Formation of muconaldehyde, an open-ring metabolite of benzene, in mouse liver microsomes: An additional pathway for toxic metabolites

(ring opening/benzene metabolism/α,β-unsaturated aldehydes/2,4-hexadienedial)

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ABSTRACT It has been proposed that a ring-opened form may be responsible for the toxicity of benzene. The present studies demonstrate that incubation of [14C]benzene with liver microsomes (obtained from male CD-1 mice treated with benzene) in the presence of NADPH results in the formation of a ring-opened product. Evidence for the identity of this product was obtained by derivatization with 2-thiobarbituric acid (TBA), which resulted in the formation of an adduct with a 490-nm absorbance maximum. This maximum is identical to that observed after authentic trans,trans-muconaldehyde has reacted with TBA. Separation of muconaldehyde, both with and without trapping with TBA, from other benzene metabolites in the incubation mixture was accomplished by HPLC. The radioactivity profile of fractions collected during HPLC analysis contained peaks that eluted with muconaldehyde and the muconaldehyde–TBA adduct. The structure of the ring-opened product was confirmed by mass spectrometry, studies in which the HPLC peak from the microsomal incubation mixture that eluted at the retention time of authentic muconaldehyde was collected and derivatized with 2,4-dinitrophenylhydrazine. The high-resolution mass spectrum of this sample contained an ion with an m/z of 291.0729, corresponding to muconaldehyde mono-dinitrophenylhydrazone. These results indicate that benzene is metabolized in vivo to a ring-opened product identified as muconaldehyde.

The search for potentially toxic metabolites of aromatic compounds has classically focused on oxidation products of the ring(s) or of side chains. This productive approach has led to evidence that intermediates, such as aromatic oxides, epoxides, oxepins, peroxides, and polyhydroxylated compounds, play a role in toxicity. Benzene is a potent hematotoxin and a known human leukemogen whose hematotoxic effects depend upon its metabolic transformation (1, 2). However, despite a focus on the many metabolites with oxygen added to the benzene ring, the search for the agent(s) causing this toxicity has thus far been unsuccessful (2). While there has been some suggestion that a reactive early intermediate, such as benzene oxide-oxepin, might be of importance, and there is good evidence indicating that oxidative metabolism to catechol, hydroquinone, and benzoquinone has some role in benzene hematotoxicity, as yet the range of hematotoxicity observed after benzene exposure has not been explained by intensive study of its known metabolites.

This focus on products containing an intact aromatic ring has ignored the possibility that toxic intermediates might be produced through the opening of the ring. Our laboratory has proposed that muconaldehyde, an α,β-unsaturated diene dialdehyde, resulting from ring opening, is a hematotoxic intermediate in benzene metabolism (3). Benzene ring opening and formation of trans,trans-muconaldehyde had previously been demonstrated to occur in irradiated solutions of benzene, presumably via the action of hydroxyl radicals (4), and more recently in our hands in a system in which a Fenton-type reagent was used to generate free radicals (5). There is indirect evidence that benzene ring opening occurs in vivo in that trans,trans-muconaldehyde, the corresponding diacid of muconaldehyde, has been found in the urine of animals administered benzene, accounting for 1–2% of the dose (6, 7). However, this finding has been thought to be due to the action of pyrocatechase, an enzyme found in intestinal bacteria, which converts catechol directly into cis,cis-muconic acid (8).

Initial toxicological studies showed that 10 μM muconaldehyde produced marked toxicity to plasma clot cultures of human erythropoietic precursors (3), and at similar or lower concentrations was toxic to other dividing cells in culture (9). Additionally, administration of trans,trans-muconaldehyde at 2 mg/kg daily for 16 days to CD-1 mice resulted in statistically significant decreases in erythrocyte count, hematocrit, and bone marrow cellularity as well as an increase in leukocyte count and spleen weight (10). This is similar to the toxicity of about a 50- to 100-fold higher concentration of benzene in this species.

The major missing link in the hypothesis that a ring-opened product of benzene is important in its toxicity is the demonstration that the known hematotoxic compound, muconaldehyde, is indeed formed during the metabolism of benzene. In the present study we have evaluated this possibility in a mouse liver microsomal system incubated with benzene. The identification of trans,trans-muconaldehyde (2, 4-hexadienedial) as a product of benzene ring fission was accomplished by several methods of analysis, including HPLC and mass spectrometry.

MATERIALS AND METHODS

Chemicals. Trans,trans-Muconaldehyde was synthesized according to the procedure of Kossmehl and Bohn (3, 11). The melting point of 119°C is in good agreement with the reported values of 117°C (12) and 121°C (13). The absorbance spectrum of trans,trans-muconaldehyde in phosphate buffer has a 278-nm maximum; its molar extinction coefficient at this wavelength is 3.15 × 10^4 M^-1cm^-1 (14). A 10 mM solution in absolute ethanol served as the stock.

Abbreviation: TBA, 2-thiobarbituric acid.
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Carbonyl-free benzene, phenol, corn oil, HPLC grade monobasic potassium phosphate, and sodium dithionate were obtained from Fisher. [14C]Benzene was obtained from ICN. Hydroquinone and 2,4-dinitrophenylhydrazine were purchased from Aldrich. trans,trans-Muconic acid, NADPH as the tetrasodium salt (type IV, 98% pure), and 2-thiobarbituric acid (TBA) were obtained from Sigma.

**Microsome Preparation and 1 ml Sample Aliquot Procedure.** Male CD-1 mice weighing 25–35 g, purchased from Charles River Breeding Laboratories, were administered benzene at 1100 mg/kg in corn oil (1:1) subcutaneously 24 and 18 hr prior to sacrifice, a treatment shown to increase benzene metabolism 2- to 3-fold (15). Control animals received corn oil. The animals were fasted for 12 hr and the microsomes were prepared (15). Cytochrome P-450 was determined by the method of Omura and Sato (16) and protein was determined by using the Lowry procedure (17).

Mouse liver microsomes (1 mg of protein per ml) were incubated with 4 mM [14C]benzene (specific activity 565 dpm/nmol) and 3 mM NADPH in a total volume of 1.5 ml of 0.1 M potassium phosphate buffer, pH 7.4. The incubations were carried out in glass-stoppered test tubes that hold a total volume of 5 ml. Benzene was added without dilution in any organic solvent. The tubes were capped immediately after the addition of benzene and incubated at 37°C in a shaking water bath for 5, 10, 15, or 20 min. Trichloroacetic acid (0.2 ml of 10%, wt/vol) was added to 1-ml samples from the microsomal suspension. After centrifugation for 30 sec at 50 × g, the supernatant solution was filtered with nylons HPLC filters and immediately injected into the HPLC column.

**Derivatization with TBA.** Previous studies in our laboratory have demonstrated that trans,trans-muconaldehyde reacts with TBA to form an adduct with a 490-nm absorbance maximum (18, 19). One milliliter of the microsomal suspension was added to 1 ml of 0.67% TBA in 10% trichloroacetic acid and centrifuged at 50 × g for 5 min, and the supernatant solution was incubated in a boiling water bath for 30 min. The muconaldehyde-TBA standard was made by mixing 10 μM muconaldehyde in 0.1 M phosphate buffer, pH 7.4, with the TBA reagent (1:1) and incubating for 30 min in a boiling water bath. Absorption spectra were recorded on a Perkin–Elmer 552 spectrophotometer.

**Analysis by HPLC.** The HPLC method for separation and detection of muconaldehyde utilized a 0.46 × 25 cm analytical column (Whatman Chemical Separations) containing 5-μm Carbowax reverse-phase packing. The mobile phase of 10 mM potassium phosphate buffer, pH 6.5, and 30–40% methanol was delivered isocratically at a flow rate of 1 ml/min. A 100-μl Rheodyne loop was used throughout. A Kratos 773 continuously variable absorbance detector was set to 280 nm for detection of muconaldehyde without derivatization. For the detection of the muconaldehyde as the TBA adduct, the detector was set to 490 nm, the absorbance maximum of this adduct (19).

**Determination of Radioactivity Profile.** The HPLC eluate was collected in 0.5-ml fractions into scintillation vials containing 5.0 ml of Liquiscint fluor (National Diagnostics, Somerville, NJ). Disintegrations per minute (dpm) were determined on a Tracer Analysis Mark III scintillation counter. Purity was assessed by injecting 4 mM labeled benzene in 0.1 M phosphate buffer, pH 7.4, into the column and using the HPLC conditions previously described.

**Derivatization with 2,4-Dinitrophenylhydrazine.** The reagent was made by dissolving 0.4 g of 2,4-dinitrophenylhydrazine in 2 ml of concentrated H2SO4, and adding 3 ml of H2O and 30 ml of 95% ethanol. A 1-ml sample from the microsome incubation mixture was treated with trichloroacetic acid and centrifuged for 30 sec as described above. A 100-μl aliquot of the supernatant was immediately injected into the HPLC column. At the retention time (tR) previously established for muconaldehyde, 1 ml of the eluate was collected and allowed to react with 1 ml of 2,4-dinitrophenylhydrazine reagent. The control sample from a microsomal incubation mixture without benzene was prepared in the same manner. The muconaldehyde dinitrophenylhydrazone standard was prepared by adding 20 μl of 10 mM muconaldehyde in ethanol to 1 ml of 2,4-dinitrophenylhydrazine reagent. After storage in the dark at room temperature for at least 24 hr, the samples were prepared for mass spectrometry by extracting twice with 1.0 ml of chloroform. The extracts were pooled and evaporated to dryness under nitrogen at room temperature. The dark orange residue was dissolved in one drop of glycerol. Analysis was performed on a VG analytical high-resolution mass spectrometer model 7070E Q.

**Recovery of Muconaldehyde from Microsomes.** In these studies, trans,trans-muconaldehyde was added to microsomes (1 mg of protein per ml) to give final concentrations of 50 and 5 μM. The samples were incubated at 37°C for 0–30 min in a shaking water bath. One-milliliter aliquots were prepared as previously described for HPLC analysis, with detection by absorbance at 280 nm.

**RESULTS**

The incubation of benzene with liver microsomes obtained from CD-1 male mice, treated with benzene, results in the formation of an open-ring metabolite, identified as trans,trans-muconaldehyde. This finding is supported by several lines of evidence employing three different methods for the detection of muconaldehyde in microsomal mixtures.

**Studies Employing TBA as a Trapping Agent.** Previous studies (18, 19) had determined that the TBA assay was useful for the detection of muconaldehyde, which forms a TBA adduct with a 490-nm absorbance maximum. A sample from microsomes incubated with [14C]benzene in the presence of NADPH and allowed to react with TBA contained a product that had a 490-nm absorbance maximum, identical to that formed by allowing authentic muconaldehyde to react with TBA. This maximum, however, was also observed after reaction of a microsomal mixture containing NADPH with TBA. Fig. 1B shows the difference spectrum of a sample from the microsomes incubated with benzene vs. a microsomal sample without benzene; both samples contained NADPH.

![Absorbance spectrum of solutions obtained from the reaction of TBA with trans,trans-muconaldehyde and microsomal incubation mixtures.](image-url)
Though the difference spectrum was consistent with the formation of muconaldehyde from benzene (Fig. 1), the confounding factor of an apparent NADPH-TBA adduct with similar spectral characteristics necessitated further studies.

The HPLC chromatogram of the supernatant from microsomes incubated with 4 mM \(^{14}C\)benzene in the presence of 3 mM NADPH for 10 min and then allowed to react with TBA is shown in Fig. 2A. When detection is by absorbance at 490 nm, a peak with \(t_R = 7.9\) min is observed. A small peak at this retention time is also present in the corresponding sample without benzene, which was also determined to be due to NADPH. Since NADPH reacts with TBA to form an adduct with the same absorption maximum and HPLC retention time as the muconaldehyde-TBA adduct, it was necessary to identify muconaldehyde derived from benzene by radioactive material eluting at the retention time previously established for muconaldehyde.

The HPLC profile of muconaldehyde and the other known metabolites of benzene, in the absence of TBA and using 280-nm detection, is shown in Fig. 2B (solid line). A chromatogram of the muconaldehyde-TBA adduct, using 490-nm detection (broken line), is also shown in Fig. 2B. The chromatograms show that the muconaldehyde-TBA adduct, in contrast to muconaldehyde, is well separated from hydroquinone, benzoquinone, catechol, and phenol, resulting in easier identification of this peak. Fig. 2C shows the radioactive profile of the HPLC eluate collected during analysis of the sample shown in Fig. 2A. Peak assignment based on standards is as follows: \(t_R = 5.0\) min, muconaldehyde (without TBA); \(t_R = 8.0\) min, muconaldehyde-TBA; \(t_R = 9.5\) min, phenol. The peaks with \(t_R = 4.0\) and 6.5 min cannot be identified with certainty. Under these HPLC conditions, trans,trans-muconic acid elutes immediately after the solvent front (\(t_R = 3.5\) min). Assessment of the purity of the radiolabeled benzene reveals a contaminant that elutes at \(t_R = 4.0\) min. Therefore, the early peak may be a contaminant, muconic acid, or another highly polar metabolite.

Detection of Muconaldehyde Without Chemical Derivatization. The studies employing radioactively labeled benzene and TBA demonstrated that trans,trans-muconaldehyde could also be detected without the use of a trapping agent. A slight adjustment of the HPLC mobile phase allowed for baseline separation from the other benzene metabolites, as shown by the HPLC profile of the standards shown in Fig. 3B. Fig. 3A shows a chromatogram of the supernatant from microsomes incubated with benzene for 5 min in the presence of NADPH. Two very small peaks absorbing at 280 nm with retention times of 6.7 and 7.0 min are present. The material with \(t_R = 7.0\) min elutes at the same time as the muconaldehyde standard (Fig. 3B) and is not present in the corresponding sample without benzene (not shown). Fig. 3C shows the radioactivity profile of the HPLC eluate that was collected during analysis of the sample shown in Fig. 3A. Peak assignment in the radioactivity profile is based on comparison with the standards: the peak at \(t_R = 5.5\) min corresponds to hydroquinone, the peak at \(t_R = 7.0\) min corresponds to muconaldehyde, and the peak at \(t_R = 14.0\) min corresponds to phenol. Assignment of the peak at \(t_R = 4.5\) min is complicated by a radioactive contaminant present in the \(^{14}C\)benzene. The radioactivity profiles of chromatograms from microsomal samples incubated with benzene for 10 and 15 min were similar except that all peaks contained more radioactivity, indicating increased product formation over time. For muconaldehyde, the difference in dpm over time was only a few counts and could not be considered significantly different from the value observed in the 5-min microsomal incubation of benzene.

In the above studies employing radioactive benzene, experiments were carried out to ascertain that the radioactivity eluting at the retention time of muconaldehyde was associated only with muconaldehyde and not with any other benzene metabolite. These studies included determining the retention times of phenol, catechol, hydroquinone, benzoquinone, 1,2,4-benzenetriol and muconic acid in both the presence and absence of TBA, with detection at both 280 and 490 nm. None of these metabolites eluted with muconaldehyde or the muconaldehyde-TBA adduct.

Detection of Muconaldehyde by Derivatization with 2,4-Dinitrophenylhydrazine. A third method for the detection of muconaldehyde employed post-column derivatization of muconaldehyde with 2,4-dinitrophenylhydrazine followed by mass spectral analysis. Fig. 4A shows the fragmentation pattern of the muconaldehyde dinitrophenylhydrzone stan...
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dard obtained by fast atom bombardment (FAB) mass spectrometry in which glycerol was the matrix. The material with an m/z of 291 corresponds to the protonated muconaldehyde mono-dinitrophenylhydrazone. The mass spectrum of the standard contains two other peaks—i.e., the peak with an m/z of 185, which corresponds to a dimer of glycerol plus one proton, and a peak with an m/z of 199, which corresponds to protonated dinitrophenylhydrazone. Fig. 4B shows the mass spectrum of a sample from the microsomal incubation mixture with benzene, collected from the HPLC column at the retention time characteristic for muconaldehyde and subsequently derivatized with dinitrophenylhydrazone. This spectrum contains a peak with an m/z of 291, which corresponds to the protonated muconaldehyde mono-dinitrophenylhydrazone. The high-precision mass value (291.0729) obtained on this sample was consistent with that of the muconaldehyde dinitrophenylhydrazone standard. Fig. 4B also contains a peak with an m/z of 281. The identity of this peak is unknown, but it is also found in the analysis of a sample from a similarly treated microsomal incubation mixture without benzene (Fig. 4C).

Quantitation of Benzene-Derived Muconaldehyde. The amount of recoverable metabolites formed during a 10-min incubation of [14C]benzene with microsomes is shown in Table 1. The peak numbers correspond to the radioactive peaks depicted in Fig. 3C. Recoverable muconaldehyde formed in this system can be directly calculated from the specific activity of the radioactively labeled benzene (565 dpm/nmol) and is expressed as benzene equivalents/mg of protein. Recoverable muconaldehyde formed from 4 mM [14C]benzene is 0.84 nmol/mg of protein or 0.87 nmol/nmol of cytochrome P-450.

![Fig. 4. Mass spectrometry of the chloroform extracts from reaction of 2,4-dinitrophenylhydrazone with samples from the microsomal incubation mixture and the trans,trans-muconaldehyde standard. (A) Standard trans,trans-muconaldehyde (0.2 mM final concentration) that had reacted with 2,4-dinitrophenylhydrazone. (B) Microsomal incubation mixture containing 15 mM benzene. (C) Microsomal incubation mixture without benzene. The background peaks were higher in this sample compared with the corresponding sample with benzene because a greater amount of sample was analyzed in the mass spectrometer.](image)

<table>
<thead>
<tr>
<th>Peak no.</th>
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<th>Benzene equivalents, nmol/mg protein</th>
<th>% of total metabolites</th>
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<td>5</td>
</tr>
<tr>
<td>2</td>
<td>Hydroquinone</td>
<td>1.12</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>Muconaldehyde</td>
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<td>9</td>
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<tr>
<td>4</td>
<td>Phenol</td>
<td>6.24</td>
<td>72</td>
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</table>

Specific activity of [14C]benzene was 565 dpm/nmol. The peak numbers describe the peaks observed in the radioactivity profile after incubating liver microsomes with [14C]benzene (Fig. 3C). Results are means of two experiments, done in duplicate. Microsomes were prepared from homogenates pooled from 11–12 animals.

The recovery studies indicated that the recoverable amount decreases as the concentration of muconaldehyde added to the microsomal suspension decreases. For example, 68% of 50 μM muconaldehyde incubated with microsomes (1 mg of protein per ml) could be recovered after 10 min, but only 20% of 5 mM muconaldehyde could be recovered at that time.

Incubation of benzene in the presence of NADPH in a system containing no microsomes or boiled microsomes resulted in no detectable formation of muconaldehyde, as determined from the radioactivity profile of the eluting material. In the absence of NADPH, no muconaldehyde was detected, nor was it detected in incubations carried out with microsomes obtained from animals that had not been treated with benzene. This latter result may very well be due to the limits of detection by radioactivity in these studies. In the present studies, the radioactivity in the peak corresponding to muconaldehyde formed from benzene by microsomes from benzene pretreated mice was 2–3 times above the background.

**DISCUSSION**

The present studies demonstrate that liver microsomes from CD-1 mice catalyze benzene ring opening, resulting in the formation of a previously unidentified metabolite. This metabolite has been identified as muconaldehyde, on the basis of comparison with an authentic standard. Detection of muconaldehyde formed from benzene in a microsomal metabolizing system was accomplished with and without chemical derivatization, employing several methods of analysis.

These studies utilized microsomes from CD-1 mice, a strain sensitive to the direct hematotoxic and leukemogenic actions of benzene (20). The overall metabolism compared well with published data from liver microsomal systems obtained from benzene-treated rats. In the present studies, 0.25% of the incubated benzene was metabolized, compared with 0.4% in a study using microsomes from benzene-treated Sprague–Dawley rats (21). In the latter study highly polar water-soluble metabolites, predominantly hydroquinone and catechol, accounted for 18% of the total metabolites (21). In the present studies, hydroquinone and an unknown compound in the solvent front also accounted for 18% of the total metabolites. In rats, the ratio of phenol to highly polar water-soluble metabolites was 5.3:1 (21), a ratio very similar to 5.4:1 observed in the present work.

The amount of recoverable muconaldehyde formed in a 10-min incubation with benzene is approximately 0.8 nmol/mg of protein (Table 1). This is 12.8% of the amount of phenol formed in this system. In recent studies on benzene metabolism in CD-1 mice administered [14C]benzene at 220 mg/kg, trans,trans-muconic acid was found to be 14% of the total amount of phenol radioactivity formed (7). As no muconaldehyde was detected in the urine of CD-1 mice administered phenol, catechol, or hydroquinone (7), it appears that a major
portion, if not all, of urinary muconic acid is derived from muconaldehyde, rather than through the action of intestinal bacteria on catechol.

Although the absolute amounts of radioactivity in metabolically derived muconaldehyde and its TBA adduct collected from the HPLC were low, in each case they were at least four standard deviations above background ($P < 0.001$). As only a small amount of benzene was recovered in the urine as muconic acid, the low amount of muconaldehyde found is not surprising in view of the extent to which benzene ring opening, as indicated by muconic acid formation, might occur in vivo, and in view of the reactive nature of muconaldehyde.

The hematotoxicity of trans,trans-muconaldehyde (3, 10) is not unexpected in view of the reported toxicity of other α,β-unsaturated aldehydes to dividing cells. It has been shown that this class of compounds reacts readily with nucleophiles, particularly sulphhydryl groups (22). The toxic effects reported include inhibition of cellular metabolism as well as inhibition of DNA, RNA, and protein synthesis.

The mechanism for the formation of muconaldehyde from benzene is not known. Fig. 5 shows a number of pathways for muconaldehyde formation. The classical pathway of benzene oxidation involving the initial formation of benzene oxide could provide two routes for muconaldehyde formation, one by way of ring opening of the dihydrodiol and one involving rearrangement of the oxidized benzene oxide-oxepin (13). Mechanisms that depend on the direct interaction of active states of oxygen with benzene may also be involved. Thus hydroxyl radicals generated by pulse radiolysis (4, 23) or in a Fenton-type system (5) cause oxidative ring opening of benzene in aqueous solutions and have been implicated in the metabolism of benzene by rabbit microsomes in vitro (24). Singlet oxygen, an active oxygen species that has been suggested to account for muconaldehyde formation from benzene during photooxidation (25), is known to form dioxetanes that decompose to aldehydes by carbon–carbon bond cleavage (26).

As in the case of benzene, hydroxyl radical-mediated ring opening and fragmentations resulting in the formation of dicarbonyl and aldehydic compounds have also been reported for toluene and xylene (27). Unsaturated dicarboxyls have also been reported to be oxidation products of polycyclic aromatic hydrocarbons, such as naphthalene (28) and phenanthrene (29). Recent reports on the carcinogenicity of benzene indicate that it is a multipotent carcinogen, capable of producing leukemia and a variety of other neoplasias. In this study, similar tumors were observed in rats administered xylene and ethyl benzene (30). The formation of muconaldehyde, a known hematotoxin, in liver microsomes suggests that a quantitatively minor metabolic route may be an important pathway leading to the formation of toxic metabolites from benzene. The probable formation of hydroxyl radicals during microsomal oxidation (31), and the propensity of hydroxyl radicals to open aromatic rings, suggest that this may be a general mechanism for the production of toxic intermediates from aromatic compounds.

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