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Review Article

POTENTIAL ROLE OF FREE RADICALS IN BENZENE-INDUCED MYELOTOXICITY AND LEUKEMIA

VANGALA V. SUBRAHMANYAM,* DAVID ROSS,† DAVID A. EASTMOND‡ and MARTYN T. SMITH*

*Department of Biomedical and Environmental Health Sciences. School of Public Health, University of California, Berkeley, CA 94720, U.S.A. †Molecular Toxicology and Environmental Health Sciences Program, School of Pharmacy, University of Colorado, Boulder, CO 80309. U.S.A. ‡Environmental Toxicology Graduate Program, Department of Entomology, University of California. Riverside. CA 92521, U.S.A.

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Abstract—Occupational exposure to benzene, a major industrial chemical, has been associated with various blood dyscrasias and increased incidence of acute myelogenous leukemia in humans. It is established that benzene requires metabolism to induce its effects. Benzene exposure in humans and animals has also ken shown to result in structural and numerical chromosomal aberrations in lymphocytes and bone marrow cells, indicating that benzene is genotoxic.

In this review we have attempted to compile the available evidence on the role of increased free radical activity in benzene-induced myelotoxic and leukemogenic effects. Benzene administration to rodents has been associated with increased lipid peroxidation in liver, plasma, and bone marrow, as shown by an increase in the formation of thiobarbituric-acid reactive products that absorb at 535 nm. Benzene administration to rodents also results in increased prostaglandin levels indicating increased arachidonic acid peroxidation. Other evidence includes the fact that bone marrow cells and their microsomal fractions isolated from rodents following benzene-treatment have a higher capacity to form oxygen free radicals.

The bone marrow contains several peroxidases, the most prevalent of which is myeloperoxidase. The peroxidatic metabolism of the benzene metabolites, phenol and hydroquinone, results in arachidonic acid peroxidation and oxygen activation to superoxide radicals. respectively. These metabolites, upon co-administration also produce a myelotoxicity similar to that observed with benzene. Recently. we have found that exposure of human promyelocytic leukemia (HL-60) cells (a cell line rich in myeloperoxidase), to the benzene metabolites, hydroquinone and 1,2,4-benzenetriol results in increased steady-state levels of 8-hydroxydeoxyguanosine a marker of oxidative DNA damage. Peroxidatic metabolism of benzene's phenolic metabolites may therefore be responsible for the increased free radical activity and toxicity produced by benzene in bone marrow. We thus hypothesize that free radicals contribute, at least in part, to the toxic and leukemogenic effects of benzene.

Keywords—Benzene, Free radicals. Lipid peroxidation. Oxidative DNA damage. Genotoxicity, Myeloroxicity, Leukemia

Address all correspondence to Martyn T. Smith Department of Biomedical and Environmental Health Sciences. School of Public Health, University of California, Berkeley, CA 94720.

Vangala Subrahmanyam obtained his Ph.D. in 1986 from Memorial University of Newfoundland, Canada. After two years of postdoctoral studies at the School of Pharmacy. University of Colorado, Boulder, CO, he joined the Department of Biomedical and Environmental Health Sciences. School of Public Health. University of California, Berkeley. CA. where he is currently appointed as Assistant Specialist Scientist. David Ross received his Ph.D. in 1982 from the University of Aston at Birmingham in England. After postdoctoral studies at the Department of Forensic Medicine. Karolinska Institute, Stockholm, Sweden. and the School of Public Health, University of California, Berkeley. CA, he moved to the School of Pharmacy, University of Colorado. Boulder, CO. where he is currently Associate Professor of Toxicology. David Eastmond received his Ph.D. in 1987 from the School of Public Health. University of California, Berkeley, CA. He spent two years as a Alexander Hollaender Distinguished Postdoctoral fellow at Lawrence Livermore National Laboratories, CA. and now is an Assistant Professor of Toxicology at the University of California, Riverside. Martyn Smith obtained his Ph.D. from the University of London in 1980, was a postdoctoral fellow at the Karolinska Institute and is now Associate Professor of Toxicology in the school of Public Health, University of California, Berkeley, CA. All investigators have a long-standing interest in oxygen radical and quinone-mediated toxicity and carcinogenicity.

INTRODUCTION

Benzene (BZ) is an ubiquitous environmental pollutant and an important industrial chemical. the annual production of which has been estimated to be over one billion gallons in the U.S. alone.[1] BZ is a constituent of gasoline and is also used in the manufacture of a wide variety of consumer goods such as plastic containers, radios, toys, sporting goods, furniture, appliances, automobiles, tires, adhesives, textiles, dyes, drugs, pesticides, lubricants, solvents and cleaning products. In addition, BZ may be found as a contaminant in drinking water, ambient air, and certain types of foods.[1–6]

Extensive epidemiological studies have linked occupational exposure to BZ with the incidence of bone marrow toxicity and a variety of leukemias in humans.[1–6] However, despite extensive research, the molecular mechanisms responsible for the induction of bone marrow toxicity and leukemia by BZ have *yet to* be fully elucidated. It is generally accepted that BZ re-

quires metabolism to induce its toxic effects.[10–14] Metabolism of BZ is very complex (Fig. 1) and is not completely understood. Numerous in vivo and in vitro studies have shown that BZ is primarily metabolized to phenol (PH). hydroquinone (HQ), catechol (CAT) and 1,2,4-benzenetriol (BT).[13–30] The majority of these phenolic metabolites are excreted in urine as glucuronide and sulfate conjugates. Some of the HQ and CAT, however, is further metabolized to reactive 1.4-benzoquinone (1.4-BQ) and 1.2-benzoquinone (1,2-BQ), respectively. Some investigators believe that these quinones are responsible for BZ-induced toxic effect~.'**BZ has also been shown to be metabolized to a ring-opened oxidation product, *trans,trans*-muconic acid, which has been detected in both bone marrow and urine after administration of BZ to mice. The corresponding dialdehyde *trans,trans*-muconaldehyde (*t,t*-MA) has also been proposed to be one of BZ's toxic metabolites. Although this aldehyde has not yet been detected in vivo, studies by Lamano et al.[32] have demonstrated that mouse liver microsomes can metabolize BZ to a product, which co-elutes with *t,t*-MA.

The myelotoxic effects of BZ have also been associated with free radical formation, either as BZ metabolites or oxygen radicals and lipid peroxidation products.[33–35] Several reviews of BZ-induced myelotoxic effects in relation to its metabolism have dealt with quinones and/or *t,t*-MA as the ultimate toxic metabolites. Little attention has been paid, however, to the toxicological significance of free radicals as ultimate toxic species. In this review we have attempted to compile the available evidence on free radical formation and associated damage during the oxidation of BZ and its metabolites and discuss their role in BZ-induced myelotoxicity and carcinogenicity.

FREE RADICALS

A free radical has been defined as *any molecule* that *contains an odd number or unpaired electron.*[36] A free radical is usually depicted as R', where the dot represents the unpaired electron. Most free radicals are highly reactive and are short lived. The formation and reactions of free radicals are classified as three steps namely initiation, propagation, and termination. Initiation of free radical formation in biological systems may occur during enzymatic- or auto-oxidation or during reduction. The extent of propagation and termination reactions depends upon the reactivity of the initiated free radical and the environment in which the radical is formed.

The biological threat from free radicals has been thought to be due to their interaction with cellular dioxygen. Lipids, thiols, proteins. and DNA.[36–42] The reactions of free d d s with dioxygen may either result in the formation of peroxyl radicals or superoxide radical anions ($O_2^{\cdot -}$). The latter are known to dismutate, either spontaneously or catalyzed by superoxide dismutase (SOD), to hydrogen peroxide (H_2O_2). Furthermore, reaction of H_2O_2 and $O_2^{\cdot -}$ in the presence of iron or other transition metals could result in the formation of hydroxyl radicals ($HO^{\cdot}$). In addition. upon supply of energy, ground state dioxygen may be activated into reactive singlet oxygen ($^1O_2$) species.

The activated oxygen species $HO^{\cdot}$ and $^1O_2$ may attack cellular DNA resulting in DNA-strand scission,[43,44] and hydroxylation of bases.[45–47] Such processes have been implicated both in the initiation and promotion phases of carcinogenesis.[48–50] In addition, reactions of drug-derived radicals or oxygen radicals with cellular lipids may initiate lipid peroxidation.[40] Free radicals of all types may interact with cellular macromolecules such as DNA, proteins. and carbohydrates and initiate or promote toxic and carcinogenic processes.

BENZENE-INDUCED FREE RADICAL FORMATION IN VIVO

Administration of BZ has been shown to induce the formation of thiobarbituric acid (TBA)-reactive products (having an absorbance maximum of 535 nm) in liver,[33] plasma,[33] and bone marrow[34] of rats, suggesting the formation of malondialdehyde-like products (TBA–MDA adducts: $\varepsilon = 1.4 \times 10^5 M^{-1} cm^{-1}$). The authors of these studies have suggested that BZ-induces lipid peroxidation. Lamano and coworkers[32,51,52] have recently demonstrated that *t,t*-MA, the open-ring oxidation product of BZ also forms an adduct with TBA having an absorbance maximum at 490 nm (TBA-*t,t*-MA-adduct: $\varepsilon = 7.3 \times 10^4$) with little or no absorbance at 535 nm. It therefore appears unlikely that these TBA-reactants absorbing at 535 nm in rats following BZ administration are due to *t,t*-MA. Since these studies did not record the whole spectrum of TBA-reactants, the formation of *t,t*-MA-TBA adducts, in BZ-exposed animals cannot be excluded. It is surprising, however, although the hplc techniques to separate TBA-*t,t*-MA adducts from TBA-*t,t*-MDA adducts have been available for some time,'' these techniques have not been applied to detect the formation of *t,t*-MA adducts in BZ exposed animals. Nevertheless, Gaido and Wierda[53] have shown that BZ administration increases PGE_2 levels in bone marrow indicating increased arachidonic acid peroxidation. Laskin et al.'' have demonstrated an increase in the phagocytic activity of bone marrow macrophages and granulocytes of Balb/c mice treated with BZ (880 mg/kg) relative to

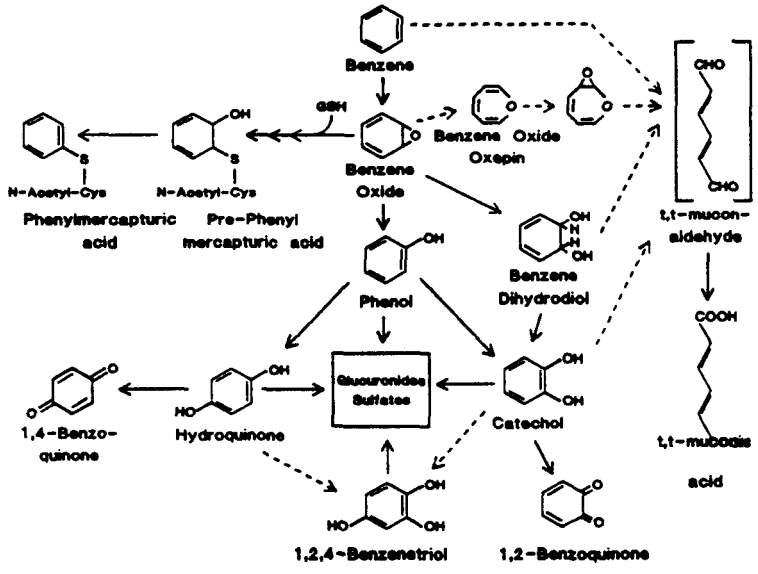

Fig. 1. Metabolic pathways of benzene (adapted from refs. 13, 14).

the control animals treated with saline or corn oil. They also indicated that a similar increase in phagocytic activity of bone marrow is observed following combined exposure of mice to HQ (50 mg/kg) and PH (50 mg/kg). Khan et al.[35] showed that the S-9 fractions isolated from bone marrow of female albino rats following the administration of BZ (0.5 ml/kg), had higher capacity to oxidize dimethyl sulfoxide (a HO' scavenger), to formaldehyde and deoxyribose to thiobarbituric acid-reactive products, indicating that BZ may increase the HO' production in bone marrow. Anwar et al.[55] have shown that dimethyl sulfoxide, can indeed reduce the formation of micronuclei in erythrocytes of BZ-exposed mice. presumably by scavenging HO'. These authors, however, suggested that this reduction of BZ-induced genotoxic effects by dimethyl sulfoxide, may be due to the inhibition of HO'-mediated BZ oxidation to genotoxic *t,t*-MA, but mechanism for dimethyl sulfoxide effects remain unclear. Taken together, these studies indicate that administration of BZ to rodents increases the generation of oxygen radicals and lipid peroxidation in bone marrow.

Recently. Ekstrom et al.[56] demonstrated excretion of malondialdehyde in urine of male Sprague–Dawley rats given HQ (100 or 200 mg/kg) by stomach tube. In a follow-up study," this group demonstrated that HQ administration to these rats also results in the depletion of hepatic glutathione (GSH). These authors suggested that the malondialdehyde excretion in urine that occurs following ingestion of HQ to rats is presumably due to lipid peroxidation that occurs following a depletion of hepatic GSH. similar to that observed in isolated hepatocytes.[58,59]

The above studies suggest that free radical formation during the metabolism of BZ in bone marrow could play a role in BZ-induced myelotoxicity. Furthermore free radical formation could play a role in the carcinogenicity of HQ to mouse liver.[60] The following sections present the evidence for free radical formation during the in vitro metabolism of BZ and its phenolic metabolites in order to understand the exact mechanisms by which BZ-exposure could lead to the formation of free radicals in bone marrow. We have also reviewed the literature on the mechanisms of interaction of free radical metabolites of BZ with dioxygen and cellular targets such as DNA, GSH, proteins and lipids and the potential significance of such reactions in BZ-induced myelotoxicity and leukemia will be discussed.

FREE RADICAL FORMATION DURING ENZYMATIC METABOLISM OF BENZENE AND ITS METABOLITES IN VITRO

Interest in free radical reactions of BZ metabolites stems primarily from the presence of high levels of heme-protein peroxidase enzymes in bone marrow and their ability to oxidize various phenolic compounds to free radicals." Myeloperoxidase (MPO) and eosinophil peroxidase (EPO) enzymes constitute the majority of the peroxidase activity detected in bone marrow.[61] However, some investigators have suggested that the peroxidase activity of prostaglandin (H) synthase (PGS) may also conmbute to the bioactivation of phenolic metabolites of BZ in bone marrow.[53,62–65] This section focuses mainlv on MPO- and PGS-catalyzed me-

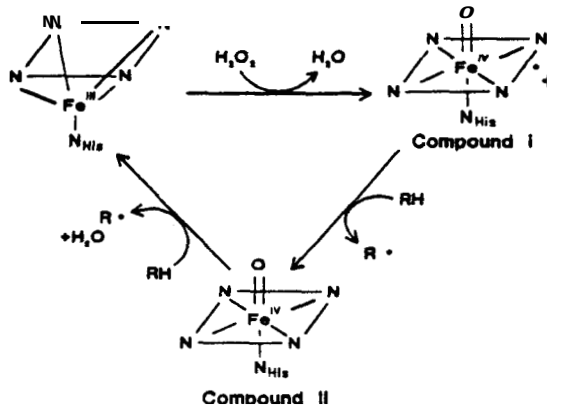

Fig. 2. Catalytic cycle of horseradish peroxidase (adapted from ref. 66). RH and R· represent electron donor and its free radical product respectively. Phenolic metabolites (phenol. hydroquinone and catechol) of benzene could also serve as electron donors to compounds I and II.[67,83]

tabolism of phenolic metabolites of BZ. The relative roles of these enzymes in catalyzing the bioactivation of BZ and its metabolites to free radical intermediates is unclear. Studies performed with neutrophils, bone marrow cells. and homogenates and the specific cell types of bone marrow are also described to indicate that peroxidase(s) present in these preparations can catalyze the bioactivation of BZ's phenolic metabolites similar to purified enzymes.

A. Mechanisms of peroxidase-catalyzed oxidations

The mechanisms of peroxidase-catalyzed oxidations of electron donors have been studied by several investigators using horseradish peroxidase (HRP), a model plant peroxidase. Peroxidase enzymes contain an iron-porphyrin complex at the active site that can reduce peroxides[e] (Fig. 2). During this process the resting enzyme which contains an Fe^{+3} heme iron is oxidized to compound I, which is believed to contain two-oxidizing equivalents above the resting state (Fe^{+5}).[67] More recent studies have demonstrated that only one oxidizing equivalent is present in the iron moiety. The other oxidizing equivalent resides on the porphyrin moiety as a porphyrin Π-cation radical.[68–70] Compound I can be reduced by a variety of electron donors to compound II. Compound II has been shown to be at Fe^{+4} oxidation state like compound I, but does not contain the porphyrin Π-cation radical.[71,72] Compound II can also be reduced by electron donors releasing the native enzyme (Fe^{+3}). During the reduction of compound I and compound II by electron donors, highly reactive free-radical metabolites of electron donors can be formed.[67] Pioneering work of Yamazaki et al.[73–75] demonstrated that phenolic compounds were excellent electron donors for the higher oxidation states of HRP. BZ. however. is not an electron donor for higher oxidation states of HRP formed from interaction with hydrogen peroxide (Subrahmanyam. V.V., unpublished). Unfortunately, the chemistry of heme-prosthetic group in MPO is not well understood. but recent studies by Dugad et al.[76] indicate that the ^1H NMR spectrum and the nuclear Overhauser effect (NOE) of cyanide-ligated ferric complex of MPO (MPO–CN) were remarkably similar to the prosthetic group of lactoperoxidase. Nicholl and coworkers,[77] showed that the prosthetic group of lactoperoxidase is an iron-porphyrin thiol. Whatever the structure of the heme prosthetic group of MPO is, it forms spectral complexes with H_2O_2[78–80] similar to that of HRP.[66] This indicates that MPO is potentially a good candidate in catalyzing the phenolic metabolites of BZ to free radicals.

B. Myeloperoxidase-catalyzed metabolism of the phenolic metabolites of benzene

Using the purified human MPO we studied the metabolism of PH,[81–83] HQ,[12,82,83] and CAT[83,84] to determine the mechanisms of their oxidation by MPO. Our studies show that HQ has the highest specificity for MPO followed by CAT and PH indicating that HQ is a better substrate for MPO than either CAT or PH. PH metabolism by human MPO results in the formation of 2,2′-biphenol and 4,4′-biphenol initially, which are further metabolized rapidly by MPO (Fig. 3). Studies with HRP as a model peroxidase indicate that 2,2′-biphenol is the major metabolite of peroxidase-mediated oxidation of PH.[85–88] The formation of biphenolic metabolites is indicative of initial phenoxy1 radical formation. the dimerization of which results in the formation of biphenols. Biphenols are also substrates for peroxidases. Our studies with both MPO and HRP show that further metabolism of 2,2′-biphenol results in the formation of insoluble melanin-like polymers as dark brown colored granular specks.[89] 4,4′-Biphenol metabolism results in the formation of 4,4′-biphenoquinone, which in the presence of GSH results in the formation of GSH-conjugates.[81,89]

Catechol metabolism by human MPO and H_2O_2 results in the formation of 1,2-BQ.[80] Kalyanaraman and associates[90,91] have shown that CAT metabolism by HRP and H_2O_2 proceeds through initial 1,2-benzosemiquinone radical (1.2-SQ·) formation. The fate of 1,2-SQ·⁻ appears to be its spontaneous disproportionation to 1,2-BQ and CAT. Using HPLC analysis, we characterized 1.2-BQ as its bromothiophenol adduct

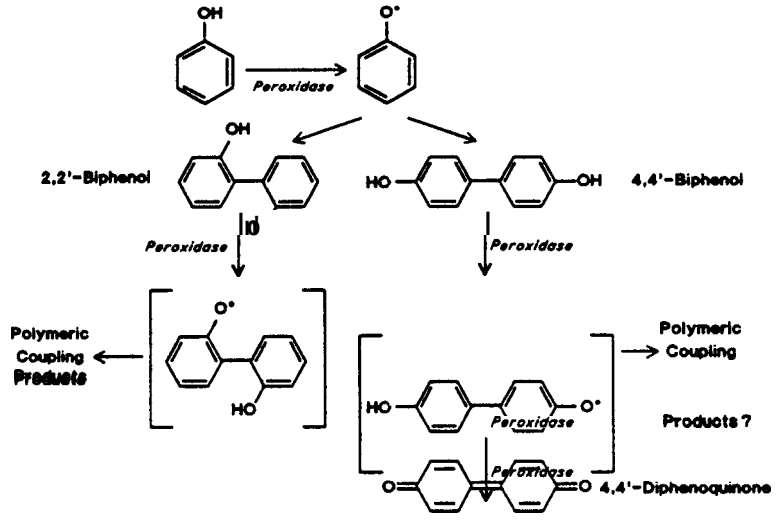

Fig. 3. Mechanisms of metabolic activation of phenol by human myeloperoxidase (adapted from ref. 12).

during the metabolism of CAT by MPO/H_2O_2 or HRP/H_2O_2.[80] 1,2-BQ could not be isolated and purified due to its instability. Subsequently, we analysed GSH-conjugates formed during CAT metabolism by MPO/H_2O_2 or HRP/H_2O_2 in the presence of GSH. Two GSH-conjugates were formed, the major conjugate appears to be the mono GSH-conjugate of 1,2-BQ (Fig. 4). The fare of GSH-conjugates of CAT, if formed in bone marrow, is unclear.

Hydroquinone metabolism by human MPO and H_2O_2 results in the formation of 1,4-BQ as the major metabolite.[81] Yamazaki and coworkers[73–75] have demonstrated that the oxidation of HQ by HRP and H_2O_2 proceeds through 1.4-benzosemiquinone radical (1,4-$SQ^{\cdot -}$) intermediate. The fate of 1.4-$SQ^{\cdot -}$ appears to be its spontaneous disproportionation to 1,4-BQ and HQ. similar to that of 1,2-$SQ^{\cdot -}$. However, 1,4-$SQ^{\cdot -}$ may, under certain conditions, also react with dioxygen resulting in the generation of $O_2^{\cdot -}$, which will be discussed later in this article.

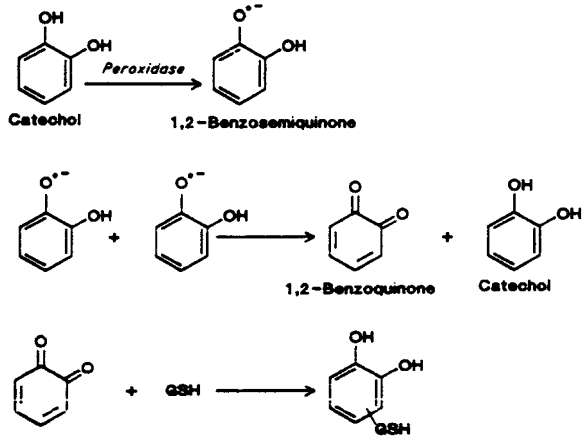

Fig. 4. Mechanisms of metabolic activation of catechol by human myeloperoxidase.

C. Metabolism of benzene and its phenolic metabolites by neutrophils

Neutrophils typically make up 50–70% of the white cell fraction of human peripheral blood.[92] Large numbers of immature neutrophils also reside in the BM.[93–96] MPO comprises 5% of the dry weight of neutrophils.[97] Neutrophils also possess the ability to be stimulated by several particulate and nonparticulate agents resulting in oxygen radical generation.[98] This process, known as the "oxidative burst" is due to the activation of a membrane-bound NADPH oxidase which is dormant in resting cells and becomes activated during phagocytosis or following interaction with suitable stimulants. Flavoproteins, cytochrome b_{558} and ubiquinone have been proposed to be the components of this oxidase-system. The presence of ubiquinone is, however, controversial. Many of the stimulants of the oxygen radical activity in neutrophils appears to mediate their actions by activating protein kinase C, either by a direct interaction or indirectly by receptor-triggered cascade (Fig. 5).[97] The receptor-triggered cascade involves the activation of phospholipase C, which acts on phosphatidyl inositol (P-inositol) and hydrolyses it to diaclyglycerol. The compounds having structural similarity to diacyl-.glycerol. such as phorbol myristate acetate (PMA; stimulant 4, Fig. 5) have been shown to directly activate protein kinase C.[99] protein kinase C appears to regu-

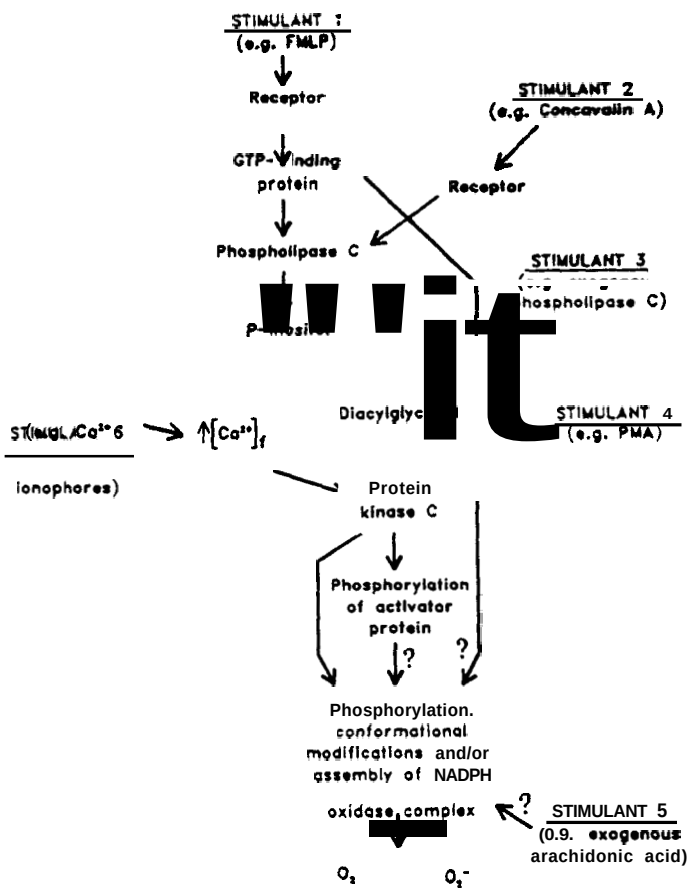

Fig. 5. pathways for stimulation of NADPH-oxidase activity (adapted **from** ref. **98). Benzene may also stimulate NADPH oxidase** activity **through protein kinase C activation.**[100,101]

late the activity of NADPH **oxidase** and result in the stimulation of oxidative burst in neutrophils. Recent studies by Castagna and **colleagues**[100,101] have shown **that** BZ can activate protein kinase **C** activity in intact platelets isolated *from* rabbit blood. but very **high** concentrations of BZ **(0.8 - 4.8%) are** required. The physiological relevance of **this** phenomenon is doubtful.

PMA is known to activate protein **kinase** C in human promyelocytic leukemia (HL-60) cells and **also** results in the differentiation of these cells to macrophages.[102–106] **HL-60** cells, however, do not possess oxidative burst, but do develop the oxidative burst during their differentiation to **macrophages.**[103] Recent studies by Kalf and colleagues have shown that incubation of **HL-60** cells with BZ induces differentiation **of** these cells into **macrophages,**[107] presumably by activating protein **kinase C.** In contrast, pre-incubation of HL-60 cells with HQ **(0.5 -5** μM) inhibits the PMA-induced differentiation of HL-60 **cells**[108] suggesting that HQ or its metabolite 1,4-BQ may act by inhibiting protein **kinase C.** It is interesting to note that BZ and its metabolite HQ have contrasting effects on HL-60 cell differentiation. **The** relevance of these studies in BZ-induced myelotoxic effects is not clear.

Kalir et **al.**[109] have shown that addition of high concentrations of BZ (1.3 mM) to human neutrophils results in a marked decrease in the number of cellular granules, a decrease in the amount of heterochromatin and a rarefication (decreased density) of ribosomes. These effects of BZ were prevented in the incubations containing 2-aminoethylthiosulfuric acid, a free radical scavenger. These authors concluded a free radical metabolite of BZ was responsible for the observed toxic effects on neutrophils, but did **not** determine the nature of the **free** radical metabolite.

We have shown that PH and HQ **are** activated to DNA and protein-binding metabolites by intact neutrophils stimulated with the tumor promoter **PMA.**[110–112] Inhibitors of MPO such **as** azide and cyanide inhibited the PH-[112] and **HQ-**[111] induced **DNA-** and protein-binding, respectively, in neutrophils, indicating that MPO is involved in the activation of these phenolics in neutrophils. **SOD,** which increases the dismutation of

$O_2^{\cdot -}$ to H_2O_2, increased the covalent binding of PH to neutrophil DNA. Conversely, catalase, which decomposes H_2O_2 to dioxygen and water, decreased the covalent binding of PH to neutrophil DNA.[111] These results indicated that H_2O_2 is required for the activation of PH and HQ to products that covalently bind to neutrophil DNA and protein.

Since H_2O_2 is required for peroxidatic metabolism of BZ's phenolic metabolites, we have attempted to determine whether they activate neutrophil NADPH-oxidase activity similar to PMA and thus generate H_2O_2 required for their metabolism by MPO. Our efforts using manometric studies (Subrahmanyam, V.V. and O'Brien. **P.J.,** unpublished) and cytochrome c reduction (Eastmond, D.A. and Smith, **M.T.,** unpublished), to determine whether the phenolic metabolites of BZ could stimulate the oxidative burst in neutrophils were negative but inconclusive. It is possible that these techniques are not sufficiently sensitive to detect small increases in oxidative burst activity in neutrophils. Alternatively, it is possible that BZ and its metabolites could prime the NADPH oxidase activity for activation by endogenous stimulants. In support of this, Laskin et al.[111] have recently demonstrated an increase in phagocytic activity of bone marrow cells of mice administered with BZ or PH and HQ, indicating that BZ or phenolic metabolites of BZ may act by priming the NADPH-oxidase activity of bone marrow cells. Unfortunately. whether such priming of NADPH oxidase activity. by BZ or its phenolic metabolites, occurs in vitro and neutrophils or bone marrow cells has not been tested.

D. Metabolism of benzene and its phenolic metabolites by bone marrow cells and homogenates

Benzene metabolism in bone marrow homogenates is mediated by the cytochrome P450 monooxygenase system.[113–115] This enzyme system has been shown to hydroxylate BZ to PH. The significance of cytochrome P450, however, in the hydroxylation of BZ in situ in bone marrow is not clear and requires further work. The mechanism(s) of BZ hydroxylation to PH has been reviewed extensively in the recent years and therefore we suggest that interested readers consult those reviews,[10,11]

We have shown that bone marrow cells and homogenates of rat. mouse. and human have the capacity to metabolize PH.[95,116] HQ[95] and CAT[117] in the presence of H_2O_2 as cofactor. In mouse bone marrow cells binding of ^{14}C-CAT metabolites to protein (20 nmol/mg protein) was found to be twice that obtained with ^{14}C-HQ (10 nmol/mg protein) and twelve-fold higher than that obtained with ^{14}C-PH (1.5 nmol/mg protein).[95]

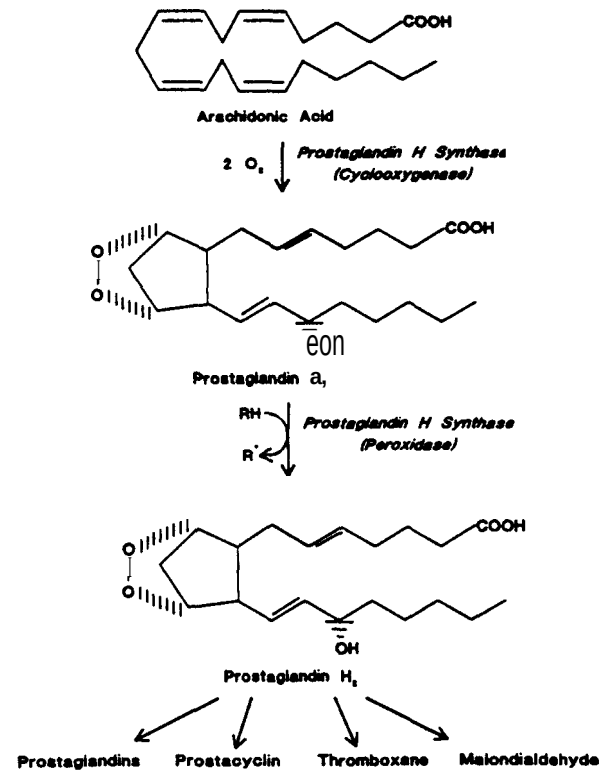

Fig. 6. Arachidonic acid metabolism by prostaglandin **H synthase. RN and R · represent** electron **donor and its free radical product. respectively. Phenolic metabolites (phenol. hydroquinone. and catechol) of benzene could also serve as electron donors for the** peroxidase **activity of** prostaglandin **H synthase.**[64,65]

Metabolism of PH by rat bone marrow homogenate in the presence of H_2O_2 has been shown to result in the formation of 2.2'- and 4,4'-biphenol as the major metabolites[116] indicating the peroxidative nature of the metabolism. Extensive protein binding of PH-equivalents to bone marrow homogenate also occurred. Using HRP and hydrogen peroxide as a model system, we have characterized the nature of products binding to bovine serum albumin. This protein-binding appears to result from a nonconvalent interaction of polymers (further oxidation products of 2,2'-biphenol) as well as a covalent interaction of 4,4'-diphenoquinone (further oxidation product of 4,4'-biphenol) with sulfhydryl groups of albumin). The binding of the polymers to protein is so strong that normal procedures for establishing covalent binding to protein were not effective in removing all of these polymers from protein. Enzymic digestion of protein, however, resulted in the release of the polymers from protein. A charge-transfer complex formation between the polymers and the protein has been tentatively suggested as an explanation for this noncovalent. yet strong interaction of these polymers with protein. The toxicological significance of this type of interaction is not clear, but such an ex-

tensive protein-binding could result in the inactivation of various cellular enzymes and conmbute to cytotoxic events. Extensive noncovalent binding of polymeric oxidation products of PH with DNA has also been observed during a peroxidatic metabolism. which will be described in later sections.

Metabolism of CAT by rat and human bone marrow cells also results in the formation of reactive metabolites that bind to protein.[117] Protein binding could be inhibited by GSH. We have shown that the GSH-conjugates are formed from the reaction of GSH with 1,2-BQ formed during the metabolism of CAT. Although H_2O_2-dependent metabolism of HQ and CAT in bone marrow could be catalyzed by either peroxidases or cytochrome P-450, indirect evidence using inhibitors suggest that cytochrome P-450 plays little role in peroxidatic bioactivation in bone marrow.[117]

E. Metabolism of phenolic metabolites of benzene by specific cell types of bone marrow

Bone marrow stroma primarily consists of two cell types (macrophages and fibroblastoid stromal cells) which appear to control the differentiation and maturation of myelopoietic cells via the production of growth factors.[116] Studies from Wierda's[119] laboratory have shown that macrophages are more sensitive than fibroblastoid stromal cells to the toxic effects of HQ. Interestingly, bone marrow macrophages contain appreciable peroxidase activity while fibroblastoid cells do not.[120] Hydrogen peroxide-dependent bioactivation of HQ occurred readily in bone marrow macrophages, but not in fibroblastoid stromal cells, indicating macrophages contain an active peroxidase.

The selective toxicity of HQ to bone marrow macrophages rather than fibroblastoid stromal cells could be a result of both increased peroxidative activation of HQ to 1,4-BQ in the macrophage and an increased deactivation of 1,4-BQ in the fibroblast by the obligate two electron reductase, DT-diaphorase.[121] Inhibition of DT-diaphorase by dicumarol in the stromal fibroblast, but not in the stromal macrophage, has been shown to lead to increased covalent binding of HQ to protein.[121] These data and previous work,[122] suggest that activation and deactivation mechanisms, such as peroxidase and DT-diaphorase, respectively, in individual cell types are an important determinant of the cell specific toxicity of phenolic metabolites of BZ.

F. Prostaglandin (H) synthase-catalyzed metabolism of phenolic metabolites of benzene

Prostaglandin (H) synthase (PGS), another hemeprotein enzyme that is mainly involved in arachidonic acid (AA) metabolism to generate prostaglandins. has also been implicated in cooxidation of several carcinogens and drugs to reactive species (Fig. 6).[123,124] This enzyme has two distinct activities, being both a cyclooxygenase and a peroxidase. It has been established that AA is oxygenated by the cyclooxygenase activity of this enzyme to generate the endoperoxy hydroperoxide (PGG_2). Two molecules of oxygen are incorporated into AA during this process. This hydroperoxide is subsequently reduced to a hydroxy derivative of AA (PGH_2) by the peroxidase activity of PGS. This peroxidase activity of PGS is similar to the peroxidase activity of other peroxidases and cooxidizes a wide variety of electron donors to free radical intermediates.[123,124] Indomethacin, a nonsteroidal anti-inflammatory drug has been shown to inhibit the cyclooxygenase activity of PGS but has no effect on its peroxidase activity.[125] Prostaglandins. such as PGE_2, have been shown to down-regulate granulocyte/monocyte colony formation and may contribute to the myelotoxic effects of BZ. Wierda and his associates[53] have demonstrated that intraperitoneal administration of BZ (200 mg/kg; twice a day for four days) to male B6C3F1 mice elevates PGE_2 levels in bone marrow. This increase was prevented by pretreatment of mice with indomethacin (2 mg/kg). Further studies by Kalf et al.[62,63] have demonstrated that indomethacin (2 mg/kg) can alleviate the genotoxic effects (micronucleus formation)[62] and bone marrow depression[63] induced in bone marrow following intraperitoneal administration of BZ (0–1200 mg/kg twice daily for two days) to Swiss–Webster male mice. These results led Kalf et al.[62,63] to suggest that PGS activity in bone marrow may be involved in the myelotoxic effects of BZ.

The major problem with this hypothesis is that indomethacin is assumed to be a specific inhibitor of PGS and to act only in the bone marrow. Indomethacin may have profound effects on the toxicokinetics of BZ and its metabolites. It can also inhibit MPO,[12] BZ-dihydrodiol dehydrogenase[126] and diglyceride lipase,[127] and ingestion of indomethacin (5 mg/kg) has also been shown to reduce intestinal peroxidase activity in rats.[128,129] Moreover, PGS has never been purified from bone marrow. To date there are only two major peroxidases isolated from bone marrow, i.e., MPO and EPO.[61] Recently, Zilletti et al.[130] have shown that HRP and bovine milk lactoperoxidase are capable of oxidizing AA to prostaglandins, but this activity has yet not been demonstrated with either MPO or EPO. However, they did show that indomethacin inhibited the peroxidase-dependent formation of PGE, from AA with an IC_{50} of 3 μM. Thus. it is possible, that the prostaglandins detected in bone marrow after BZ administration are the result of peroxidase activity and not that of PGS.

Early studies by Christ and Van Dorp[131] indicated that guinea pig bone marrow contains no detectable PGS activity. Ziboh et al.[132] incubated bone marrow microsomes (isolated from Sprague–Dawley rats) with ^{14}C-AA and found that PGE, and PGF, (another prostaglandin metabolite of AA) were the major metabolites. These studies contained at least 6-mg microsomal protein in 2 ml of reaction mixture. The total conversion of AA into the products was found to be less than 2%. These incubations also contained HQ (0.55 mM), presumably to prevent the rapid inactivation of cyclooxygenase activity by AA metabolites. However, no studies were performed by these authors to determine the fate of added HQ. Kojima et al.[133] incubated ^{14}C-AA with 15 mg of whole bone marrow homogenate (prepared from male Wistar rats) in 0.5 ml of incubation mixture and found that approximately *80%* of the added AA was converted into three products. The major product, which contained approximately 60% of the incubated AA, was not identified and was assumed to be a nonprostaglandin derivative due to its distinct chromatographic properties. The other major product, which contained approximately 20% of the incubated AA, was tentatively identified as prostaglandin D_2. Formation of these metabolites from AA is completely inhibited by indomethacin (*25* μM).

The requirement of large amounts of bone marrow protein to study the metabolism of AA by bone marrow suggests that bone marrow contains very low levels of prostaglandin biosynthetic capability. Ziboh et al.[132] reported that microsomes isolated from hyperplastic bone marrow (turpentine-induced) contained 2.5-fold higher prostaglandin biosynthetic activity compared to the activity in normal bone marrow microsomes. However, the total formation of PGE, and PGF, in hyperplastic bone marrow represented only 4.2% of incubated AA.

Gaido and Wierda[53] suggested that macrophages present in adherent bone marrow stromal cells could be the source of PGS activity in bone marrow. These authors found that incubation of HQ with adherent bone marrow stromal cell cultures induced PGE, synthesis in these cells. They also showed that the stromal cells incubated with HQ also prevented granulocyte/monocyte colony formation in co-culture. The presence of indomethacin in these incubations ameliorated the toxic effects of HQ on granulocyte/monocyte colony formation. Interestingly. indomethacin was not able to reduce the PGE_2 levels induced by HQ in these cultures. These authors concluded that HQ-induced stromal cell toxicity was not solely due to increased PGS activity.

Kalf et al.,[62–65] however, maintain that bioactivation of phenolic metabolites of BZ by PGS is an important step in BZ-induced bone marrow myelotoxicity. These authors conducted a series of experiments using purified PGS (from ram seminal vesicles) and isolated peritoneal macrophages (from the peritoneal cavity of male C57BL/6 mice).[64,65] Their experiments showed that purified PGS, either in the presence of AA or H_2O_2, readily catalyzes the bioactivation of ^{14}C-PH and ^{14}C-HQ to reactive protein-binding metabolites. These authors also showed that a similar H_2O_2-dependent bioactivation of HQ and PH occurs in peritoneal macrophages, indicating a peroxidase is responsible. The observation that purified PGS can catalyze activation of phenolics via cooxidative mechanisms is not surprising, and the critical question is the role of PGS in bioactivation of phenolics in bone marrow cells in general and macrophages in particular. Using white bone marrow cell fractions from either mouse (Ross, D., unpublished) or rat[117] we have not been able to demonstrate a major role for PGS in bioactivation of phenolics to protein binding species. Studies in purified bone marrow cell types are of more relevance, however, and using bone marrow macrophages, rather than peritoneal macrophages, we have shown that they *are* indeed capable of peroxidatic bioactivation of HQ to 1,4-BQ, which can either form a GSH-conjugate and/or covalently bind to protein.[120] We have been unable to demonstrate arachidonate stimulated covalent binding of ^{14}C-HQ to protein, which would implicate PGS in the binding process, in bone mmow macrophages (Ganaosis, L. and Ross, D., unpublished). Schlosser and Kalf,[64,65] however, have reported such a stimulation, albeit marginal, in peritoneal macrophages. Michel et al.[134] found that mouse bone marrow macrophages can catalyze the synthesis of PGD, from AA that is associated with endogenous peroxidase activity. The nature of peroxidase present in the bone marrow macrophages may therefore be *MPO* or an, as yet, uncharacterized peroxidase. Since peroxidases other than PGS have also been shown to transform AA to prostaglandins, it remains to be determined whether the prostaglandin biosynthetic activity of bone marrow macrophages is due to PGS or other peroxidases.

Clearly much more work needs to be done to determine whether PGS plays a role in modulating BZ toxicity. Whether the role, if any, of PGS in BZ toxicity is at the level of peroxidatic activation of phenolic metabolites to reactive species or is a result of altered levels of eicosanoids in bone marrow due to the presence of BZ metabolites remains to be determined.

MOLECULAR AND CELLULAR EFFECTS OF BENZENE AND ITS METABOLITES

In order to understand the mechanisms by which BZ exerts its toxic and leukemogenic effects, numerous studies have attempted to identify the cellular and mo-

Table 1. A Comparison of HRP/H_2O_2-Mediated Binding of Oxidation Products of ^{14}C-phenol, ^{14}C-catechol and ^{14}C-hydroquinone to Calf Thymus DNA

Condition[a]	^{14}C-bound to DNA (nmol/mg)
Phosphate Buffer (0.1 *M*, pH 7.4)	
^{14}C-PH, HRP, H_2O_2, DNA	66.8 ± 15.7[b]
minus HRP	0.6 ± 0.2
^{14}C-CAT, HRP, H_2O_2, DNA	14.6 ± 1.2
minus HRP	0.5 ± 0.2
^{14}C-HQ, HRP, H_2O_2, DNA	0.56 ± 0.18
minus HRP	0.46 ± 0.2
Tris HCl Buffer (0.1 M; pH 7.4)	
^{14}C-PH, HRP, H_2O_2, DNA	42.1 ± 8.4
minus HRP	0.6 ± 0.1
^{14}C-CAT, HRP, H_2O_2, DNA	4.89 ± 0.12
minus HRP	0.38 ± 0.04
^{14}C-HQ, HRP, H_2O_2, DNA	0.33 ± 0.08
minus HRP	0.24 ± 0.05

[a]Incubations of either phosphate or tris-HCl buffer, contained phenolic substrate (200 μM), HRP (10 μg) DNA (1 mg). The rextions were initiated with the addition of H_2O_2 (400 @) and incubated for 30 min at room temperature. DNA was purified as described previously by Subrahmanyam and O'Brien.[87,88]

[b]Values represent mean ± S.E.M. (n = 3).

lecular effects that occur following exposure to BZ and its metabolites. Most research has focused on the effects of 1,4-BQ and BZ's phenolic metabolites due to evidence for their involvement in myelotoxicity, their commercial availability and their stability in aqueous solutions. Exposure to other reactive BZ metabolites such as 1,2-BQ, *t,t*-MA, or BZ-epoxide may induce similar effects although much less data is available on the adverse effects produced by these compounds. Indeed, Witz et al.[135] have shown that *t,t*-MA is a potent myelotoxicant in CD-1 male mice. The adverse effects of the benzoquinones and *t,t*-MA are primarily related to their ability to react with nucleophilic sulfhydryl and amino groups at key functional sites on proteins and with nucleophilic sites on DNA. In addition, the ability of quinones to undergo cyclic reduction and oxidation reactions (redox cycling), producing oxygen- and lipid-derived radicals, has also been implicated in the induction of pathological effects by these compounds. The following sections will overview the research efforts in five areas, DNA damage, protein damage, oxygen activation, GSH oxidation and lipid peroxidation, which have been proposed as being involved in the hematotoxic effects of BZ.

A. DNA-damage

Due to an association between neoplastic development and the ability of many chemical carcinogens to bind to DNA,[136] considerable research has focused on the interaction of BZ and its metabolites with DNA. Initial studies by Lua and Schlatter[137] recovered radioactivity bound to rat liver DNA following the administration of tritiated and ^{14}C-BZ to male rats. Additional studies by Gill and Ahmed[138] and Arfellini *et al.*[139] demonstrated that BZ (or metabolites) was capable of binding to bone marrow DNA following in vivo administration. More recently, a series of investigations has focused on the ability of the individual BZ metabolites to bind to deoxynucleotides and DNA in vitro.[140–145] These studies have indicated that 1.4-BQ, HQ, PH. BT. *t,t*-MA, and CAT are all capable of forming adducts with deoxynucleotides and DNA during in vitro incubations. However, the nature of the binding in the bone marrow and the identity of the reactive species involved remain largely unknown.

We have performed extensive in vitro studies to determine whether free radicals or other oxidation products of BZ could bind to the DNA. Table 1 shows a comparison of binding of reactive metabolites of ^{14}C-PH, ^{14}C-CAT, and ^{14}C-HQ formed during a peroxidase-catalyzed reaction. The reactions were performed in phosphate buffer as well as in tris-HCl buffer at pH 7.4. Extensive binding of PH and CAT oxidation products to DNA was observed. The binding of ^{14}C-PH, or ‘“C-CAT oxidation products to DNA is better in phosphate buffer than in tris-HCl buffer. Oxidation of “C-HQ, either in phosphate buffer or in ms-HC1-buffer, however, resulted in no significant increase in the binding of ^{14}C-HQ oxidation products to DNA compared to that in the absence of enzyme. Nonenzymic binding to DNA, of HQ-autooxidation products, is also not significantly different in both buffers. Previous studies aimed at determining the ability of 1,4-BQ to bind DNA and RNA. during microsomal activation of ^{14}C-BZ or ‘“C-PH, have resulted in contradictory results. The studies by Subrahmanyarn and O'Brien[88] and Tunek et al.'s[29] failed to detect any covalent binding of ^{14}C-PH oxidation products with DNA or RNA, during microsome-mediated activation although PH metabolism occurred. Arfellini et al.,[139] however, found a significant association of radioactivity with DNA during microsomal activation of ^{14}C-BZ, which was attributed to a covalent interaction of 1.4-BQ with DNA. In addition, Snyder and coworkers[141,142] found that 1.4-BQ covalently interacts with DNA following their incubation together. While our studies were performed in tris-HCl buffer,[88] Arfellini,[139] and Snyder[141,142] groups performed their studies in phosphate buffer. The data presented in Table 1, however, shows that there is no significant difference in the DNA-binding of ^{14}C-HQ oxidation products, in either buffer. Therefore, it is clear that the differences in our results with the other groups were not due to the different buffers used. Nevertheless. the data in Table I indicated that some oxidation products of ^{14}C-HQ are capable of binding

to DNA. presumably due to autooxidation, since the peroxidatic metabolism of ^{14}C-HQ did not increase this binding that occurred in the absence of the enzyme. On the other hand, the reason for the increased binding of ^{14}C-PH or ^{14}C-CAT oxidation products in phosphate buffer is not clear and remains to be determined. However, an analysis of the DNA-binding of PH oxidation products revealed that most of the binding is due to a noncovalent, charge-transfer complex formation between the melanin-like polymers and DNA.[87] DNA had no effect on the formation of biphenols which indicated that phenoxy radicals were not capable of binding to DNA. HRP-catalyzed oxidation of CAT also resulted in the formation of black melanin-like products, the formation of which was not inhibited by the presence of DNA (Howell, M.; Ross, D., and Subrahmanyam, V.V., unpublished). Similarly, the peroxidatic metabolism of the bladder carcinogens benzidine,[111] phenylenediamine[146] and methylaminoazobenzene,[146] and the uterine carcinogen diethylstilbestrol.[147] have been shown to form polymeric oxidation products that interact noncovalently with DNA. Peroxidatic metabolism of acetaminophen has also shown to result in the formation of polymers,[148] but whether these products bind to DNA has not been investigated. The interactions of polymers with DNA may result in irrepairable mutagenic changes. but this remains to be established. But, if this type of binding occurs in the cells, repair may be difficult and the cell may die readily. Cell proliferation may be initiated in neighbouring less damaged cells.

Snyder and coworkers[141,142] have isolated DNA adducts formed following chemical oxidation of HQ using $FeCl_3$ in the presence of DNA or following the incubation of 1.4-BQ with DNA and have identified these adducts as (3'OH) benzethenol(1,N^2)deoxyguanosine. These investigators have recently reported, using a ^{32}P-postlabelling technique, formation of several DNA adducts in the liver DNA of rabbits following the in vivo administration of BZ.[149] However, neither the chemical structure of this adduct nor its ability to co-chromatograph with the known standard 1,4-BQ-adducts was determined. In contrast, Reddy and associates.[150,151] using a nuclease P_1-enhanced ^{32}P-post-labelling technique, have been unable to detect DNA adducts in the liver. kidney, bone marrow, and mammary gland of rats treated with BZ or PH and HQ. Some preliminary evidence of the formation of adducts in the Zymbal gland of BZ-treated rats was observed during these experiments.

Studies by Norpoth *et* al.[152] have identified N^7-phenylguanine in the urine of rats following the intraperitoneal administration of BZ *at* 330-400 mg/kg. These authors suggested that this adduct was formed through the reaction of BZ-epoxide with guanine residues in DNA and that its presence in the urine was the result of the excision repair of this DNA adduct.

In spite of the considerable effort which has been made to identify DNA binding following BZ administration and to characterize the nature of the DNA adducts, there is no direct evidence for the involvement of these events in BZ-induced leukemia. The degree of covalent DNA binding recovered following BZ administration is low and rankings of carcinogenic agents by DNA binding generally rank BZ among the weakest initiating agents, approaching agents that act through indirect genotoxic mechanisms.[136,139] Subrahmanyam and O'Brien,[87] several years ago, found that an in vitro peroxidative metabolism of PH leads to the formation of polymeric products that strongly bind to DNA but do not form covalent adducts. The binding of these polymers to DNA is so strong that normal procedures for establishing covalent binding to DNA were not effective in removing all of these polymers from DNA. Therefore, a strong association of some radioactivity with DNA, following administration of radio-labelled BZ to animals, should not be taken as indicating covalent interaction of BZ metabolites with DNA. In addition, BZ and its metabolites are weakly or nonmutagenic in specific gene mutation assays.[153] These observations suggest that other types of molecular events are likely to be involved in the development of leukemia by BZ.

B. Protein-damage

One consistent observation that has been made in BZ-exposed humans and animals is the appearance of structural and numerical chromosomal aberrations and sister chromatid exchanges in lymphocytes and bone marrow cells indicating that BZ is genotoxic.[153–157] Chromosome and chromatid deletions, gaps, chromatid exchanges, hyperdiploidy and micronuclei induction, all have been detected to some extent. Since the induction of chromosomal aberrations in humans have been associated with a variety of neoplastic development,[158,159] these chromosomal aberrations could also contribute to the myelotoxic and leukemogenic effects of BZ in humans. BZ administration to animals is also accompanied with an inhibition of DNA, RNA, and protein synthesis (reviewed[10,11]). BZ metabolites interfere with DNA replication and transcription in rat liver and BM mitochondria in vitro that has been attributed to the inhibition of DNA polymerase gamma activity by HQ and 1.4-BQ. Studies by Lee and coworkers[160] have confiied that an inhibition of DNA synthesis occurs in bone marrow following the in vivo administration of BZ to mice and have indicated that inhibition occurred *at* doses that had no inhibitory effect on the synthesis of protein or heme. These authors reported that BZ itself had *the* ability to inhibit DNA synthesis in vitro in

cell-free incubations following the addition of BZ. Additional studies by these investigators studying the effects of individual BZ metabolites indicated that CAT, BT, HQ, and 1.4-BQ were able to inhibit nuclear DNA synthesis in cultured mouse bone marrow cells.[161] However, only 1.4-BQ and BT were able to inhibit DNA synthesis in a cell-free system. This effect appeared to be due to an interaction of these compounds with DNA polymerase-*alpha*.

The inhibition of DNA polymerase-alpha or -*gamma* activity by HQ and 1.4-BQ was suggested to be due to the arylation of 1.4-BQ with a critical sulfhydryl group on these proteins.[10,11,161] Due to the arylating activity of quinones with sulfhydryl groups, Irons[31] several years ago postulated that quinone metabolites of BZ may act as spindle poisons during cell division and disrupt microtubule assembly. This may cause lagging chromosomes and result in numerical chromosomal aberrations (aneuploidy). Indeed, Irons and colleagues[162] have demonstrated that administration of BZ to animals results in the block of cell division in the G_2 or M phase of the cell cycle, supporting a role for disruption of microtubule assembly. These investigators also found that HQ and 1,4-BQ are highly effective inhibitors of microtubule assembly in mouse lymphocytes[163] and isolated microtubule preparations.[164] The mechanism of this inhibition appeared to involve the 1,4-BQ-dependent arylation of extremely nucleophilic sulfhydryl groups on tubulin, which function in GTP binding and serve as a control site for the regulation of microtubule assembly. The inhibition of microtubule assembly by 1,4-BQ appeared to parallel the inhibition of cell growth and microtubule assembly induced by the T suppressor cell lymphokine, soluble immune response suppressor (SIRS) suggesting that 1,4-BQ may act by mimicking this intracellular messenger in vivo.[160] Although the spindle-poisoning effects by BZ metabolites appears to be a likely mechanism for the induction of numerical chromosomal aberrations, it cannot explain the induction of structural chromosomal aberrations that have been observed following BZ exposure. Such events probably occur via DNA damage, either through oxidative mechanisms or adduct formation.

C. *Active oxygen formation*

Ground state dioxygen itself is a radical species since it has two unpaired electrons with the same spin number (i.e., mplet state). Supply of energy can activate the triplet oxygen to highly reactive singlet oxygen in which the two-electrons have opposite signs. Other active oxygen species such as $O_2^{\cdot -}$, H_2O_2, and HO' can also be generated during successive one-electron reduction of dioxygen to water [eqn (1)].[46,50]

$$O_2 \xrightarrow{e^-} O_2^{\cdot -} \xrightarrow{e^- 2H^+} H_2O_2 \xrightarrow{e^-} HO^- + HO^{\cdot} \xrightarrow{e^-} HO^- + H_2O \quad (1)$$

Oxygen radicals may attack cellular DNA resulting in DNA-strand scission[43,44] and base damage.[45-47,165] Some of the basedarnaged products are hydroxylated bases which include 8-hydroxydeoxyguanosine (8-OHdG), thymine glycol (5,6-dihydroxydihydrothymine) and hydroxymethyluracil. Presence of 8-OHdG in DNA has been recently shown to cause DNA-polymerase to miscode nucleotide incorporation in the replicated strand and result in mutagenesis.[166,167]

Some xenobiotic free radicals have the ability to interact with dioxygen, resulting in $O_2^{\cdot -}$ formation.[168] However, oxidation of PH, CAT. or HQ by peroxidase and H_2O_2, which results in the formation of phenoxyl- or 1,2-$SQ^{\cdot -}$ or 1,4-$SQ^{\cdot -}$ radicals, respectively, did not result in any oxygen consumption.[167] This indicates that these radicals may not be reactive enough to interact with dioxygen. Sawada et al.[170] have, however, demonstrated increased 1,4-BQ formation during peroxidase-catalyzed oxidation of HQ in the presence of SOD. They suggested that the reaction of 1,4-$SQ^{\cdot -}$ with dioxygen could indeed occur if the $O_2^{\cdot -}$ are removed from the reaction. This appears to be due to faster back reaction, i.e., reaction of 1,4-BQ and $O_2^{\cdot -}$ forming 1,4-$SQ^{\cdot -}$ and dioxygen [eqn (2)]

$$1,4\text{-}SQ^{\cdot -} + O_2 \rightleftharpoons 1,4\text{-}BQ + O_2^{\cdot -}. \quad (2)$$

Our studies[W] confirmed Sawada et al.'s[170] study showing an increased production of 1.4-BQ during peroxidatic metabolism of HQ in the presence of SOD. Interestingly, we found no increased production of 1,2-BQ during peroxidatic metabolism of CAT in the presence of SOD.[84]

It has been shown, however, by Kasai and Nishimura[171] that incubation of either HQ or CAT with H_2O_2, femc iron (Fe^{+3}) and deoxyguanosine results in the formation of 8-OHdG, presumably due to the addition of HO' to deoxyguanosine. Recently, Iwahashi et al.[172] showed that a variety of catechols, including CAT in the presence of femc iron, EDTA and H_2O_2, could result in the formation of HO'. The mechanism appears to be due to the initial reduction of iron from the femc (Fe^{+3}) state to ferrous (Fe^{+2}) state by CAT. The reaction of Fe^{+2} with H_2O_2 thus generates the reactive HO' as shown below:

$$CAT + Fe^{+3} \rightarrow 1,2\text{-}SQ^{\cdot -} + Fe^{+2}, \quad (3)$$

$$Fe^{+2} + H_2O_2 \rightarrow HO^{\cdot} + OH^- + Fe^{+3}. \quad (4)$$

Similarly, HO' formation was described by Rao and

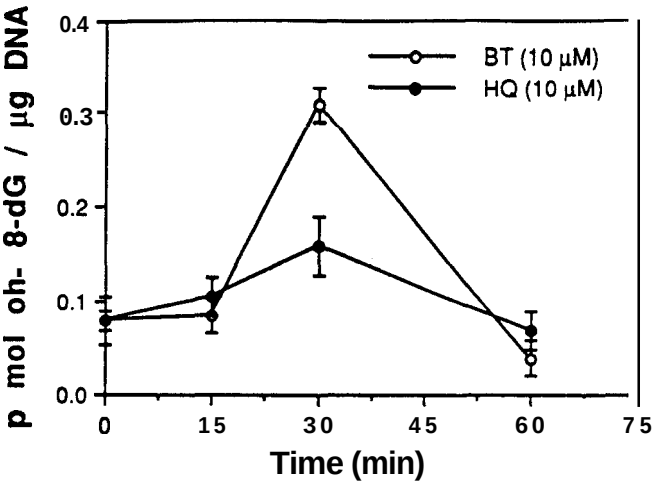

Fig. 7. Formation of 8-hydroxydeoxyguanosinein DNA of human promyelocytic leukemia cells cells exposed to benzene metabolites.

Pandya,[173] who showed that incubation of HQ or BT with DNA in the presence of copper ions (Cu^{+2}) results in the formation of thiobarbituric acid reactive products. This process was inhibited to some extent by mannitol, a HO' scavenger, and also by catalase and SOD, which prevent the HO' formation by decomposing H_2O_2 and removing $O_2^{\cdot -}$, respectively. In contrast, Kawanishi et al.[174] did not detect HO' formation using electron spin resonance during auto-oxidation of BT in the presence of copper ions, although they detected HO' formation in the presence of iron (Fe^{+3}). They indicated that the DNA-damage induced by BT and copper ions in their studies may not be due to HO'. Lewis et al.[175] have shown that DNA-damage also occurred during the auto-oxidation of HQ and BT at physiological pH but only the damage induced by the latter was inhibited by scavengers of $O_2^{\cdot -}$, H_2O_2, and HO'. It is likely that the differences in these studies are due to differences in the concentrations employed. The relevance of metal-catalyzed oxygen radical production by BZ metabolites in myelotoxicity induced by BZ is not known, but, it is worth emphasizing that iron accumulation in bone marrow has been observed following administration of BZ to rats.[34,176] This iron accumulation may be due to the induction of heme oxygenase,[177] which also occurs in rats exposed to BZ. Heme oxygenase is an enzyme responsible for heme degradation to biliverdin.[178,179] Biliverdin can be further reduced to bilirubin by biliverdin reductase. Iron may be released from heme during its degradation. It is also interesting to note that biliverdin and bilirubin are considered to be good antioxidants.[178] Therefore, it is difficult at this point to emphasize whether induction of heme oxygenase by BZ in bone marrow increases myelotoxicity by releasing iron from heme or prevents the free radical reactions in bone marrow by increasing the levels of the antioxidants, biliverdin, and bilirubin.

Pellack-Walker et al.[180] have shown that HQ, BT, CAT, and PH are cytotoxic to L5178YS cells, inhibit DNA synthesis and induce DNA-strand breaks in these cells. The inhibition of DNA synthesis by these BZ metabolites correlated inversely with their respective one-electron redox potentials: HQ is most potent followed by BT, CAT, and PH. These authors suggested that oxygen radicals play a major role in the cytotoxicity and DNA strand breaks induced by these BZ metabolites in these cells.

Human promyelocytic leukemia (HL-60) cells contain appreciable levels of MPO.[181] We found that incubation of HL-60 cells with HQ (10 µM) or BT (10 µM) can result in alkylation of cellular proteins and cytotoxicity.[182,183] This cytotoxicity and alkylation of HL-60 cells was potentiated in the presence of hydrogen peroxide, indicating that MPO may be involved in the bioactivation of HQ to the reactive 1,4-BQ. Formation of 1.4-BQ was confirmed by the presence of a GSH-conjugate that co-elutes with the standard 1,4-BQ-GSH conjugate during HPLC analysis. We also found that incubation of HL-60 cells with HQ (10 µM) or BT (10 µM) results in a 2- and Cfold increase in 8-OHdG formation in DNA, respectively[183] (Fig. 7). The increase in 8-OHdG formation, however, is transient, and prolonged incubation of these cells results in a drop in 8-OHdG levels in DNA to steady-state levels, indicating that these cells are also equipped with an efficient repair mechanism for oxidative DNA damage. These results suggest that alkylation of cellular proteins and oxidative DNA-damage induced by BZ-metabolites in HL-60 cells may contribute to cytotoxic and gentoxic events in these cells. They also provide the first direct evidence that BZ metabolites could produce mutations via oxidative modification of DNA.

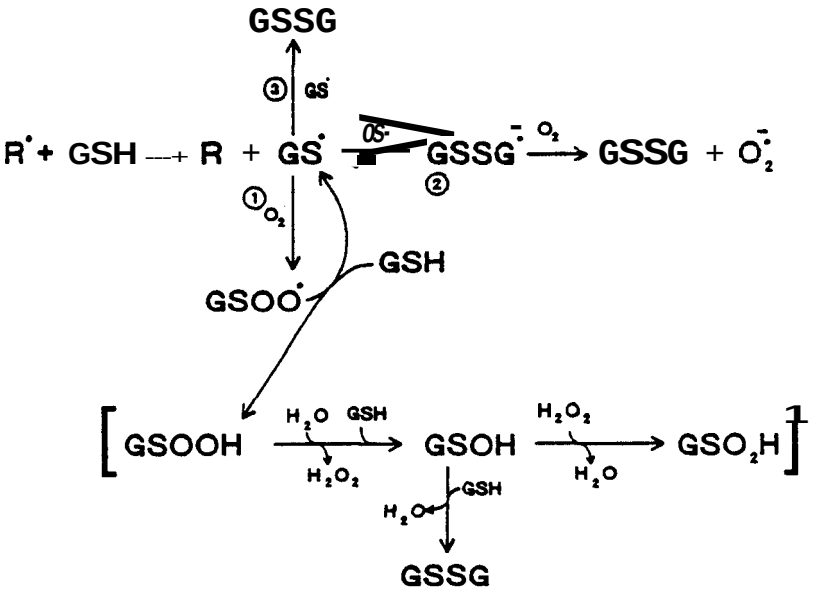

Fig. 8. Interaction of free radicals (denoted as R˙ in figure) with glutathione and further reactions of the glutathionyl radical. Similarly, phenoxy radicals could act as R˙ initiating glutathione oxidation and subsequent oxygen activation.[169] (Adapted from ref. 185. See text for a complete description of the figure.)

D. Glutathione oxidation

Glutathione (GSH; γ-glutamylcysteinylglycine) is the major cellular soluble thiol. It is involved in the detoxification of electrophilic or free radical metabolites of xenobiotics.[184–186] The detoxification of electrophilic metabolites by GSH appears to involve direct conjugation of the nucleophilic sulfhydryl group with the electrophilic metabolite, which may require GSH-transferases. GSH-conjugates of electrophilic metabolites of toxic chemicals that are formed in the liver are further metabolized to mercapturic acids and excreted in urine.[187] BZ administration to rabbits results in the excretion of phenylmercapturic acid, which suggests that BZ-epoxide conjugation with GSH occurs in the liver of these animals.[16] Jerina et al.[188] have shown that BZ-epoxide-GSH-conjugates can be formed in vitro. Studies in vitro also revealed that 1,4-BQ,[22,26] 1,2-BQ,[84] and 1,4-diphenoquinone,[81,89] all react with GSH, forming one or more GSH-conjugates. A mercapturate derivative of HQ has recently been isolated as one of the urinary metabolites of BZ, PH, and HQ, indicating that 1,4-BQ-GSH conjugate formation also occurs in vivo.[189]

The detoxification of certain free radical metabolites of xenobiotics was shown to be due to the interaction of xenobiotic-derived free radicals with GSH forming thiyl radicals (GS˙)[185,186] (Fig. 8). These GS˙ can readily interact with dioxygen, forming a peroxy sulfenyl radical (GSOO˙; 1, Fig. 8), in the presence of excess GSH result in the formation of dimerized radical ($GSSG^{\cdot-}$; 2, Fig. 8) or dimerize to yield GSSG (3, Fig. 8). The reaction of dimerized thiyl radicals with oxygen yields $O_2^{\cdot-}$, which subsequently dismutates to H_2O_2. Further reactions of GSOO˙ with excess GSH also eventually results in the formation of GSSG. The GSSG and H_2O_2 can subsequently be detoxified by GSH peroxidase/GSSG reductase system.[185]

The oxidation of PH by HRP and H_2O_2 results in the formation of phenoxyl radicals.[67] These radicals in the presence of GSH are reduced back to PH, resulting in the formation of thiyl radicals.[84,169,190] Extensive oxygen uptake occurs during this process (Table 2), which results in the formation of superoxide radical. Catalytic amounts of PH and H_2O_2 oxidize large excess of GSH to GSSG. The amount of GSH oxidation to GSSG was increased in the presence of

Table 2. Phenol-induced Glutathione Oxidation and Arachidonate Peroxidation During Peroxidative Metabolism of Phenol

Phenolic Substrate	Oxygen Uptake (μM) (+GSH)[a]	Thiyl Radical Formation[b]	Oxygen Uptake (μM) (+ arachidonate)[a]
None	0.05 ± 0.05		0.6 ± 0.5
Phenol	124 ± 14	+	13 ± 2
Catechol	*0.5 ± 0.5*		0.6 ± *0.5*
Hydroquinone	0.5 ± 0.5	-	0.6 ± *0.5*

[a]Reaction system contained 3 ml of 10-mM tris-HCl buffer (pH 7.4), phenolic substrate (100 μM), HRP (10 μg) and GSH (200 μM) or AA (500 μM). The reactions were initiated with the addition of H_2O_2 (50 μM).

[b]Thiyl radicals were trapped with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO; 0.1 M) in a reaction system containing phenolic substrate (100 μM) in 1 ml of 0.1-M tris-HCl buffer containing 1-mm EDTA (pH 8.0). ESR spectra were recorded on a Varian E9 spectrometer.

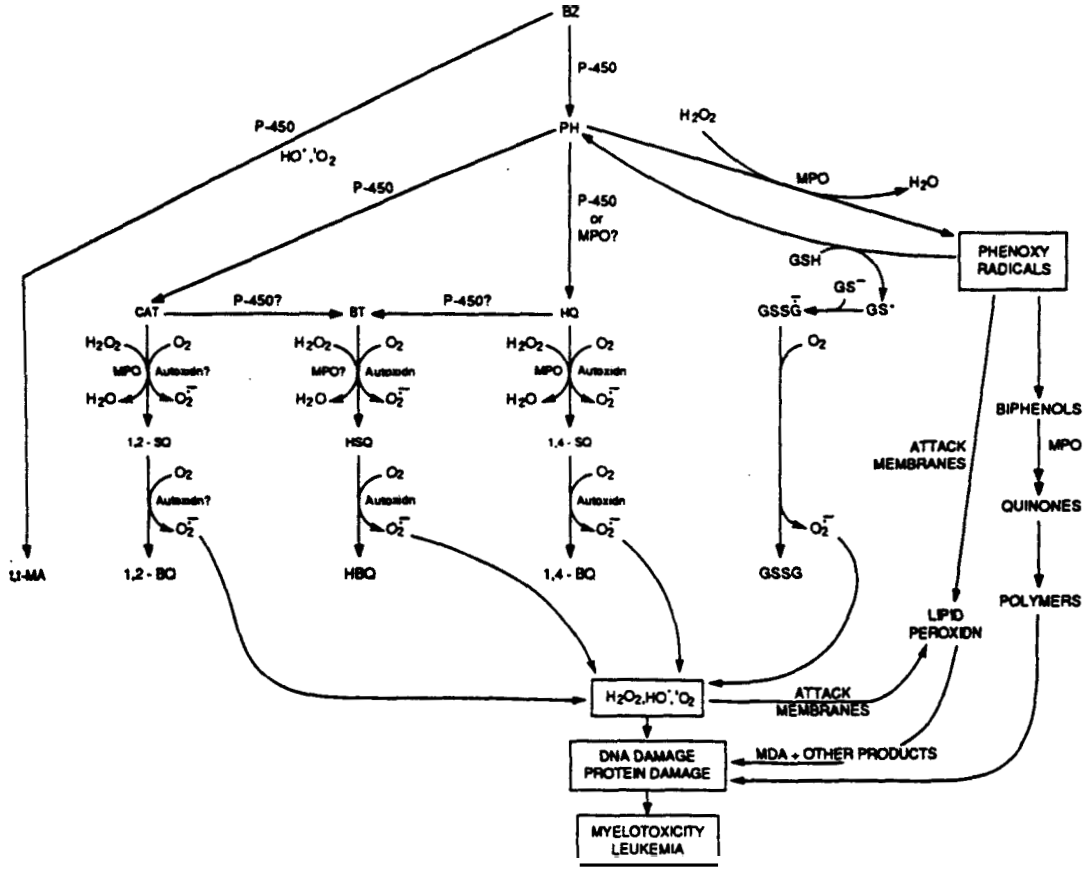

Fig. 9. Hypothetical scheme for **the possible pathways** of **free radical formation during** benzene **metabolism that induce oxygen activation. lipid peroxidation. glutathione oxidation and** DNA/protein **damage. (See Abbreviations** for **the exact** names of **the** abbreviated forms **used** in **this figure.)**

SOD. This may be due to the increased dismutation of $O_2^{\cdot-}$ *to* H_2O_2. **This** H_2O_2 *can* efficiently oxidize more **PH** by **peroxidase. No** PH removal **occurs** until all the GSH is converted to GSSG. HQ and CAT oxidation by the HRP/H_2O_2 system in the presence of GSH. however, do not result in oxygen activation, indicating that the semiquinone radicals of HQ and CAT may not react readily with GSH.[169]

The physiological significance of the reactions of phenoxy1 radicals with GSH and the subsequent redox cycling of thiyl radicals, in BZ-induced toxicity remains to be determined. We found that red blood cell free bone **marrow** cells contain low levels of GSH (1 **nmol/10^6 cells).**" **Smart** and **Zannoni**[191] found that the total GSH **content** of guinea pig bone **marrow** (unper*fused)* is approximately in the range of 20–25 nmol/mg **protein. which** is similar **to the** hepatic GSH levels **(27–28-nmol/mg protein). This indicates** that **the majority of bone marrow GSH may** be **associated with the red blood cell fraction. It** is **also possible** that species **differences exist in the GSH levels of bone marrow, which remains to be determined.**

E. Lipid peroxidation

The peroxidation of lipids is commonly described as **an** oxidative, oxygen-dependent deterioration of fats, particularly unsaturated fatty **acids.**[192,193] Lipid peroxidation can be initiated by abstraction of an H atom from unsaturated fatty acids. **This** can be achieved either enzymatically **as** described earlier for **AA** oxygenation by PGS, or by lipoxygenase or by reaction with another free radical. The addition of oxygen yields a lipid peroxyl radical, which is considered a hallmark of peroxidizing lipids. The peroxy radical combines with the hydrogen atom that it abstracts **to** give a lipid hydroperoxide, R–OOH. *An* alternative fate of peroxyl radicals is to **form** cyclic peroxides. Pure lipid hydroperoxides are fairly stable molecules at physiologic temperatures, but they rapidly decompose in the presence of transition-metal complexes. **This** results in the fission of O–O bond **to** form **an** alkoxyl radical.

$$\text{R-O-O-H} + \text{Fe}^{+2}\text{-complex} \rightarrow \text{Fe}^{+3}\text{-complex} + \text{OH}' + \text{R–O}^{\cdot} \quad (5)$$

Peroxyl radicals seem to be less reactive than alkoxyl radicals.[194] In both cases, however, the formation of radical products will stimulate the chain reaction of lipid peroxidation by causing more initiation. β-Scission. a well-known reaction of alkoxyl radicals. leads to the production of compounds containing the carbonyl group C = O, particularly aldehydes such as malondialdehyde and 4-hydroxy nonenal. These aldehydes can also readily attack macromolecules such as DNA and protein, which then results in covalent binding as well as intra- and inter-molecular crosslinks of DNA and proteins.[192,193,195–198] In addition. lipid peroxidation products such as PGE_2 are potent regulators of hematopoiesis.[53]

It is interesting that HRP-catalyzed oxidation of PH in the presence of AA results in rapid oxygen uptake (Table 2) and conjugated-diene formation.[40,190] However, HQ and CAT were found to be inactive in this system. It appears that the phenoxyl radicals formed in this system can efficiently abstract an hydrogen atom from AA and induce lipid peroxidation. Baumann et al.[199] recently analyzed the products of arachidonic acid in this reaction and found that 90% of the products co-chromatographed with 15-HPETE and 15-HETE, resembling a 15-lipoxygenase-catalyzed pathway.

Benzene administration to rats has been shown to induce lipid peroxidation in both liver and bone marrow of rats.[33,34,53] It is possible that the phenoxy radicals and/or oxygen radicals generated during BZ metabolism could contribute to the observed lipid peroxidation following BZ administration.

CONCLUSIONS

This review indicates that a good deal of information is already available in the literature that documents the ability of BZ to induce free radical formation in vivo. However, the role these free radicals play in BZ-induced myelotoxicity and carcinogenesis remains to be determined. Figure 9 summarizes the possible mechanisms of free radical formation, during BZ metabolism that can result in damage to the cellular macromolecules. Free radical formation in bone marrow may occur either during peroxidatic metabolism (catalyzed by MPO, EPO, and perhaps PGS) or during auto-oxidation of BZ's phenolic metabolites. The interactions of these radicals with cellular thiols and lipids may initiate oxidative damage and lipid peroxidation. Oxygen free radicals may also be formed by stimulation of the membrane-bound NADPH-oxidase activity of phagocytic bone marrow cells. Active oxygen species such as HO' and $^1O_2$ may bind to DNA, resulting in the formation of hydroxylated adducts of bases such as 8-OHdG. Therefore, free radical-mediated DNA damage may conmbute to the production of the chromosomal aberrations that have been observed in humans following exposure to BZ and the subsequent development of leukemia. Free radical formation in liver. a secondary target organ for BZ-toxicity, *can* also *occur* provided cellular defenses such as GSH are depleted.

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ABBREVIATIONS

AA — Arachidonic acid
1.2-BO — 1,2-Benzoquinone
1,4-BQ — 1,4-Benzoquinone
BT — 1,2,4-Benzenetriol
BZ — Benzene
CAT — Catechol
DMPO — 5,5-Dimethyl-1-pyrroline-N-oxide
DNA — Deoxyribonucleic acid
EPO — Eosinophil peroxidase
GSH — Reduced glutathione
GSSG — Oxidized glutathione
HL-60 — Human promyelocytic leukemia cells
H_2O_2 — Hydrogen peroxide
HPLC — High pressure liquid chromotography
HQ — Hydroquinone
HRP — Horseradish peroxidase
HBQ — 2-hydroxy, 1.4-benzoquinone
HSQ — 2-hydroxy, 1,4-benzosemiquinone anion radical
MPO — Myeloperoxidase
1,2-$SQ^{\bullet-}$ — 1,2-Benzosemiquinone anion radical
1,4-$SQ^{\bullet-}$ — 1,4-Benzosemiquinone anion radical
t,t-MA — trans,trans-muconaldehyde
NADPH — Reduced nicotinamide adenine dinucleotide phosphate
NOE — Nuclear overhausser effect
$O_2^{\bullet-}$ — Superoxide anion radical
$HO^{\bullet}$ — Hydroxyl radical
$^1O_2$ — Singlet oxygen
8-OHdG — 8-hydroxy-2′-deoxyguanosine
PGG_2 — 15-hydroperoxy-9,11-endoperoxide
PGH_2 — 15-hydroxy-9.11-endowroxide
PGS — Prostaglandin H synthase
PMA — Phorbol myristate acetate
RNA — Ribonucleic acid
SIRS — Soluble immune response suppressor
SOD — Superoxide dismutase
TBA — Thiobarbituric acid