Hydroxyl Radical-mediated, Cytochrome P-450-dependent Metabolic Activation of Benzene in Microsomes and Reconstituted Enzyme Systems from Rabbit Liver*

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The mechanism of benzene oxygenation in liver microsomes and in reconstituted enzyme systems from rabbit liver was investigated. It was found that the NADPH-dependent transformation of benzene to water-soluble metabolites and to phenol catalyzed by cytochrome P-450 LM2 in membrane vesicles was inhibited by catalase, horseradish peroxidase, superoxide dismutase, and hydroxyl radical scavengers such as mannitol, dimethyl sulfoxide, and catechol, indicating the participation of hydrogen peroxide, superoxide anions, and hydroxyl radicals in the process. The cytochrome P-450 LM2-dependent, hydroxyl radical-mediated destruction of deoxyribose was inhibited comcomitantly to the benzene oxidation. Also the microsomal benzene metabolism, which did not exhibit Michaelis-Menten kinetics, was effectively inhibited by six different hydroxyl radical scavengers. Biphenyl was formed in the reconstituted system, indicating the cytochrome P-450-dependent production of a hydroxy-cyclohexadienyl radical as a consequence of interactions between hydroxyl radicals and benzene. The formation of benzene metabolites covalently bound to protein was efficiently inhibited by radical scavengers but not by epoxide hydrolase. The results indicate that the microsomal cytochrome P-450-dependent oxidation of benzene is mediated by hydroxyl radicals formed in a modified Haber-Weiss reaction between hydrogen peroxide and superoxide anions and suggest that any cellular superoxide-generating system may be sufficient for the metabolic activation of benzene and structurally related compounds.

Benzene, a known leukemogen, produces leucopenia and aplastic anemia in animals and in man (1–4). Bioactivation of benzene is believed to be important for its leukemogenic and toxic effects (2, 3, 5, 6). The microsomal hydroxylase system in bone marrow (5) and in liver (7–9) has been suggested to participate in this reaction, yielding metabolites binding covalently to cellular macromolecules (6, 11–12).

The liver microsomal benzene monoxygenase is known to have unique properties (7). It is not inactivated by metyrapone and is activated upon treatment of the membranes with detergent, in contrast to ordinary microsomal monoxygenase activities. It is known that hydroxyl radicals may react with the benzene ring at a rate of $3 \times 10^{10} \text{ M}^{-1} \text{s}^{-1}$ (13), yielding a hydroxycyclohexadienyl radical, which in the presence of oxygen primarily gives phenol (14, 15). Liver microsomes can produce hydroxyl radicals (16), mediating the microsomal oxidation of ethanol (17), and therefore a hydroxyl radical-mediated benzene monoxygenation mechanism in microsomes was postulated.

We present here results indicating that benzene monoxygenase (7), constituting the first enzymatic step in the metabolic activation process, mediates hydroxylation by hydroxyl radicals produced in an iron-catalyzed Haber-Weiss reaction between superoxide ions and hydrogen peroxide. The results may indicate that any cellular system generating superoxide anions will be sufficient for metabolic activation of benzene and structurally related compounds.

**EXPERIMENTAL PROCEDURES**

**Materials**—Catalase (11,500 units/mg), catechol, dihydroxyfumaric acid, d-mannitol, superoxide dismutase (2,410 units/mg), and hydroquinone were purchased from Sigma. Dimethyl sulfoxide was obtained from Merck, and [14C]benzene (specific activity, 57 mcg/mmol) was from New England Nuclear. Microsomal phospholipids were extracted from liver microsomes obtained from phenobarbital-treated rabbits according to Bligh and Dyer (18) and stored under nitrogen in sealed tubes at $-20^\circ$ C. Cytochrome P-450 LM2 and NADPH-cytochrome P-450 reductase was purified to apparent homogeneity from liver microsomes of phenobarbital-treated rabbits essentially as described by Haugen and Coon (19) and Yasukochi and Masters (20), respectively. The cytochrome P-450 LM2 preparations used had specific contents of 10.5–13 nmol/mg, and the specific content of flavin in the NADPH-cytochrome P-450 reductase preparations was 20–22 nmol/mg, when flavin was determined according to Iyanagi and Mason (21). Epoxide hydrolase (specific activity, 450 nmol of styrene oxide hydrolyzed per mg/min) was purified from liver microsomes of phenobarbital-treated rabbits essentially as described by Halpert et al. (22).

**Methods**—Unilamellar phospholipid vesicles containing cytochrome P-450 LM2 and NADPH-cytochrome P-450 reductase were prepared by the cholate gel filtration method (23). The vesicles contained cytochrome P-450, reductase, and phospholipids in a molar ratio of about 10:3:1. The incubation mixtures were incubated for 10 min. The ethyl acetate layer was removed, and the residue was applied on TLC plates which were developed using either ethyl acetate or toluenechloroform:methyl acetate (1:11, by volume) as mobile phases.

1 The abbreviations used are: P-450 LM2, the phenobarbital-inducible form of rabbit liver microsomal cytochrome P-450 (19); Me$_3$SO, dimethyl sulfoxide.
RESULTS

Incubations of benzene (17 μM) in the presence of NADPH with reconstituted membrane vesicles containing NADPH-cytochrome P-450 reductase and cytochrome P-450 LM2 resulted in the production of several lipid- and water-soluble metabolites, phenol constituting the major product (Fig. 1). At least 11 different metabolites were formed, and among the most predominant ones were phenol, hydroquinone, and catechol. As shown in Fig. 1, the formation of both phenol and the other metabolites was strongly inhibited by the addition of the hydroxyl radical scavenger MeSO₃ or of catalase to the incubation system. This indicates that hydrogen peroxide and hydroxyl radicals participate in the cytochrome P-450-dependent metabolic transformation of benzene. In the absence of cytochrome P-450 or NADPH, no or negligible metabolism of benzene was observed.

Halliwell and Gutteridge (25) recently described a simple assay for hydroxyl radicals involving detection of fluorescent products formed by reaction of thiobarbituric acid with radical-destroyed deoxyribose. The cytochrome P-450 LM2-dependent conversion of benzene to water-soluble products, including the formation of covalently protein-bound benzene metabolites, was compared with respect to the inhibition profile obtained by addition of scavengers of hydrogen peroxide, superoxide anions, and hydroxyl radicals with the hydroxyl radical-mediated destruction of the deoxyribose. As shown in Fig. 2, concomitant inhibition of benzene metabolism and deoxyribose destruction was observed following the addition of either catalase, superoxide dismutase, MeSO₃ or mannitol to the system. By contrast, an ordinary cytochrome P-450 LM2-catalyzed reaction, the O-demethylation of para-nitroanisole, was not affected by the additions.

As shown in Table I, also the formation of the primary benzene metabolite, phenol, was concomitantly inhibited upon the addition of these scavengers to the incubation system. In addition, also catechol and horseradish peroxidase inhibited phenol production and the formation of water-soluble metabolites. The scavengers mannitol and catechol

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**Fig. 1.** Effect of MeSO₃ (A) and catalase (B) on cytochrome P-450 LM2-dependent oxidation of benzene in reconstituted membrane vesicles. [³⁵Cl]Benzene metabolites were separated on TLC in the solvent system chloroform:methyl acetate:toluene (1:1:1, by volume) (A) or in ethyl acetate (B) and visualized by autoradiography. Incubations were performed with vesicles corresponding to 0.4 nmol of cytochrome P-450 LM2 as described under "Experimental Procedures." Control incubations were carried out in the absence of NADPH and in the absence (C₁) or in the presence (C₂) of 100 mM MeSO₃ (DMSO). The positions of the metabolites phenol (P) and catechol (C) are indicated.

**Fig. 2.** Effect of MeSO₃, catalase, superoxide dismutase, and mannitol on cytochrome P-450 LM2-dependent oxidation of benzene to water-soluble products (X), cytochrome P-450 LM2-dependent destruction of deoxyribose (D), and cytochrome P-450 LM2-catalyzed O-demethylation of para-nitroanisole (Θ). Incubations were carried out as described under "Experimental Procedures" using 17 μM benzene, 0.7 mM deoxyribose, or 1 mM para-nitroanisole in the presence of 0.5 mM NADPH. DMSO, dimethyl sulfoxide; Θ, superoxide dismutase.
TABLE I

Effect of hydroxyl radical scavengers, superoxide dismutase, catalase, and horseradish peroxidase on cytochrome P-450 LM2-dependent oxidation of benzene in reconstituted membrane vesicles

Incubations were performed with liposomes corresponding to 0.4 nmol of cytochrome P-450 as described under "Experimental Procedures."

| Additions | Water-soluble metabolites | inhibition | Phenol inhibition | Metabolites covalently bound to protein | pmol/nmol LM2/min | % pmol/nmol LM2/min | % pmol/nmol LM2/min |
|-----------|---------------------------|-----------|------------------|----------------------------------------|-------------------|--------------------|--------------------|
| None      |                           |           |                  |                                        | 5.1               | 0.88               | 0.45               |
| Mannitol  |                           |           |                  |                                        | 5.3               | 0.82               | 0.41               |
| 0.5 mM    |                           |           |                  |                                        | 4.2               | 0.55               | 0.28               |
| 5 mM      |                           |           |                  |                                        | 1.9               | 0.30               | 0.28               |
| 50 mM     |                           |           |                  |                                        | 0.5               | 0.05               | 0.14               |
| Me2SO     |                           |           |                  |                                        | 4.8               | 0.81               | 0.8               |
| 0.1 mM    |                           |           |                  |                                        | 4.1               | 0.72               | 0.18               |
| 1 mM      |                           |           |                  |                                        | 1.3               | 0.12               | 0.07               |
| 10 mM     |                           |           |                  |                                        | 0.3               | 0.10               | 0.07               |
| 50 mM     |                           |           |                  |                                        | 0.05              | 0.07               | 0.07               |
| Catechol  |                           |           |                  |                                        | 3.4               | 0.33               | 0.41               |
| 0.01 mM   |                           |           |                  |                                        | 1.9               | 0.63               | 0.17               |
| 0.05 mM   |                           |           |                  |                                        | 2.2               | 0.57               | 0.17               |
| 0.1 mM    |                           |           |                  |                                        | 1.3               | 0.75               | 0.07               |
| 0.5 mM    |                           |           |                  |                                        | 1.0               | 0.80               | 0.07               |
| Superoxide dismutase | | | | | | | |
| 0.5 µg    |                           |           |                  |                                        | 2.5               | 0.51               | 0.45               |
| 2 µg      |                           |           |                  |                                        | 1.7               | 0.29               | 0.67               |
| 9 µg      |                           |           |                  |                                        | 1.1               | 0.26               | 0.70               |
| 50 µg     |                           |           |                  |                                        | 0.9               | 0.25               | 0.72               |
| 150 µg    |                           |           |                  |                                        | 0.9               | 0.16               | 0.82               |
| Catalase  |                           |           |                  |                                        | 4.7               | 0.54               | 0.39               |
| 0.1 unit  |                           |           |                  |                                        | 3.9               | 0.46               | 0.48               |
| 1 unit    |                           |           |                  |                                        | 1.6               | 0.18               | 0.80               |
| 10 units  |                           |           |                  |                                        | 0.2               | 0.05               | 0.91               |
| 100 units |                           |           |                  |                                        | 2.2               | 0.13               | 0.55               |
| Hydroquinone, 0.5 mM | | | | | | | |
| + 0.001 unit horse-radish peroxidase | | | | | | | |
| + 0.01 unit horse-radish peroxidase | | | | | | | |
| + 0.1 unit horse-radish peroxidase | | | | | | | |
| + 1 unit horse-radish peroxidase | | | | | | | |

also effectively inhibited the formation of benzene metabolites covalently bound to protein (Table I).

In addition, liver microsomal metabolism of benzene was reduced by scavengers of hydroxyl radicals, i.e. the production of phenol and water-soluble benzene metabolites was inhibited by the addition of either mannitol, Me2SO, catechol, benzoate, ketomethylthiolbutyric acid, thiourea, aniline, or ethanol to the microsomal system (Table II). Catechol inhibited the formation of covalently bound benzene metabolites in liver microsomes (Fig. 3).

In liposomes containing NADPH-cytochrome P-450 reductase, cytochrome P-450, and cytochrome b5 in a molar ratio of about 0.3:1:1, only about 50% of the rate of formation of water-soluble benzene metabolites was observed and only about 60% of the amount of protein-bound adducts were isolated compared to vesicles depleted of cytochrome b5 (data not shown). In reconstituted phospholipid vesicles containing cytochrome b5, instead of cytochrome P-450 LM2, the metabolic transformation of benzene was 0.58 pmol/nmol of cytochrome b5/min using otherwise similar conditions, whereas in vesicles only containing equivalent amounts of P-450 reductase, 0.37 pmol of benzene metabolites was formed per 0.3 nmol of reductase/min.

TABLE II

Effect of hydroxyl radical scavengers on the NADPH-dependent oxidation of benzene to phenol and water-soluble metabolites in liver microsomes from control rabbits

Incubation conditions were as described under "Experimental Procedures" and in the legend to Fig. 3.

| Scavenger used | To phenol inhibition | To water-soluble metabolites inhibition | pmol/mg/min | % pmol/mg/min | % pmol/mg/min |
|----------------|----------------------|---------------------------------------|-------------|---------------|---------------|
| None           | 1.0                  | 0.83                                  | 1.0         | 0.83          |               |
| Mannitol, 500 mM | 0.72                 | 0.52                                 | 0.44        | 0.14          | 0.14          |
| Me2SO, 300 mM  | 0.86                 | 0.52                                 | 0.37        | 0.11          | 0.11          |
| Catechol       | 0.86                 | 0.52                                 | 0.05        | 0.02          | 0.02          |
| Thiourea       | 1.0                  | 0.83                                  | 0.37        | 0.11          | 0.11          |
| 0.2 mM         | 0.86                 | 0.52                                 | 0.05        | 0.02          | 0.02          |
| 2 mM           | 0.69                 | 0.52                                 | 0.30        | 0.11          | 0.11          |
| 20 mM          | 0.49                 | 0.52                                 | 0.05        | 0.02          | 0.02          |
| Aniline        | 0.86                 | 0.52                                 | 0.37        | 0.11          | 0.11          |
| 1 mM           | 0.86                 | 0.52                                 | 0.05        | 0.02          | 0.02          |
| 10 mM          | 0.63                 | 0.52                                 | 0.37        | 0.11          | 0.11          |
| 100 mM         | 0.86                 | 0.52                                 | 0.05        | 0.02          | 0.02          |

Fig. 3. Effect of catechol on the rate of oxidation of benzene in liver microsomes isolated from phenobarbital-treated rabbits to water-soluble metabolites and to metabolites covalently bound to proteins. Liver microsomes, corresponding to 2.3 mg of proteins, were incubated in the presence of 0.5 mM NADPH in 50 mM potassium phosphate buffer, pH 7.4, with 17 µM benzene, containing 1 µCi of [14C]benzene for 30 min at 37°C. The products were analyzed as described under "Experimental Procedures." One thousand cpm correspond to the formation of 0.2 pmol of metabolites/mg of protein/min.
The results hitherto presented indicate that the NADPH-
dependent metabolic transformation of benzene in liver mi-
crosomes is mediated by hydroxyl radicals as the active oxy-
genating species. It seems plausible that the radicals are
formed in a iron-catalyzed Haber-Weiss reaction between
hydrogen peroxide and superoxide anions. If this is the case,
incubation of microsomes with increasing benzene concentra-
tions would not result in normal Michaelis-Menten kinetics
of the product formation. As shown in Fig. 4, elevated amounts
of benzene in the incubation mixtures resulted in a propor-
tionally increased rate of the production of water-soluble
metabolites from benzene by the liver microsomes; no satu-
ration of the activity was reached despite 10 mM benzene
concentration.

Dihydroxyfumarate is known to autooxidize spontaneously
in water, thereby generating superoxide anions (27). Incuba-
tion of benzene with 10 mM dihydroxyfumarate for
30 min at 37 °C resulted in the conversion of 2.7% of the
added benzene to water-soluble metabolites, i.e. 7.7 pmol of
products/min (Fig. 5). In the presence of superoxide dismu-
tase, inhibition of the reaction was achieved; only 0.91 pmol
of water-soluble products was formed per min.

Interaction of benzene with hydroxyl radicals will lead to
the formation of hydroxyl radical adducts, which in
the presence of oxygen is converted to phenol (14, 15) or in
the presence of itself is converted to biphenyl (15). However,
this type of radical is very reactive, and it may be expected
that this intermediate may be responsible for covalent binding
to proteins. In order to exclude the possibility that benzene
oxide is the reactive metabolite binding to protein, reconsti-
tuted vesicles containing cytochrome P-450 LM2 were incu-
bated with benzene in the presence of epoxide hydrolase. As
shown in Table III, the introduction of the epoxide hydrolase did not have any protective effect against the formation of water-

### TABLE III

| Enzyme | Water-soluble metabolites (pmol/nmol LM2/min) |
|--------|---------------------------------------------|
| P-450 LM2 | 2.70 | 0.37 |
| P-450 LM2 + epoxide hydrolase | 2.88 | 0.36 |
| P-450 LM2 + 500 mM mannitol | 0.09 | <0.01 |

Reconstituted membrane vesicles corresponding to 1 nmol of
cytochrome P-450 LM2 and, if indicated, 1 nmol of epoxide hydrolase
were incubated at 37 °C for 1 h in the presence of 25 µM benzene,
containing 1.5 µCi of [14C]benzene and 0.5 mM NADPH in 50 mM
potassium phosphate buffer. Bovine serum albumin (2 mg) was added
after the incubations as a protein carrier. Other procedures as de-
scribed under “Experimental Procedures.”

![Fig. 4. Effect of the benzene concentration on the formation of water-soluble benzene metabolites in liver microsomes iso-
lated from control rabbits.](image)

In incubations were performed for 20 min with liver microsomes corresponding to 3 mg of microsomal proteins.
Other conditions are as described under “Experimental Procedures.”

![Fig. 5. Oxidation of benzene by dihydroxyfumarate. [14C]Benzene (17 µM) was incubated in 1 ml of 50 mM potassium phosphate buffer, pH 7.4, for 1 h at 37 °C with 10 mM dihydroxyfumarate in the absence or in the presence of 100 µg of superoxide dismutase (SOD)/ml. The ethyl acetate-extractable metabolites were separated on TLC in the solvent system chloroform:methyl acetate:toluene (1:1:1, by volume), and the TLC plates were subjected to autoradiography. C, zero time incubations.](image)

![Fig. 6. High pressure liquid chromatographic analysis of benzene metabolites formed by cytochrome P-450 LM2-conta-
taining membrane vesicles. Reconstituted liposomes corresponding to 1 nmol of cytochrome P-450 LM2 were incubated for 1 h at 37 °C in 1 ml of 50 mM potassium phosphate buffer, pH 7.4, containing 1.6 µCi of [14C]benzene (10 mM) and 0.5 mM NADPH. Biphenyl (5 µg) was added as carrier to the ethyl acetate phase obtained after extraction, and this phase was taken to dryness under nitrogen. The residue was subsequently dissolved in a small amount of methanol and injected onto the high pressure liquid chromatography column. Separation was achieved using a µBondapak C18 column (1.2 x 25 cm) and two pumps (Waters M 45 and 6000). Elution (1.2 ml/min) was performed using a linear gradient (7 min) of 60% (v/v) methanol in water to 100% methanol. Fractions were collected every 20 sec directly into counting vials to an Intertechnique SL-30 spectrometer. Counting was performed using Aquoluma Plus as scintillator liquid. Control incubations were performed in the absence of NADPH or membrane vesicles.](image)
soluble benzene metabolites or metabolites covalently bound to protein, whereas mannitol completely inhibited both processes.

In order to obtain further evidence for the formation of the hydroxycyclohexadienyl radical as a product of hydroxyl radicals and benzene, experiments were designed to evaluate any formation of biphenyl in the reconstituted enzyme system. Using a concentration of benzene of 10 mM and reversed phase high pressure liquid chromatography for detection of benzene metabolites (Fig. 6), it was found that biphenyl was formed at the rate of 6.2 pmol/nmol of cytochrome P-450/min.

**DISCUSSION**

Based upon the results obtained, one may postulate the following mechanism for the cytochrome P-450-dependent benzene monooxygenase (Fig. 7). Ferric cytochrome P-450 is reduced by NADPH-cytochrome P-450 reductase and upon binding of oxygen, the oxyctytochrome P-450 complex is formed (£FeO2−.O2) which upon autooxidation releases superoxide anions (28–30). These will spontaneously dismutate to hydrogen peroxide but also be able to reduce ferric iron to a ferrous complex or to a species like (£FeO2−)2+ active in cleaving hydrogen peroxide into OH− and ·OH−. The result will be a Haber-Weiss reaction

\[ \text{O}_2 + \text{H}_2\text{O}_2 \rightarrow \cdot \text{OH} + \text{OH}^− + \text{O}_2 \]

The hydroxyl radicals generated will interact with benzene and produce the hydroxycyclohexadienyl radical (13) which in the presence of oxygen will give phenol. At high concentrations, the radical may also interact with itself, yielding biphenyl after elimination of water (15). This radical-induced reaction will probably not be specific for cytochrome P-450; any system producing superoxide anions containing trace amounts of iron should be capable of mediating the process. Accordingly, superoxide anions generated from dihydroxyfumarate were able to metabolize benzene to water-soluble products. In line with this argumentation, it may not be surprising that following injection of radioactively labeled benzene into mice, the main part of the covalently bound benzene metabolites in liver was found in mitochondria (31), known to produce substantial amounts of ′O2 (32, 33), rather than in microsomes or cytosol (37).

Irons et al. (34) found that upon administration of benzene to perfused rat bone marrow, phenol, hydroquinone, and catechol as well as covalent binding of benzene metabolites to tissue constituents could be detected, indicating the capability of the bone marrow cells to metabolize benzene. However, the hydroxylase capacity of the microsomal system in bone marrow is very low (8) and may not explain the pronounced retention and covalent binding of benzene metabolites observed in the bone marrow (6, 31, 35, 36). The superoxide anions released from leukocytes and granulocytes (37) may therefore be of importance in this respect.

Recently, it was found that benzene has the capability to induce the same type of cytochrome P-450 in rabbit liver microsomes as is observed following exposure of ethanol to animals (38, 39). This may be of certain interest since ethanol has previously been described to be metabolized by cytochrome P-450 LM2 according to the same mechanism as found presently for the benzene monooxygenase (17). Accumulating evidence suggests that the ethanol- and benzene-inducible form of cytochrome P-450 partly utilizes a similar reaction mechanism for ethanol oxidation as cytochrome P-450 LM2 (38, 39). This may indicate that the enhanced rate of microsomal benzene monooxygenase after exposure of benzene to animals (7, 39) may be inherent in an induction of this or similar types of microsomal cytochrome P-450 participating in the metabolism of benzene according to this radical-mediated oxygenation mechanism.

Originally, benzene oxide was proposed to be the reactive metabolite of benzene (3). However, recent studies have failed to show covalent binding to macromolecules or toxic effects of this compound (10, 12). Since, in contrast to benzene, benzene metabolites such as phenol, benzene dihydrodiol, and hydroquinone or metabolites formed from these compounds, do not produce hemotoxic effects (40, 41), it seems plausible that another primary metabolite of benzene is toxic to the cell. Our results indicate that the hydroxycyclohexadienyl radical is the intermediate responsible for the covalent binding and the harmful effects.

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