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# IRON-STIMULATED RING-OPENING OF BENZENE IN A MOUSE LIVER MICROSOMAL SYSTEM

## MECHANISTIC STUDIES AND FORMATION OF A NEW METABOLITE

ZHIHUA ZHANG, BERNARD D. GOLDSTEIN and GISELA WITZ*

Joint Graduate Program in Toxicology, Rutgers University/UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ 08855 U.S.A.

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**Abstract**—In the present study, we investigated the mechanism(s) of ring-opening of benzene in a mouse liver microsomal system in the presence of $Fe^{2+}$. HPLC analysis based on coelution with authentic standards and on-line UV spectra obtained using a diode array detector indicated that benzene is metabolized to phenol, hydroquinone (HQ), *trans,trans*-muconaldehyde (muconaldehyde, MUC), 6-oxo-*trans,trans*-2,4-hexadienoic acid (COOH-M-CHO), 6-hydroxy-*trans,trans*-2,4-hexadienal (CHO-M-OH), and 6-hydroxy-*trans,trans*-2,4-hexadienoic acid (COOH-M-OH). CHO-M-OH was confirmed by mass spectrometry. Muconaldehyde was also metabolized to CHO-M-OH, COOH-M-CHO and COOH-M-OH, in the same microsomal system. The inhibition of muconaldehyde metabolism by microsomes in the presence of pyrazole indicates that there is cytosolic alcohol dehydrogenase (ADH) activity in the microsomes. Metabolism by contaminating ADH of muconaldehyde formed during microsomal incubation of benzene could be involved in the formation of CHO-M-OH and COOH-M-OH. The ring-opening of benzene was stimulated by added $Fe^{2+}$. Hydrogen peroxide was produced in the microsomal system and consumed in the presence of added $Fe^{2+}$. Addition of catalase inhibited the formation of ring-opened products, while superoxide dismutase increased their formation in the presence of azide. Singlet oxygen scavengers, i.e. histidine, deoxyguanosine, Tris and azide (at concentrations above 1.0 mM), dramatically decreased the ring-opening of benzene. Hydroxyl radical scavengers, DMSO, mannitol and formate, but not ethanol, also decreased the ring-opening of benzene. The data indicate that Fenton chemistry plays an important role in benzene ring-opening by microsomes. An unknown peak with UV absorption maxima at 275 and 345 nm was also detected. Based on pH sensitivity of the UV spectrum, the reactivity with thiobarbituric acid (giving a chromogen with absorption maximum at 532 nm) and the molecular weight (126), this compound was identified tentatively as α- or β-hydroxymuconaldehyde.

*Key words:* benzene; muconaldehyde; ring-opening; iron; benzene metabolism; reactive oxygen species; hydroxymuconaldehyde

Chronic exposure of humans and experimental animals to benzene produces pancytopenia and aplastic leukemia, and is associated with an increased incidence of myelogenous leukemia in humans [1, 2]. Although hematotoxic and leukemogenic effects of benzene are generally believed to be caused by its metabolites as opposed to the parent compound, the ultimate reactive intermediate(s) responsible for benzene toxicity is not known [3, 4]. The major benzene-metabolizing organ is the liver, where cytochrome P450 is involved in the metabolism of benzene to phenol, HQ†, catechol and 1,2,4- benzenetriol [5]. Since no single ring-hydroxylatd metabolite of benzene can produce the whole spectrum of benzene toxicity, more and more attention has focused on the interactions of benzene metabolites [6–8], reactive ring-opened metabolites, i.e. *trans,trans*-muconaldehyde (muconaldehyde) [9–12], and the toxicological significance of free radicals as ultimate toxic species mediating benzene toxicity [13]. It is believed that the effect of benzene is likely to be exerted through the action of multiple metabolites on multiple targets through multiple biological pathways [14].

Our laboratory has proposed that muconaldehyde, a six-carbon α,β-unsaturated diene dialdehyde, is a hematotoxic intermediate in benzene metabolism [9]. Muconaldehyde was shown to be a potent hemotoxin in CD-1 mice and a genotoxin in a variety of mammalian cell cultures [10, 15–17]. It is an electrophilic compound that reacts readily with glutathione [18].

The ring-opening of benzene has been studied since Parke and Williams [19] found *trans,trans*-muconic acid in the urine of rabbits administered [$^{14}$C]benzene. Pulse radiolysis of aqueous solutions saturated with benzene results in the opening of the benzene ring and formation of *trans,trans*-muconaldehyde [20], the corresponding aldehyde of *trans,trans*-muconic acid. Using similar radiolysis of an aerated aqueous benzene system, Sirnivasan *et al.* [21] detected β-hydroxymuconaldehyde and Balakrishnan and Reddy [22] found both α- and β-hydroxymuconaldehyde. Latriano *et al.* [23] demonstrated that muconaldehyde is formed from benzene in a Fenton system, presumably via hydroxyl radical-mediated ring-opening. Using the same Fenton system, Zhang *et al.* [24] found that benzene forms *cis,trans*-muconaldehyde as well as *trans,trans*-muconaldehyde, suggesting that *trans,trans*-muconaldehydye may not be the original ring-opened product of benzene. Pan *et al.* [25],

* Corresponding author: Dr. Gisela Witz, Toxicology Division, Environmental Occupational Health Sciences Institute, 681 Frelinghuysen Road, P.O. Box 1179, Piscataway, NJ 08855-1179. Tel. (908) 445-0170; FAX (908) 445-0119.

† Abbreviations: PH, phenol; HQ, p-hydroquinone; MUC or CHO-M-CHO, *trans,trans*-muconaldehyde (muconaldehyde); COOH-M-CHO, 6-oxo-*trans,trans*-2,4-hexadienoic acid; CHO-M-OH, 6-hydroxy-*trans,trans*-2,4-hexadienal; COOH-M-OH, 6-hydroxy-*trans,trans*-2,4-hexadienoic acid; and SOD, superoxide dismutase.

however, failed to detect *trans,trans*-muconaldehyde when benzene was reacted with hydroxyl radicals generated radiolytically in $N_2O/O_2$-saturated aqueous solutions. Although the mechanism by which muconaldehyde is formed is not known, Latriano *et al.* [11] detected muconaldehyde formation from benzene in a mouse liver microsomal system, and the results implicated metabolism by cytochrome P450 in the ring-opening reaction.

Previous studies have shown that benzene exposure results in iron accumulation in the liver and bone marrow of rats [26]. In the same study, it was found that iron added to mouse bone marrow cells *in vitro* potentiated lipid peroxidation caused by benzene, presumably via the generation of reactive oxygen species. Iron is distributed throughout the body, especially in liver and bone marrow [26], and it is essential as a redox-active center in many enzymes, such as cytochrome P450, catalase, and myeloperoxidase. As a transition metal, iron catalyzes lipid, protein and DNA oxidation [27]. Physiologically, iron is well-regulated by the body, where it is stored in cells by being bound to ferritin [28]. Although there is no "free" iron under normal conditions in cells, a low-molecular-weight-iron pool, which induces lipid peroxidation in hepatocytes, has been detected in liver cytosol [29, 30]. Moreover, the iron in ferritin can be released and promotes lipid peroxidation in a rat liver microsomal system [31], presumably via a superoxide-driven Fenton reaction [32].

The present studies were undertaken to assess the role of iron in the mechanism of ring-opening of benzene in a mouse liver microsomal system. The results show that benzene ring fission is stimulated by iron. The data further suggest that Fenton chemistry may be involved in benzene ring-opening by microsomes, with singlet oxygen possibly being involved in the reaction. The studies provide evidence for the formation of α- or β-hydroxymuconaldehyde, a new ring-opened metabolite of benzene formed in a biological system.

## MATERIALS AND METHODS

### *Animals*

Male 9-week-old CD-1 mice were purchased from Charles River (Wilmington, MA). They were maintained in a climate-controlled animal care facility on a 12-hr light cycle with free access to food (Purina Mouse Chow) and water for 1 week. Then they were given one dose of acetone (50% aqueous solution, 4.0 mL acetone/kg) intragastrically to induce cytochrome P450IIE1 [33]. The induced mice were killed 24 hr after benzene administration.

### *Chemicals*

CHO-M-CHO was custom-synthesized by Calbiochem (San Diego, CA) according to a method used previously in our laboratory [9]. COOH-M-CHO was synthesized using a modification of a published method [34]. CHO-M-OH was synthesized by reduction of muconaldehyde using limited sodium borohydride [18]. COOH-M-OH was prepared by reduction of COOH-M-CHO [35]. Ferrous sulfate, acetic acid, monobasic and dibasic sodium phosphate, ethyl acetate, and HPLC grade methanol were obtained from the Fisher Scientific Co. (Fair Lawn, NJ). EDTA was bought from Mallinckrodt (Paris, KY). HQ and catechol were obtained from the Aldrich Chemical Co. (Milwaukee, WI). Carbonyl free benzene was purchased from Burdick & Jackson Laboratories (Muskegon, MI). SOD, catalase, and the rest of the chemicals were bought from the Sigma Chemical Co. (St. Louis, MO).

### *Preparation of mouse liver microsomes*

Mice were killed by cervical dislocation, and microsomes were prepared by the method of Thomas *et al.* [36]. The isolated microsomes were washed with 10 mM EDTA and centrifuged at 105,000 *g* for 60 min at 4°. The pellet (microsomes) was suspended in sucrose (0.25 M, 0.4 mL/g liver) and divided into 0.3- to 0.5-ml aliquots and stored at −70°. Protein concentration was determined by the method of Lowry *et al.* [37]. The activity of nitrosodimethylamine demethylase of the mouse liver microsomes, an indicator of cytochrome P450IIE1 activity, was determined in the microsomes according to Peng *et al.* [38].

### *Metabolism of benzene and muconaldehyde by mouse liver microsomes*

Benzene solutions were prepared in phosphate buffer (pH 7.4) prior to use, and the concentration was determined using an extinction coefficient of 0.124 $mM^{-1}$ $cm^{-1}$ at 254 nm. A stock solution of ferrous sulfate was freshly prepared in deionized water since precipitation was observed when it was prepared in phosphate buffer. Incubations were carried out as follows: samples containing mouse liver microsomes (1.0 mg protein/mL incubation mixture) in 100 mM phosphate buffer (pH 7.4) with 50 μM ferrous sulfate were equilibrated at 37° for 3 min, followed by the addition of 2.0 mM benzene or 0.1 mM muconaldehyde, and 2.0 mM NADPH in final concentrations. The final volume was 2.0 mL in 5-mL glass vials. The vials were capped tightly and incubated in a 37° shaking water bath for the designated time. The reaction was stopped with 50 μL concentrated HCl. The samples were extracted three times with 2.0 mL ethyl acetate. The organic layers were separated from the aqueous phase by centrifugation, pooled, and dried with $MgSO_4$. The ethyl acetate samples were centrifuged and evaporated almost to dryness under $N_2$ at 37° in a water bath. The residues were dissolved in 1.0 mL water and filtered before HPLC analysis through Gelman Acrodisc HPLC filters (0.2 μm) obtained from Fisher Scientific.

### *High performance liquid chromatographic analysis of benzene metabolites*

Benzene metabolites were analyzed by reverse-phase HPLC using a Kratos solvent delivery system equipped with a Perkin-Elmer LC-235C auto scan diode array UV detector. The samples were analyzed on a Lichrosorb RP-18 (5 μm) 25 × 0.40 cm analytical column (E. Merck, Darmstadt, Germany) with an RP-18 Supelco guard column. The wavelength of detection was 275 nm, and the flow rate was 1.0 mL/min. Elution solvents were solvent A (1.0% acetic acid in water) and solvent B (10% methanol in 1.0% acetic acid). The sample (100 μL) was applied to the column and eluted with a linear gradient of 0–35% solvent B over 45.0 min. After washing with solvent B for 5 min, the column was returned to the starting conditions and equilibrated for 10 min.

Concentrations of benzene metabolites were calculated from the corresponding integrated chromatogram peak areas using calibration curves generated as follows:

samples containing boiled mouse liver microsomes (1.0 mg protein/mL) in 100 mM phosphate buffer (pH 7.4) with 50 μM ferrous sulfate were equilibrated at 37° for 3.0 min followed by addition of known concentrations of standards and NADPH (2.0 mM). These samples were acidified, extracted, and analyzed by HPLC as described above. Calibration curves were constructed and extraction efficiencies were calculated from the concentration of standards before and after extraction.

*Characterization of benzene metabolites*

Mixtures of benzene incubated for 30 min with mouse liver microsomes supplemented with 2.0 mM NADPH and 50 μM ferrous sulfate were spiked with authentic standards. The samples were processed and analyzed by HPLC as described above. To form sufficient amounts of benzene metabolites for further characterization and structure proof, the microsomal mixture incubated with benzene, ferrous sulfate and NADPH was scaled up to a final volume of 20 mL. After incubation at 37° for 30 min, the reaction was stopped with 500 μL concentrated HCl. The incubation mixture was extracted three times with 20 mL ethyl acetate and processed as described above. The residue was dissolved in 2.0 mL water and filtered through HPLC filters as described above. Positive-ion discharge mass spectrometry of the extract was performed by thermospray LC-MS on a model Vestec 201 (Houston, TX). HPLC conditions were the same as described above except that 200-μL samples were injected. The eluate under the 17.7 min peak (Fig. 1) was collected, and UV spectra were taken at various pH levels adjusted with NaOH or HCl. One milliliter of the same eluate was reacted with 1.0 mL of 0.67% (w/v in 1.0 N acetic acid) thiobarbituric acid in boiling water for 60 min. The absorption spectrum was recorded from 400–600 nm against 1.0% acetic acid.

*Effects of antioxidant enzymes and other chemicals on benzene metabolism*

Stock solutions of antioxidant enzymes (i.e. catalase and SOD) and other chemicals were freshly prepared by dissolving in phosphate buffer (pH 7.4) at a concentration 10.0 times higher than the final concentration in the incubation mixture. Microsomal samples were constituted as described above except that the antioxidant or antioxidant enzyme was added prior to addition of benzene and NADPH. Incubation, ethyl acetate extraction, and HPLC analysis were carried out as described above.

*Hydrogen peroxide measurement*

Hydrogen peroxide concentration in the benzene incubation mixtures was measured using a ferrithiocyanate method [39]. Briefly, after reaction was stopped, a 1.0-mL incubation mixture was subjected to extraction with ethyl acetate for HPLC analysis of benzene metabolites. The precipitated protein in the remaining 1.0-mL incubation mixture was removed by centrifugation. Ferrous ammonium sulfate (0.2 mL aqueous solution, 10 mM) followed by potassium thiocyanate (0.1 mL aqueous solution, 2.5 M) were added to 0.8-mL aliquots of the supernatant. The absorbance of the red ferrithiocyanate complex formed in the presence of hydrogen peroxide was measured at 480 nm using a Perkin-Elmer spectrophotometer, and the concentration of $H_2O_2$ was calculated from a standard curve obtained with hydrogen peroxide.

*Statistical analysis*

One-way analysis of variance, followed by the Fisher multiple comparison test, was performed on all data analyzed using the StatView program.

## RESULTS

*Extraction efficiencies and cytochrome P450 activity of microsomes*

The extraction efficiencies for COOH-M-OH, CHO-M-OH, COOH-M-CHO, muconaldehyde and phenol were 89.6 ± 7.4% (concentration range, 0.2 to 4.0 μM), 76.2 ± 8.9% (0.8 to 8.0 μM), 99.7 ± 9.7% (0.25 to 5.0 μM), 56.3 ± 9.2% (0.25 to 5.0 μM), and 44.7 ± 3.6% (5.0 to 100 μM), respectively. These extraction efficiencies were determined from four different concentrations with ranges indicated above in parentheses. Since the extraction efficiency for HQ was low and inconsistent (3.5 to 73.7%) under the conditions used in the present studies, HQ was not analyzed quantitatively. The activity of nitrosodimethylamine demethylase, an indicator of P450IIE1 [32], in the mouse liver microsomes was 2.56 ± 0.16 nmol/mg protein/min (N = 4).

*Characterization of benzene metabolites*

Figure 1 shows the HPLC chromatogram obtained for the metabolites formed from benzene incubated with mouse liver microsomes supplemented with NADPH in the presence of $Fe^{2+}$. Based on coelution with authentic standards and on-line absorption spectra, the peaks were identified as follows: HQ, 9.9 min ($\lambda_{max}$ = 290 nm); COOH-M-OH, 23.5 min (258 nm); CHO-M-OH, 27.5 min (274 nm); COOH-M-CHO, 34.0 min (274 nm); muconaldehyde, 38.1 min (279 nm) and phenol, 40.0 min (270 nm). The peak that eluted at 17.7 min had two absorption maxima, a major one at 345 nm and a minor one at 275 nm. The material under the peak which eluted at 32.0 min was not derived from benzene since it was still present in the extract when microsomes were boiled or when benzene was omitted from the incubation system (Fig. 1). Positive ion discharge LC-MS studies yielded a M + 1 = 127 for the material eluting at 17.7 min and a M + 1 = 113 for the material eluting at 27.5 min. These data confirm that the material under the peak which eluted at 27.5 min is CHO-M-OH, and they suggest that the material that eluted at 17.7 min is α- or β-hydroxymuconaldehyde. To further characterize the material under the 17.7 min peak, the eluate under this peak was collected, and UV spectra were recorded at various pH levels. At pH 3.2, two UV absorbance maxima, i.e. 275 and 345 nm, were observed. At a pH of about 1.0, the UV absorption maximum at 345 nm disappeared, and the absorbance at 275 nm shifted to shorter wavelengths (255 nm, data not shown). The eluate collected under this peak reacted with thiobarbituric acid and formed a chromogen with an absorption maximum at 532 nm, suggesting that this compound is an α,β-unsaturated aldehyde. Based on its mass, UV spectral properties, and reactivity with thiobarbituric acid, the material under the peak eluting at 17.7 min was tentatively identified as α- or β-hydroxymuconaldehydes.

Figure 2 shows the time course of benzene metabolite formation in the presence of added $Fe^{2+}$ (50 μM). The amounts of CHO-M-OH and phenol increased rapidly

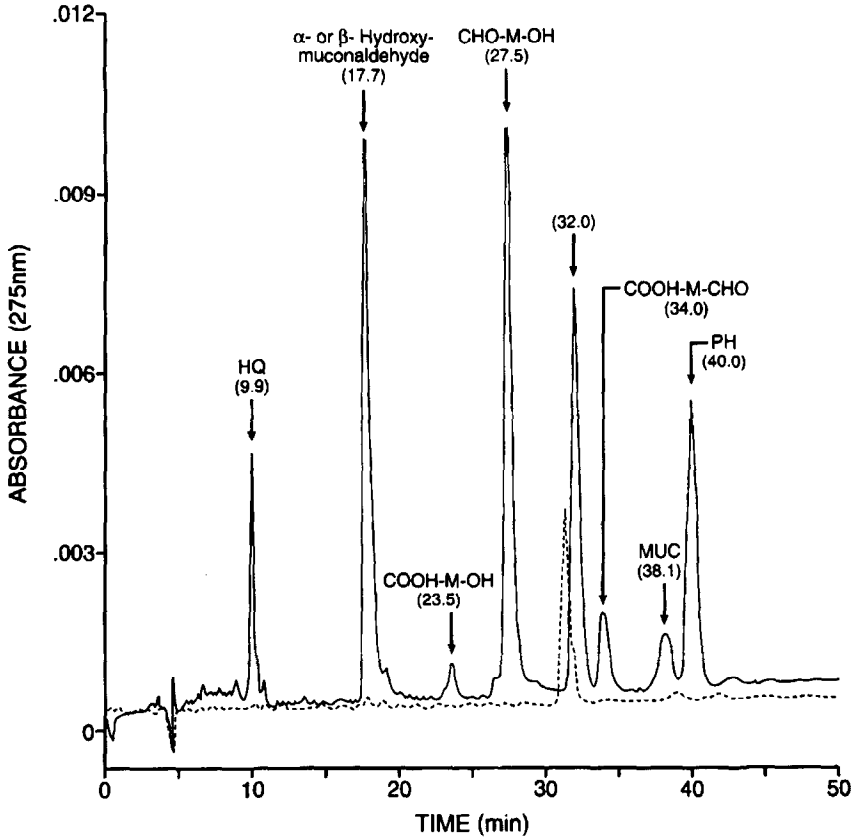


Fig. 1. High performance liquid chromatogram of products formed from benzene. Benzene (2.0 mM) was incubated with mouse liver microsomes supplemented with NADPH in the presence of added $Fe^{2+}$ (50 μM) at 37° for 30 min. Products extracted with ethyl acetate were analyzed by HPLC using 275 nm as the wavelength of detection (solid line). The dashed line is the HPLC chromatogram of compounds extracted with ethyl acetate when benzene was omitted or boiled microsomes were used in the incubation mixtures. Abbreviations: PH, phenol; MUC, muconaldehyde; HQ, hydroquinone.

for the whole incubation period. The amounts of CHO-M-CHO and COOH-M-CHO increased quickly in the first 5 min and then increased slowly during the next 15 min and reached a plateau at 20 min. The compound COOH-M-OH was not formed until about 10.0 min and it increased slowly. To assess the effect of $Fe^{2+}$ on the ring-opening of benzene, the latter was incubated with added $Fe^{2+}$ (0.0 to 100 μM) in a mouse liver microsomal system. Figure 3 reveals that the ring-opening of benzene was stimulated by added $Fe^{2+}$. The total identified ring-opened metabolites (=the sum of COOH-M-OH, CHO-M-CHO, COOH-M-CHO and CHO-M-OH throughout this paper unless otherwise stated) increased dramatically when added $Fe^{2+}$ increased from 0.0 to 10 to 25.0 μM and then reached a plateau starting at 50 μM. The compound CHO-M-CHO was not detected until the added $Fe^{2+}$ concentration was at 25.0 μM. Phenol formation also increased when added $Fe^{2+}$ was at 25.0 μM. The metabolism of benzene, including ring-opening, decreased when the added $Fe^{2+}$ concentration was higher than 50.0 μM.

*Muconaldehyde metabolism in a mouse liver microsomal system*

Muconaldehyde was incubated with microsomes supplemented with NADPH in the presence of added $Fe^{2+}$. The data in Table 1 indicated that COOH-M-CHO, CHO-M-OH and COOH-M-OH are formed from muconaldehyde in the microsomal system. Pyrazole, an alcohol dehydrogenase inhibitor, significantly inhibited muconaldehyde monoreduction and monooxidation. Cyanamide, an aldehyde dehydrogenase inhibitor, did not decrease muconaldehyde metabolism significantly.

*Effect of antioxidant enzymes, EDTA, and azide on benzene metabolism in the presence of added iron*

Hydrogen peroxide is generated by a liver microsomal system in the presence of NADPH and oxygen [39]. Since liver microsomes contain catalase, the $H_2O_2$ concentration is usually low in the microsomal system in the absence of a catalase inhibitor [39]. To assess the effect of $H_2O_2$ on benzene metabolism, it was measured in the present studies. In the microsomal system containing 1.0 mg protein/mL and 2.0 mM NADPH, the $H_2O_2$ concentration was low, i.e. 17.8 μM (Table 2). In the presence of 1.0 mM azide, a catalase inhibitor, the $H_2O_2$ concentration increased 5-fold. These data confirm that appreciable amounts of $H_2O_2$ are formed by liver microsomes supplemented with NADPH, and that $H_2O_2$ is metabolized quickly by catalase in the microsomes. Hydrogen peroxide concentrations decreased in the presence of added $Fe^{2+}$ and azide, relative to microsomes with added $NaN_3$, indicating that $H_2O_2$ is consumed in the presence of added $Fe^{2+}$.

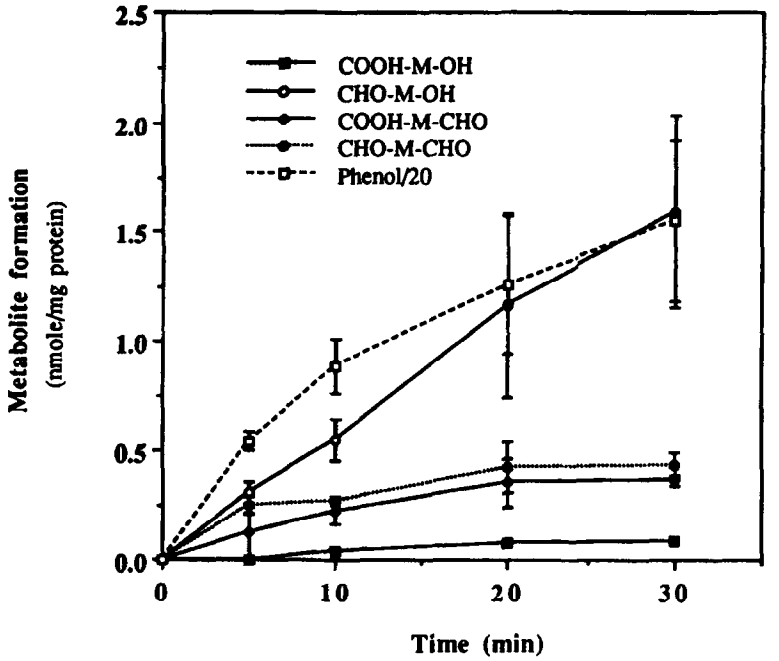


Fig. 2. Time course study of benzene metabolism. Benzene was incubated with mouse liver microsomes supplemented with NADPH in the presence of added $Fe^{2+}$ (50 μM) at 37° for the designated time periods. The products extracted with ethyl acetate were analyzed by HPLC as described in Materials and Methods. Values are the means ± SD of three experiments.

Table 3 shows the effect of added antioxidant enzymes, EDTA and $NaN_3$ on the formation of ring-opened compounds and phenol from benzene by microsomes in the presence of added iron. In the presence of added $Fe^{2+}$, the total amount of ring-opened metabolites but not phenol increased significantly compared with the amount formed in the absence of added $Fe^{2+}$. In the presence of added $Fe^{2+}$ and EDTA, the formation of

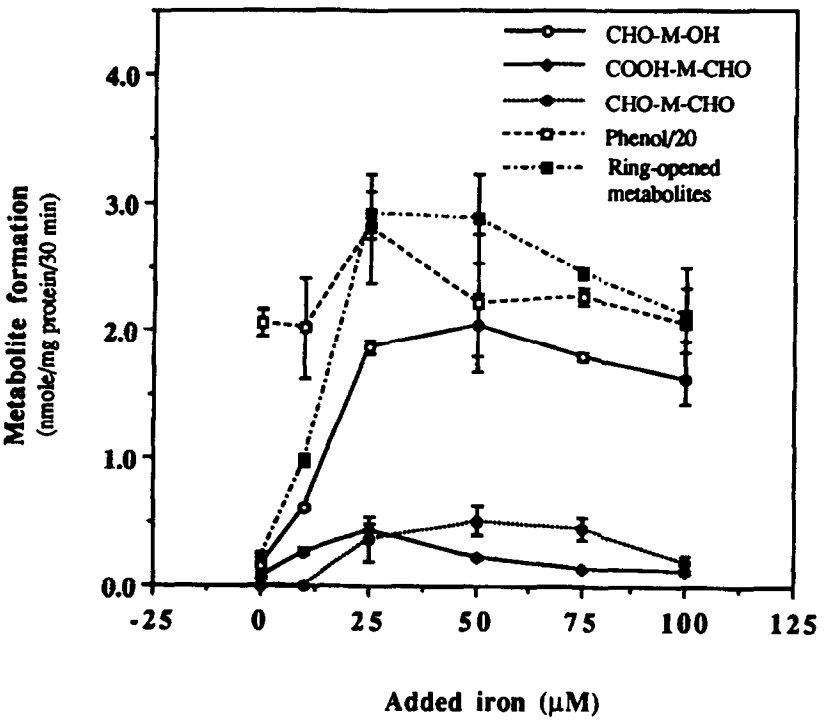


Fig. 3. Iron concentration dependency study of benzene metabolism. Benzene was incubated with mouse liver microsomes supplemented with NADPH in the presence of added $Fe^{2+}$ at 37° for 30 min. The products extracted with ethyl acetate were analyzed by HPLC as described in Materials and Methods. Values are the means ± SD of four experiments.

Table 1. Muconaldehyde metabolism in a mouse liver microsomal system*

| Inhibitor | Metabolite formation (nmol/mg protein/10 min) | | | |
|---|---|---|---|---|
| | COOH-M-OH | CHO-M-OH | COOH-M-CHO | CHO-M-CHO† |
| None | 1.92 ± 0.35 | 59.41 ± 2.11 | 9.12 ± 1.19 | 43.70 ± 8.62 |
| Pyrazole (3.0 mM) | 0.66 ± 0.26‡ | 9.67 ± 0.29‡ | 5.69 ± 0.14‡ | 76.57 ± 2.16‡ |
| Cyanamide (3.0 mM) | 2.52 ± 0.13 | 55.28 ± 2.54 | 9.99 ± 0.18 | 50.76 ± 2.50 |

* Mouse liver microsomes (1.0 mg protein/mL) supplemented with NADPH (2.0 mM) were incubated with muconaldehyde (0.1 mM) in the presence of $Fe^{2+}$ (50 μM) with or without the inhibitors at 37°C for 10 min. After extraction with ethyl acetate, samples were analyzed by HPLC as described in Materials and Methods. Values are the means ± SD of four experiments.

† Muconaldehyde remaining.

‡ Significantly different from samples incubated without inhibitor, $P < 0.05$.

the total ring-opened metabolites as well as phenol increased significantly. In the presence of azide and added $Fe^{2+}$, the formation of ring-opened metabolites and phenol was increased significantly, suggesting that there is an association between benzene metabolism and the formation and consumption of $H_2O_2$ in the presence of azide and $Fe^{2+}$. Addition of catalase decreased the formation of ring-opened metabolites, further suggesting that $H_2O_2$ is involved in the ring-opening of benzene. Addition of SOD did not increase the ring-opening of benzene, but in the presence of azide, SOD increased benzene metabolism, including ring-opening as well as ring-hydroxylation, suggesting that excess catalase exists in the mouse liver microsomes.

*Effect of copper on benzene metabolism in comparison with iron*

To further understand the effects of metal ions on benzene metabolism, $Cu^{2+}$, another transition metal, was incubated with benzene and microsomes supplemented with NADPH. Table 4 shows that $Cu^{2+}$ did not increase ring-opening or ring-hydroxylation of benzene, whereas the presence of $Fe^{2+}$ significantly increased the formation of ring-opened products but did not increase phenol formation. The ratio of ring-opened products and phenol was about 1:15 after incubation for 30 min in the presence of added $Fe^{2+}$.

Table 2. Effects of azide and iron on hydrogen peroxide formation in a mouse liver microsomal system

| Compounds | $H_2O_2$ concentration* (μM) |
|---|---|
| Microsomes + NADPH | 17.8 ± 3.1 |
| + $NaN_3$ | 86.7 ± 4.6† |
| + $NaN_3$ + $FeSO_4$ | 59.0 ± 6.8†‡ |
| + $FESO_4$ | 22.3 ± 0.6 |

* Mouse liver microsomes (1.0 mg protein/mL) were incubated with NADPH (2.0 mM) in the presence of $NaN_3$ (1.0 mM), or $FeSO_4$ (50 μM), or both at 37° for 20 min. Hydrogen peroxide was measured as described in Materials and Methods. Values are the means ± SD of four experiments.

† Significantly different from the first group, $P < 0.05$.

‡ Significantly different from the second group, $P < 0.05$.

*Effect of reactive oxygen species scavengers on benzene metabolism*

To further understand the mechanism of benzene ring-opening, hydroxyl radical scavengers and singlet oxygen scavengers were incubated with benzene and mouse liver microsomes supplemented with NADPH in the presence of added $Fe^{2+}$. The results in Table 5 show that the hydroxyl radical scavengers DMSO, formate and mannitol, but not ethanol, significantly decreased the ring-opening of benzene. Histidine, dGMP and Tris, which are singlet oxygen scavengers, dramatically decreased the ring-opening of benzene. All chemicals discussed above did not alter significantly the formation of phenol as compared with that in the absence of scavengers.

Azide, a catalase inhibitor as well as a singlet oxygen scavenger (or quencher), markedly increased ring-opening as well as ring-hydroxylation of benzene at lower

Table 3. Effects of azide, EDTA, catalase and SOD on benzene metabolism in a mouse liver microsomal system

| Compounds | Product formation* (nmol/mg protein/20 min) | |
|---|---|---|
| | Total ring-opened metabolites | Phenol |
| Microsomes + NADPH + benzene† | 0.41 ± 0.16 | 22.0 ± 4.0 |
| + $NaN_3$ | 0.37 ± 0.05 | 48.0 ± 8.7‡ |
| + $FeSO_4$ | 2.03 ± 0.36‡ | 26.8 ± 7.8 |
| + $FeSO_4$ + EDTA | 3.04 ± 0.51§ | 69.1 ± 11.6§ |
| + $NaN_3$ + $FeSO_4$ | 5.12 ± 1.00§ | 53.6 ± 8.1§ |
| + $FeSO_4$ + catalase‖ | 0.77 ± 0.11§ | 25.3 ± 12.1 |
| + $FeSO_4$ + SOD¶ | 2.05 ± 0.73 | 34.6 ± 2.9 |
| + $NaN_3$ + $FeSO_4$ + SOD | 2.85 ± 0.35§ | 51.3 ± 7.9§ |

* Samples in a total volume of 2.0 mL were incubated at 37° for 20 min. After extraction with ethyl acetate, samples were analyzed by HPLC as described in Materials and Methods. Values are the means ± SD of four experiments.

† The concentrations were 1.0 mg protein/mL for mouse liver microsomes, 2.0 mM NADPH, and 2.0 mM benzene.

‡ Significantly different from the first group, $P < 0.05$.

§ Significantly different from the third group, $P < 0.05$.

‖ Catalase, 560 units/mL.

¶ SOD = superoxide dismutase, 315 units/mL.

Table 4. Effects of metal ions on benzene metabolism in a mouse liver microsomal system

| Ion added | Metabolite formation* (nmol/mg protein/30 min) | | | | |
|---|---|---|---|---|---|
| | CHO-M-CHO | CHO-M-OH | COOH-M-CHO | Total ring-opened metabolites | Phenol |
| None | ND† | 0.15 ± 0.01 | 0.08 ± 0.02 | 0.22 ± 0.02 | 41.2 ± 1.9 |
| $Cu^{2+}$ (50 μM) | ND | 0.22 ± 0.06 | 0.07 ± 0.01 | 0.29 ± 0.05 | 46.3 ± 4.4 |
| $Fe^{2+}$ (50 μM) | 0.51 ± 0.11‡ | 2.04 ± 0.25‡ | 0.22 ± 0.02‡ | 2.88 ± 0.40‡ | 44.3 ± 10.9 |

* Benzene (2.0 mM) was incubated with mouse liver microsomes supplemented with NADPH (2.0 mM) at 37° for 30 min in the presence or absence of added metal ions. After extraction with ethyl acetate, samples were analyzed by HPLC as described in Materials and Methods. Data are the means ± SD of four experiments.

† ND = not detected.

‡ Significantly different from benzene incubated in the microsomal system in the absence or in the presence of $Cu^{2+}$, $P < 0.05$.

concentrations (0.01 and 0.10 mM) (Fig. 4). Phenol formation continued to increase with increasing azide concentration up to 1.0 mM. In contrast, the formation of ring-opened products decreased at an azide concentration higher than 0.1 mM. At high concentrations (>1.0 mM or above), azide dramatically inhibited benzene metabolism by decreasing the formation of ring-opened metabolites as well as phenol.

## DISCUSSION

The present studies confirm our previous findings which showed that incubation of benzene with liver microsomes supplemented with NADPH leads to ring-opening [11]. In our previous studies, using [$^{14}$C]benzene, muconaldehyde was identified as a ring-opened microsomal metabolite of benzene. In the present studies, using cold benzene, muconaldehyde and the ring-opened compounds CHO-M-OH and COOH-M-CHO were identified.

Table 5. Effects of reactive oxygen species scavengers on benzene metabolism in a mouse liver microsomal system

| Compounds | Metabolite formation* (nmol/mg protein/20 min) | |
|---|---|---|
| | Total ring-opened metabolites | Phenol |
| None | 1.79 ± 0.43 | 30.6 ± 5.7 |
| Formate† (10 mM) | 1.35 ± 0.39‡ | 39.9 ± 9.3 |
| Ethanol† (10 mM) | 1.46 ± 0.28 | 34.7 ± 2.4 |
| DMSO† (10 mM) | 0.83 ± 0.27‡ | 39.7 ± 10.9 |
| Mannitol† (10 mM) | 1.11 ± 0.22‡ | 32.4 ± 4.1 |
| Histidine§ (10 mM) | 0.60 ± 0.06‡ | 28.9 ± 3.8 |
| dGMP§ (1.0 mM) | 0.42 ± 0.05‡ | 28.2 ± 2.8 |
| Tris§ (10 mM) | 1.27 ± 0.37‡ | 37.4 ± 10.0 |

* Mouse liver microsomes (1.0 mg protein/mL) supplemented with NADPH (2.0 mM) were incubated with benzene (2.0 mM) in the presence of $Fe^{2+}$ (50 μM) with or without the compounds at 37° for 20 min. After extraction with ethyl acetate, samples were analyzed by HPLC as described in Materials and Methods. Values are the means ± SD of four experiments.

† Hydroxyl radical scavengers.

‡ Significantly different from the samples incubated in the absence of scavenger, $P < 0.05$.

§ Singlet oxygen scavengers.

At present, the mechanism of benzene ring-opening to muconaldehyde is not known. Muconaldehyde could be formed by cytochrome P450-mediated reactions that involve the action of reactive oxygen species, such as hydroxyl radicals and/or singlet oxygen [11]. The compound CHO-M-OH could have been formed from muconaldehyde via metabolism of the latter by alcohol dehydrogenase (ADH). Since CHO-M-OH formation was inhibited significantly by the addition of pyrazole, an ADH inhibitor (Table 1), the data suggest contamination of the microsomes with cytosolic ADH, which catalyzes the reduction of CHO-M-CHO to CHO-M-OH. It cannot be ruled out, however, that there may be more than one mechanism of ring-opening, each leading to a specific product, i.e. CHO-M-CHO and CHO-M-OH, or that there is one reactive intermediate that forms two products via two distinct pathways. Thus, muconaldehyde may not be the primary product of benzene ring-opening. The metabolite COOH-M-CHO can be formed from the oxidation of muconaldehyde by microsomal aldehyde dehydrogenase. A potential route for the formation of COOH-M-CHO is cytochrome P450-dependent metabolism of muconaldehyde, similar to that reported for the oxidation of retinal to retinoic acid by cytochrome P4501A2 [40].

The ring-opening of benzene incubated in a microsomal system was stimulated by added iron (Fig. 3, Tables 3 and 4). The results indicate that the total amount of ring-opened metabolites increases with increasing amounts of added iron, i.e. up to 50 μM $Fe^{2+}$ added. Muconaldehyde was not detected at 10 μM added iron, but was present at 25 μM added iron. As discussed above, it is possible that muconaldehyde, once formed from benzene, is converted quickly to the other ring-opened compounds, i.e. CHO-M-OH, COOH-M-OH, COOH-M-CHO (Fig. 1, Table 4). The metabolism of muconaldehyde to other oxidized and/or reduced compounds by mouse liver microsomes supports this hypothesis (Table 1).

Incubation of benzene in a microsomal system in the presence of added iron resulted in the formation of a new ring-opening compound that was tentatively identified as α- or β-hydroxymuconaldehyde based on its molecular weight (126), UV absorption spectral characteristics that were identical to those reported for α- or β-hydroxymuconaldehyde [21, 22] and its reactivity with thiobarbituric acid to form a chromogen that has an absorption maximum at 532 nm (data not shown). This compound

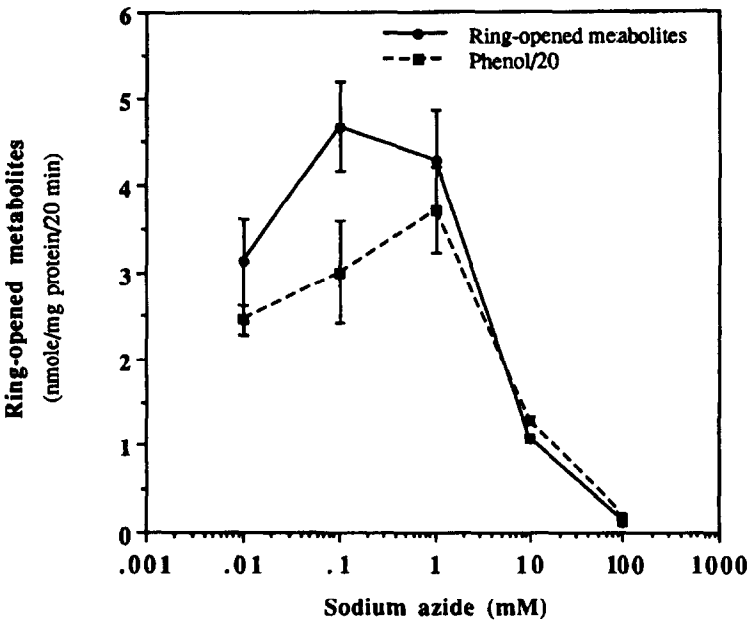


Fig. 4. Azide concentration dependency study of benzene metabolism. Benzene was incubated at 37° with mouse liver microsomes supplemented with NADPH in the presence of added $Fe^{2+}$ (50 μM) and sodium azide for 30 min. The products extracted with ethyl acetate were analyzed by HPLC as described in Materials and Methods. Values are the means ± SD of four experiments.

is not formed in the microsomal system by oxidation of muconaldehyde since it was not detected in microsomes supplemented with iron and incubated with muconaldehyde. Due to the lack of a synthetic standard, definitive identification of this compound has not been possible and requires further studies.

Cytochrome P450 is the major enzyme involved in benzene metabolism [5]. The present results suggest that Fenton chemistry is involved, in part, in benzene metabolism in a microsomal system supplemented with NADPH. Evidence from several experimental approaches supports this conclusion. First, the results (Table 2) clearly demonstrate that $H_2O_2$ is generated in the microsomal system supplemented with NADPH. The amount of $H_2O_2$ present increased in the presence of azide. An increase in $H_2O_2$ with added azide is expected if the microsomes contain catalase. These findings are similar to those reported by Thurman *et al.* [39]. The $H_2O_2$ concentration decreased in the presence of added $Fe^{2+}$, suggesting that a Fenton reaction had occurred. Second, the formation of ring-opened products increased with increasing iron concentration (Fig. 3) and dramatically increased in the presence of azide (Table 3). Third, added catalase significantly decreased the formation of ring-opened products of benzene. Furthermore, SOD, which dismutates superoxide anion to $H_2O_2$, increased both ring-opened products and phenol in the presence of azide. Finally, EDTA, a known chelator that enhances the Fenton reaction efficiency [41], significantly increased the formation of ring-opened products as well as of phenol (Table 3).

The Fenton reaction requires hydrogen peroxide and transition metals, i.e. $Fe^{2+}$. Thurman *et al.* [39] demonstrated that $H_2O_2$ is generated by rat liver microsomes in the presence of NADPH. They concluded that about one-third of microsomal NADPH-dependent hydrogen peroxide formation is cytochrome P450 dependent and the remaining two-thirds is formed by "NADPH oxidase" activity. Similar findings were reported by Kuthan and Ullrich [42]. The $Fe^{2+}$ is oxidized rapidly to $Fe^{3+}$ in phosphate buffer solution at neutral pH [27], but it can be reduced in a microsomal system by the following reactions:

$$Fe^{3+} + O_2^{\cdot-} \rightarrow O_2 + Fe^{2+} \quad (1)$$

$$Fe^{3+} + NADPH \rightarrow Fe^{2+} + NADP^+ + H^+ \quad (2)$$

Superoxide anion can serve either as an oxidant or a reductant depending on the system, and it can reduce $Fe^{3+}$ to $Fe^{2+}$ [43]. Reaction 2 also can be catalyzed by cytochrome P450 reductase [44]. The most likely precursor of $H_2O_2$ generated by the microsomal system is superoxide anion. The latter can be generated by autooxidation of $Fe^{2+}$, reduction of oxygen mediated by cytochrome P450 reductase in the presence of NADPH, and by uncoupled oxidation of substrates by cytochrome P450. In addition, polyhydroxylated benzene metabolites, i.e. HQ, are redox active [13] and can redox-cycle with NADPH cytochrome P450 reductase to generate superoxide anions [45].

The findings by Johansson and Ingelman-Sundberg [46] suggested that the cytochrome P450-dependent formation of phenol may be mediated by hydroxyl radicals most likely generated from hydrogen peroxide. A role for hydroxyl radicals in the oxidation of benzene to phenol also has been suggested by Gorsky and Coon [47], who concluded that the hydroxyl radical-mediated formation of phenol is the major pathway at low benzene concentrations (μM), whereas at millimolar concentrations, the direct oxidation by P450 is of much greater importance. It is generally accepted that hydroxyl radicals play a major role in the oxidation of benzene to phenol in the Fenton system. That hydroxyl radicals also play a role in benzene ring-opening is indirectly supported by the findings which showed that hydroxyl radical scavengers significantly decrease the formation of ring-opened products of benzene incubated in the mi-

crosomal system (Table 5). Several observations from the present study and other studies, however, suggest that ·OH may not play a major role in benzene ring-opening. First, added $Fe^{2+}$ significantly increased benzene ring-opening (Fig. 3, Table 4), but copper, another transition metal that also drives the Fenton reaction to produce ·OH [48], did not increase ring-opening (Table 4). Second, the disproportional increase of phenol (158% above control) compared with that of the ring-opening products (50% above control) in the presence of EDTA (Table 3) suggests that the ring-opening and ring-hydroxylation may occur, in part, by different mechanisms via different reactive oxygen species. Finally, the observations by Pan *et al.* [25] do not support the ·OH hypothesis of benzene ring opening. These investigators did not detect muconaldehyde formation from benzene reacted with hydroxyl radicals that were generated radiolytically in $N_2O/O_2$-saturated aqueous solutions.

The results shown in Table 5 and Fig. 4 suggest that singlet oxygen may be involved in the ring-opening of benzene. Histidine, dGMP and Tris (singlet oxygen scavengers) dramatically inhibited benzene ring-opening (Table 5). Azide, a catalase inhibitor as well as a singlet oxygen scavenger, increased benzene ring-opening at lower concentrations (1.0 mM or below), while at higher concentrations (above 1.0 mM) it significantly decreased benzene metabolism (Fig. 4). One interpretation of the results is that at low concentrations azide mainly inhibits catalase, and, at higher concentrations it acts as a singlet oxygen scavenger (or quencher) or alternatively, as an inhibitor of cytochrome P450 since the formation of phenol from benzene was also decreased in the presence of azide (10 mM or higher, Fig. 4). Many studies indicate that an iron–oxygen complex, probably a tetravalent iron, is generated and serves as a final two-electron oxidant in Fenton reactions [41, 49–52]. In addition to the classic Fenton reaction (Reaction 3) proposed by Walling [48], Sugimoto and Sawyer [53] proposed another mechanism shown in Reaction 4:

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

$$H_2O_2 + Fe^{2+} \rightarrow [FeO]^{2+} + H_2O \quad (4)$$

The $[FeO]^{2+}$, a two-electron oxidant, may be responsible for the ring-opening of benzene. The proposed mechanistic pathways for benzene oxidation by mouse liver microsomes supplemented with NADPH in the presence of $Fe^{2+}$ are shown in Fig. 5.

The significance of Fenton chemistry demonstrated for the microsomal metabolism of benzene, including ring-opening, in this study may be of biological relevance. Although it is generally believed that there is no "free" iron *in vivo*, there is a large body of evidence that supports the existence of a so-called "low-molecular-weight-iron (LMW-iron) pool" in cells, including hepatocytes. Nielsen *et al.* [30] showed that the concentration of LMW-iron in normal rat liver can be as high as 23.7 μg iron/g wet liver (equivalent to 0.5 mM iron). This LMW-iron pool exists in the cytoplasm in the form of nucleotide or amino acid complexes. These complexes participate in Fenton reactions to produce lipid peroxidation [29, 54]. More importantly, LMW-iron can be

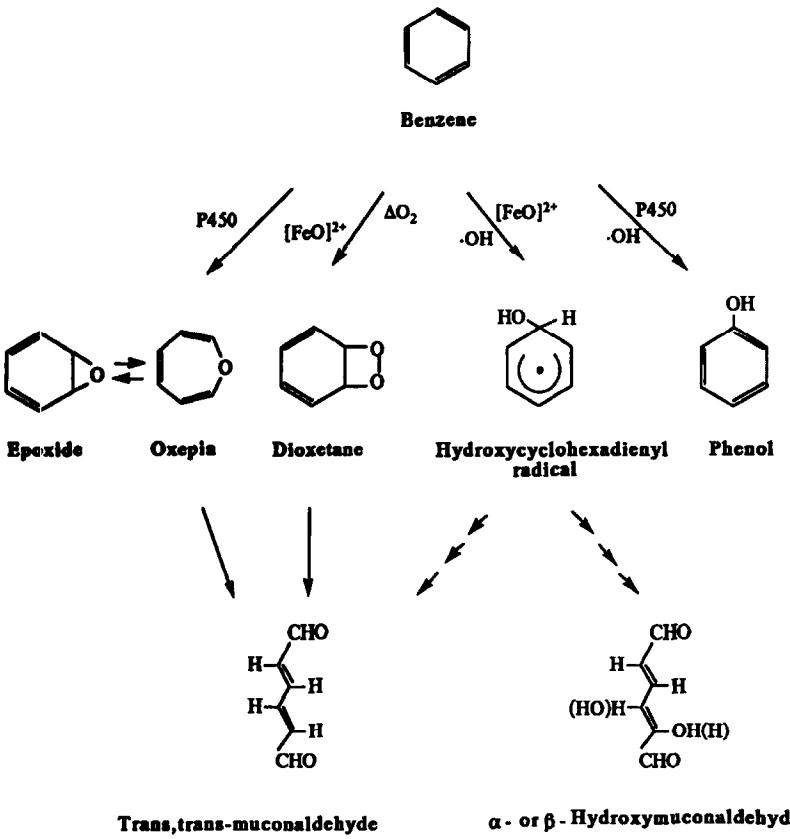


Fig. 5. Proposed mechanistic pathways for benzene metabolism in a mouse liver microsomal system in the presence of added iron.

found in the cytosol isolated from rat and guinea pig reticulocytes [55, 56]. Inhibition of heme synthesis and uncoupling of oxidative phosphorylation, a process that generates $H_2O_2$, significantly increase the levels of LMW-iron. Another concept relevant to benzene ring-opening is intracellular iron mobilization in the hepatocyte [57]. The liver is a tissue rich in iron, which is stored in ferritin and present in many heme-containing proteins. Under normal conditions, the mobilization of iron is strictly regulated. Oxidative stress produces conditions that interfere with this regulation, and which may result in the release of iron from ferritin. Since there is an animal model for $Fe^{2+}$ accumulation [30] and since methods for assessing benzene hematotoxicity and for measuring metabolism are available, it would be of interest to evaluate the role of $Fe^{2+}$ in benzene hematotoxocity *in vivo*.

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