Comparison of Protein Adducts of Benzene Oxide and Benzoquinone in the Blood and Bone Marrow of Rats and Mice Exposed to $^{14}$C-$^{13}$C$_6$Benzene

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ABSTRACT

Protein binding of hemoglobin (Hb) and bone marrow was used to compare in vivo reactions of 3 electrophilic metabolites of benzene, i.e., benzene oxide and 1,2- and 1,4-benzoquinone (1, 2-BQ and 1, 4-BQ), in F344 rats and B6C3F1 mice. Following a single p.o. administration of a mixture of $^{14}$C- and $^{13}$C$_6$Benzene between 50 and 400 mg/kg body weight, cysteine adducts of benzene oxide, 1,2-BQ, and 1,4-BQ were assayed, and the proportions of cysteine-bound adducts to total protein binding were estimated. Although dose-related production of each adduct was seen, large differences were observed between species and tissues. With rat Hb, benzene oxide adducts represented 27% of the total Hb binding and 73% of the cysteinyl binding, whereas quinone adducts represented relatively small proportions. However, with mouse Hb, the 1,4-BQ adducts accounted for 5.5% of the total Hb binding and 12.2% of the cysteinyl binding, while 1,2-BQ and benzene oxide each accounted for less than 3% of the total. In the bone marrow of both rats and mice, BQ adducts were more abundant than those of benzene oxide. However, adducts of 1,2-BQ predominated in rat marrow (9% of binding), whereas adducts of 1,4-BQ were more abundant in the mouse (21% of binding). The average blood concentrations of 1,4-BQ were estimated from the control and reaction-rate constants to be 2-5-fold higher in the mouse than in the rat. This work suggests that BQ binding is favored over that of benzene oxide in the bone marrow; however, high background levels of BQ adducts, observed with Hb and bone marrow proteins, suggest that any toxic effects of the quinones should only arise from high exposures to benzene.

INTRODUCTION

Although benzene is known to cause leukemia in humans and tumors at multiple sites in rats and mice, the mechanism of carcinogenesis remains elusive. The complex metabolism of benzene gives rise to a number of electrophilic metabolites including the following: 1,2-, 1,4-, and hydroxy-1,4-BQ (or corresponding semiquinones), benzene oxide, trans,trans-muconaldehyde, epoxides of hydroquinone and catechol, benzene dihydrodiol epoxide, benzene oxide oxepin, oxygen and glutathione free radicals, products of lipid peroxidation (1–3), and possibly conjugated quinones.

Despite reports that radiolabeled benzene binds to cellular macromolecules in vivo (4–6), only a few investigators have quantified specific binding products. Bechtold et al. (7, 8) reported that SPC, the presumed reaction product of benzene oxide with cysteinyl residues of Hb and albumin, accounted for 32% of the Hb binding following administration of benzene to rats. Melikian et al. (9) quantified cysteine-bound adducts of benzene oxide and BQ with Hb after administration of benzene in the rat (9). We reported the formation of adducts of 1,2-BQ and 1,4-BQ with cysteine residues of Hb and bone marrow proteins following administration of $^{13}$C$_6$Benzene to rats and noted that high levels of these same adducts arose from endogenous and/or environmental sources (10). DNA adducts of 1,4-BQ (semiquinone) in bone marrow and WBC of mice treated with multiple high dosages of benzene were measured by the 32P-postlabeling method (11). Various products of reactive intermediates of benzene have also been observed in the urine, including N7-phenylguanine in rats (12), glutathione conjugates of 1,4-BQ in rats (13), and glutathione conjugates of benzene oxide in rats and humans exposed to benzene (14–17).

In order to elucidate the relative abundance of different reactive intermediates of benzene, we utilized binding to cysteinyl residues of proteins to compare in vivo levels of 3 reactive intermediates, i.e., benzene oxide, 1,2-BQ, and 1,4-BQ, between species and tissues. To accomplish this, we administered a mixture of $^{14}$C$_6$Benzene and $^{13}$C$_6$Benzene to both F344 rats and B6C3F1 mice. Following isolation and purification of Hb and bone marrow proteins, the adducts of benzene oxide, 1,2-BQ, and 1,4-BQ were assayed by methods published previously (7, 10, 18). The proportion of each adduct to the total radiobinding was estimated. Moreover, since our assay (10, 18) utilizes a catalyst that specifically cleaves sulfur-bound adducts, we were also able to estimate the fraction of total cysteinyl binding associated with each of the genotoxic metabolites.

MATERIALS AND METHODS

Chemicals. S-Benzyl-l-cysteine (Sigma grade) was obtained from Sigma Chemical Company (St. Louis, MO). Acetonitrile (ChromAR HPLC grade) was obtained from Mallinckrodt (Paris, KY). $^{14}$C$_6$Benzene (128 mCi/mmol) was purchased from Amersham Corporation (Arlington Heights, IL). Reagents used for SDS-PAGE analysis were obtained from Bio-Rad (Richmond, CA). All other chemicals were the same as reported previously (7, 10, 18).

Synthesis of SPC. SPC was kindly provided by Dr. A. Gold and Dr. R. Sangaiah. S-Phenyl mercapturic acid was synthesized based on a procedure described by Behringer and Fackler (19). SPC was acid hydrolyzed from the mercapturic acid, purified by selective crystallization, and characterized by high resolution $^1$H-labeled nuclear magnetic resonance using methods to be published. No impurities could be detected by nuclear magnetic resonance or by HPLC and GC-MS (using systems described below for analysis of benzene oxide adducts).

Reactions of 1,4-BQ with Mouse Blood in Vitro. Mouse blood was reacted with 1,4-BQ in order to estimate the first-order elimination rate constant and the second-order reaction rate constant of 1,4-BQ with Hb. Blood from 6 B6C3F1 mice, anesthetized with methoxyfluorane, was removed by cardiac puncture into a heparinized syringe. One hundred 1 2ml of a freshly prepared solution of 1,4-BQ in saline (0.9% NaCl) were added to 3.5 ml of fresh whole blood (37°C) to achieve a final concentration of either 50 or 100 μM. The blood was incubated at 37°C and mixed by gentle inversion of the vial every 5 min. At time points, 1, 10, 20, 40, 60, and 80 min, a 0.25-ml aliquot of the reaction mixture was removed and added to a vial containing 0.5 ml ethyl acetate. The 1,4-BQ was extracted from the blood by vortex mixing for 30 s and then separated by centrifugation. The ethyl acetate layer was transferred to a vial containing Na$_2$SO$_4$ (anhydrous), of which 2 ml were immediately analyzed by GC-MS as described previously (18). After a total incubation...
time of 3 h, Hb was isolated from the remaining blood and analyzed for BO adducts as described below.

Administration of [14C] Benzene to F344 Rats and B6C3F1 Mice. Twenty male F344 rats (average weight, 308 ± 15 g, SD) were divided into 5 groups and administered a single dosage via gastric intubation. Three animals per dose group received [14C] benzene in corn oil at 50, 100, 200, and 400 mg/kg body weight. To minimize the amount of radioactive benzene used, the ratio of [14C] benzene to [14C] benzene was adjusted so that the rats were given 25, 50, 50, and 50 μCi/animal in the 50-, 100-, 200-, and 400-mg/kg groups, respectively. The activity of the dosing solutions was measured by scintillation counting prior to administration. Since the variability of the GC-MS assays was anticipated to be higher than that of the radiobinding, one animal in each of the 50- and 100-mg/kg groups was also given a single dosage of [13C] benzene in corn oil. The remaining rats were placed in the control group and given corn oil (vehicle control).

Twenty male B6C3F1 mice (average weight, 31.1 ± 2.5 g, SD) were equally divided into 5 groups: 0 (vehicle control), 50, 100, 200, and 400 mg/kg body weight. Three mice in each group were administered a single dosage of [14C] benzene in corn oil via gastric intubation. The ratio of [14C] benzene to [14C] benzene was adjusted so that the mice were given 0, 12.5, 25, and 50 μCi/animal in the 0-, 50-, 100-, 200-, and 400-mg/kg groups, respectively. The activity of the dosing solutions was measured by scintillation counting prior to administration. Additionally, one mouse in each dose group was given [13C] benzene in corn oil.

Both rats and mice were sacrificed 24 h after the administration of labeled benzene. The animals were anesthetized with methoxyfluorane and their blood was removed via cardiac puncture into a heparinized syringe. Blood was processed as described below.

Isolation of Globin and Bone Marrow Proteins. Globin (apohemoglobin) was isolated as described previously (18). To summarize, after separation of the RBCs from the plasma, the cells were lysed and the membranes were removed by centrifugation. The lysate was passed through 2 Sephadex size-exclusion-chromatography columns to remove small molecules from the Hb. Globin was precipitated by adding the Hb solution to cold, acidified acetone. The precipitate was washed with acetone and dried to constant weight.

Bone marrow proteins were isolated as described previously (10). Briefly, after isolation of the leg bones, the ends of the bones were cut and the marrow was flushed out with 10 mM solution of ascorbic acid. After the marrow cells were lysed and dialyzed against 1 mM ascorbic acid, the marrow proteins were precipitated with trichloroacetic acid. The acid-precipitable proteins were washed 3 times with a 1:1 mixture of ethanol and diethyl ether and dried to constant weight in a vacuum oven. For this experiment, bone marrow was isolated from the tibia and femur of both hind legs in the rat and from the femur and tibia of both hind legs and from the humerus of both front legs in the mouse. Due to the small amount of protein that was obtained from each animal, 19 ± 2.9 (SD) mg from the rat and 5.0 ± 1.4 (SD) mg from the mouse, it was necessary to pool bone marrow proteins from rats within each dosage group in order to perform the BQ and radiobinding analyses. Mouse marrow proteins were pooled by dosage group for all analyses.

Analysis of SPC. SPC, the adduct presumed to arise from reaction of benzene oxide with cysteinyl residues of Hb and bone marrow proteins, was measured using the method developed by Bechtold et al. (7) with some modifications. Purified proteins and 6 N HCl were added to a protein hydrolysis tube (Pierce, Rockford, IL). Samples were degassed, purged with N2, sealed under vacuum, and incubated for 24 h at 110°C. The amount of protein used for each analysis was as follows: rat globin, 5 mg; mouse globin, 7.5 mg; rat marrow protein, 5 mg; and mouse marrow protein, 6–10 mg. Hydrolysates were dried under N2 and resuspended in 950 μl water. After addition of 0.5 nmol of S-benzyl-l-cysteine (internal standard), the samples were filtered through a 0.8-μm cellulose acetate filter (Millipore). SPC was then separated from the unmodified amino acids by preparative HPLC (model 200; BAS, West Lafayette, IN) using a Partisil M9 50-cm x 10-mm ODS-2 column (Whatman, Hillsboro, OR). Since this column was twice the length of that used by Bechtold et al. (7), the percentage of acetonitrile in the mobile phase was increased from 10 to 20% and the flow rate was increased from 2.0 to 2.5 ml/min. The fraction of the HPLC effluent containing SPC and S-benzyl-l-cysteine was collected and dried in a vacuum oven (40°C, 15 mm Hg). The samples were dissolved in 0.5 ml of 0.2 N HCl and derivatized for GC-MS analysis with an amino acid derivatization kit (ALLTECH Associates, Inc., Deerfield, IL). (It is important to note that, since the ALLTECH kit was designed for analysis of mg quantities of amino acids, we scaled down the derivatization procedure by reacting the samples in 1-ml reaction vials and using one-tenth of the volumes specified in the ALLTECH kit.) After transferring the sample to a 1-ml reaction vial (thick-walled glass), the samples were sealed with a Teflon-lined cap and heated at 110°C for 5 min. The cap was removed and the samples were taken to dryness under N2 at 100°C. After the vials were cooled to room temperature, 500 μl of isobutane were added. Acetyl chloride (125 μl) was slowly added dropwise to the vial. (It is important to note that if the acetyl chloride is added too quickly, the samples can be lost because of the speed of the reaction.) The vial was recapped and heated at 100°C for 45 min, to esterify the carboxyl group with an isobutyl moiety, after which the sample was dried with N2 at 115°C. The vials were cooled by placing them in a freezer (−20°C) for 5 min. Three hundred μl methylene chloride and 200 μl heptfluorobutyric acid anhydride were added, and the vial was sealed and heated for 15 min at 100°C, to fluoroacetylate the amino group of SPC. The vials were cooled to room temperature, the caps removed, and the excess reagents were removed with N2 at room temperature. Derivatives were then extracted with a 10 ml solution of isobutanol and stored at −20°C until analysis. Prior to analysis, the sample was concentrated to 25 μl; 2 μl were injected into the GC in the splitless mode. Standards were prepared by dissolving SPC (0–5 nmol) and S-benzyl-l-cysteine (0.5 nmol) in 0.2 N HCl and derivatizing them with the ALLTECH amino acid derivatization kit according to the same procedures. If the recovery of the internal standard was found to be low, the sample was rejected and the analysis was repeated. However, for 6 samples, insufficient protein remained to repeat the analysis. These samples were quantitated by external standard calibration and were consistent with the rest of the data.

GC-MS analysis of SPC derivatives was carried out with a Hewlett Packard 5890 gas chromatograph equipped with a HP5971A mass selective detector. A DB5 fused silica capillary column (30-m x 0.242-mm inner diameter, 1 mm phase thickness) was obtained from J & W Scientific, Inc. (Folsom, CA). The carrier gas was He at a flow rate of 1.0 ml/min. The temperatures of the port and detector transfer line were 220° and 280°C, respectively. The electron multiplier voltage was increased to 400 units above the autotune value, since this change increased the sensitivity of the analytes. The molecular ions of unlabeled SPC, [14C]SPC and S-benzyl-l-cysteine derivatives (449, 455, and 463 mass units/charge, respectively) were monitored.

Analysis of 1,2-BQ and 1,4-BQ Adducts. Adducts of 1,2-BQ and 1,4-BQ with cysteinyl residues (cysteol-cysteine and hydroquinono-cysteine, respectively) of Hb and bone marrow proteins were analyzed as described previously (10, 18). Briefly, ascorbic acid, bis-tris-propan buffer, and the bound internal standard ([H3]hydroquinono-globin) were added to a solution of purified protein. The pH of the solution was adjusted to 7.0 and the protein was digested with protease XIV (Pronase E, 8% by weight of protein; Sigma) for 10 h at 37°C with constant stirring. After adding additional ascorbic acid and a catalyzed agent, the protein solution was extracted with diethyl ether to remove lipophilic contaminants and protease-hydrolyzed adducts. The solution was reacted with Raney nickel to catalytically cleave the adducts from the cysteinyl residues. The adducts were then extracted with diethyl ether, dried, and derivatized with heptfluorobutyrylmimidazol. The adducts were measured by GC-MS using negative chemical ionization detection. Use of mass spectrometry allowed for simultaneous measurement of SPC-labeled doce-specific adducts, 13C6 background adducts, as well as the 2H3-labeled internal standard. Two small aliquots of the protein solution were removed for scintillation counting, one before the Raney nickel reaction and one after the adducts had been cleaved and extracted. The difference in activity observed between these 2 measurements was used to estimate the proportion of adducts released by Raney nickel.

Radiobinding. Small aliquots of the [14C] benzene dosing solutions or the purified 14C-labeled proteins, dissolved in water, were added to 10 ml of scintillant (Optima-Gold; Packard Instrument Company, Meriden, CT) and counted on a Packard 2500 TR liquid scintillation analyzer.

SDS-PAGE Analysis of Bone Marrow Proteins. The protein composition of the acid-precipitated bone marrow lysates was analyzed using a 12% separating gel. The electrophoresis was performed under constant current conditions (30 mA) on a Protein II xi slab cell (Bio-Rad) according to the method of Laemmli (20), except that dithiothreitol was used instead of 2-mercaptoethanol. Aliquots (10–100 μg) of the bone marrow proteins were compared to a mixture of low molecular weight markers (Bio-Rad) and to samples
of purified rat albumin and globin. After staining with Coomassie blue, the gel was scanned with an LKB Ultrosean XL laser densitometer. Additionally, the isolated marrow proteins and standards were run on a 16% acrylamide gel to confirm that there were no additional low molecular weight proteins.

RESULTS

Reaction of 1,4-BQ with fresh mouse blood, in vitro, was found to be pseudo-first order with an elimination rate constant, \( K_a \), of 1.87 h\(^{-1}\), corresponding to a half-life of 0.37 h. Additionally, adducts of 1,4-BQ with cysteinyl residues of Hb were estimated from reactions of whole mouse blood modified with 0, 50, and 100 \( \mu \)M 1,4-BQ. The production of adducts was observed to be linear with a slope of 4.71 (pmol adduct/mg Hb)/\( \mu \)M [BQ] (initial concentration) and an intercept (background level) of 10.9 pmol/mg. We estimated the second-order reaction rate constant, \( k_{\text{BQ-Hb}} \), using the formula developed by Rappaport et al. (21), based upon a formula provided by Ehrenberg and Hussain (22):

\[
k_{\text{BQ-Hb}} = \frac{\beta (k)}{[\text{Hb}] (1 - e^{-k}))}
\]

where \( \beta \) is the coefficient of the regression of adduct level on [BQ].

By utilizing standard values for the concentration of Hb in mouse blood (2.08 \( \times \) 10\(^{-3}\) M) (23), we converted the adduct results to units of \( \mu \)M to obtain a regression coefficient, \( \beta \), of 0.632 (\( \mu \)M BQ-Hb/\( \mu \)M BQ) from which \( k_{\text{BQ-Hb}} \) was estimated to be 570 m\(^{-1}\) h\(^{-1}\).

The production of total adducts, as indicated by radiobinding to Hb and bone marrow proteins, is shown in Fig. 1, A and B. Increasing production of Hb adducts was observed over all dosages tested, i.e., 0–400 mg/kg, in both rats and mice. The shapes of the curves of Hb binding versus dosage were nonlinear in both species, suggesting that the rates of adduct formation in the blood decreased at higher dosages. Formation of benzene-Hb adducts was approximately 120-fold greater in the rat than in the mouse over the dosages tested. In both species, the relationships between marrow adducts and dosage were linear with slopes of 0.116 (pmol/mg protein)/(mg/kg) (\( r^2 = 0.901 \)) in the rat and 0.150 (pmol/mg protein)/(mg/kg) (\( r^2 = 0.970 \)) in the mouse.

Protein adduct levels in the bone marrow were about 20% of the levels in rat Hb and about 60% of the levels in mouse Hb.

The proportion of total protein binding which can be attributed to reaction with free cysteinyl residues was estimated from the fractions of radioactivity released following treatment with Raney nickel, as part of the BQ assay (18). Table 1 shows the percentage of the \(^{14}\)C-labeled adducts released by Raney nickel. The proportions of cysteinyl binding remained constant over the range of dosages within species for both rat and mouse Hb (\( F \) tests, \( P > 0.05 \) in both cases). The proportional binding to cysteinyl residues in bone marrow proteins likewise appears to be constant, as there is no significant linear trend with dosage (\( P > 0.05 \)). However, differences were again apparent between species since significantly greater proportions of cysteinyl binding were observed in mice than those in rats for Hb (mouse, 45.2%; rat, 37.6%; paired \( t \) test, \( P < 0.05 \)); however, the differences only approached significance for the bone marrow proteins (mouse, 81.1%; rat, 63.1%; paired \( t \) test, \( P = 0.09 \)).

Levels of SPC, the presumed reaction product of benzene oxide with cysteinyl residues of proteins, are shown as functions of the dosage of \(^{13}\)C\(_6\)-benzene in Fig. 2, A, B, and C. The formation of adducts of benzene oxide was much greater with rat Hb than with the other proteins tested. Our results with rat Hb agree with those of Bechhold et al. (7), who showed that the levels of SPC in Hb increased with the benzene dosage but were less than proportionate to dosage over this range. Formation of \(^{13}\)C\(_6\)-SPC with mouse Hb was roughly 100-fold lower at the 50-mg/kg dosage and approximately 17-fold lower at 400 mg/kg than that observed with rat Hb. The production of benzene oxide adducts in mouse Hb was proportional to the dosage of benzene with a slope of 4.17 \( \times \) 10\(^{-3}\) (pmol/mg Hb)/(mg/kg) (\( r^2 = 0.918 \)) (Fig. 2C). Similarly, the production of benzene oxide adducts with rat marrow proteins increased in a linear fashion with benzene dosage with a slope of 3.74 \( \times \) 10\(^{-3}\) (pmol/mg protein)/(mg/kg) (\( r^2 = 0.976 \)) (Fig. 2B). Since the benzene-oxide adducts with mouse marrow-proteins were below the limit of detection at the 50- and 100-mg/kg dosage levels, the relationship of adduct formation was presumed to be linear, with a slope of 2.15 \( \times \) 10\(^{-3}\) (pmol/mg protein)/(mg/kg) (\( r^2 = 0.906 \)) on the basis of the 0-, 200-, and 400-mg/kg dosages (Fig. 2C).

The benzene-specific production of cysteinyl adducts of \(^{13}\)C\(_6\)-1,2-BQ and \(^{13}\)C\(_6\)-1,4-BQ is represented in Fig. 3, A, B, C, and D. Although increasing levels of BQ adducts were seen with increasing dosages in both species and proteins, the relative magnitudes of the isomeric forms of the adducts were highly species dependent. In the rat, adducts of \(^{13}\)C\(_6\)-1,2-BQ were more abundant (Fig. 3, A and C), consistent with earlier work (10), while those of \(^{13}\)C\(_6\)-1,4-BQ predominated in the mouse (Fig. 3, B and D). Non-linearities were observed with benzene dosage for 1,2-BQ and 1,4-BQ adducts with Hb of mice (Fig. 3B), while linear relation-

![Fig. 1. Total binding of \(^{14}\)C-benzene equivalents with Hb and bone marrow proteins following a single p.o. administration of \(^{14}\)C/\(^{13}\)C\(_6\)-benzene in (A) F344 rats and (B) B6C3F, mice. Bars for the Hb data, SEM (n = 3). Some bars are within the symbols. Data for bone marrow-protein adducts are single estimates of protein pooled by dosage group.](image-url)
Table 1  Percentage of the [14C]benzene-protein adducts bound to cysteine

<table>
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<tr>
<th>Dosage (mg/kg)</th>
<th>Rat Hb</th>
<th>Mouse Hb</th>
<th>Rat marrow</th>
<th>Mouse marrow</th>
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<tr>
<td>50</td>
<td>38.8 ± 1.5</td>
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<tr>
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<td>200</td>
<td>35.5 ± 1.6</td>
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<td>400</td>
<td>37.1 ± 0.5</td>
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Mean 37.6± 45.2 ± 63.1 ± 81.1

*a As estimated by treatment with Raney nickel.
*b Mean ± SE.
*c No error estimates are available within dosage groups since the marrow proteins were pooled.
*d Significantly different by paired t test, P < 0.05.

Levels of background adducts ([14C]-adducts) of 1,2-BQ and 1,4-BQ with Hb and bone marrow proteins of both rats and mice were much greater than those of the benzene-specific adducts ([13C]-labeled adducts). Levels of background adducts (pmol adduct/mg protein) in Hb were as follows: rat, 15.7 ± 2.39 (SE) and 13.9 ± 0.83 (SE) (n = 20) for the 1,2- and 1,4-BQ isomers, respectively; mouse, 4.06 ± 0.47 (SE) and 17.1 ± 2.53 (SE) (n = 20) for the 1,2- and 1,4-BQ isomers, respectively. Estimates of background levels (pmol adduct/mg protein) in 1,2-BQ and 1,4-BQ in bone marrow proteins were, respectively, 15.7 ± 7.36 (SE) and 11.3 ± 0.82 (SE) (n = 5) in the rat and 21.9 ± 7.61 (SE) and 8.94 ± 1.33 (SE) (n = 5) in the mouse. When the levels of BQ adducts, arising from administration of [13C]benzene, were expressed as ratios of the corresponding background values (Table 2), the levels of benzene-specific adducts only approached background levels at the highest dosages tested (200 and 400 mg/kg).

The fractions of the total adducts (based on 14C-labeled radiobinding) that were attributable to the cysteiny] adducts of benzene oxide, 1,2-BQ and 1,4-BQ with Hb and bone marrow proteins, were estimated. Moreover, estimates of total cysteine radiobinding, resulting from Raney nickel cleavage of sulfur-bound adducts, were used to calculate the proportions of the total cysteine-bound adducts accounted for by benzene oxide, 1,2-BQ and 1,4-BQ. These results are summarized in Tables 3-5. In Table 3, it is observed that benzene oxide adducts of cysteinyl residues of rat Hb accounted for 27.1% of the total Hb binding and 72.6% of the total cysteinyl binding. However, benzene oxide adducts constituted much smaller percentages of the binding with rat bone marrow proteins, i.e., 2.7% of the total protein binding and 4.3% of the total cysteinyl binding. Benzene oxide binding with Hb and bone marrow proteins in the mouse also made up a small percentage of the total adducts and cysteine adducts.

The percentages of the dose-specific binding associated with 1,2-BQ and 1,4-BQ are shown in Tables 4 and 5. These results point to large differences in BQ adduct production between the rat and mouse. In the rat, the 1,2-BQ adduct accounted for a greater percentage of the binding than did the 1,4-BQ adduct. The proportions of total protein adduction measured as 1,2-BQ were 0.5 and 8.5% for rat and mouse proteins (1.4 and 13.7% of the cysteinyl binding), respectively. Interestingly, the proportions of binding of 1,4-BQ with both Hb and bone marrow proteins in the rat were always small but showed linear trends (P < 0.05) towards higher levels with increasing dosage of administered benzene, i.e., rat Hb, 0.05 to 0.26% of total binding (0.12 to 0.72% of cysteinyl binding); rat bone marrow, 1.4 to 11.9% of total binding (2.2 to 21.9% of cysteinyl binding). In the mouse, on the other hand, the 1,4-BQ species was by far the predominant adduct. The percentage of protein binding measured as the 1,4-BQ adduct was 5.5% with Hb and 21.2% with bone marrow proteins, representing ~12 and 26.6% of the cysteinyl binding with these proteins, respectively. Binding of 1,2-BQ with cysteinyl residues of mouse Hb and bone marrow proteins accounted for only 2.5 and 0.7% of the total protein adducts and 5.6 and 0.9% of the cysteine adducts, respectively.
Table 2 Comparison of benzene-specific protein adducts (\(^{13}C_6\)) to background adducts (\(^{12}C_6\)) of 1,2-BQ and 1,4-BQ at various dosages of \(^{13}C_6\) benzene

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<th>Dosage (mg/kg)</th>
<th>Ratio of 1,2-BQs (^{12}C_6)/(^{13}C_6)</th>
<th>Ratio of 1,4-BQs (^{12}C_6)/(^{13}C_6)</th>
<th>Ratio of 1,2-BQs (^{12}C_6)/(^{13}C_6)</th>
<th>Ratio of 1,4-BQs (^{12}C_6)/(^{13}C_6)</th>
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Table 3 Percentage of total protein adducts and total cysteine-bound adducts accounted for by benzene oxide adducts with cysteinyl residues

<table>
<thead>
<tr>
<th>Dosage (mg/kg)</th>
<th>Rat Hb</th>
<th>Mouse Hb</th>
<th>Rat Marrow</th>
<th>Mouse Marrow</th>
<th>% total binding as benzene oxide</th>
<th>% cysteinyl binding as benzene oxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>27.5</td>
<td>1.5</td>
<td>2.5</td>
<td>ND</td>
<td>70.7</td>
<td>3.4</td>
</tr>
<tr>
<td>100</td>
<td>26.5</td>
<td>1.8</td>
<td>2.2</td>
<td>ND</td>
<td>68.4</td>
<td>4.1</td>
</tr>
<tr>
<td>200</td>
<td>28.1</td>
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<td>2.5</td>
<td>1.7</td>
<td>80.2</td>
<td>1.8</td>
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<tr>
<td>400</td>
<td>26.4</td>
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<td>3.6</td>
<td>1.3</td>
<td>71.2</td>
<td>5.6</td>
</tr>
<tr>
<td>Mean</td>
<td>27.1</td>
<td>1.7</td>
<td>2.7</td>
<td>1.4</td>
<td>72.6</td>
<td>3.7</td>
</tr>
</tbody>
</table>

\(^{a}\) Measured as SPC.

\(^{b}\) ND, not detected.

Table 4 Percentage of total protein adducts and total cysteine-bound adducts accounted for by 1,2-BQ adducts with cysteinyl residues

<table>
<thead>
<tr>
<th>Dosage (mg/kg)</th>
<th>Rat Hb</th>
<th>Mouse Hb</th>
<th>Rat Marrow</th>
<th>Mouse Marrow</th>
<th>% total binding as 1,2-BQ</th>
<th>% cysteinyl binding as 1,2-BQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>0.4</td>
<td>3.9</td>
<td>1.1</td>
<td>0.6</td>
<td>0.9</td>
<td>9.0</td>
</tr>
<tr>
<td>100</td>
<td>0.7</td>
<td>2.1</td>
<td>12.4</td>
<td>1.1</td>
<td>1.7</td>
<td>4.9</td>
</tr>
<tr>
<td>200</td>
<td>0.4</td>
<td>1.9</td>
<td>7.1</td>
<td>0.9</td>
<td>1.2</td>
<td>3.9</td>
</tr>
<tr>
<td>400</td>
<td>0.7</td>
<td>2.0</td>
<td>13.4</td>
<td>0.2</td>
<td>1.8</td>
<td>4.5</td>
</tr>
<tr>
<td>Mean</td>
<td>0.5</td>
<td>2.5</td>
<td>8.5</td>
<td>0.7</td>
<td>1.4</td>
<td>5.6</td>
</tr>
</tbody>
</table>

Separation of bone marrow proteins by SDS-PAGE indicated that a significant portion of the protein comigrated with standards of Hb and albumin. The α- and β-globin chains of Hb were well resolved. The number of bands and banding patterns for bone marrow proteins from both rats and mice were roughly equivalent. Estimates of the percent albumin and Hb by densitometry were 9 and 36% for the rat and <5 and 41% for the mouse, respectively. Of the balance of bone marrow proteins detected, 3 constituted less than 10% each of the total protein and migrated with approximately \(M_r\), 50,000, 33,000, and 30,000. The remaining 35–40% of the bone marrow proteins migrated as 3 closely migrating bands with an average \(M_r\), 17,000. No additional proteins were detected using a 16% gel.
DISCUSSION

In this study, we compared the levels of protein adducts produced by 3 electrophilic intermediates arising from metabolism of benzene, namely, benzene oxide, 1,2-BQ, and 1,4-BQ, in the blood and bone marrow of rats and mice. It should be noted that throughout this paper SPC has been presumed to arise from reaction of cysteine with benzene oxide. However, this product may arise from other hypothesized mechanisms such as by hydroxycyclohexadienyl-free radical reaction with cysteine (9, 24), or possibly by reaction of a cystine-thiol radical with benzene. (The latter possibility was pointed out by a reviewer of this paper.) The demonstration of dose-related increases in the levels of adducts of 1,2-BQ and 1,4-BQ with Hb and marrow proteins in the mouse extends our earlier work in the rat (10). Likewise, the measurement of dose-related adducts of benzenoxide with marrow proteins in both species extends the work of Bechtold et al. (7). By administering a mixture of [14C]benzene and [13C]benzene, it was possible to determine not only the levels of these adducts in the blood and bone marrow but also the proportion of total covalent binding which was represented by each reactive metabolite.

The elimination rate of 1,4-BQ from whole blood, in vitro, was found to be greater in B6C3F1 mice (half-life, 0.37 h) than those measured previously in F344 rats (half-life, 0.68 h) and humans (half-life, 3.5 h) (18). Interestingly, the estimated second-order rate constant for reaction of 1,4-BQ with sulphydryl groups in mouse Hb (570 m–1 h–1) was 3.2 times larger than that measured in rat blood (180 m–1 h–1) (18), despite the fact that rats have a free cysteinyl residue at position β-125 (25), which is not present in either mice (26–28) or humans (29). The unexpectedly large rate of reaction between 1,4-BQ and cysteinyl residues of Hb in mouse blood could reflect either differences in the microenvironment of the blood, such as antioxidant levels, or the presence of one or more additional free cysteinyl residues in mouse Hb. Although we could not locate the primary amino acid sequence for Hb of the B6C3F1 mouse, we note that the Hb of some stains of mice, namely BALB/c and the “house mouse” (mus musculus), have an additional free cysteine at β3-13(26, 27) or the rat (32), suggesting an even greater preference for the catechol pathway. This difference may be accounted for by the increased ratio of epoxide hydrolase activity to P-450 activity (using styrene oxide and styrene as substrates) in the livers of the 2 species. However, as noted earlier, the possibility that formation of SPC in Hb involved an intermediate other than benzene oxide should be considered.

Regarding the BQs, Tables 4 and 5 indicate that the metabolic pathways leading to the formation of hydroquinone (following 2 oxidations of benzene by P-4502E1) (1, 3) were favored in the mouse, whereas the pathways leading to catechol (via hydration of benzene oxide by epoxide hydrolase to benzene-1,2-dihydrodiol followed by oxidation by dehydrogenases) (1) were favored in the rat. This difference may be accounted for by the increased ratio of epoxide hydrolase activity to P-450 activity (using styrene oxide and styrene as substrates) in the livers of rats relative to that of mice (32). Interestingly, the ratio of activities (epoxide hydrolase/P-450) in the livers of humans is significantly greater than that of either the mouse or the rat (32), suggesting an even greater preference for the catechol pathway.) Furthermore, it appears that the BQs contributed much greater proportions of total binding than benzene oxide in the bone marrow of both species. This increased activation of catechol or hydroquinone and subsequent binding of BQs may be due to the high peroxidative activity in the bone marrow by enzymes such as myeloperoxidase and eosinophil peroxidase (2).

The integrated dose of [14C]1,4-BQ in the blood can be estimated using the following relationship given by Osterman-Golkar et al. (33):

\[ D = \frac{[\text{BQ} - \text{Hb}]}{k_{\text{BQ-Hb}} - k_{\text{Hb}}} \]

where \( D \) is the dose given in nm 1,4-BQ-h, \( [\text{BQ-Hb}] \) is the concentration of the [14C]1,4-BQ adduct (nm), \( k_{\text{BQ-Hb}} \) is the second-order

<table>
<thead>
<tr>
<th>Dosage (mg/kg)</th>
<th>% total binding as 1,4-BQ</th>
<th>% cysteinyl binding as 1,4-BQ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rat Hb</td>
<td>Mouse Hb</td>
</tr>
<tr>
<td>50</td>
<td>0.05</td>
<td>5.9</td>
</tr>
<tr>
<td>100</td>
<td>0.06</td>
<td>5.6</td>
</tr>
<tr>
<td>200</td>
<td>0.13</td>
<td>5.9</td>
</tr>
<tr>
<td>400</td>
<td>0.26</td>
<td>4.4</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>5.5</td>
</tr>
</tbody>
</table>

* Linear trend with dosage \( (P < 0.05) \).
rate constant ($M^{-1} h^{-1}$), and [Hb] is the concentration of Hb in blood (rat, 2.32 \times 10^{-3} M; mouse, 2.08 \times 10^{-3} M) (23, 34). Values of $k_{BO-Hb}$ for the reaction of 1,4-BQ and Hb were estimated to be 180 $M^{-1} h^{-1}$ in rat blood (18) and 570 $M^{-1} h^{-1}$ in mouse blood (described herein). Values of $D$ were estimated for each group of animals and regressed upon the dosage of benzene. In the rat, a significant linear relationship was found (r = 0.912) with an estimated slope of 0.452 nm 1,4-BQ-h/mg benzene/kg body weight. Since a linear relationship was not found for Hb adducts in the mouse, values of D per mg/kg body weight were calculated at each dosage of benzene. These values ranged from 2.21 nm 1,4-BQ-h/mg benzene/kg body weight at the lowest dosage (50 mg/kg) to 0.925 nm 1,4-BQ-h/mg benzene/kg body weight at the highest dosage (400 mg/kg). Since the duration of the time of each in vivo experiment was 24 h, the average blood concentration of 1,4-BQ ($D/t$) was estimated to be 18.9 pm 1,4-BQ/mg benzene/kg body weight for the rat and between 38.6 and 92.3 pm/mg benzene/kg body weight in the mouse. Thus, we estimate that mice had a body weight in the mouse. Thus, we estimate that mice had a body weight 6.3—18-fold greater than 3C6-labeled 1,2-BQ and 50-mg/kg body weight dosage, and 11- and 25-fold higher at the 400-mg/kg body weight. Similarly, in mouse HB, background levels of 1,2-BQ and 1,4-BQ adducts were 68-fold and 473-fold higher than those observed that background levels of 1,2-BQ and 1,4-BQ adducts were 68-fold and 473-fold higher than those 

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Comparison of Protein Adducts of Benzene Oxide and Benzoquinone in the Blood and Bone Marrow of Rats and Mice Exposed to $^{14}$C/$^{13}$C$_6$Benzene

Thomas A. McDonald, Karen Yeowell-O'Connell and Stephen M. Rappaport


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