy, the effect of BT on microtubule assembly was therefore tested in HL60 cells using an immunofluorescence staining assay with anti-tubulin antibody. The normal patterns of microtubule distribution in interphase and metaphase of untreated cells are shown in Figure 2. The full complement of cytoplasmic microtubules in the interphase cell (Fig. 2A) and spindle microtubules in the mitotic cell (Fig. 2B) was observed. Experiments on microtubule disruption by BT were performed for 1 h in complete medium. Treatment of HL60 cells with BT and colchicine resulted in decreased microtubule integrity as indicated by decreased fluorescence intensity as compared with controls (Fig. 3). At the lowest dose of

BT tested (20 μM), the microtubules were intact and the fluorescence intensity slightly increased, but abnormal tripolar configurations were observed (Fig. 3A). An intermediate concentration of BT (50 μM) dramatically decreased the fluorescence staining intensity and disrupted most microtubules (Fig. 3B). At the maximal dose tested (100 μM BT), microtubules were essentially absent due to complete disruption, and only microtubule organizing centers were visible (Fig. 3C). Colchicine (1 μM), used as a positive control (Fig. 3D), produced complete microtubule disruption in both mitotic and interphase cells. Thus, both BT and colchicine disrupt microtubule assembly in HL60 cells.

DISCUSSION

In spite of the evidence implicating the involvement of aneuploidy and aneuploidy-inducing agents in the carcinogenic process, rapid methods for detecting aneuploidy in systems relevant to humans have not been well established (Oshimura and Barett, 1986). Standard cytogenetic techniques are time consuming and labor intensive, require highly trained personnel, and are prone to technical artifacts. In addition, karyotypic analyses are restricted to cells and tissues from which good metaphase spreads can be obtained. In recent years, a new method, named fluorescence *in situ* hybridization (FISH) for detecting numerical and structural chromosomal changes in both interphase nuclei and metaphase spreads has become available (Gray and Pinkel, 1992). This methodology utilizes *in situ* hybridization with DNA probes specific to blocks of repetitive DNA sequences on defined regions of specific chromosomes (Willard and Waye, 1987; Pinkel et al., 1986; 1988). The

visualization of chemically-modified probes involves the use of non-radioactive fluorescent antibodies. The determination of aneuploidy is performed by simply counting the number of labeled regions representing a particular chromosome of interest within the isolated nucleus. This method has been widely used for the molecular cytogenetic analysis of cancer cells (Cremer et al., 1988a, b; Hopman et al., 1991), but has also been used in cultured human lymphocytes (Eastmond and Pinkel, 1990) and bone marrow (Poddighe et al., 1991; Jenkins et al., 1992). Recently, our laboratory has also adapted this procedure so that it can be used in exfoliated human cells (Moore et al., 1993).

In this study, we have used FISH to determine if the benzene metabolite, 1,2,4-benzenetriol (BT), can induce aneuploidy in a model cell system, HL60 cells. These cells contain high levels of the enzyme myeloperoxidase, which activates BT (Subrahmanyam et al., 1992) and are representative of immature myeloid cells. Although the background level of aneusomy was slightly higher in HL60 cells than that in human lymphocytes, use of a positive control (colchicine) showed that it increased the frequency of hyperdiploidy in HL60 cells in a manner similar to that found in lymphocytes. Thus, HL60 cells are a suitable model for studying the aneuploidogenic effects of BT. Further, our data show that BT induces a dose-related increase in aneuploidy of chromosomes 7 and 9 in HL60 cells.

Virtually all of the BT-induced aneuploidy in HL60 cells detected by FISH was in the form of hyperdiploidy. No BT-induced change in hypodiploidy was detected. However, no significant change in the level of hypodiploidy was observed after treatment with the known aneuploidy-inducing agent, colchicine. This is because the background level of hypodiploidy (monosomy) detected by

FISH is artificially high due to probe overlap or close juxtaposition of signals. Indeed, with the larger sized classical-satellite probe of chromosome 9, the amount of overlap would be expected to be greater than that seen with the smaller α-satellite centromeric probe for chromosome 7. This most likely explains the higher frequency of monosomy detected by FISH for chromosome 9 as compared with chromosome 7 in this study. Eastmond and Pinkel (1990) have calculated that as much as 85% of the apparent monosomy detected by FISH is due to probe overlap. FISH is, therefore, unsuitable for the detection of monosomy in interphase nuclei. It is, however, a sensitive method for the detection of hyperdiploidy, and both colchicine and BT increased the level of hyperdiploidy significantly in HL60 cells.

Although an increase in hyperdiploidy of chromosomes 9 was observed after BT treatment, this could have been due to chromosomal breakage occurring within the region stained by the DNA probe. For example, the target region 9q12 for the classical-satellite chromosome 9 probe used in this study, has been reported to be susceptible to non-random chromosomal breakage by γ-rays (Dubos et al., 1978). To determine whether the effects of BT on hyperdiploidy of chromosome 9 were caused by true aneuploid events or non-random chromosome breakage, we used a smaller α-satellite probe specific to another chromosome, chromosome 7. A similar increase in hyperdiploidy of chromosome 7 was observed in HL60 cells (Table 4) as that detected using the chromosome 9 probe (Table 2). We, therefore, conclude that the BT-induced hyperdiploidy is real and not artificial. Moreover, our results also show the effects of BT on inducing hyperdiploidy are not chromosome specific, since they are observed in both chromosome 7 and 9 probes. Chromosome 7 and 9 are, however, both C-group chromosomes which benzene has been shown to

specifically affect (Erdogan and Aksoy, 1973; Sasiadek, 1992). Thus, further work is needed to determine if benzene's metabolites produce specific effects on the C-group chromosomes as compared to chromosomes from other groups.

Trisomy of group C chromosomes has also been observed in myeloid metaplasia (Sandberg et al., 1964), myeloproliferative disorders (Winkelstein et al., 1966), and in benzene-induced leukemia (Erdogan and Aksoy, 1973). Although possible mechanisms of trisomy induction are obscure, it is clear that trisomy of a chromosome with a dominant-acting gene will result in an increased expression of the gene product. Here, we have shown the majority of BT-induced hyperdiploidy of chromosome 7 and 9 in HL60 cells is in the form of trisomy. The ratio of trisomy to tetrasomy induced by BT was around 3:1 (Table 5).

The BT-induced increases in hyperdiploidy of chromosomes 7 and 9 in HL60 cells were dose-dependent and independent of whether the treatment was performed in media or in PBS (Fig. 1). The presence of complete medium was protective, however, and incubations with BT had to be much longer in media than in PBS for an effect to be observed (Fig. 1A). Interestingly, incubation of HL60 cells in PBS for one hour elevated the baseline of hyperdiploid frequency by two-fold, but BT still markedly increased this level. The enhanced background may be explained by our unexpected finding that microtubular structures were unstable when HL60 cells were suspended in PBS for 1 h (data not shown). Thus, PBS may act as a possible aneuploidy-inducer under these circumstances.

Since the mitotic apparatus consists predominantly of microtubules, any compound that affects microtubules might be a potential aneuploidy-inducer. We, therefore, hypothesized that the mechanism by which BT induces hyperdiploidy in HL60 cells most likely involves microtubule disruption and dysfunction. Microtubules are composed primarily of tubulin polymers which are rich in nucleophilic sulfhydryl groups important for microtubule assembly. Their integrity is required for spindle formation and proper segregation of chromosomes into the daughter nuclei during cell division. It has been shown that the polyhydroxy benzene metabolites, BT and HQ, inhibit tubulin polymerization *in vitro* , with BT being approximately twice as potent as HQ (Irons et al., 1981). These effects are oxygen dependent and significantly inhibited under anaerobic conditions, showing that the oxidation of BT and HQ to their semiquinone intermediates and/or quinone metabolites must be responsible for their effects on microtubules (Irons, 1985; Pfieffer and Irons, 1983). BT can be spontaneously (Zhang and Davison, 1990) or enzymatically (Subrahmanyam et al., 1992) oxidized to electrophilic quinone and semiquinone intermediates which may interact with nucleophilic sulfhydryls of tubulin and cause mitotic abnormalities resulting in aneuploidy. This may also explain our finding of a dose-related increase in hyperdiploidy of chromosome 9 was found in HL60 cells treated with BT 1 h in PBS, whereas a plateau phase was observed at increasing doses of BT treated 1 h in medium (Fig. 1B). The difference may be due to the presence of serum or other SH-rich proteins and glutathione (GSH) in the medium which act as additional targets for BT's quinone metabolites.

A new immunocytochemical assay (Leung, et al., 1992) was used to allow us to visualize the microtubular structure within HL60 cells and showed

that BT causes disruption of the intercellular microtubular apparatus. Although BT almost completely disrupted the microtubular structure at high aneuploidogenic concentrations, it unexpectedly produced a small increase in antitubulin fluorescent intensity and abnormal mitotic figures, such as tripolar spindle formation, at relatively low concentrations (Fig. 3A). Similar results were obtained in another study, in which human lymphocytes were treated with other benzene metabolites, HQ and catechol alone and in combination. These treatments decreased microtubule integrity at higher concentrations but increased fluorescent intensity at lower doses (Robertson, 1992). A previous study on BT-induced genotoxic damage also showed that BT increased micronucleus formation predominantly in the form of kinetochore-positive micronuclei, indicating whole chromosomes, in both lymphocytes and HL60 cells (Zhang, et al., 1993). Since microtubule disruption is observed in HL60 cells treated with BT at the same concentrations as those which produce aneuploidy and micronuclei, we conclude that the effect of BT on microtubule disruption is most likely involved in its production of hyperdiploidy and kinetochore-positive micronuclei.

In summary, we have shown that the benzene metabolite BT is able to induce aneuploidy and microtubular disruption in human myeloid HL60 cells. The disruption of microtubules may be involved in the production of BT-induced aneuploidy. The observed aneuploidy was mainly in the form of hyperdiploidy. Specifically, BT induced hyperdiploidy of the C-group chromosomes 7 and 9. Moreover, the majority of the hyperdiploidy induced was due to trisomy. Since trisomy of C-group chromosomes is commonly found in the leukemias, we suggest that BT-induced aneuploidy could play a role in benzene-induced leukemia.

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Legends for Figures:

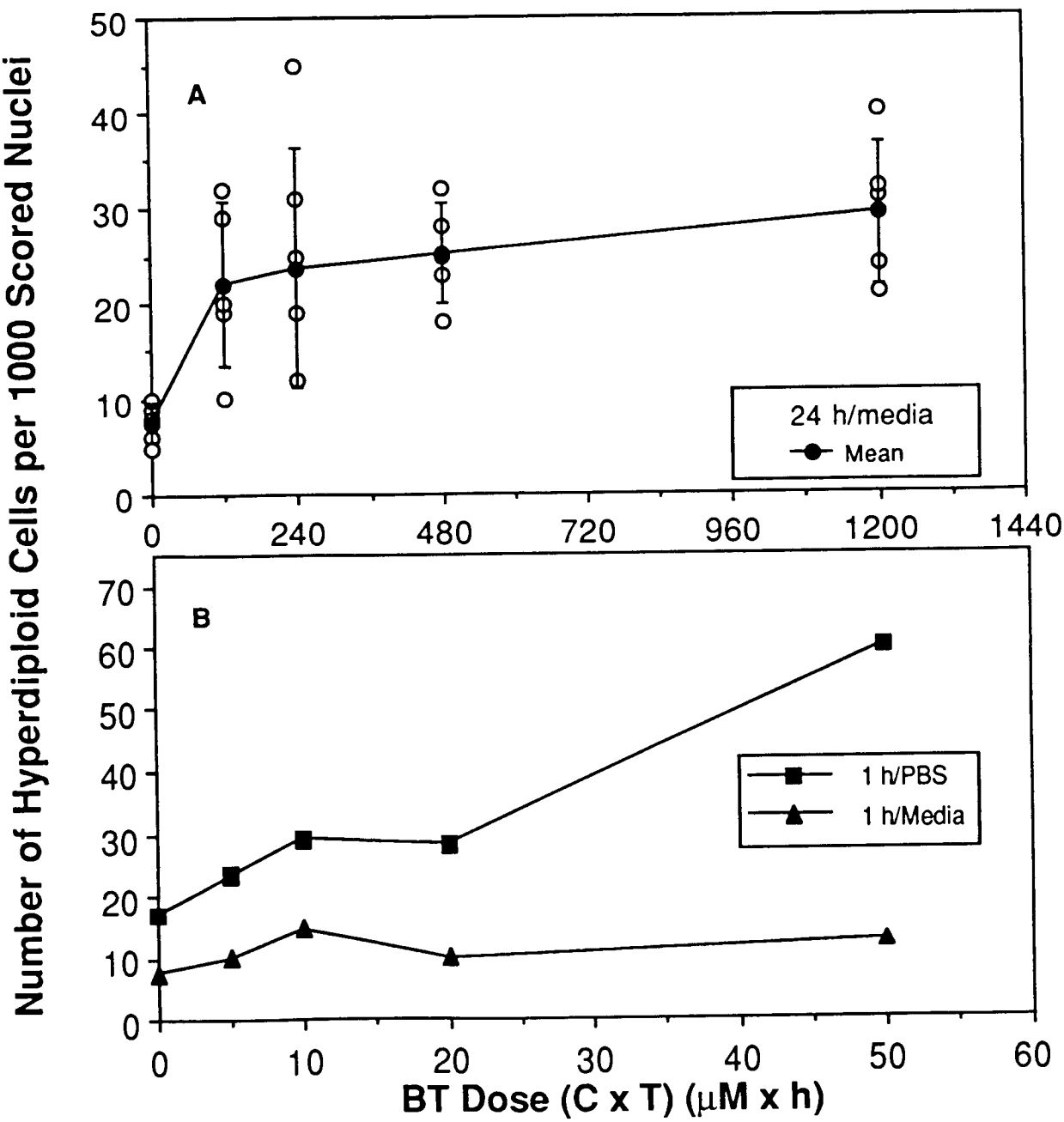
Figure 1. Dose-response for 1,2,4-benzenetriol-induced hyperdiploidy in HL60 cells following treatment with 5 to 50 μM 1,2,4-benzenetriol in complete medium for 24 hours (A) and in the medium or PBS for 1 hour (B). Doses are expressed as concentration x time (C x T) so that 24 and 1 hour exposures can be directly compared. Figure 1A also shows the level of hyperdiploidy from five separate experiments. Means (solid dots) ± standard deviation (error bars) are shown. Details of the procedures are described in Material and Methods.

Figure 2. Immunocytochemical anti-tubulin antibody staining of control HL60 cells. Microtubules are clearly visible in the interphase cell (A) and the cell undergoing mitosis (B).

Figure 3. Effects of 1,2,4-benzenetriol and colchicine on microtubule integrity and cytoskeletal organization in HL60 cells. The fluorescence intensity of treated HL60 cells stained with anti-tubulin antibodies was decreased as the concentration of 1,2,4-benzenetriol was increased. HL60 cells treated with 1,2,4-benzenetriol at 20 μM (A), 50 μM (B) and 100 μM (C), and treated with 1 μM colchicine as a positive control (D) are shown.

Figure 1

Dose-response for Benzenetriol-induced Hyperdiploidy in HL60 Cells



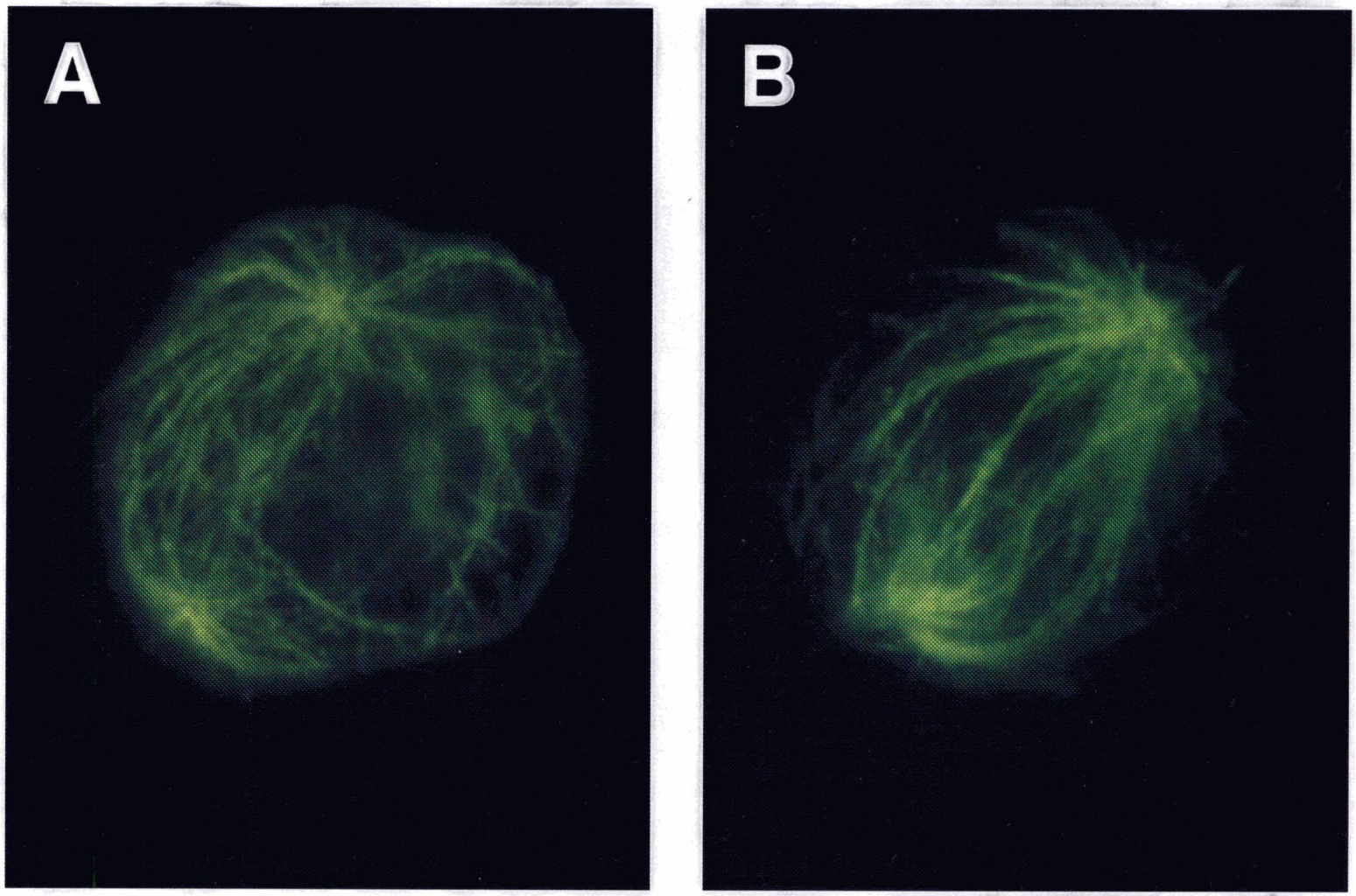


Figure 2. Immunocytochemical anti-tubulin antibody staining of control HL60 cells.

(A) The interphase and **(B)** the metaphase.

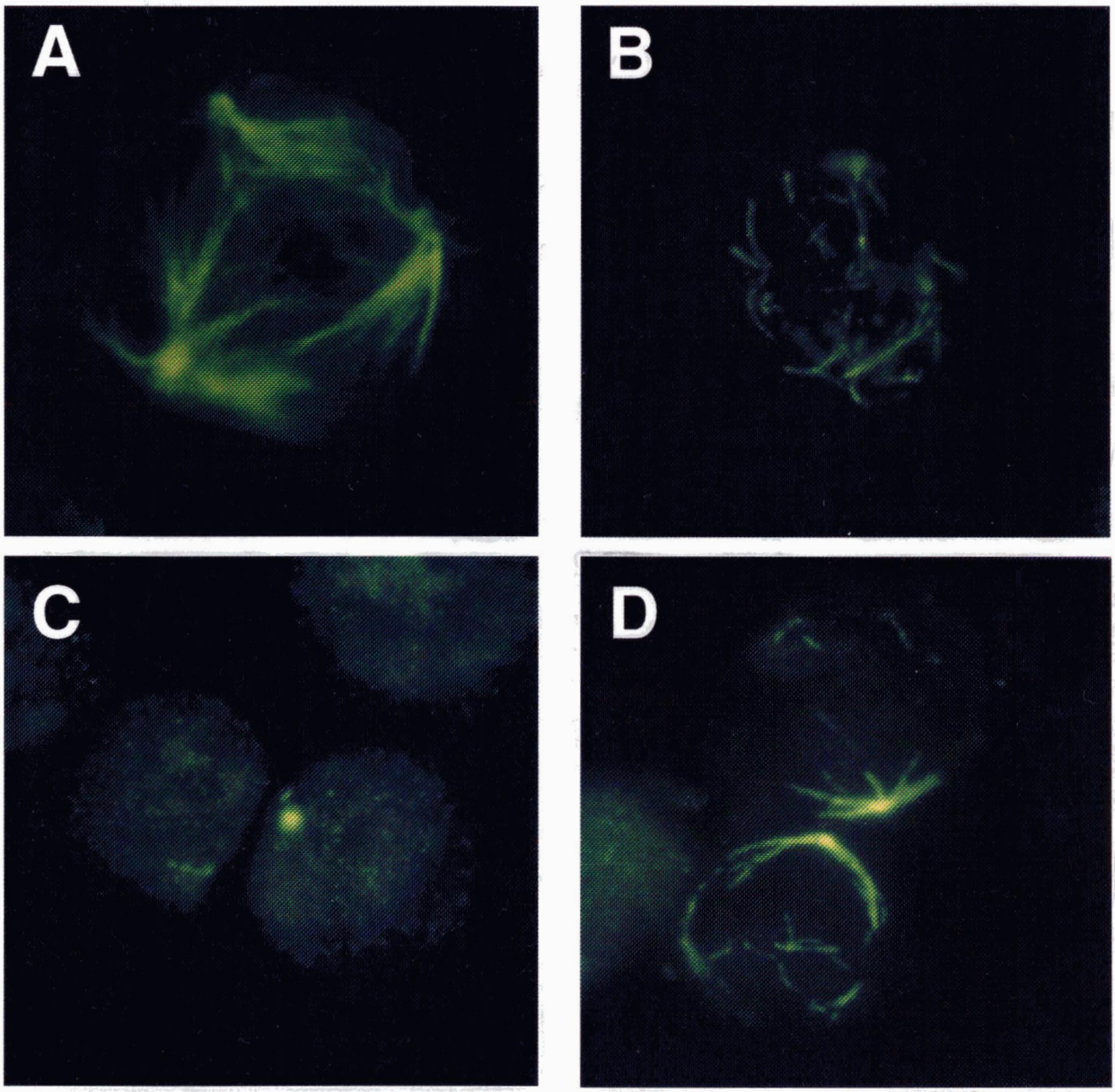


Figure 3. **Effects of 1,2,4-benzenetriol and colchicine on microtubule integrity and cytoskeletal organization in HL60 cells.**

(A) 20 μM 1,2,4-benzenetriol, **(B)** 50 μM 1,2,4-benzenetriol, **(C)** 100 μM 1,2,4-benzenetriol, and **(D)** 1 μM colchicine.

Table 1 Baseline and effect of colchicine on nuclear spot frequencies in HL60 cells using a centromeric probe specific for chromosome 9.

Treatment	Total Cells Scored	Number of spots / 1000 cells						Total Hyperdiploidy per 1000 Nuclei
		0	1	2	3	4	≥5	
Controls	10,000 (S.D.)*	0.2 (0.3)	99.2 (14.0)	893.4 (13.1)	6.3 (3.0)	0.9 (0.7)	0 (0)	7.2 (2.9)
Colchicine (0.1μM)	5,000 (S.D.)*	0.8 (1.3)	101.8 (13.3)	867.0 (25.9)	22.8 (9.4)	7.4 (5.7)	0.2 (0.4)	30.4 (12.6)

* Standard Deviation (S.D.) of 5 separate experiments is shown in parentheses. 2000 cells each for controls and 1000 cells each for colchicine treatments were scored.

Table 2 Number of hybridization domains of chromosome 9 in interphase nuclei of HL60 cells following 1, 2, 4-benzenetriol treatment for 24 h in media.

[BT] μM	Total number of scored cells	Number of domains / 1000 nuclei *						Total Hyperdiploidy per 1000 Nuclei
		0	1	2	3	4	≥5	
0	5000	0	93.2 ± 18.3	899.2 ± 16.6	5.6 ± 1.8	1.6 ± 0.5	0.4 ± 0.5	7.6 ± 2.1
5	5000	0.2 ± 0.4	84.4 ± 21.1	893.4 ± 12.6	17.0 ± 5.6	4.4 ± 4.7	0.6 ± 1.3	22.0 ± 8.7
10	5000	0	89.2 ± 12.4	884.4 ± 16.4	21.8 ± 12.4	4.4 ± 2.4	0.2 ± 0.4	26.4 ± 12.6
20	5000	0	88.4 ± 3.4	886.4 ± 3.2	19.4 ± 3.3	5.6 ± 2.4	0.2 ± 0.4	25.2 ± 5.3
50	5000	0	75.4 ± 9.7	895.0 ± 15.6	22.0 ± 5.5	6.6 ± 2.9	1.0 ± 1.2	29.6 ± 7.4
80	2000	0.5 ± 0.7	87.5 ± 9.2	888.0 ± 9.9	17.5 ± 0.7	6.5 ± 0.7	0	24.0 ± 1.4
100	1000	0	89	877	28	6	0	34

* The data presented here are the mean ± S.D. (Standard Deviation).

Table 3 Number of hybridization domains of chromosome 9 in interphase nuclei of HL60 cells following 1, 2, 4-benzenetriol treatment for 1 h in PBS.

[BT] μM	Total number of scored cells	Number of hybridization domains						Total Hyperdiploidy per 1000 Cells
		0	1	2	3	4	≥5	
0	1000	0	88	895	11	6	0	17
5	1000	0	70	907	18	4	1	23
10	1000	0	109	862	22	7	0	29
20	1000	0	90	882	22	6	0	28
50	1000	2	140	798	57	3	0	60

Table 4 Nuclear spot frequencies in HL60 cells treated with 1,2,4-benzenetriol for 24 h in media using a centromeric probe specific for chromosome 7.

[BT] μM	Total number of scored cells	Number of domains / 1000 nuclei					Total number of hyperdiploidy per 1000 cells
		0	1	2	3	4	
0	1000	0	64	929	5	2	7
5	1000	1	47	937	12	3	15
10	1000	0	53	930	17	0	17
20	1000	0	43	931	24	2	26
50	1000	0	31	950	13	6	19

Table 5 Proportion of trisomy and tetrasomy in total hyperdiploidy induced by 1, 2, 4-benzenetriol in HL60 cells.

[BT] μM	Chromosome 9				Chromosome 7	
	BT (24 h in Media) *		BT (1 h in PBS)		BT (24 h in Media)	
	Trisomy (%)	Tetrasomy (%)	Trisomy (%)	Tetrasomy (%)	Trisomy (%)	Tetrasomy (%)
0	73.4 ± 8.2	22.1 ± 8.4	64.7	35.3	71.4	28.6
5	81.6 ± 18.8	16.5 ± 15.4	78.3	17.4	80.0	20.0
10	81.4 ± 10.7	17.6 ± 9.3	75.9	24.1	100	0
20	77.6 ± 6.4	21.7 ± 5.7	78.6	21.4	92.3	7.7
50	74.8 ± 8.2	22.1 ± 7.7	95.0	5.0	68.4	31.6

* The data presented here are the mean ± S.D. (Standard Deviation).

CHAPTER FOUR

Effect of Metals, Ligands and Antioxidants on the Reactions of Oxygen with 1,2,4-Benzenetriol

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ABSTRACT

1,2,4-Benzenetriol is a reactive metabolite of the human leukemogen benzene. The genotoxicity of benzenetriol reportedly results from its ability to reduce molecular oxygen (O_2) to active species in the course of its oxidation to its corresponding quinones. The mechanism of reaction of O_2 with benzenetriol is poorly understood and little is known of the effects of metals, metal chelators, radical scavengers and antioxidants on the rate of reduction of O_2. We therefore compared candidate free radical propagators of benzenetriol's reaction with O_2 at pH 7.4 in the presence or absence of selected metal ions (Cu^{2+} and Fe^{3+}). Catalytic amounts of Cu^{2+} and Fe^{3+} accelerated the oxidation of benzenetriol (250 μM) in a dose-dependent manner. Fe^{3+} (50 μM) increased the rate of autoxidation by approximately 91% and Cu^{2+} (10 μM) increased it by 1076%. In the absence of metals, superoxide dismutase inhibited and desferrioxamine stimulated the autoxidation. In the Cu^{2+}-catalyzed reaction, superoxide dismutase neither inhibited nor stimulated, but desferrioxamine abolished the catalysis by Cu^{2+}. In the presence of Fe^{3+}, superoxide dismutase slowed the reaction, but desferrioxamine, surprisingly, did not. When both superoxide dismutase and desferrioxamine were present, the autoxidation was blocked regardless independent of the presence or absence of metals. We therefore conclude: (1) Superoxide is a propagator of sequential one-electron transfer reactions in the absence of added metals. (2) Addition of Cu^{2+} removes the dependence of the reaction on propagation by superoxide. Apparently Cu^{2+} changes the free radical propagated chain reaction to a concerted two-electron transfer, but Fe^{3+} does not. (3) The further addition of desferrioxamine restores superoxide-dependent propagation.

INTRODUCTION

Benzene is metabolized in the liver via benzene oxide to phenol, which is further hydroxylated to catechol, hydroquinone and 1,2,4-benzenetriol [Inoue et al., 1989a, b]. Each of these metabolites and a ring-opened metabolite, *t,t*-muconaldehyde, may mediate the myelotoxicity and carcinogenicity of benzene [Yardley-Jones, et al., 1991]. The chemistry of benzene metabolites are therefore of interest. Among these, the triphenolic metabolite of benzene, benzenetriol, is the most reactive toward molecular oxygen (O_2) [Greenlee et al., 1981].

Benzenetriol is a strong reductant and rapidly autoxidizes to its corresponding quinone via semiquinone radical intermediates [Greenlee et al., 1981; Bandy et al., 1990]. Its strong chemical reactivity toward O_2 is consistent with a high biological potency. Benzenetriol induces both single and double strand breaks in DNA [Lewis et al., 1988; Kawanishi et al., 1989; Li, 1992], causes sister chromatid exchanges in human lymphocytes [Erexson et al., 1985] and gene mutations (6-thioguanine resistance) in V79 cells [Glatt et al., 1989].

The current research is the third in a series of studies on the roles of quinones, oxygen and transition metal ions in the genotoxicity of benzenetriol. It complements previous studies in which benzenetriol induces micronucleus formation, aneuploidy and microtubule disruption in cultured human cells [Zhang et al., 1993a, b]. In these studies, we demonstrated that benzenetriol induces oxidative DNA damage (formation of 8-hydroxy-2'-deoxyguanosine)

both in cultured human cells *in vitro* [Zhang et al., 1993a] and in mouse bone marrow *in vivo* [Kolachana et al., 1993]. These manifestations of genotoxicity are presumed to result, at least in part, from benzenetriol's ability to reduce O_2 to reactive species during its autoxidation. Despite the evidence that benzenetriol-induced active species are cytotoxic and genotoxic, surprisingly little is known regarding the mechanisms of its reaction with O_2.

Autoxidation is the uncatalyzed oxidation of a substance exposed to O_2 in air (Uri, 1961). However, because the direct transfer of an electron pair to O_2 is spin forbidden, the sample reaction $QH_2 + O_2 \longrightarrow Q + H_2O_2$ does not occur directly. Instead, reduction of oxygen is sometimes depicted as sequence of two single-electron transfers:

(1) $QH_2 + O_2 \longrightarrow s\text{-}Q^{\bullet -} + O_2^{\bullet -} + 2H^+$

(2) $s\text{-}Q^{\bullet -} + O_2^{\bullet -} + 2H^+ \longrightarrow Q + H_2O_2$

The first of these reactions is, however, plausible only in the presence of metal ions. Transition metals are efficient catalysts of redox reactions in part because they contain unpaired electrons, and therefore their reactions with O_2 are not spin restricted (Miller et al., 1990). The "autoxidation" observed for many compounds usually turns out on closer examination to be metal catalyzed. Redox active metals can serve as free radical initiators, bypassing the unfavorable reaction (1) as shown below:

(3) $Cu^{2+}/Fe^{3+} + QH_2 \longrightarrow s\text{-}Q^{\bullet -} + Cu^{+}/Fe^{2+} + 2H^+$

(4) $Cu^{+}/Fe^{2+} + O_2 \longrightarrow O_2^{\bullet -} + Cu^{2+}/Fe^{3+}$

Iron and copper are the predominant among the transition metals which, if decompartmentalized, can become prooxidant *in vivo*. Their addition to the culture medium increases the genotoxicity of benzenetriol as measured by DNA strand breaks [Lewis et al., 1988; Kawanishi et al., 1989; Li, 1992], oxidative DNA damage and micronucleus formation [Zhang et al., 1993a].

The autoxidation of benzenetriol is mediated by reactive oxygen species including superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\bullet}$) [Greenlee et al., 1981]. In the process, benzenetriol is oxidized to its major oxidative product, 2-hydroxy-1,4-benzoquinone (2-OH-BQ) [Mason, 1949]. The reduction of O_2 by benzenetriol can occur either by sequential transfer of individual electrons (one-electron transfer), or by concerted transfer of two electrons within the same solvent cage (two-electron transfer) [Bandy et al., 1990]. The one-electron propagators, in theory, are $O_2^{\bullet -}$ from the reduction of O_2, the corresponding semiquinone radical anion ($s\text{-}Q^{\bullet -}$) from the oxidation of benzenetriol, and perhaps $HO^{\bullet}$ in the further reduction of H_2O_2. The concerted two-electron reaction, in theory, requires metal ions to release the spin restriction.

The observation of a stimulation or inhibition by a given scavenger or ligand indicates which propagators are accessible to scavenging. We therefore undertook a systematic investigation of the effects of metals, metal chelators, scavengers and antioxidants on the autoxidation of benzenetriol. Specifically we sought to learn the roles of metals and chelators in determining: (1) whether one-electron transfer or two-electron transfer is dominant; (2) which propagators are predominantly involved in the reaction; and, (3) under what circumstances a

given scavenger or ligand will stimulate or inhibit the autoxidation of benzenetriol.

We therefore examined the acceleration of the autoxidation of benzenetriol by Cu^{2+} and Fe^{3+}, and the influence of superoxide dismutase or desferrioxamine on the pathway for the reduction of oxygen. These results are interpreted in the light of our earlier finding that Cu^{2+} changes the mechanism of benzenetriol-induced micronucleus formation from aneuploidy to clastogenicity. The data have implications regarding the roles of transition metals and benzenetriol in benzene-induced human leukemia.

MATERIALS AND METHODS

Reagents. 1,2,4-Benzenetriol (99%) was purchased from Aldrich Chemical Co. (Milwaukee, WI). All the chemicals for phosphate buffers (analytic purity) were obtainable from American Scientific and Chemical (Seattle, WA). Cupric sulfate and ferric chloride were purchased from Fisher Scientific Co. (Fair Lawn, NJ). Superoxide dismutase (4050 U/mg, E. C. 1.15.1.1) was from Pharmaceutical Inc. (Mountain View, CA). Catalase (65,000 U/mg, E. C. 1.11.1.6) was from Boehringer Mannheim Co. (Indianapolis, IN). Desferrioxamine was a gift from CIBA Pharmaceutical Co. (Summit, NJ). Sodium formate was purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ) and mannitol was from Sigma Chemical Co. (St. Louis, MO).

Anaerobic solution of 1,2,4-Benzenetriol. 1,2,4-Benzenetriol stock solution (25 mM) was prepared daily by dissolving benzenetriol in argon-saturated deionized-distilled water, and then flushing with argon for 5 min. Rubber stoppers used to seal the Virtis vials were boiled and evacuated to remove any air in the rubber. A gas-tight syringe was used to inject aliquots of the benzenetriol solution into a measured amount of aerated buffer solution.

Assay Procedures. Experiments were conducted in air-saturated 50 mM phosphate buffer, pH 7.4, at 25 °C. 2.5 ml of the buffer was transferred to a cuvette and appropriate combinations of metals, chelators and scavengers (indicated in legends of the figures) were added. Reactions were initiated by addition of a 25 μl aliquot of the anaerobic preparation of benzenetriol with simultaneous agitation by a cuvette mixer. Benzenetriol (λ_{max} = 290 nm) at

physiological pH (7.4) spontaneously autoxidizes to its corresponding quinone product, 2-OH-BQ. The oxidized product absorbs in the ultraviolet (λ_{max} = 267 nm) and in the visible (λ_{max} = 490 nm). The formation of 2-OH-BQ was monitored spectrophotometrically at 490 nm in this study. Further details are given in the legends of the figures.

Data Analyses. Data were collected on both a linear strip chart recorder and simultaneously digitized using a twelve bit analogue-digital converter, and were transferred to an IBM mainframe computer using a microprocessor data buffer/coupler locally designed and constructed. Digitized voltages were converted to absorbance values at 490 nm. Subsequent data analyses were performed using Excel programme. To determine the molar extinction coefficient (ε), it was assumed that benzenetriol was completely converted into 2-OH-BQ when the reaction approached substantial completion. The ε at pH 7.4 was then derived from the slope of the plot of maximal absorption at 490 nm as a function of the initial concentration of benzenetriol. Rate constants (*k*) were obtained from the slope of ln ($\underline{a}$ / $\underline{a}$-$\underline{x}$) as a function of time, where "$\underline{a}$" is the initial concentration of benzenetriol and "$\underline{x}$" is the concentration of 2-OH-BQ. The initial rates of benzenetriol autoxidation were the slopes determined by linear regression over the *p*-quinone product (μM) formation as function of time (s) during the first 60 s reactions.

Statistical Analysis The statistical comparisons of individual means were compared using a one-tailed Student t-test. The level for significance was chosen in advance to be $p < 0.05$.

RESULTS

Characterization of Benzenetriol Autoxidation

Since the autoxidation of benzenetriol has not been well characterized, we determined selected characteristics of the reaction including: the molar extinction coefficient of the quinonoid product, rate constants and order of the reaction. The molar extinction coefficient (ε) at λ_{max} = 490 nm, pH 7.4 was 2079 M^{-1} cm^{-1} from regression analysis of the plot of absorbance versus benzenetriol concentration. This ε value is in agreement with the earlier reports for ε of 2051 M^{-1} cm^{-1} (λ_{max} = 480 - 485 nm, pH 5.4) (Mason, 1949) and 2042 M^{-1} cm^{-1} (λ_{max} = 483 nm, pH > 12) (Corbett, 1970).

Next, the linearity of a plot of ln (a / a-x) against time (t) (Fig. 1a) indicates that benzenetriol autoxidation followed the first order kinetics with respect to time. From the slope, the apparent first order rate constant (k) for 2-OH-BQ formation was 3.08 x 10^{-3} s^{-1}. Initial rates of benzenetriol autoxidation (10 - 500 μM) were estimated from the slopes of the reaction during the first 60 s following initiation. The initial rate increased as the concentration of benzenetriol increased (Fig. 1b). Figure 1b shows that the dependence of rate on concentration corresponds to a fractional order reaction with the apparent order being 0.6 with respect to benzenetriol.

Catalytic Effect of Cu^{2+} and Fe^{3+}

Since cuprous copper or ferrous iron can generate active oxygen directly, only cupric copper and the ferric form of iron were tested in this study. The dependence of the rate on the concentrations of Cu^{2+} and Fe^{3+} is shown in Figure 2a. The oxidation rate increased as the concentration of Cu^{2+}

increased, following saturation kinetics. When the concentration of Fe^{3+} increased, the rate linearly increased in a dose-dependent manner (Fig. 2a). Fe^{3+} (50 μM) and Cu^{2+} (10 μM) catalyzed the oxidation of benzenetriol in a time-dependent manner (Fig. 2b). As shown in Table 1, 50 μM Fe^{3+} ($t_{1/2}$ =104 s) doubled the rate of oxidation compared with the control (203 s), while 10 μM Cu^{2+} enhanced the oxidation by a factor of 12 (17 s). Further, Fe^{3+} (50 μM) accelerated the initial rate of 2-OH-BQ formation by approximately 91% in comparison with control, and at only 10 μM, Cu^{2+} accelerated it by 1076% (Table 1). Thus, Cu^{2+} was a more efficient catalyst of benzenetriol oxidation than Fe^{3+}, by about 12-fold.

Inhibitory Effects of Superoxide Dismutase and Catalase

In the absence of metal ions, both superoxide dismutase (25 U/ml) and catalase (25 U/ml) inhibited the autoxidation of benzenetriol by 74% and 27%, respectively (Table 2). At 2 U/ml, superoxide dismutase still slowed the reaction by 60%, but catalase did not (data not shown). Thus inhibition by superoxide dismutase was considerably more effective than that by catalase. A combination of both enzymes (each 2 U/ml, or 5 or 12.5 U/ml) inhibited synergistically, inducing a prolonged latent period (Fig. 3). Inactivated superoxide dismutase and catalase failed to inhibit (data not shown).

However, in the presence of Cu^{2+} (10 μM), catalase (25 U/ml) inhibited the reaction by 35% (Table 2), but superoxide dismutase (2 and 25 U/ml) neither inhibited nor accelerated it (Fig. 4). Moreover, a combination of both enzymes (each 12.5 U/ml) did not significantly inhibit the reaction (Table 2). In contrast, superoxide dismutase (2 or 25 U/ml) significantly inhibited the Fe^{3+} (50 μM) catalyzed reaction by 28% or 64% compared with the metal control,

respectively (Fig. 4). Therefore, Cu^{2+} removes the sensitivity of benzenetriol oxidation to superoxide dismutase, but Fe^{3+} does not.

Stimulation or inhibition by Desferrioxamine

Unexpectedly, desferrioxamine (0.5 mM) stimulated the autoxidation by 83% when no metals were added (Fig. 5). It counteracted catalysis by Cu^{2+} (10 μM) but did not diminish the effect of Fe^{3+} (50 μM). Desferrioxamine inhibited the Cu^{2+} catalyzed oxidation by 94% but stimulated the Fe^{3+} catalyzed reaction by only 20% in comparison with their own metal controls (Table 2). Thus, stimulation rate by desferrioxamine was blocked by the addition of Cu^{2+}, whereas the addition of Fe^{3+} rendered desferrioxamine inhibiting.

Whether or not Cu^{2+} or Fe^{3+} were added, the oxidation of benzenetriol was inhibited virtually completely (no detectable progress in 24 h) when both superoxide dismutase and desferrioxamine were present simultaneously (Fig. 5). Catalase combined with desferrioxamine did not block the reaction but merely diminished approximately 30% of the stimulatory effect of desferrioxamine with metals absent (Table 2).

Effects of Formate and Mannitol

Formate and mannitol neither inhibited nor stimulated benzenetriol oxidation significantly whether or not metals were added (Table 2). In the absence of metals, however, formate (50 mM) combined with superoxide dismutase (25 U/ml) or both superoxide dismutase and catalase (each 12.5 U/ml) inhibited the reaction by 63% or 79%, respectively (Table 2). In contrast, a combination of catalase (25 U/ml) and formate (50 mM) did not significantly inhibit it. Thus, $HO^{\bullet}$ plays no kinetically detectable roles in the reaction.

DISCUSSION

Cu^{2+} is a more effective catalyst than Fe^{3+}.

Oxidation of benzenetriol at physiological pH occurs both spontaneously (autoxidation) or enzymatically (through the action of myeloperoxidase) [Subrahmanyam, et al., 1992]. In addition, the oxidation is catalyzed by iron and copper salts. Cu^{2+} was a more effective catalyst than Fe^{3+} (Fig. 2b), which is consistent with findings from the previous biological studies. For example, Cu^{2+} was more efficient than Fe^{3+} at stimulating benzenetriol-induced DNA strand breaks [Kawanishi et al., 1989; Li, 1992] and at causing DNA base damage in the presence of H_2O_2 [Aruoma et al., 1991]. The differences between iron and copper *in vivo* may reflect differences in the effectiveness of biological compartmentation, or different rates of reduction of the metal complexes or rate constants for the Fenton reaction [Koppenol and Butler, 1985]. The rate constant for the reaction of Cu^{+} with H_2O_2 is much higher than the corresponding one for iron [Walling, 1982].

Superoxide propagates the redox reactions in the absence of added metals.

When metal ions were omitted from the system (Fig. 3), the ability of superoxide dismutase to inhibit benzenetriol autoxidation conforms the findings by Greenlee et al. (1981) and Bandy et al. (1990). The extent of inhibition reflects the importance of superoxide as a propagator of free radical chain reactions:

Initiation: (1) $QH_2 + O_2 \Longleftrightarrow s\text{-}Q^{\bullet -} + O_2^{\bullet -} + 2H^+$

Propagation: (5) $QH_2 + O_2^{\bullet -} \Longleftrightarrow s\text{-}Q^{\bullet -} + H_2O_2$

(6) $s\text{-}Q^{\bullet -} + O_2 \Longleftrightarrow Q + O_2^{\bullet -}$

Termination: (7) $s\text{-}Q^{\bullet -} + s\text{-}Q^{\bullet -} + 2H^+ \Longleftrightarrow Q + QH_2$

(8) $O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \Longleftrightarrow O_2 + H_2O_2$

(2) $s\text{-}Q^{\bullet -} + O_2^{\bullet -} + 2H^+ \Longleftrightarrow Q + H_2O_2$

(QH_2: benzenetriol; $s\text{-}Q^{\bullet -}$: semiquinone; Q: 2-OH-benzoquinone)

The initiation step (1) involves redox cycling of metal ions (see reaction (3) and (4) in Introduction). Reactions (5) and (6) show how superoxide can propagate a sequential one-electron transfer pathway. In addition, semiquinone and hydroxyl radicals are candidate propagators of one-electron transfer chain reactions. Since neither formate nor mannitol influence the rate of benzenetriol autoxidation, $HO^{\bullet}$ is excluded as a kinetically significant propagator. Since the initial rate was not significantly enhanced when benzenetriol was exposed to O_2 in the presence of reaction products (data not shown), we conclude that $s\text{-}Q^{\bullet -}$ is not a significant propagator of the one-electron transfer in autoxidation of benzenetriol. Note that semiquinone propagation is reflected in the same reactions (5 and 6) as superoxide propagation, but in the reverse order.

Effect of Cu^{2+} on the free radical-propagated chain reactions

We interpret the failure of superoxide dismutase (25 U/ml) to alter the rate of benzenetriol oxidation in the presence of Cu^{2+}, on the basis that Cu^{2+} changes the sequential one-electron transfer pathway to a concerted two-electron transfer pathway*. The *o*-quinol of benzenetriol provides for strong binding of metal ions, encouraging its oxidation by such an inner sphere mechanism. Electron transfer within a [BT-Cu-O_2] inner sphere complex may well preclude superoxide release by allowing synchronous transfer of two electrons, from the bound benzenetriol via Cu^{2+} to O_2 (Fig. 6). The possible mechanisms as follows:

(a) Metal-binding: $BT + Cu^{2+} \longrightarrow BT\text{-}Cu + 2H^+$

(b) Ternary complex formation: $BT\text{-}Cu + O_2 \longrightarrow [BT\text{-}Cu\text{-}O_2]$ **

(c) Two-electron transfer: $[BT\text{-}Cu\text{-}O_2] + 2H^+ \longrightarrow 2\text{-}OH\text{-}BQ + H_2O_2 + Cu^{2+}$

Taken together, the sum of reactions (a) + (b) + (c) is equivalent to reactions (1)+(8), the overall reaction being:

$$BT + O_2 \longrightarrow 2\text{-}OH\text{-}BQ + H_2O_2$$

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* By a two-electron pathway we mean that any one electron intermediates are too transient, or too tightly sequestered in the solvent cage to be accessible to scavengers.

** This may be a true reaction intermediate or merely an inner sphere "collision complex".

Figure 6 shows the putative chemical structure of a benzenetriol-Cu-O_2 complex and the schematic flow of two electrons from benzenetriol via Cu^{2+} to O_2. In the long term, H_2O_2 released from the ternary complex by the concerted inner sphere transfer of two electrons is also a candidate propagator. However, catalase inhibited the Cu^{2+}-catalyzed reaction by 35% (Table 2), suggesting that although Cu^{2+} may well facilitate peroxidative oxidation of benzenetriol, the processes are largely subsequent to the rate determining step.

Desferrioxamine restores superoxide sensitivity to the Cu^{2+}-stimulated reaction. The concerted two-electron reaction is disrupted by the presence of metal chelators, presumably because they remove the metal from association with partly reduced O_2, forcing the propagators into the solvent where they are accessible to scavengers.

Cu^{2+} also accelerates the autoxidation of hydroquinone, another metabolite of benzene, and increases the cytotoxicity of hydroquinone in bone marrow stromal cells [Li and Trush, 1993]. Superoxide dismutase stimulates the oxidation of hydroquinone to benzoquinone [Greenlee et al., 1981; Bandy et al., 1991]. To the extent that hydroquinone resembles benzenetriol, however, Cu^{2+}-mediated oxidation of hydroquinone should be superoxide dismutase inhibitable. Catalase, on the other hand, fails to protect against cytotoxicity induced by hydroquinone with Cu^{2+} present [Li and Trush, 1993].

Effect of Fe^{3+} on the reaction mechanism

In contrast to the actions of added Cu^{2+}, addition of Fe^{3+} fails to diminish dependence on the propagation by superoxide in one-electron transfer steps. Thus, the hypothetical ternary complex, [BT-Fe-O_2] apparently does not permit

inner sphere electron transfer to O_2. A partial explanation might be that superoxide dismutase binds to Fe^{3+} more effectively than Cu^{2+}. Further studies are needed to explore this and other possibilities.

Cu^{2+} also changes mechanisms of benzenetriol-induced micronucleus formation.

Cu^{2+} enhances benzenetriol-induced oxidative DNA damage and micronucleus formation. Moreover, Cu^{2+} changes the mechanism of benzenetriol-induced micronucleus formation from kinetochore-positive (mitotic aneuploidy) to kinetochore-negative (clastogenicity) [Zhang et al., 1993a]. It presumably reflects Cu^{2+}-induced changes in the mechanism of autoxidation of benzenetriol. We have seen that the presence of Cu^{2+} decreases superoxide-propagated one-electron transfer, while the Cu^{2+}-induced increase in overall reaction rate stimulates production of H_2O_2 by Cu^{2+}-mediated two-electron transfer.

Benzenetriol, therefore, causes chromosomal aberrations by two distinct mechanisms. It can disrupt microtubules by oxygen-derived autoxidation products (its corresponding quinones and active oxygen species) [Zhang, et al., 1993b], and can oxidatively damage DNA by reactive oxygen species generated during its autoxidation. Added Cu^{2+} increases chromosomal DNA (as opposed to microtubular) damage by increasing the yield of H_2O_2 at the expense of $O_2^{\bullet -}$. On this basis, the presence of Cu^{2+} directs the H_2O_2-mediated damage toward the chromosomes, whereas the absence of Cu^{2+} directs the $O_2^{\bullet -}$-mediated damage toward the microtubules. One can assume that Cu^{2+}-catalyzed site specific formation of $HO^{\bullet}$, which in turn mediates: DNA base modification, strand breaks and the fragmentation of DNA [Halliwell and

Aruoma, 1991]. The participation of H_2O_2 in microtubular breakage in the absence of added Cu^{2+} is consistent with protection by catalase against destruction of tubulin by the benzenetriol analog, 6-hydroxydopamine (Davison et al., 1986). Taken together, these observations suggest that the additional genotoxicity of benzenetriol in the presence of Cu^{2+} results from the ability of Cu^{2+} to reduce O_2 to H_2O_2 and further to produce $HO^{\bullet}$ radicals. The roles of copper are likely: 1) to accelerate H_2O_2 production, 2) to catalyze formation of active species in a Fenton-type reaction, and 3) to direct the site-specific radical production to a site where the copper is bound (i.e. to the chromosomal DNA in the case of copper mediated micronucleus formation) [Chevion, 1988].

Desferrioxamine stimulates benzenetriol autoxidation by lowering the reduction potential of iron.

The effect of desferrioxamine is conditioned by the presence or absence of metal ions. Thus desferrioxamine stimulated autoxidation (Fig. 5) when the metals were not added but inhibited by counteracting the effects of added Cu^{2+} (but not Fe^{3+}). Desferrioxamine is an avid chelator of both Fe^{3+} and Cu^{2+}. The chelation of iron diminishes reducibility and accessibility, but simultaneously increases the ability of iron to reduce O_2 by lowering its reduction potential. The optimal reduction potential for catalysis by a redox cycling metal is approximately midway between those of the reduction potentials of electron donor and electron acceptor [Davison et al., manuscript in preparation]. Since benzenetriol is a very strong reductant, even though the reduction potential of unchelated iron is high, desferrioxamine may move it closer to this optimal value between O_2 and benzenetriol. In contrast, the [Cu^{2+}-desferrioxamine] complex has a reduction potential incompatible with redox cycling.

Combination of superoxide dismutase and desferrioxamine blocks both superoxide-propagated and Cu^{2+}-mediated pathways.

Although desferrioxamine stimulates autoxidation of benzenetriol when present alone, it strongly inhibits in the presence of small quantities (2 U / ml) of superoxide dismutase (Fig. 5). Thus desferrioxamine acted synergistically with superoxide dismutase to prevent autoxidation of benzenetriol, irregardless of the presence or absence of the metal ions. The presence of desferrioxamine blocks the metal propagated inner sphere mechanism, while the presence of superoxide dismutase blocks the propagation by superoxide. Arrest of the reaction when both one-electron and two-electron pathways were blocked implies that there is no additional pathway independent route for the aerobic oxidation of benzenetriol. In the simultaneous presence of desferrioxamine and superoxide dismutase, benzenetriol is inert toward molecular oxygen.

Conclusion

In summary, oxidant-mediated genotoxicity of benzenetriol is a complicated and multifactorial process. It involves the reactions of oxygen with benzenetriol, and production of oxygen-derived active species and quinones. Cu^{2+} stimulates the oxidation of benzenetriol, which is consistent with its effect on enhancing the benzenetriol-induced genotoxicity. Cu^{2+} further changes mechanisms of the reaction from $O_2^{\bullet -}$-propagated one-electron transfer to Cu^{2+}-mediated two-electron transfer, which correlates with the changes in the pattern of benzenetriol-induced micronucleus formation from kinetochore-positive to kinetochore-negative. We, therefore, conclude that the oxidation of benzenetriol and the catalysis of Cu^{2+} may play an important role in the benzenetriol-induced genotoxicity.

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Legends for Figures:

Figure 1. Kinetics of 1,2,4-benzenetriol autoxidation.

Accumulation of product was monitored by the increase in absorption at 490 nm. The reaction was initiated by addition of stated concentrations of benzenetriol to 50 mM phosphate buffer solution pH 7.4, 25°C. Details of the procedures are described in Material and Methods.

Fig. 1a: **Rate constant (*k*) and order of the reaction with respect to time.** Ln (*a* / *a-x*) is plotted as a function of time (*t*) during the first 3 min of the reaction at 10 s interval. The ***k*** is the slope of this linear curve from the regression analysis. The reaction is first order kinetics with respect to time. The initial concentration of benzenetriol is 250 μM.

Fig. 1b: **Dependence of the autoxidation rate on concentration of benzenetriol.** Log of initial oxidation rate is plotted against log concentration of benzenetriol. The concentrations tested were 10, 30, 50, 100, 200, 250 and 500 μM of benzenetriol. The fractional order with respect to benzenetriol was determined as the slope of the linear curve.

Figure 2. Catalysis of autoxidation of benzenetriol by copper and iron salts.

Following the addition of stated concentrations of metal ions, the reaction was initiated by the addition of 250 μM benzenetriol as described in Figure 1.

Fig. 2a: **Cu^{2+} and Fe^{3+} accelerate the autoxidation of benzenetriol in a dose-dependent manner.** Reactions were conducted in the presence of 2, 4, 10, 20 and 40 μM Cu^{2+} or 10, 20, 50, 100, and 200 μM Fe^{3+}. Absorbance of the *p*-quinone product at 30 s after initiation of the reaction is plotted on the log scale of the vertical axis against log concentration of metal ions.

Fig. 2b: **Cu^{2+} and Fe^{3+} accelerate the autoxidation of benzenetriol in a time-dependent manner.** Where indicated, the concentrations of Cu^{2+} and Fe^{3+} were 10 and 50 μM, respectively. Benzenetriol (BT) at 250 μM was used in both the absence and the presence of metal ions. The absorbance at 490 nm is plotted as a function of time. The half-life time ($t_{1/2}$) was obtained from this figure.

Figure 3. Inhibition of autoxidation of benzenetriol by superoxide dismutase or catalase individually or in combination.

In the absence or presence of antioxidant enzymes, the reaction was initiated by the addition of 250 μM benzenetriol (BT) as described in Figure 1. Where indicated, 25 U / ml of superoxide dismutase (SOD) or catalase (CAT) were present in the reaction medium prior to initiating the reaction. In combination, both enzymes were present in the medium at equal concentrations (2, 5, and 12.5 U / ml).

Figure 4. Effects of superoxide dismutase in the presence of Cu^{2+} and Fe^{3+} on the autoxidation of benzenetriol.

Percentage of control ($\{A490_{[test]} / A490_{[control]}\} \times 100$) was calculated by dividing A490 nm after 30 s for the test reaction by A490 nm (0.0412 ± 0.0016)

for the control reaction (250 μM benzenetriol with no scavengers or metals present). The percentage of control is plotted on the log scale of the vertical axis. The error bars represent SEM (the standard error of means), and the star (*) indicates the estimated SEM from only one observation. The solid dot indicates a significant difference from the Fe^{3+} control ($p < 0.005$). Reaction conditions and abbreviations used are the same as indicated in the legend of Figure 3.

Figure 5. Autoxidation of benzenetriol: Stimulation and inhibition by desferrioxamine in the presence and absence of superoxide dismutase and metal ions.

Reaction conditions were as in Figures 3 and 4 except that 0.5 mM desferrioxamine (DEF), and 25 U / ml superoxide dismutase (SOD) were included where indicated. Percentage of control was calculated as in Figure 4. The black bar indicates benzenetriol with or without metal ions, in the absence of both desferrioxamine and superoxide dismutase (BT ± metals). The slant shaded bar represents the presence of 0.5 mM desferrioxamine without superoxide dismutase (+DEF). The dot shaded bar stands for the presence of both 0.5 mM desferrioxamine and 25 U / ml superoxide dismutase (+SOD+DEF) in the reaction. The error bars and the stars were as described in Figure 4. The solid dot ($p < 0.005$) and the open circle ($p<0.01$) indicate significant differences from their own controls.

Figure 6. Possible chemical structure of [benzenetriol-Cu-oxygen] and the schematic electron flow from 1,2,4-benzenetriol via Cu^{2+} to oxygen. The arrows from oxygen to Cu (O -----> Cu) indicate coordinate covalent bonds. Other arrows show the possible electron transformation.

Figure 1a

The rate constant of BT autoxidation is 3.08 x 10(-3).

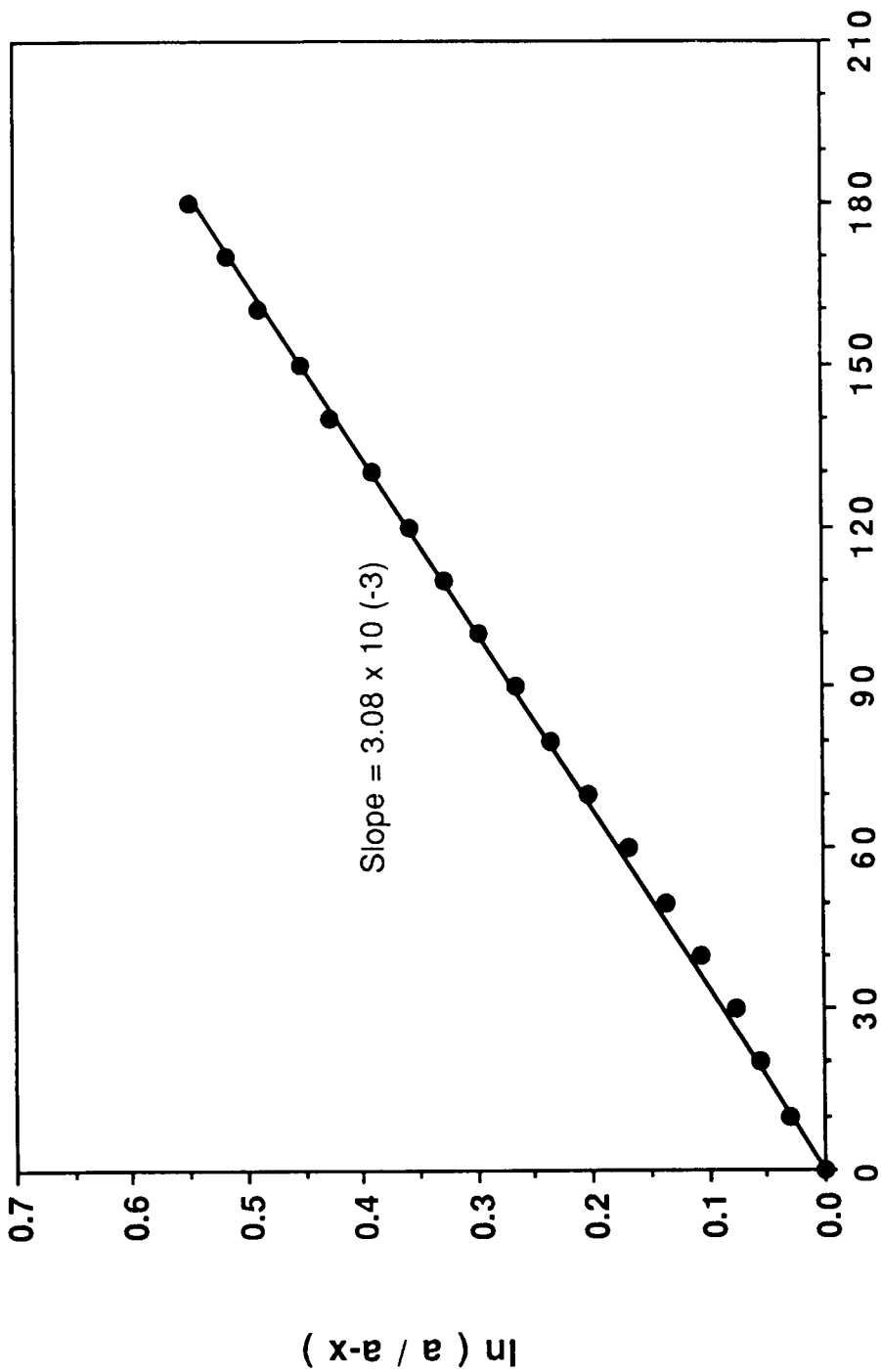


Figure 1b

Increasing concentrations of BT increase initial rate of the reaction.

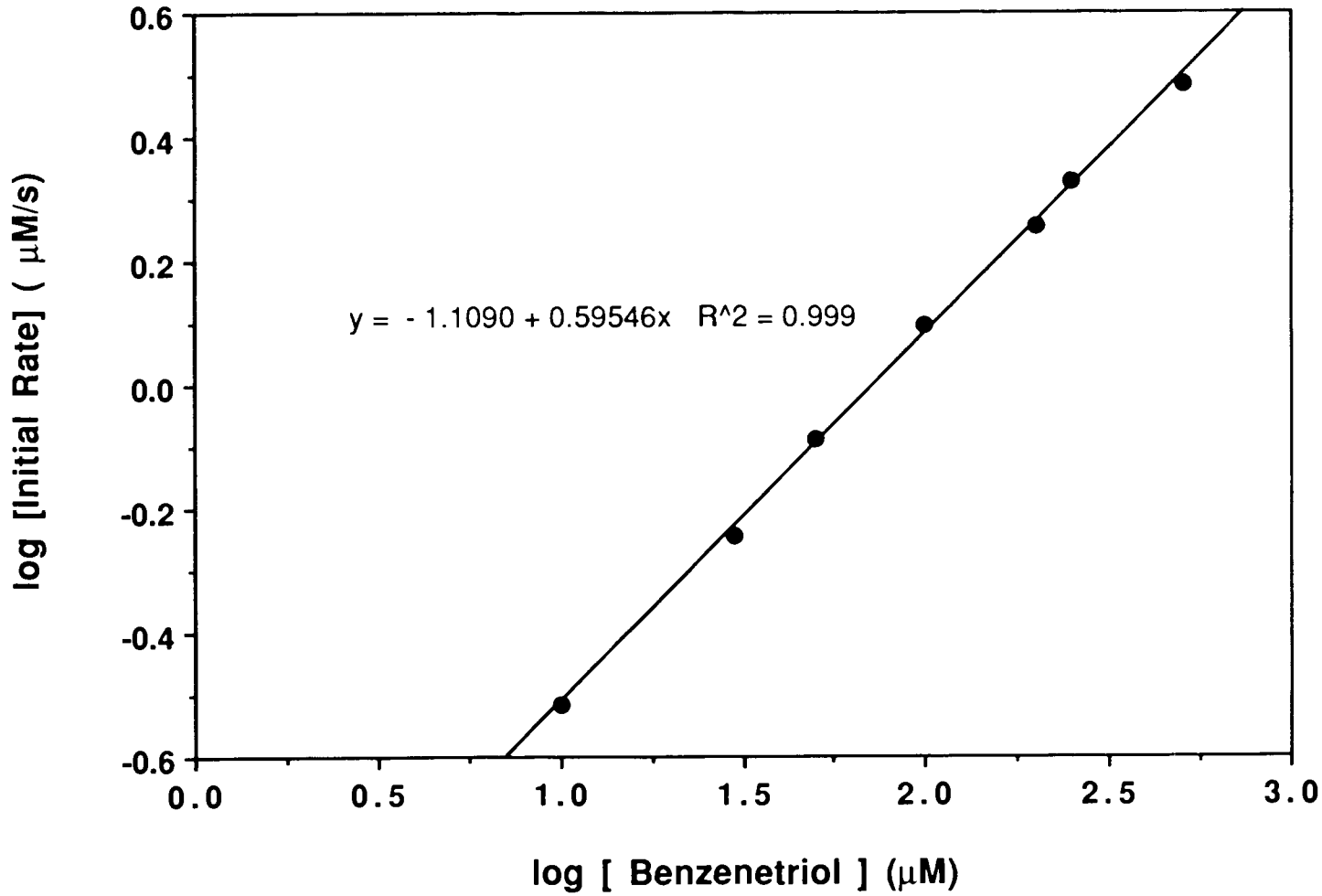


Figure 2a

Cu and Fe accelerate BT autoxidation in a dose-dependent manner.

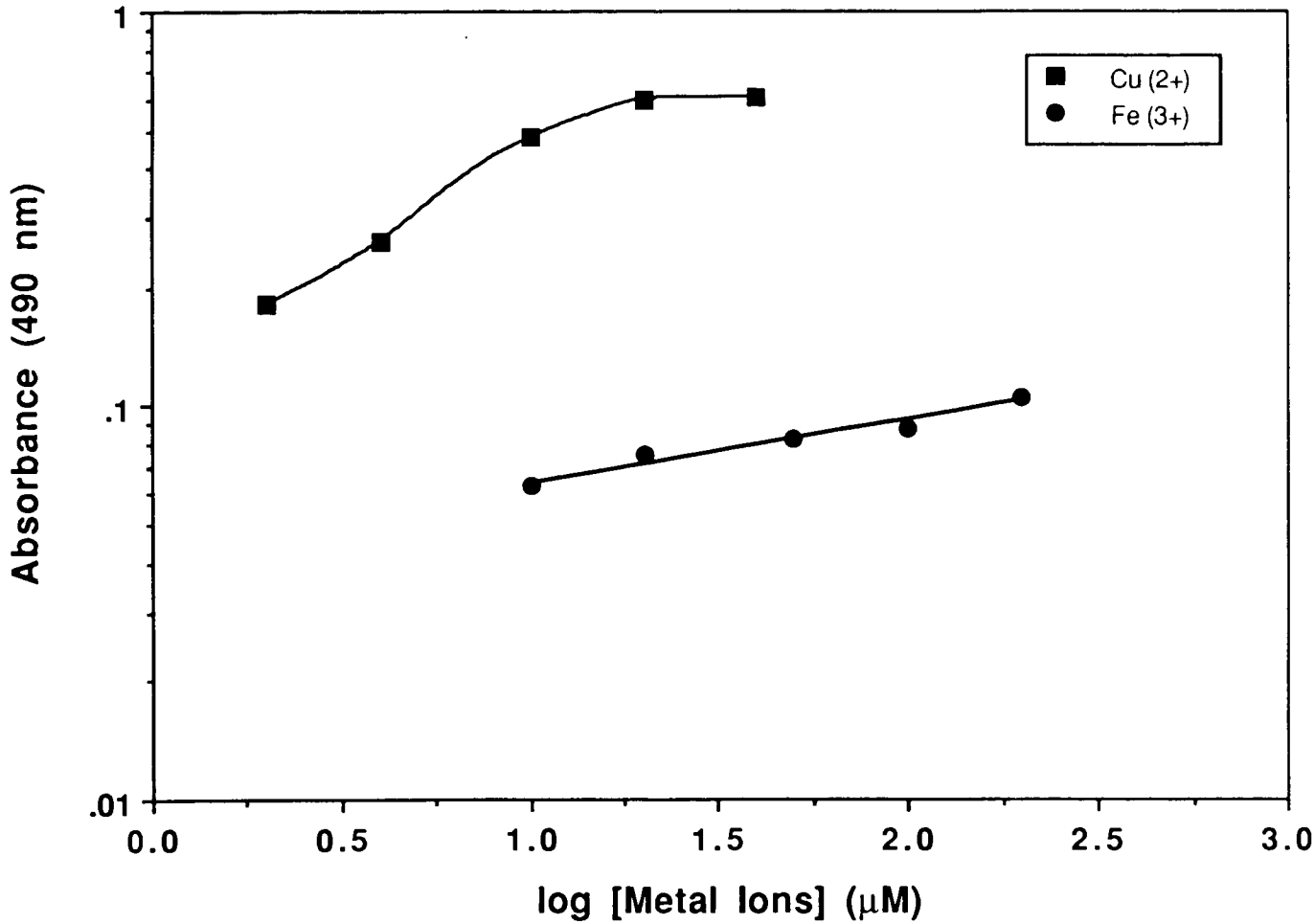


Figure 2b

Cu is more efficient than Fe in the catalysis of BT autoxidation.

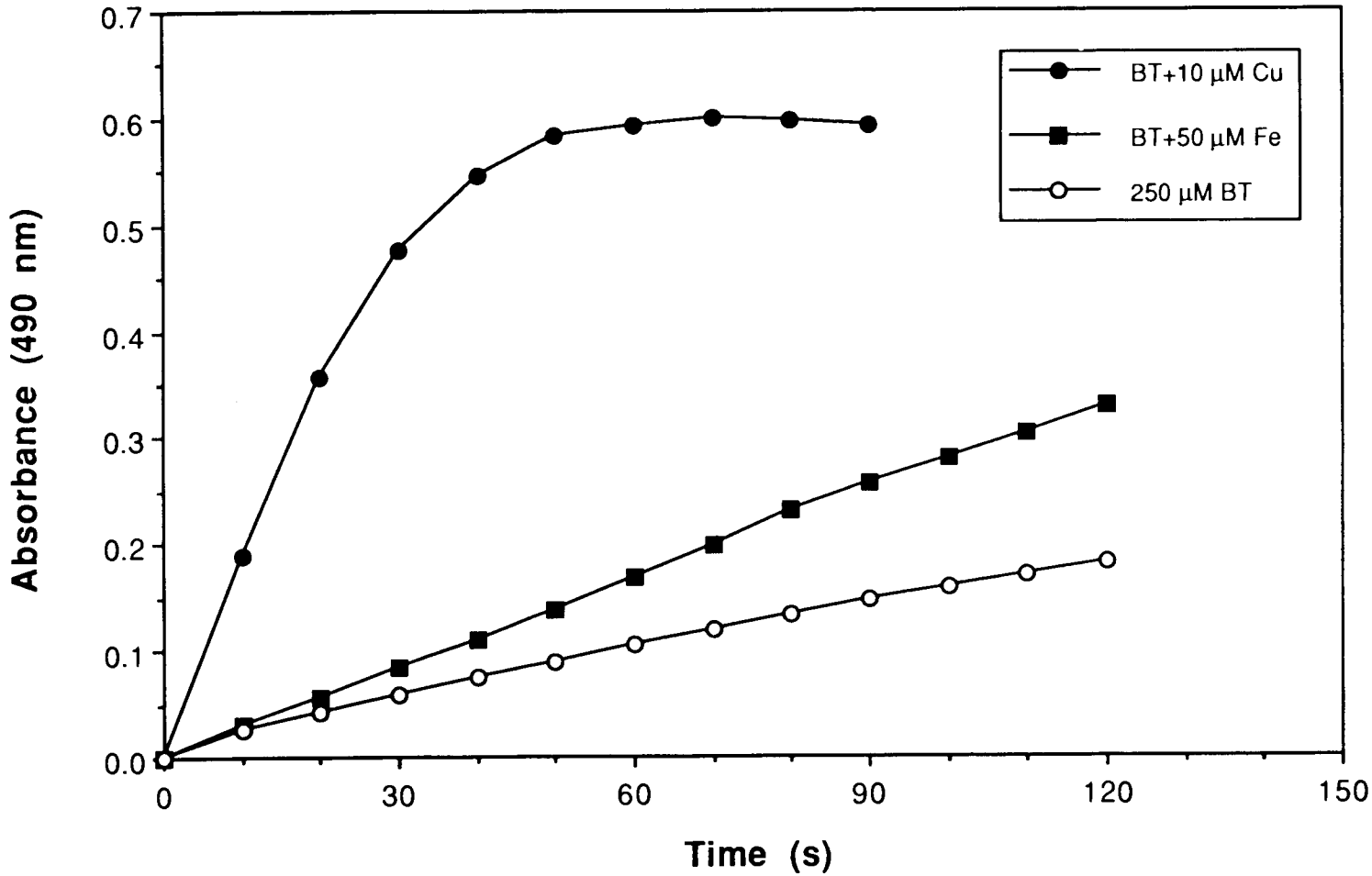


Figure 3

Superoxide dismutase and catalase inhibit autoxidation of benzenetriol.

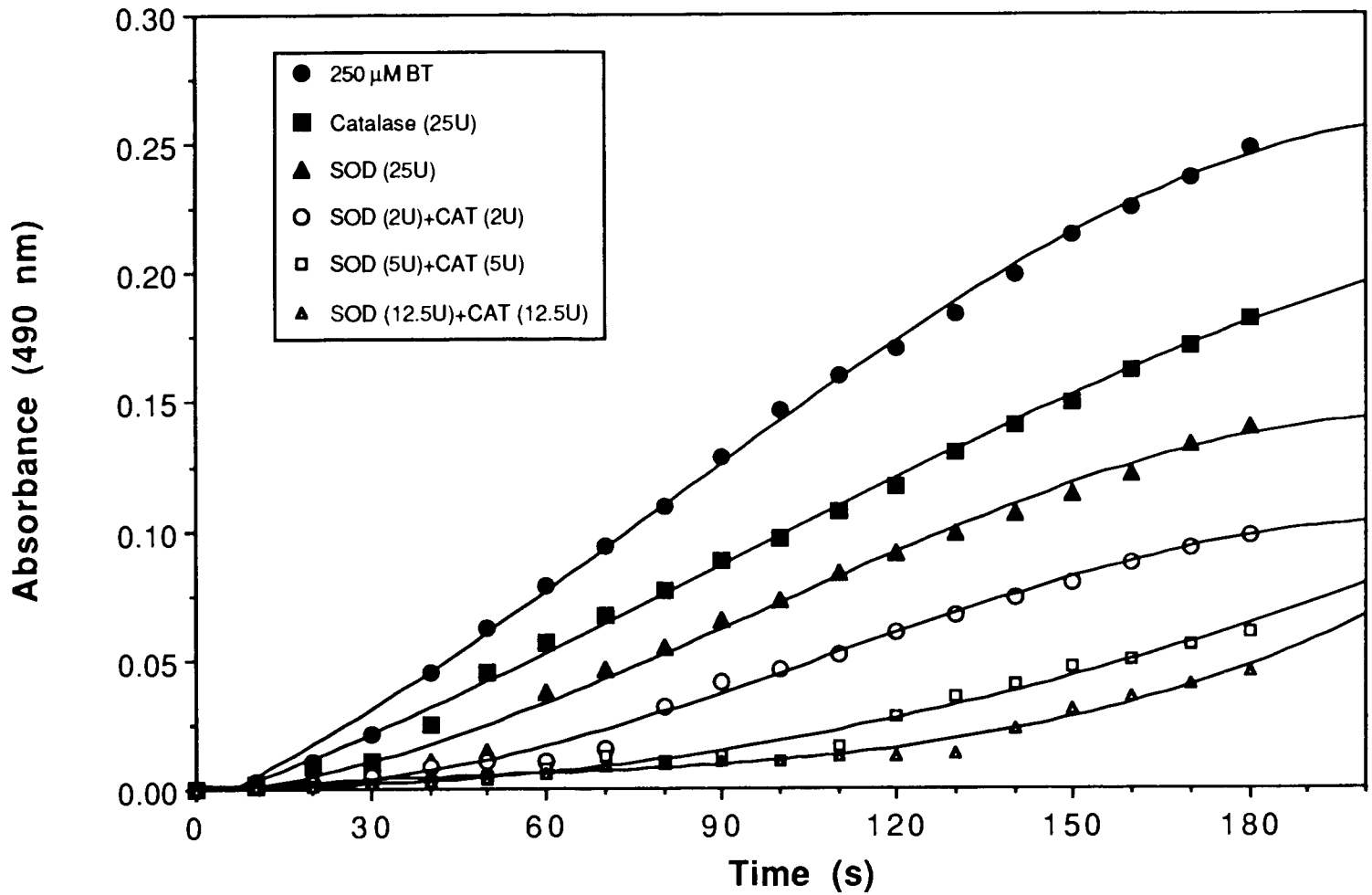


Figure 4

Effects of superoxide dismutase in the presence of metal ions

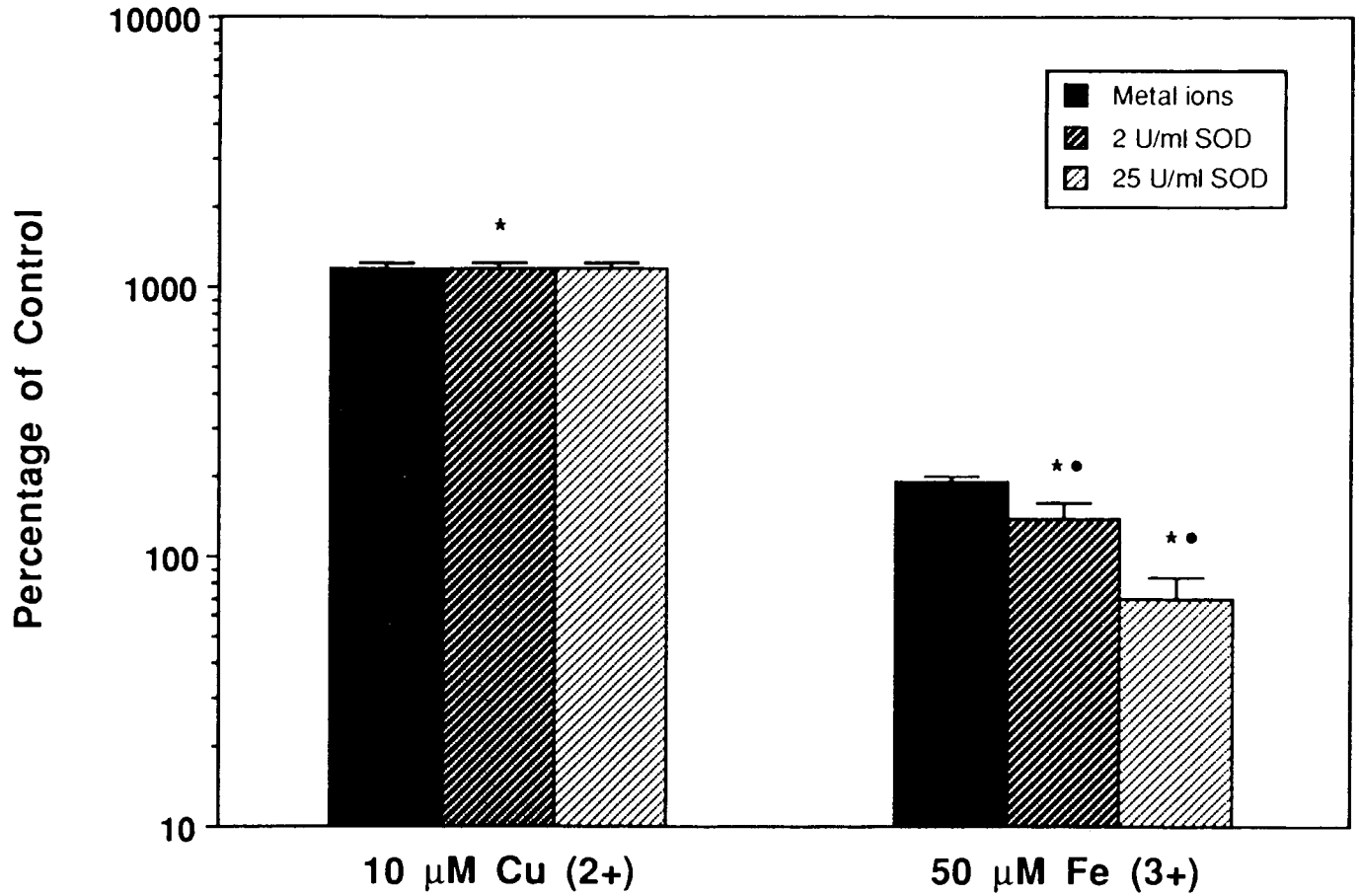
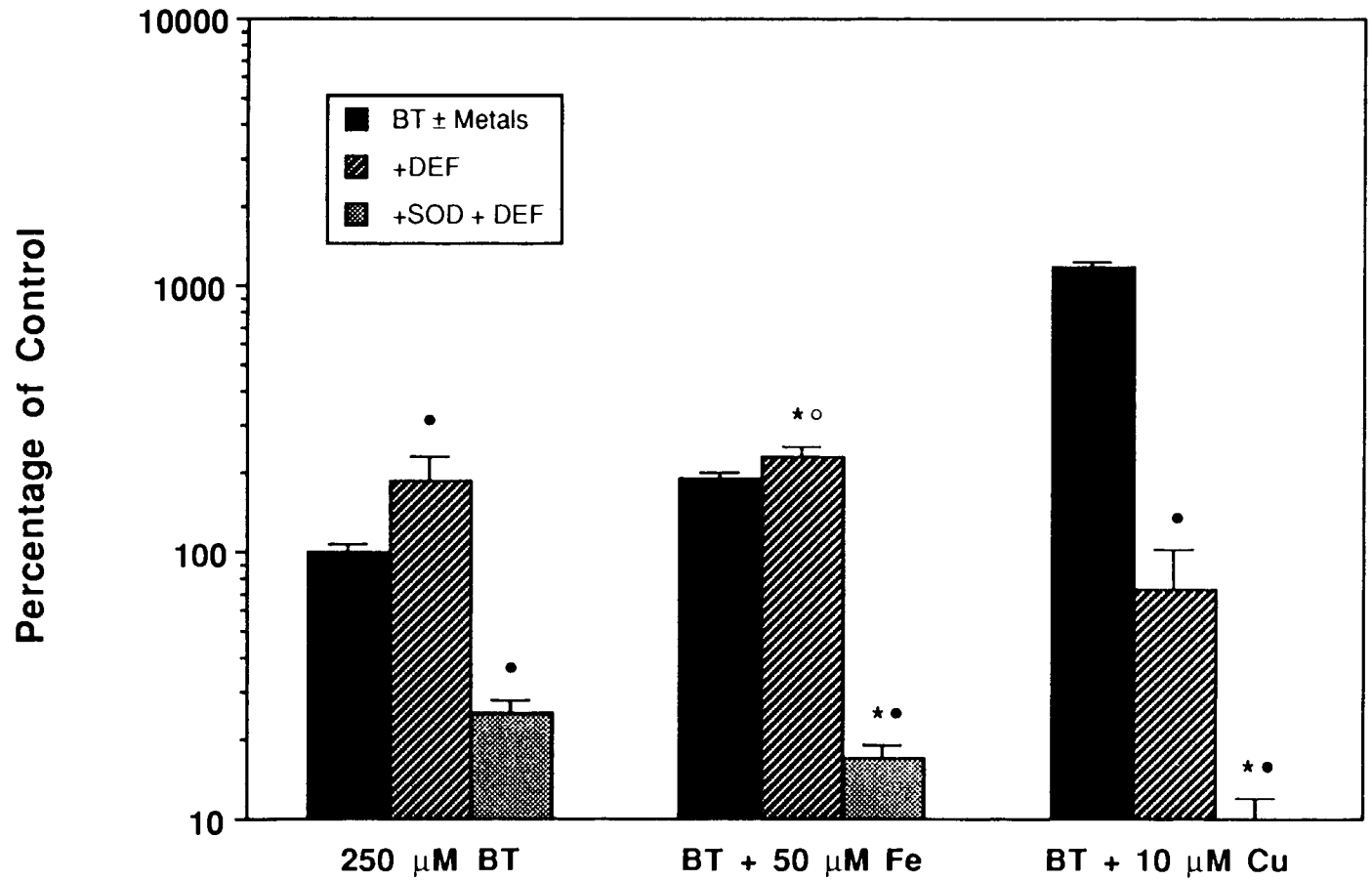


Figure 5

Effects of desferrioxamine on autoxidation of benzenetriol in the presence and absence of metal ions



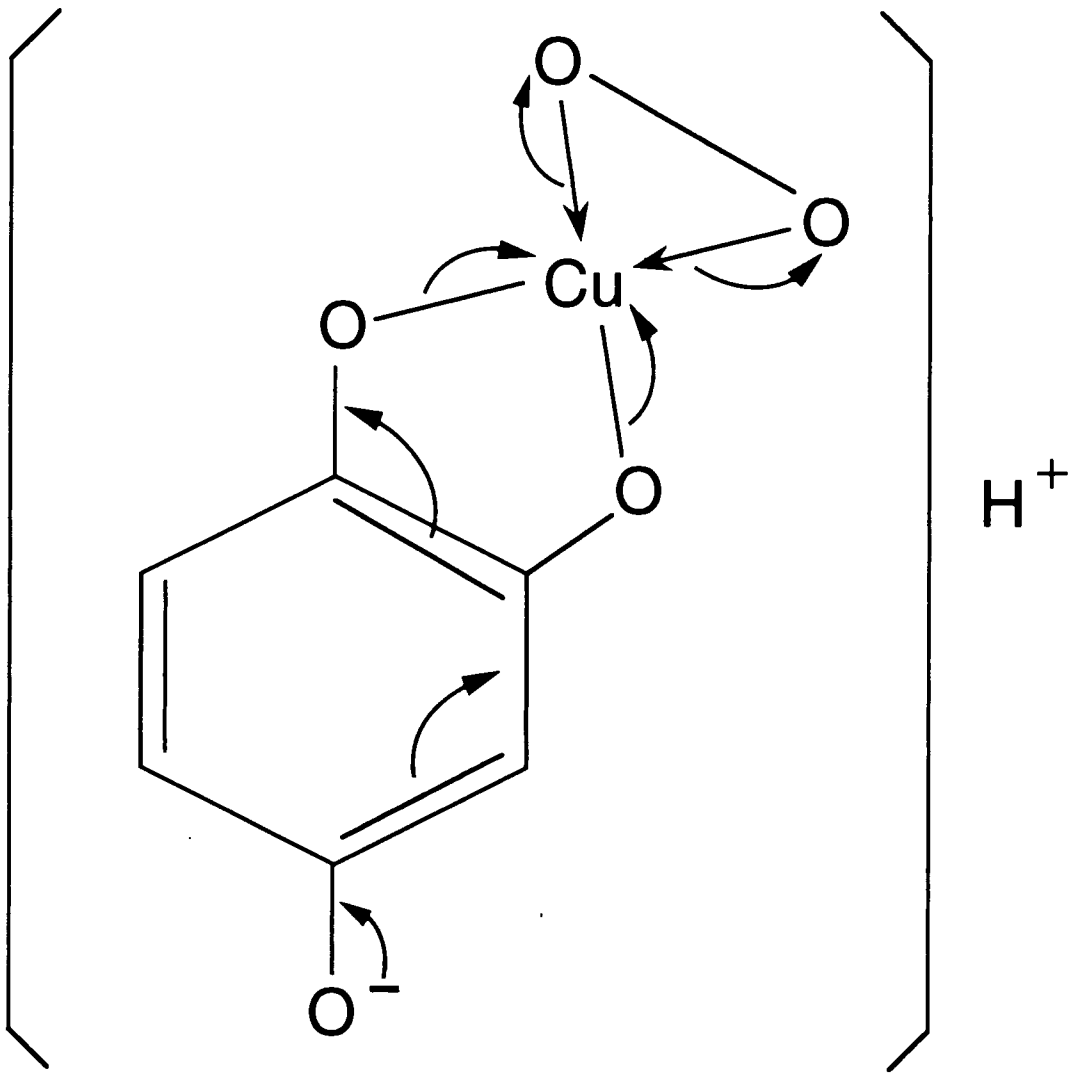


FIGURE 6. Possible chemical structure of [benzenetriol-Cu-oxygen] and the schematic electron flow from 1,2,4-benzenetriol via Cu(2+) to oxygen

Table 1 Effects of Cu^{2+} and Fe^{3+} on the oxidation of 1,2,4-benzenetriol.

	250 μM BT	BT + 10 μM Cu^{2+}	BT + 50 μM Fe^{3+}
Half-life $T_{1/2}$ (s)	203	17	104
Absorbance (490 nm)*	0.0412 ± 0.0016**	0.4847 ± 0.0043	0.0787 ± 0.0008
Percentage of Control	100 ± 8***	1176 ± 56	191 ± 9

* Absorbance (490 nm) at 30s after initiation of the reaction.

** The data presented as mean ± SEM (Standard Error of Means).

*** The variance shown as a relative error of the ratio: x/y (a/x + b/y); (a = x's SEM, b = y's SEM).

Table 2 Summary of Results:

Percentage of Inhibition (-) or Activation (+) [1]	250 μM BT	BT + 10 μM Cu^{2+}	BT + 50 μM Fe^{3+}
Benzenetriol (BT) Controls [2]	**100 ± 8**	**100 ± 2**	**100 ± 2**
Superoxide Dismutase(25U/ml)	-74	0	-64
Catalase (25 U/ml)	-27	-35	N/A[4]
SOD+Catalase(12.5+12.5U/ml)	-88	-2	N/A
Desferrioxamine (0.5mM)	+83	-94	+20
SOD + Desferrioxamine[3]	-75	-99	-91
Catalase + Desferrioxamine	+50	N/A	N/A
Formate (50 mM)	-3	+9	N/A
SOD + Formate	-63	+4	N/A
Catalase + Formate	-5	0	N/A
SOD + Catalase + Formate	-79	+8	N/A
Mannitol (50 mM)	+9	+3	N/A
Catalase + Mannitol	N/A	-4	N/A

1) The data are presented as percentage of inhibition (-) or activation (+) in this table.

2) Absolute values for the controls are presented in Table 1 as absorbance at 490 nm.

3) The concentration of antioxidants or chelators is the same as stated at first time unless restated it again.

4) N/A: Data are not available.

CHAPTER FIVE

CONCLUSION AND PERSPECTIVE

In summary, the results presented in this dissertation demonstrate that 1,2,4-benzenetriol is genotoxic in cultured human lymphocytes and HL60 cells on the basis of increased micronucleus formation, increased frequency of aneuploidy, and increased levels of 8-hydroxy-2'-deoxyguanosine (8-OH-dG). It is concluded that benzenetriol induces numerical and structural chromosomal changes and point mutations by two distinct mechanisms.

Benzenetriol, by itself, induces predominantly kinetochore-positive micronuclei, which have a high probability of containing a entire chromosome. It was confirmed by FISH that benzenetriol induces numerical aneuploidy of chromosomes 7 and 9, mainly in the form of hyperdiploidy. On this basis, benzenetriol is a potent aneuploidogen. The induction of both kinetochore-positive micronuclei and hyperdiploidy is likely to result from lagging chromosomes which fail to be incorporated into the daughter nuclei during cell division.

Proper chromosomal segregation requires microtubule integrity, and microtubule disruption can lead to aneuploidy. Thus, the effect of benzenetriol on microtubule integrity was investigated. It was shown that benzenetriol interferes with microtubule assembly during mitosis. The oxidative products of benzenetriol, its quinone and semiquinone, are the most likely toxicants involved. Since they are electrophilic, quinones can interact with the

nucleophilic sulfhydryls of tubulin, the component protein of microtubules. The sulfhydryl groups are important for microtubule assembly and the covalent addition of quinones may block their function thereby causing mitotic abnormalities. The disruption of microtubules is therefore most likely responsible for the production of benzenetriol-induced aneuploidy.

In addition, benzenetriol-induced micronuclei may also result from oxidative DNA damage caused by reactive oxygen species, which are generated during oxidation of benzenetriol. Catalytic amounts of copper (Cu^{2+}) or other transition metal ions accelerate the reaction of benzenetriol with oxygen and stimulate the formation of these reactive oxygen species in a cell-free system. We further tested the effect of benzenetriol in combination with Cu^{2+} on the induction of micronuclei in cultured human cells. Addition of Cu^{2+} increases the total micronuclei induced by benzenetriol and also alters the pattern of micronucleus formation from kinetochore-positive to kinetochore-negative. Kinetochore-negative micronuclei indicate chromosome breakage. Thus, benzenetriol gains another mechanism for clastogenicity in the presence of metal ions.

The change in the mechanism of micronucleus formation from numerical aneuploidy (kinetochore-positive) to clastogenicity (kinetochore-negative) in the presence of Cu^{2+} correlates with our findings in the cell-free system. Cu^{2+} changes the pathway of benzenetriol oxidation from superoxide-propagated one-electron transfer to Cu^{2+}-mediated two-electron transfer. By accelerating the oxidation of benzenetriol, Cu^{2+} stimulates the generation of hydrogen peroxide which can be further reduced to hydroxyl radicals via Fenton-type reactions. Moreover, Cu^{2+} catalyzes the site-specific formation of hydroxyl

radicals, which are the immediate DNA-damaging clastogens in the presence of benzenetriol.

Hydroxyl radicals cause DNA base modification, strand breaks and fragmentation of DNA or deoxyribose. 8-OH-dG represents the covalent addition of $HO^{\bullet}$ or $^1O_2$ to guanine in DNA and is a sensitive marker of oxidative DNA damage. Benzenetriol increases the level of 8-OH-dG in cell DNA, particularly in the presence of Cu^{2+}. Because 8-OH-dG causes DNA base G ——> T substitution, the misreading at the modified and adjacent base residues can lead to point mutations. These observations illustrate that the genotoxicity of benzenetriol in the presence of Cu^{2+} results from the ability of Cu^{2+} to activate oxygen to H_2O_2 and further to produce $HO^{\bullet}$ radicals. Taken together, the genotoxicity of benzenetriol has been characterized by its ability to cause aneuploidy, clastogenicity and point mutations. The high potency of benzenetriol suggests that it may play a role in benzene-induced genetic damage and perhaps human leukemogenesis.

Our findings open new avenues for investigation of the impact of oxygen-derived active species and toxic quinones on cytotoxicity, genotoxicity and tumorigenesis. Further studies should consider detailed mechanisms by which benzenetriol induces distinct cellular damage. The future goal is to distinguish the role of reactive oxygen species from that of quinones in benzenetriol-induced genotoxicity. For example, we can apply H_2O_2 with or without metal ions to the cell system to test whether microtubule disruption results from quinones or from oxygen radicals. It will be also important to measure the effects of antioxidants on the formation of micronuclei and 8-OH-dG in order to understand the mechanisms involved.

APPENDICES

Appendix 1 Description of HL60 Cells from American Type Culture Collection

ATCC CCL 240 HL-60 (Peripheral Blood, Human) Promyelocytic Leukemia

Current medium for propagation: RPMI 1640, 80%; fetal bovine serum, 20%. Initial recovery and growth is improved using Iscove's modified Dulbecco's medium with 20% fetal bovine serum.

HL-60 is a promyelocytic cell line derived by S.J. Collins *et al.* (Nature *270:* 347-349, 1977). Peripheral blood leukocytes were obtained by leukopheresis from a 36 year-old Caucasian female with acute promyelocytic leukemia. The cells were cultured in suspensions in the presence of conditioned medium from human embryonic lung cultures. Subsequently, it was found that continued growth of the cells did not require the conditioned medium. The predominate cell population that has been maintained consists of neutrophilic promyelocytes with prominent nuclear/cytoplasmic asynchrony. Up to 10% of the cultured cells spontaneously differentiate beyond the promyelocytic stages and the proportion is markedly enhanced by polar-planar compounds such as DMSO. A wide variety of other compounds, including butyrate, hypoxanthine, TPA, actinomycin D, and retinoic acid also induce differentiation.

The HL-60 cells lack specific markers for lymphoid cells but express surface receptors for Fc fragment and complement (Gallagher *et al.,* Blood *54:* 713-733, 1979). They exhibit phagocytic activity and responsiveness to chemotactic stimuli. HL-60 cells form colonies in semi-solid media and produce subcutaneous myeloid tumors in nude mice.

An initial culture designated passage 8 was obtained from Dr. Robert Gallo, National Cancer Institute, in March, 1982. These cultures were continuously maintained in suspension.

DESCRIPTION OF REPOSITORY REFERENCE SEED STOCK

Number of Serial Subcultures from Tissue of Origin: 10.
Freeze Medium: Culture medium, 90%; dimethyl sulfoxide (DMSO), 10%; antibiotic-free.
Viability: Approximately 85% (dye exclusion).
Culture Medium: RPMI medium 1640, 80%; fetal bovine serum (heat-inactivated), 20%.
Growth Characteristics of Thawed Cells: An inoculum of 10^5 viable cells/ml in the above culture medium will increase 6-7 fold within 7 days when incubated at 37C, provided fresh medium (30% by volume) is added every 3-4 days.
Plating Efficiency: The cells cannot be plated.
Morphology: Lymphoblast-like.
Karyology: Chromosome Frequency Distribution 50 Cells: 2n = 46

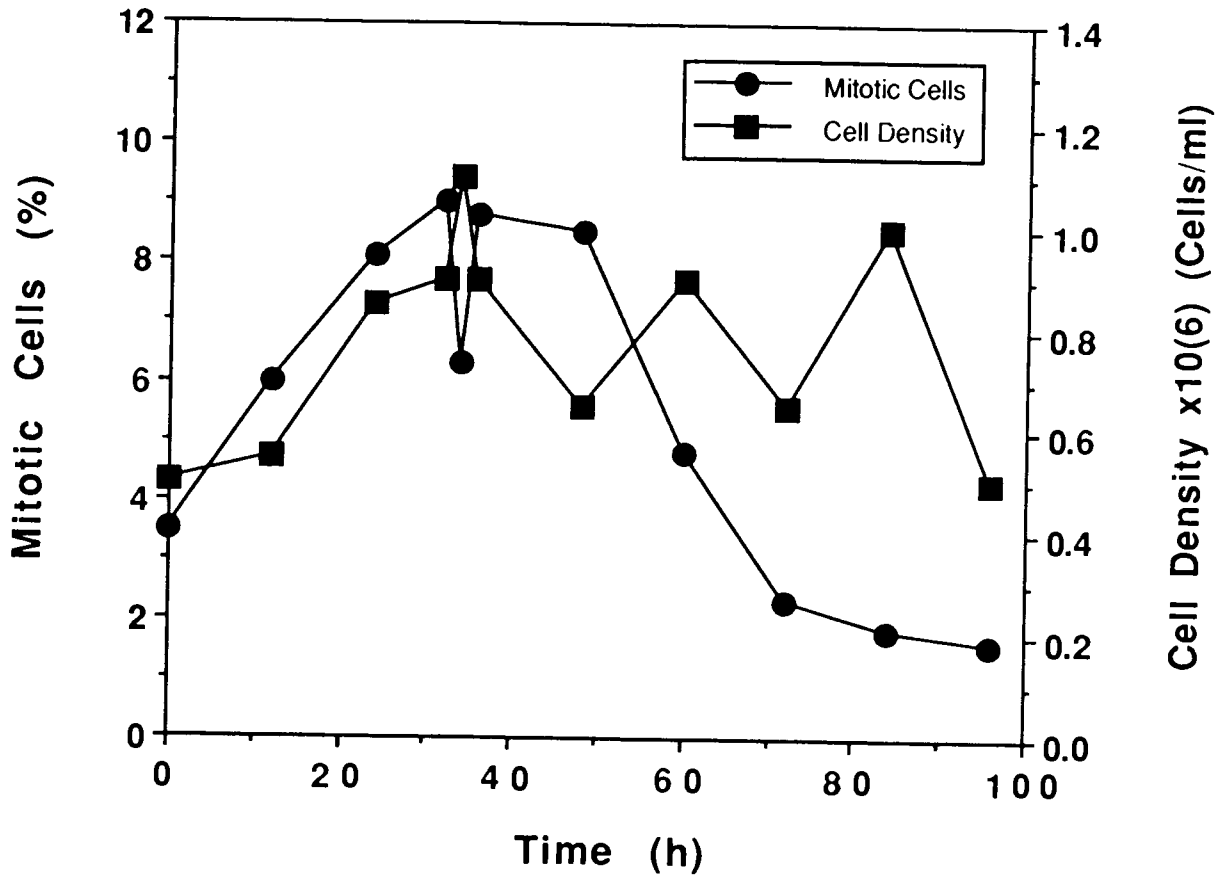
Cells:	1	3	44	1	1
Chromosomes:	43	45	46	47	48

The stemline chromosome number is pseudodiploid with the 2S component occurring at 6.2%. Five markers (M2 through M6) were common to most S metaphases. DM's, which varied in numbers per cell, occurred in all metaphases karyotyped. HSR chromosomes were not detected.

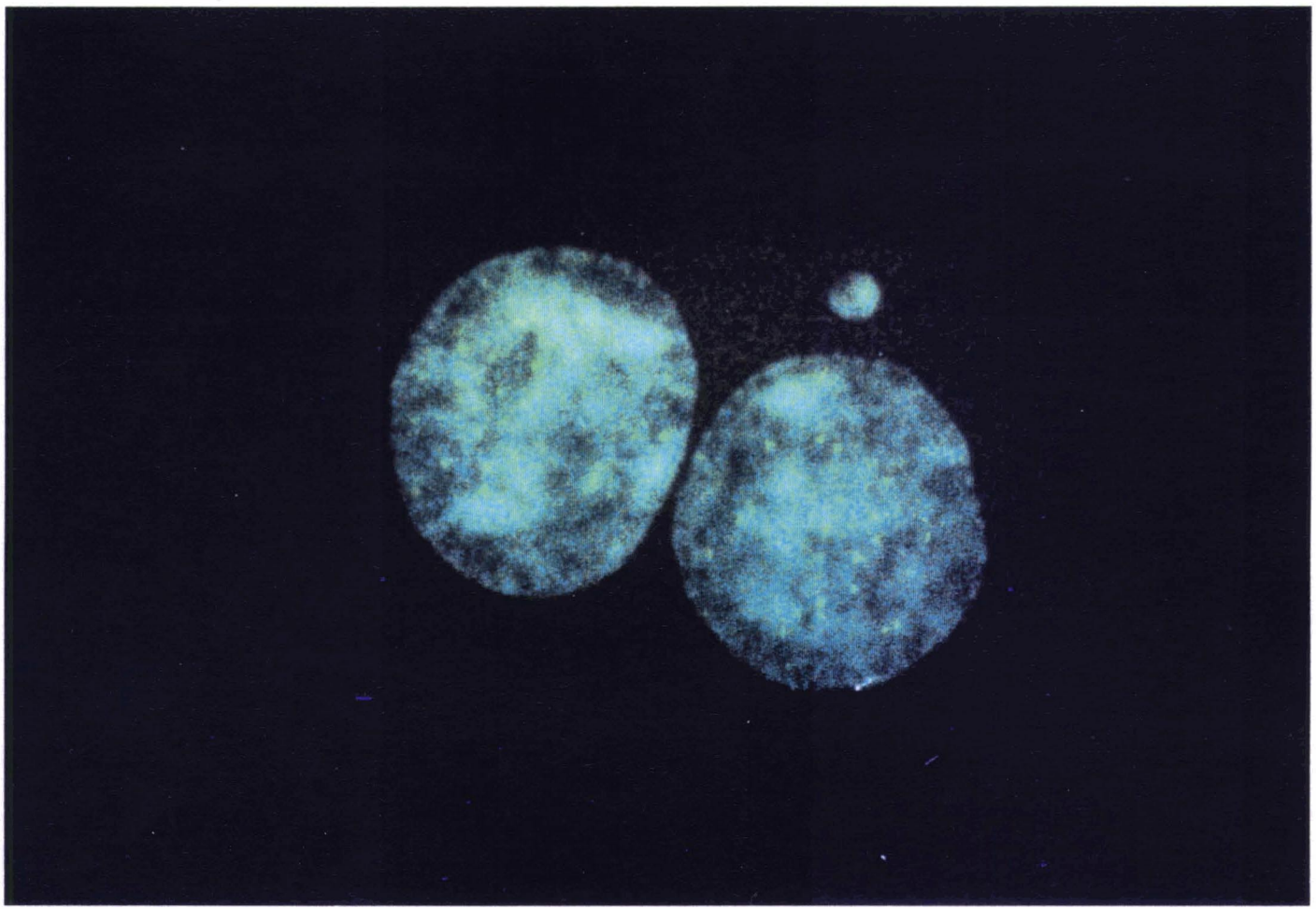
Sterility: Tests for mycoplasma, bacteria and fungi were negative.
Species: Confirmed as human by isoenzyme analysis.
Erthrocyte Rosette Test: E, 4%; EA, 17%; EAC, 1%.
Surface Immunoglobulins: Negative.
EBNA: Negative.
Reverse Transcriptase: Not detected.
Isoenzymes: G6PD, B; PGM_1, 1; PGM_3, 1; EST-D, 1; Me-2, 1; AK1, 1; GLO-1, 1.
Submitted by: R.C. Gallo, National Cancer Institute, NIH, Bethesda, Maryland
Prepared and characterized by: American Type Culture Collection, Rockville, Maryland.

Appendix 2

Mitotic Index and Growth of HL60 Cells

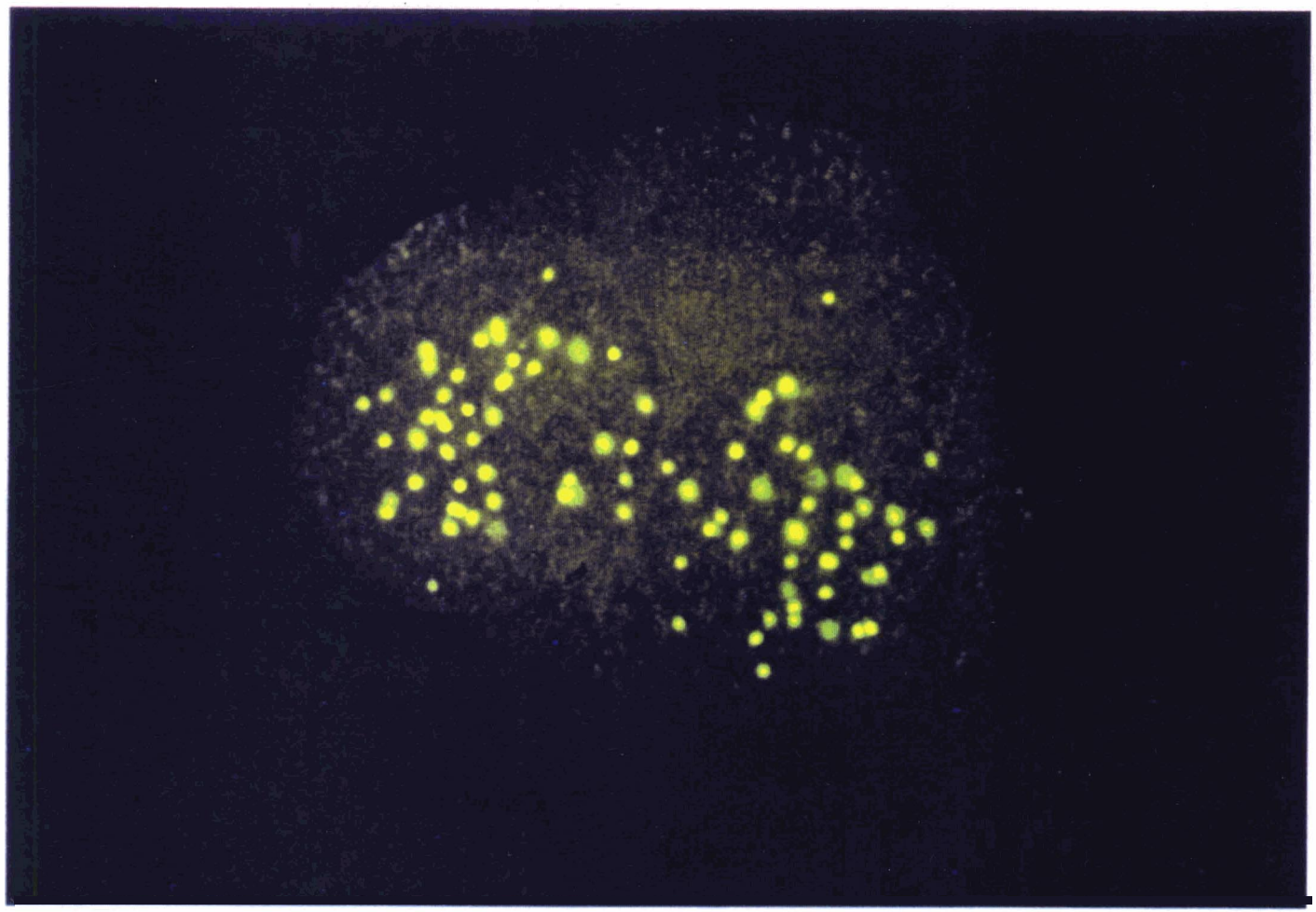


Appendix 3A



Binucleate Cell with a Micronucleus (DAPI Filter)

Appendix 3B



Binucleate Cell with a Kinetochore-Positive Micronucleus (FITC Filter)

Appendix 4: Sample of Score Sheet for Micronucleus Assay

Modified MN Score Sheet

Sample Code: Date: Decode: Scorer:

Slide	# BN Cell	# MN	# MW w/ Kinetochore (+) (U) if Unscorable	Location	Comments

Summary	Culture #	# BN Cells	# MN	# BN Cells w/ MN	# MN Cells w/ K+	K-	U
Totals							

Replicative Index

	MONO	BI	>BI	TOTAL
Dup. 1				200
Dup. 2				200
Total				400
Percent				100

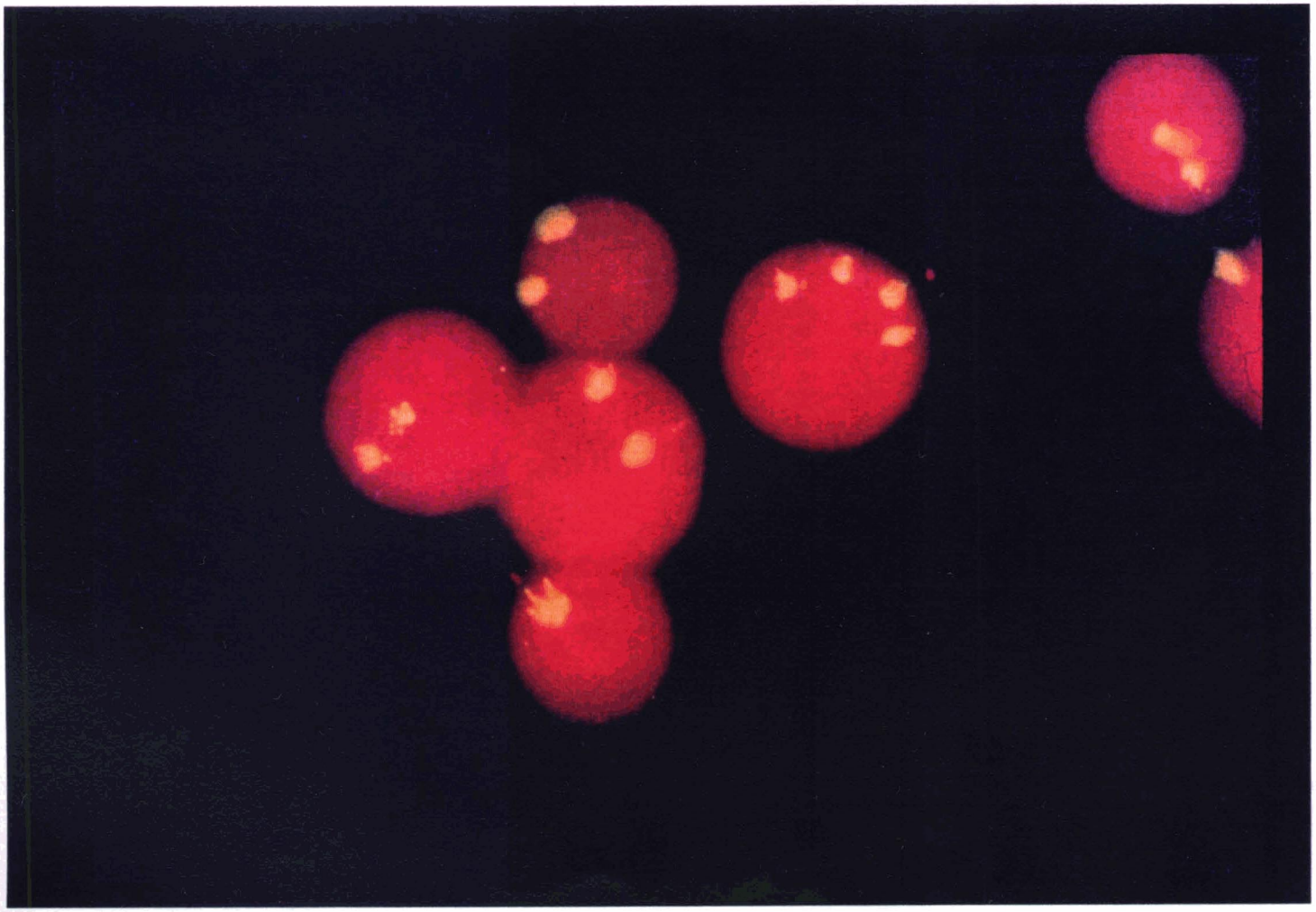
R.I.=

Appendix 5 Sample of Score Sheet for FISH Assay

Experiment: _____ Probe #: _____ Scorer: _____ Scored Date: _____

Slide #	Sample #	Location	Number of Domains / Nucleus							Total # of Scored Cells
			0	1	2	3	4	≥5	U	
Total Number										
Frequency (%)										

Appendix 6



Fluorescence *in situ* Hybridization Staining of Chromosome 9 in HL60 Cells.

Appendix 7

Respective Contributions of the Author and Co-authors

This study was proposed and designed by the author. The author of this dissertation, collected and analyzed most of data from the micronucleus assay, FISH technique, microtubule staining and chemical system. The major contributions from the author in this research are: (1) designing, initiating and organizing all these major studies; (2) designing the experimental procedures and applying these techniques; (3) treating HL60 cells with BT and Cu^{2+} in PBS simultaneously; (4) interpreting the results of the chemical and cellular studies; and (5) writing up this dissertation and the three publications. Other co-authors participated in part of the study as follows:

In Chapter II, the experiment of benzenetriol-induced micronucleus formation in human lymphocytes was set up in collaboration with Moire Robertson. Dr. Prema Kolachana determined the benzenetriol-induced 8-OH-dG formation (by HPLC) in Dr. Bruce Ames' laboratory. Dr. Allan Davison did most of the statistical analysis. Dr. Martyn Smith, Dr. Allan Davison, and Moire Robertson critically read a few versions of the manuscript and made suggestions for improving the writing.

In Chapter III, the immunocytochemical assay with anti-tubulin antibody staining in human lymphocytes and HL60 cells was set up in collaboration with Moire Robertson. Pravina Venkatesh scored some of the stained slides for FISH. Dr. Martyn Smith critically read the final manuscript and provided suggestions for the discussion.

In Chapter IV, Dr. Allan Davison proposed the topic of this research, provided suggestions and critically read the previous version and final manuscript.