The hepatic metabolism of benzene is thought to be a prerequisite for its bone marrow toxicity. However, the complete pattern of benzene metabolites formed in the liver and their role in bone marrow toxicity are not fully understood. Therefore, benzene metabolism was studied in isolated rodent hepatocytes. Rat hepatocytes released benzene-1,2-dihydrodiol, hydroquinone (HQ), catechol (CT), phenol (PH), trans-trans-muconic acid, and a number of phase II metabolites such as PH sulfate and PH glucuronide. Pretreatment of animals with 3-methylcolanthrene (3-MC) markedly increased PH glucuronide formation while PH sulfate formation was decreased. Likewise, V79 cells transfected with the 3-MC-inducible rat UGT1.6 cDNA showed a considerable rate of PH and HQ glucuronidation. In addition to inducing glucuronidation of phenols, 3-MC treatment (reported to protect rats from the myelotoxicity of benzene) resulted in a decrease of hepatic CYP2E1. In contrast, pretreatment of rats with the CYP2E1-inducer isopropanol strongly enhanced benzene metabolism and the formation of phenolic metabolites. Mouse hepatocytes formed much higher amounts of HQ than rat hepatocytes and considerable amounts of 1,2,4-trihydroxybenzene (THB) sulfate and HQ sulfate. In conclusion, the protective effect of 3-MC in rats is probably due to a shift from the labile PH sulfate to the more stable PH glucuronide, and to a decrease in hepatic CYP2E1. The higher susceptibility of mice toward benzene may be related to the high rate of formation of the myelotoxic metabolite HQ and the semistable phase II metabolites HQ sulfate and THB sulfate. — Environ Health Perspect 104(Suppl 6):1183–1188 (1996)

Key words: benzene metabolism, cytochrome P450E1, drug-metabolizing enzymes, glucuronidation, hepatocytes, hydroquinone formation, inducers of drug metabolism, sulfate conjugates, UDP-glucuronosyltransferase

**Introduction**

It is widely accepted that the bone marrow toxicity and carcinogenicity of benzene results from the action of reactive metabolites that damage essential cellular macromolecules in the target organ, thus leading to decreased proliferation, cell death, and genotoxicity. A number of findings indicate that the metabolism of benzene in the liver plays an important role in this scenario by releasing myelotoxic or pro-myelotoxic benzene metabolites into the systemic circulation (1,2). Principal pathways of hepatic benzene metabolism include the formation of phenolic metabolites and their conjugates, and of the ring-opened trans-trans-muconaldehyde and its oxidation products (3). However, the metabolites ultimately responsible for bone marrow toxicity still have to be identified. Application of single metabolites of benzene such as phenol (PH), catechol (CT), hydroquinone (HQ), and 1,2,4-trihydroxybenzene (THB) to rodents failed to reproduce the characteristic toxic effects of benzene in the bone marrow (4,5). In several studies the possible synergistic action of certain metabolites of benzene on the bone marrow was investigated. It was shown that PH enhanced the conversion of HQ into 1,4-benzoquinone catalyzed in vitro by myeloperoxidase (6,7), an enzyme present in abundance in the bone marrow (8). The electrophilic 1,4-benzoquinone thus formed is able to bind to cellular proteins and DNA. Binding to critical proteins such as tubulin (9) or DNA polymerase-α (10) may play an important role in benzene toxicity.

In vivo experiments by Eastmond et al. (11) demonstrated that combined but not single treatment of rats with HQ and PH led to a decrease in bone marrow cellularity, while CT was ineffective when combined with either PH or HQ. In a report by Guy et al. (12) the combination of muconaldehyde and HQ was reported to inhibit erythroid iron utilization in mice in a synergistic manner. These studies demonstrate the central role of phenolic metabolites in the myelotoxicity of benzene.

In this report we show that pretreatment of rats with 3-methylcolanthrene (3-MC) leads to a shift from sulfation to glucuronidation of phenol, the major phase I metabolite of benzene. Induction of a phenol glucuronosyltransferase (UGT 1.6) may thus protect the organism by forming stable glucuronides instead of semistable sulfates. Since inhalation experiments had revealed significantly higher levels of various benzene metabolites such as HQ, HQ glucuronide, and trans-trans-muconic acid in mice compared to rats (13), we investigated the pattern of benzene metabolites released from isolated hepatocytes of NMRI mice to elucidate whether differences in benzene metabolism might contribute to the observed differences in metabolite levels and myelotoxicity (14). Major differences were found in the formation of sulfate conjugates of phenolic benzene metabolites between mouse and rat hepatocytes.

**Materials and Methods**

**Chemicals**

14C-Benzene was obtained from Amersham (Braunschweig, Germany) at a radiochemical
and chemical purity of > 99.5%. It was diluted with unlabeled HPLC-grade benzene (Baker, Gross-Gerau, Germany) to a specific activity of 37 MBq/mmol. Collagenase type IV was from Sigma (Taufkirchen, Germany) or from Boehringer (Mannheim, Germany). HQ sulfate was obtained from TCI Chemicals (Tokyo, Japan). Benzene-1,2-dihydrodiol was a generous gift of Prof. F. Oesch (Institute of Toxicology, University of Mainz, Mainz, Germany).

**Animals**

Male Wistar rats weighing 220 to 240 g, and male NMRI mice weighing 20 to 24 g, were obtained from Savo (Kisslegg, Germany) and the breeding station of the GSF (Neuherberg, Germany), respectively. The animals were kept under standard conditions, and had free access to laboratory chow (Altromin, Lage, Germany) and drinking water. Isopropanol (2.5 ml/kg) was applied by gavage as dilution in saline 24 hr before sacrifice. 3-MC (40 mg/kg, ip, dissolved in corn oil) was administered 3 days before sacrifice, and phenobarbital was given by a single ip injection of 100 mg/kg (dissolved in saline) followed by application in drinking water (0.1%, w/v) over 3 days. Control rats received saline or corn oil injections, respectively.

**Hepatocyte Preparation and Incubation**

For hepatocyte preparation animals were anesthetized with pentobarbital sodium (100 mg/kg bw), and the liver was perfused in situ via the portal vein using the sequential ethylenediaminetetraacetic acid (EDTA)/calcium–collagenase method. Rat hepatocytes were prepared as described as described by Schrenk and Bock (15). Only cell preparations exceeding a viability of 90% were used for incubations.

For preparation of mouse hepatocytes, livers were perfused sequentially with 50 ml preperfusion buffer 1 (Ca^2+–free modified Hank’s medium containing 100 µM [EGTA]), with 50 ml pre-perfusion buffer 2 (Ca^2+–free modified Hank’s medium, without EGTA), and with 100 ml of Dulbecco’s minimal essential medium (DMEM) containing 1.8 mM CaCl_2 and 12 U collagenase/100 ml at a flow rate of 10 ml/min. Then cells were dispersed in DMEM containing 1% albumin. After filtration through 80-µm and subsequently through 40-µm nylon mesh filters, the cells were washed 3 times in DMEM at 50°C for 45 sec. Viability, as determined by exclusion of 0.4% Trypan Blue, was greater than 85%.

14C-Benzene (0.5 mCi; final specific activity 37 MBq/mmol) was incubated with 20 x 10^6 freshly isolated hepatocytes for 1 hr in 20 ml Krebs-Ringer buffer (NaCl 7.0 g/liter, KCl 0.36 g/liter, MgSO_4 x H_2O 0.295 g/liter, KH_2PO_4 0.163 g/liter, NaHCO_3 2.016 g/liter) supplemented with 5 mM HEPES, 10 mM d-glucose, 5 mM Na_2SO_4, and 20 g bovine serum albumin per liter as previously described (15). Incubations were performed in airtight Erlenmeyer flasks (250 ml) which were gently shaken.

**Separation and Quantification of Metabolites**

After addition of 10 µM ascorbic acid to avoid oxidation of metabolites, the incubations were stopped by adding 2 vol of ice-cold methanol, flushed with nitrogen, and kept on ice for 30 min. Then, precipitated protein was removed by centrifugation. Supernatants were evaporated to dryness, redissolved in 1 ml methanol, and aliquots were analyzed on a high performance liquid chromatography (HPLC) system as described by Schrenk and Bock (15). Metabolites were detected using a Lambda-Max 491 UV-detector (Millipore, Dreieich, Germany) at 280 nm and a Beckmann 171 radiodetector (Beckman Instruments, Fullerton, CA). The radiodetector was set for liquid scintillation using Ulmita Gold scintillation cocktail (Packard Instruments, Meriden, CT) at a flow rate of 3 ml/min. Alternatively, fractions of 0.5 min were collected and quantified by liquid scintillation counting.

**Identification of Metabolites**

The metabolites PH sulfate, PH glucuronide, S-phenylglutathione, CT, HQ, PH, benzene-1,2-dihydrodiol, and metabolite G (presumably trans-trans-trans-acidic) were identified after preparative HPLC as described by Schrenk and Bock (15). Two novel benzene metabolites separated by HPLC analysis of supernatants from mouse hepatocyte incubations were isolated by preparative HPLC. Fractions eluting at the respective retention intervals were collected. After adjustment to pH 7.8, fractions were evaporated to dryness, kept under nitrogen, and were analyzed by Fast atom bombardment (FAB) mass spectrometry using a Varian MAT 711A mass spectrometer (Varian, Bremen, Germany) after dissolving the sample in a glycerol matrix. Alternatively, the samples were analyzed in a Finnigan 4021 mass spectrometer (Finnigan, San Jose, CA) using electron impact (EI) mass spectrometry, after derivatization with N-methyl-N-(trimethyl-silyl)trifluoroaceticamide (MSTFA).

**Immunoblotting of CYP2E1**

Microsomes were prepared from isolated hepatocytes, and microsomal proteins were separated by SDSPAGE, transferred to Immobilon P sheets (Millipore, Dreieich, Germany), and incubated with rabbit anti-CYP2E1 IgG as described by Schrenk and Bock (15). Immunoreactive bands were visualized by incubation with peroxidase-conjugated swine anti-rabbit IgG and subsequent reaction with 4-chloro-1-naphthol/H_2O_2.

**Glucuronidation Experiments**

Glucuronidation of phenols was studied in rat liver microsomes or in V79 Chinese hamster lung fibroblasts. Liver microsomes were prepared as described by Bock and White (16) and were incubated with 200 µM HQ under the same conditions as described for PH (15). After addition of 10 µM ascorbic acid to avoid oxidation, the incubations were stopped by adding 2 vol of ice-cold methanol, flushed with nitrogen, and kept on ice for 30 min. Then, precipitated protein was removed by centrifugation. Supernatants were evaporated to dryness, redissolved in 1 ml methanol, and aliquots were analyzed on the HPLC system used for benzene metabolites. A distinct peak of HQ monoglucuronide eluting slightly earlier than HQ was detected by UV absorbance (280 nm) and was quantified by peak area integration.

Both wild-type V79 cells and a clone stably transfected with the rat UGT1.6 cDNA were cultured as described by Bock et al. (17). HQ or PH was freshly dissolved in sterile phosphate-buffered saline and was added to the culture dishes. After various time points, aliquots of the supernatants were removed and HQ glucuronide or phenylglucuronide were separated and quantified by HPLC/UV detection. Both glucuronides could be identified by FAB–MS.

**Results**

In addition to a number of highly polar metabolites designated as fraction A, seven radioactive peaks could be separated from supernatants of rat hepatocytes incubated with 14C-labeled benzene (Figure 1). Further analysis of peak 3 revealed the presence of two metabolites that were identified
by mass spectrometry (not shown) as phenylsulfate and S-phenylglutathione (15). Based on the amount of radioactivity, a quantitative pattern of metabolites was obtained (Table 1). In hepatocytes from untreated rats, PH sulfate, PH glucuronide, and S-phenylglutathione represented the major metabolites, while benzene-1,2-dihydrodiol, HQ, HQ glucuronide, CT, and unconjugated phenol (PH) were formed in smaller amounts. Another metabolite, possibly trans-trans-muconic acid, could not be identified because the amount formed was insufficient for mass spectrometric identification.

While total metabolism (at a substrate concentration of 0.1 mM) was not affected by pretreatment with 3-MC or phenobarbital (PB), 3-MC caused a significant increase in PH glucuronide formation at the expense of PH sulfate and unconjugated PH. In contrast, isopropanol pretreatment resulted in a pronounced increase in total metabolism and in the release of a number of benzene metabolites. The most dramatic effects were obtained for HQ/HQ glucuronide (8-fold) and for benzene-1,2-dihydrodiol (5-fold). Enhanced glucuronidation of PH in liver microsomes from 3-MC-pretreated rats has been shown (15). In a similar experiment, glucuronidation of HQ was also found to be markedly inducible by 3-MC and, to a lower extent, by PB (Figure 2). From Lineweaver-Burk diagrams an apparent Km value for the high-affinity UGT activity toward HQ of approximately 0.1 mM was calculated (not shown). Figure 3 shows the time course of glucuronidation of PH and HQ in V79 cells stably expressing rat UGT 1.6 cDNA, an isozyme that is induced in rat liver by 3-MC treatment. While almost no

![Figure 1](image1.png)

Figure 1. HPLC radiochromatogram of 14C-benzene metabolites formed in isolated rat hepatocytes. Hepatocytes (20×10^6/20 ml) were incubated for 1 hr with 0.5 mM 14C-benzene (specific activity, 37 MBq/nmol). Fractions were collected at 0.3-min intervals and radioactivity was determined by liquid scintillation counting. Numbers represent polar metabolites (A), benzene-1,2-dihydrodiol (1), hydroquinone/hydroquinone glucuronide (2), phenylsulfate/S-phenylglutathione (3), catechol (4), phenylglucuronide (5), unidentified metabolite coeluting with trans-trans-muconic acid (6), phenol (7).

![Figure 2](image2.png)

Table 1. Pattern of benzene metabolites formed in isolated rat hepatocytes: effects of inducers.

| Metabolite                     | Control | Phenobarbital | 3-MC | Isopropoanal |
|-------------------------------|---------|---------------|------|-------------|
| Polar metabolites             | 0.19 ± 0.04 | 0.22 ± 0.05   | 0.30 ± 0.06 | 0.37 ± 0.09* |
| Benzene-1,2-dihydrodiol       | 0.07 ± 0.04 | 0.07 ± 0.03   | 0.06 ± 0.03 | 0.38 ± 0.06* |
| Hydroquinone/HQ glucuronide   | 0.20 ± 0.06 | 0.20 ± 0.07   | 0.13 ± 0.06 | 1.76 ± 0.31* |
| Phenylsulfate                 | 0.94 ± 0.15 | 0.71 ± 0.11   | 0.63 ± 0.10* | 1.51 ± 0.27* |
| Phenylglutathione             | 0.32 ± 0.09 | 0.27 ± 0.10   | 0.25 ± 0.09 | 1.60 ± 0.24* |
| Catechol                      | 0.27 ± 0.05 | 0.32 ± 0.06   | 0.21 ± 0.05 | 0.45 ± 0.07* |
| Phenylglucuronide             | 0.41 ± 0.07 | 0.60 ± 0.07   | 0.56 ± 0.09* | 0.67 ± 0.12 |
| (Muconic acid)                | 0.03 ± 0.02 | 0.06 ± 0.03   | 0.05 ± 0.02 | 0.07 ± 0.04 |
| Phenol                        | 0.16 ± 0.05 | 0.09 ± 0.04   | 0.08 ± 0.02* | 0.24 ± 0.08 |
| Others                        | 0.14 ± 0.06 | 0.11 ± 0.06   | 0.06 ± 0.04 | 0.48 ± 0.08* |
| Total                         | 2.67      | 2.62          | 2.79  | 7.51        |

*Flat hepatocytes were incubated with 0.1 mM benzene. Mean ± SD from four experiments. Incorporates conjugates of catechol. Characterized on the basis of retention. *Significantly different from controls at p < 0.05.

![Figure 3](image3.png)

Figure 3. Glucuronidation of hydroquinone and phenol in V79 cells expressing rat UGT1.6. V79 wild type (○,△) and stably UGT1.6-transfected (●,▲) V79 cells were incubated with 200 μM phenol (■,●) or hydroquinone (○,▲). Glucuronides were analyzed by HPLC as described in "Materials and Methods." Data represent mean ± SD from three experiments.
glucuronides were formed in untransfected cells, UGT1.6-expressing cells showed a considerable rate of glucuronidation of both PH and HQ.

The most important phase I enzyme involved in benzene metabolism is CYP2E1 (18,19). Western blot analysis of microsomes prepared from freshly isolated hepatocytes showed that both 3-MC and PB treatment decreased hepatic CYP2E1, whereas the well-known inducing action of short-chained aliphatic alcohols could be confirmed in parallel experiments with isopropanol (Figure 4).

Comparatively, the metabolism of benzene in mouse hepatocytes was investigated. At least nine peaks detectable by liquid scintillation counting could be separated by HPLC (Figure 5). Most of the metabolites were identical to those found in rat hepatocytes, as revealed by mass spectrometric analysis. However, two additional peaks were observed at a retention time of 7.0 min (peak 1) and around 10.0 min (peak 3). The compound eluting at 7.0 min could be isolated and identified by FAB-MS as THB sulfate (not shown). The compound eluting at around 10.0 min coeluted with synthetic HQ sulfate but did not reveal a concisive FAB signal. However, after treatment with diluted hydrochloric acid overnight, HQ was detected in the El mass spectrum (not shown).

Mouse hepatocytes metabolized 0.5 mM benzene at a 3-fold higher rate than rat hepatocytes (Table 2). Furthermore, considerable differences were found when the quantitative patterns of metabolites were compared. Rat hepatocytes did not show formation of THB sulfate or HQ sulfate, whereas in mouse hepatocytes benzene-1,2-dihydrodiol was not found and catechol formation was significantly lower. The most pronounced differences were seen with HQ (including HQ glucuronide), which was found at a 10-fold higher amount in mouse hepatocytes. The HQ fraction probably comprises both HQ and HQ glucuronide, which were not clearly distinguishable by radiodetection. Furthermore, PH and metabolite 7, presumably trans-trans-muconic acid, were found in mouse hepatocytes at a 4-fold and 3-fold higher level, respectively.

**Discussion**

This study’s major aim was to explore the pattern of benzene metabolites in hepatocytes, including phase II metabolism. The combined radiodetection/direct mass spectrometry methods allowed the sensitive detection, identification and quantification of phase I metabolites and of intact conjugates. The overall pattern of metabolites was similar to that found in urine of laboratory animals treated with benzene (20,21), a finding that emphasizes the role of the liver in benzene metabolism.

Treatment of rats with 3-MC, leading to the induction of drug-metabolizing enzymes that belong to the Ah gene battery, did not result in dramatic changes in total benzene metabolism. However, increased glucuronidation of PH at the expense of PH sulfate formation suggest involvement of UGT1.6 (a member of the Ah receptor gene battery that catalyzes the
glucuronidation of planar phenols with high affinity). Both 3-MC induction of PH and HQ glucuronidation in liver microsomes and the high glucuronidation rates of both substrates in V79 cells expressing rat UGT1.6 suggest that UGT1.6 induction is the cause of the metabolic shift toward PH glucuronidation. Apparent \( K_m \) values of 0.14 mM for PH (15) and of 0.1 mM for HQ characterize both compounds as intermediate-affinity substrates of the enzyme when compared to other planar phenols (17,22). No glucuronide was detectable in incubations of liver microsomes with THB, possibly as a result of the high polarity of THB.

Another major effect of inducing agents was the pronounced enhancement of benzene metabolism by isopropanol. Similar observations were made by measuring benzene metabolism in laboratory animals and liver microsomes (23,24). In fact, the isopropanol-inducible isozyme CYP2E1 acts as a high-affinity benzene monooxygenase (18,19), and its induction thus led to an increased formation of a broad spectrum of benzene metabolites, which are derived directly or indirectly from benzene oxide. A surprising result was the clear suppression of CYP2E1 expression in hepatocytes freshly isolated from 3-MC- (and PB-) treated rats. Although the molecular basis of this suppression is not known, it may contribute to the protection toward the myelotoxicity of benzene provided by 3-MC-type inducers (25,26). At a substrate concentration of 0.1 mM, this suppression was not reflected by a decreased rate of total benzene metabolism in rat hepatocytes. However, incubations at lower substrate concentrations showed a decrease in total benzene metabolism after 3-MC or PB treatment (27). The low yield of metabolites, however, prevented the quantification and identification of individual metabolites. Apparently, the concomitant induction of low-affinity benzene monooxygenases such as CYP1A2 or CYP2B1 by these inducers at 0.1 mM benzene counterbalances the decrease in CYP2E1. It can be speculated that modifications of the hepatic CYP2E1 level may have considerable consequences for the toxicity of benzene at lower concentrations in the range of human exposure.

Since mice have been reported to be more sensitive toward the carcinogenic action of benzene (14), the patterns of benzene metabolism in rat and mouse hepatocytes were compared. A major finding was that hepatocytes isolated from NMRI mice metabolize benzene at a 3-fold higher rate than hepatocytes from Wistar rats (Table 2). This agrees with data published by Henderson et al. (13) showing that mice had considerably higher levels of various benzene metabolites in liver, lung, and blood after a 6-hr inhalation of 50 ppm benzene.

The comparison of metabolite patterns formed in isolated hepatocytes from both species also revealed qualitative differences. A benzene metabolite detectable in supernatants from mouse hepatocytes only was identified as THB sulfate. Marked quantitative differences were also found for HQ and its conjugates. HQ (including HQ glucuronide) and HQ sulfate taken together accounted for more than 50% of total metabolites in mouse hepatocyte incubations, whereas HQ was only a minor metabolite in rat hepatocytes. In rats, evidence for the excretion of HQ sulfate in bile was obtained in a previous study (27). Nevertheless, the rate of formation in rat hepatocyte incubations was probably too low to allow a clear separation and identification of this compound. No evidence was obtained for the formation of HQ disulfate in accordance with a report by Divincenzo et al. (28).

Benzene-1,2-dihydrodiol, usually formed in small amounts in rat hepatocytes, was completely absent in mouse hepatocytes. Similarly, rat hepatocytes produced more catechol, which is in agreement with the suggestion that benzene-1,2-dihydrodiol, which was detected only in rat hepatocytes, may be a precursor of catechol (3). The fact that some catechol was also found in mouse hepatocytes, however, suggests that benzene-1,2-dihydrodiol is converted more effectively to catechol in mouse hepatocytes.

The most prominent differences found by Henderson et al. (13) in an in vivo study are basically in agreement with the data presented here. The authors report much higher levels of the putative myelotoxic metabolites HQ and HQ glucuronide in mouse tissues. However, the reported considerable formation of trans-trans-muconic acid was not confirmed in the present study. This discrepancy may be due to limitations in the comparison between whole animals and cell cultures.

The pronounced differences in benzene metabolism between rat and mouse hepatocytes may have an important impact on benzene toxicity. Since HQ is suspected to mediate the myelotoxicity of benzene in concert with other benzene metabolites such as phenol or trans-trans-muconic acid, a higher risk of myelotoxicity in mice could be concluded from our experiments. Furthermore, the higher rate of formation of sulfate conjugates may represent an additional pro-myelotoxic factor. Experimental evidence in our laboratory suggests, at least for HQ sulfate, a tendency for hydrolysis and oxidation in an aqueous environment. Recently, the instability of the sulfate conjugate of 1,4-dihydroxy-naphthalene in mice was demonstrated by Tsuruda et al. (29). Similarly, THB sulfate may provide a semistable transport form for THB. THB was shown to be genotoxic in Chinese hamster V79 cells (30); it may also play a role in benzene myelotoxicity (31). Thus, the role of HQ sulfate and THB sulfate as putative pre-myelotoxic metabolites and their activation in bone marrow cells warrants further investigation.

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