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COVALENT BINDING OF [^{14}C]BENZENE TO CELLULAR ORGANELLES AND BONE MARROW NUCLEIC ACIDS

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Abstract—Our objective was to determine whether reactive metabolites of benzene covalently bind to cellular proteins and nucleic acids of the hematopoietic organs and liver of the mouse. The distribution and binding of [^{14}C]benzene after a single dose (880 mg/kg, s.c., 0.75 mCi/kg) were investigated at 3, 6, 12, and 24 hr. Maximum levels of radioactivity were found at 3 hr in liver, spleen, bone marrow, and blood. Maximum covalent binding occurred at 6 hr in liver, spleen, and bone marrow. Chemical fractionation of label derived from [^{14}C]benzene indicated that 0.5, 7, and 17 per cent of the covalently bound label in liver, spleen, and bone marrow was recovered in the nucleic acids. Upon subcellular fractionation of liver, total radioactivity was localized chiefly in the cytosol (1723 dpm/mg protein) and mitochondria (1433 dpm/mg protein). Of the total radioactivity recovered in various organelles, mitochondria had the highest proportion covalently bound (25 per cent). These studies indicate that label derived from [^{14}C]benzene covalently binds to nucleic acids of hematopoietic cells, the major site of toxicity. In addition, the high levels of covalent binding with macromolecules of the mitochondria suggest that their functions may be impaired by benzene.

Benzene, a known leukemogen, produces leucopenia and aplastic anemia in laboratory animals and man [1–4], but the mechanism by which it produces toxicity is unknown. Bioactivation of benzene in hematopoietic cells may be important for its leukemogenic effect [3,5,6], so it is significant that bone marrow microsomal enzyme systems [5] produce the same metabolites of benzene as liver [7, 8]. Reactions of other chemicals with cellular nucleic acid are considered to be a basis for carcinogenesis and mutagenesis [9,10], and reactive benzene metabolites produced by hepatic microsomes bound to microsomal proteins [11]. Chromosomal abnormalities produced in blood cells by benzene [3,12–15] suggest that such reactions may occur in man and indicate the necessity of studying the interaction of benzene with nucleic acids in bone marrow cells. Snyder *et al.* [6] have shown irreversible binding of [^{3}H]benzene and metabolites to acid-and alcohol-insoluble marrow residues, while Lutz and Schlatter [16] have shown binding of [^{14}C]benzene metabolites to liver nucleic acids. Direct labeling of bone marrow nucleic acids and cellular organelles, however, has not been demonstrated previously.

The present studies were performed in mice receiving [^{14}C]benzene subcutaneously. The magnitude of uptake and covalent binding of radioactivity to macromolecular fractions, protein, and nucleic acids was determined in the hematopoietic organs and liver at early periods after treatment. The magnitude of irreversible interaction with various subcellular organelles of the liver was also investigated at the time when labeling of macromolecules was a maximum.

MATERIALS AND METHODS

Animals. DBF_1 male mice (18–20 g) were obtained from the Charles River Co. (Wilmington, MA). Animals were maintained on laboratory chow and water *ad lib.* and were acclimated to our animal environment for 7 days prior to treatment. Experimental groups contained four to six animals per group, unless otherwise stated.

Chemicals. [^{14}C]Benzene (sp. act. 60 mCi/mmole, and 99% + purity) was obtained from the New England Nuclear Corp. (Boston, MA). The radioactive material was diluted with unlabeled analytical grade benzene (Aldrich Chemical Co., Milwaukee WI). Purity was checked on a gas chromatograph that was equipped with a flame ionization detector. The purity of the diluted samples was 99.89%. Specific activity was adjusted with unlabeled chemical so that each animal received 10 or 18 μCi of radioactivity.

Administration of benzene. Undiluted benzene was administered subcutaneously at a dose of 880 mg/kg. This dose has been shown previously in our laboratory (unpublished data) and by other investigators [17,18] to produce hematopoietic toxicity, but no mortality.

Collection of specimens. After benzene administration, animals were placed in metabolic cages to allow collection of urine uncontaminated by feces. At 3, 6, 12, and 24 hr, groups of animals were anesthetized with diethyl ether and blood was obtained in a heparinized tube via the orbital sinus. Livers and spleens were then excised and immediately frozen on dry ice. Urine was aspirated from the bladders and combined with the cage collections for analysis. Gall bladders were excised and frozen prior to analysis. Bone marrow was collected by opening both ends of the femur with a 23 gauge needle and flushing the cavity five times with 0.25 ml of 0.9% saline. Specimens were then frozen until ready for assays. This method provides quantitative recovery of hematopoietic cells from the femur [19,20].

Analysis of [^{14}C]*benzene metabolites in tissues.* Livers and spleens were homogenized in 5 vol. of 0.25 M sucrose and aliquots were extracted as described below. Bone marrow was dispersed by repeatedly aspirating it through a 27 gauge needle. Exhaled benzene was determined by gas chromatography with a flame ionization detector and a Poropak Q column.

Chemical fractionation was performed by the Schmidt–Thannhauser technique [21] as adapted by Reynolds *et al.* [22] and Reynolds and Moslen [23,24]. All analyses were performed in triplicate and averaged. The variation between specimens was less than 10 per cent in all tissues and fractions.

Acid soluble fraction. Tissue homogenate (3.0 ml) was mixed with cold 0.6 M perchloric acid (PCA) (3.0 ml) and then centrifuged. The precipitate was resuspended in chilled 0.3 M PCA and centrifuged. The supernatant fractions were combined and an aliquot was counted for ^{14}C-activity. All steps were carried out at 4°.

Lipid fraction. The insoluble material obtained above was suspended in 3 ml of absolute ethanol, cooled to −16° for 12–16 hr, and centrifuged. The residue was washed once with 3 ml absolute alcohol at 0° and resuspended in 3.0 ml of alcohol–ether (3:1). This suspension was heated at 70° for 3 min and then centrifuged. The combined supernatant fractions constituted the lipid fraction.

Macromolecular fraction. The remaining solid residue was washed twice with ethanol and was designated the macromolecular fraction. This was dissolved with gentle heating in KOH for scintillation counting. In experiments where nucleic acid and protein fractions were separated, the washed residue was suspended in 1.5 ml of 10% NaCl containing phenol red, and the pH was made alkaline (pH 8.5) with 0.1 N NaOH. The reaction vessels were loosely capped and heated in a boiling water bath for 20 min. After cooling, the specimens were centrifuged and the precipitate was subjected to the same procedure once again. The final precipitate was washed with saline and then dissolved in KOH for scintillation counting. This represented the protein fraction.

The combined saline supernatant fractions were added to 2 vol. of ethyl alcohol and maintained at −40° for several days. The resulting precipitate represented nucleic acids [21] and was dissolved in 0.1 N KOH for scintillation counting.

Subcellular fractionation. Liver tissue was homogenized in 5 vol. of 0.25 M sucrose at 4°. Differential centrifugation was performed as described elsewhere [23, 25] and radioactivity was determined in the membrane–nuclear fraction, mitochrondria, microsomes, and cytosol. Protein content was determined by the method of Lowry *et al.* [26].

Liquid scintillation counting. Specimens were dissolved in PCS (Phase Combining System) liquid scintillation counting solutions (Amersham Radiochemicals, Arlington Heights, IL). Alkaline digests of various fractions were first neutralized with HCl. All counts were made on a Searle Mark III liquid scintillation counter with an external standard and automatic correction for quenching and autofluorescence. Background counts were 14–15 dpm and were subtracted from all values reported. Results are expressed as disintegrations per minute (dpm). Counting times were extended as necessary for specimens of low activity.

RESULTS

Time course of elimination of a subcutaneous dose of benzene. Each animal received a single subcutaneous dose of benzene of 880 mg/kg containing 10 μCi [^{14}C]benzene. The plasma levels of radioactivity were a maximum at 3 hr, and then they gradually declined to a low level at 12 hr (Fig. 1A). The cumulative urinary excretion of benzene metabolites is shown in Fig. 1B. Large amounts of radioactivity were detectable at 3 hr, and excretion of labeled compounds was completed at 6 hr. The minor loss

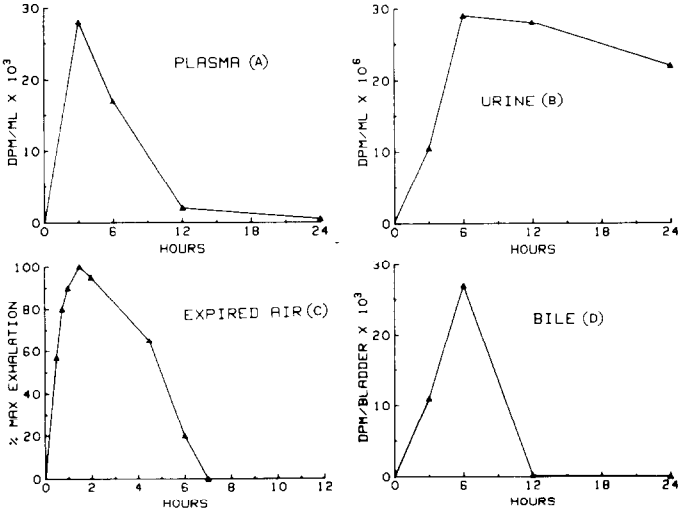

Fig. 1. Time course of ^{14}C in the blood and various excretory fluids after a single subcutaneous dose of [^{14}C]benzene: (A) plasma, (B) urine, (C) air, and (D) bile. Each data point is the mean of values obtained from three animals killed at the time indicated.

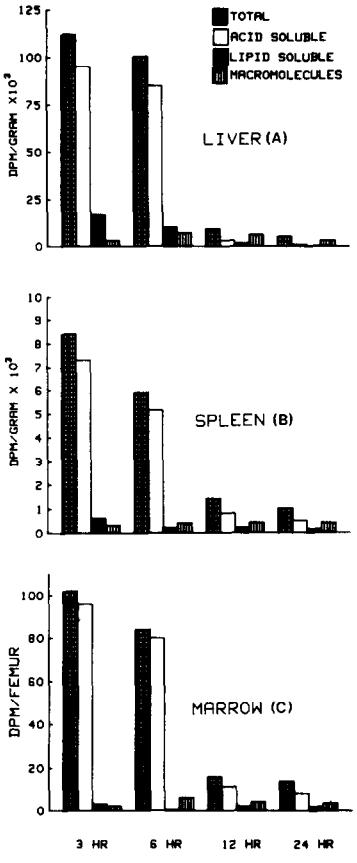

Fig. 2. Content of radiolabel in various chemical fractions of liver, (A), spleen (B), and bone marrow (C) after a single subcutaneous dose of [^{14}C]benzene. Results are mean values obtained from three animals killed at the time indicated. Liver and spleen values are in thousands of dpm per g of liver or spleen; marrow counts are in dpm per femur.

of activity between 12 and 24 hr represented evaporative losses or adsorption of labeled compounds to the plastic collection containers or conversion of [^{14}C]-products to [^{14}C]CO_2. Gall bladders from animals killed at 3 or 6 hr (Fig. 1D) contained large quantities of bile and the amount of radiolabel increased until 6 hr. Gall bladders of animals killed at 12 and 24 hr contained bile with no detectable radioactivity (Fig. 1D).

The rate of exhalation of labeled compounds was determined using gas chromatography. The ^{14}C content of expired air increased rapidly to peak levels at approximately 1 hr, remained elevated for 1 hr, and then declined over the subsequent 4–5 hr (Fig. 1C).

The distribution of radiolabel in various chemical fractions of liver, spleen, and bone marrow was determined 3, 6, 12, and 24 hr after treatment. The greatest amount of radioactivity was found in each tissue 3 hr after subcutaneous [^{14}C]benzene administration (Fig. 2).

In liver and spleen (Fig. 2, A and B), both the acid and lipid extractable compounds fell progressively from the 3 hr level. In the bone marrow, acid

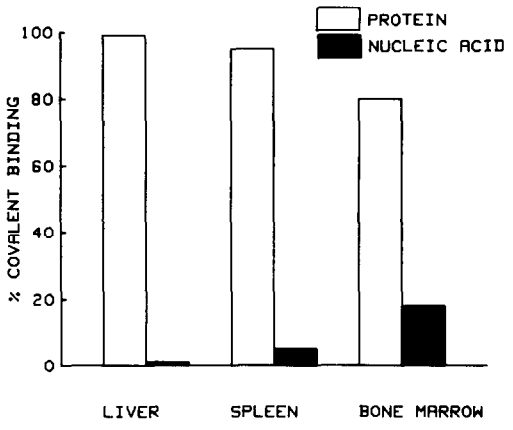

Fig. 3. Distribution of covalently bound ^{14}C in the macromolecular fractions of liver, spleen, and bone marrow 6 hr after a subcutaneous dose of [^{14}C]benzene. Results were obtained from pooled tissues. For marrow, N = 20 femurs; for spleens, N = 5; for livers, N = 4. Total radioactivity in each: liver, 3082 dpm; spleen, 408 dpm, and marrow, 86 dpm.

extractable substances fell after 3 hr, while lipid extractable label remained quite low and showed little variation (Fig. 2C). In each of these tissues, the largest portion of the radioactivity was acid extractable.

The times at which the peak levels of radioactivity were detected in the macromolecular fraction were about the same for each of the tissues studied. In liver (Fig. 2A), macromolecular binding was a maximum at 6 hr and was over 6 per cent of total tissue radioactivity. In the spleen, the level at 12 hr was only slightly higher than those observed at 6 or 24 hr (Fig. 2B) Bone marrow, like liver, showed the greatest macromolecular labeling at 6 hr (Fig. 2C).

Binding of [^{14}C]*benzene metabolites to nucleic acids and cellular organelles.* Based on the findings above, a group of ten animals was treated with 880 mg/kg of benzene containing 18 μCi [^{14}C]benzene, and the

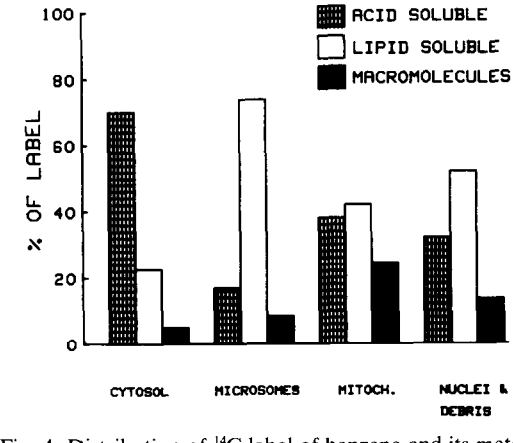

Fig. 4. Distribution of ^{14}C label of benzene and its metabolites in various chemical fractions of the subcellular organelles of liver 6 hr after a subcutaneous dose. Results are percentages of total radioactivity in each organelle. Total radioactivity in each subcellular fraction: cytosol, 267,00 dpm; microsomes, 4550 dpm; mitochondria, 22,220 dpm; and nuclei, 11,300 dpm.

Table 1. Distribution of ^{14}C label from [^{14}C]benzene in various subcellular fractions of the liver, 6 hr after a subcutaneous dose*

	Subcellular distribution (dpm/g tissue)	(dpm/mg protein)
Cytosol	26,700	1,723
Microsomes	455	144
Mitochondria	2,220	1,433
Nuclei and debris	1,130	137

* Values are the means of triplicate determinations of six pooled livers.

organs were harvested after 6 hr, at the time of peak macromolecular labeling. The macromolecular fraction was isolated as before, and then separated further into protein and nucleic acid fractions. The percentage of label fixed in the macromolecular fraction was the same as above, the proportions in the nucleic acid and protein fractions are shown in Fig. 3. The nucleic acid fraction in each of the tissues was found to contain ^{14}C, and in bone marrow 17 per cent of the radioactivity in macromolecules was in the nucleic acid subfraction. In spleen and liver 7 and 0.5 per cent of label in the macromolecular fraction was in nucleic acid subfraction.

Subcellular fractionation by differential centrifugation of livers, obtained 6 hr after [^{14}C]benzene administration, demonstrated that the cytosol contained the largest total amount of radiolabel. Seventy per cent of the label in the cytosol was acid extractable (Fig. 4.); much smaller amounts of radioactivity were detected in the cytosolic lipid soluble and macromolecular subfractions. The microsomal fraction contained far less activity, and it was concentrated mostly in the lipid soluble subfraction. The nuclei and membrane debris fraction was also most heavily labeled in the lipid soluble subfraction, but it did show considerable activity associated with the macromolecules. Approximately 20 per cent of the macromolecular radioactivity in the nuclei-membrane fraction was specifically associated with nucleic acids. The mitochondrial fraction contained labeled compounds in the three subfractions examined and showed a percentage of activity associated with macromolecules that was higher than in the other organelles. Insufficient material was obtained to allow further separation of the mitochondrial macromolecules into protein and nucleic acid fractions.

The relative amounts of label in the various organelles is of more interest than the absolute amounts of benzene metabolites. Therefore, ^{14}C content per milligram of protein was determined for each fraction, the results are shown in Table 1. It is apparent that the cytosolic and mitochrondrial fractions were most heavily labeled with 1723 dpm/mg protein and 1433 dpm/mg protein respectively.

DISCUSSION

A myelotoxic dose of benzene labeled with ^{14}C was used to provide a more specific marker for benzene binding than is achieved with the ^{3}H label [16]. In the present studies, maximum covalent binding occurred early after benzene administration—before repair processes or resynthesis of macromolecules would be expected to occur. A distinct peak in covalently bound [^{14}C]benzene or metabolites, occurred at 6 hr in the liver, and less pronounced peaks were evident at 6 and 12 hr in the marrow and spleen. Persistence of label in the macromolecular fraction 24 hr after treatment is in agreement with previous reports [6].

This time course suggested that the chemical and subcellular locations of bound [^{14}C]benzene and its metabolites would be best studied 6 hr after a subcutaneous dose. Chemical fractionation showed extensive protein binding in each tissue studied (Fig. 3), but this is of uncertain significance. Lutz and Schlatter [16] have shown nucleic acid labeling in the rat liver, this organ, however, is not a major site of toxicity. We verified this observation and, in addition, found that the nucleic acids were more heavily labeled by [^{14}C]benzene and metabolites, in bone marrow than in liver. This suggests that the extensive detoxification mechanisms in liver may have decreased labeling in that tissue.

Previous studies have not examined the distribution of benzene in subcellular organelles. Reactive, short-lived, epoxide intermediates would be expected to fix to sites in close proximity to their production. The cytosol contained large amounts of acid extractable compounds, representing [^{14}C] benzene metabolites and their conjugates. The labeled cytosolic macromolecules were most likely, proteins. Predictable from their membranous nature, microsomes contained a high percentage of label in the lipid extractable material. The small percentage of label irreversibly bound to microsomal macromolecules is consistent with studies of other compounds activated by microsomal enzymes [24]. A fraction containing membrane fragments and nuclei showed considerable labeling of the nucleic acids, proteins, and lipid soluble compounds.

The data in Fig. 4 shows that the mitochondrial fraction had the highest percentage of radioactivity irreversibly bound to the macromolecular fraction. The implications of mitochondrial binding are uncertain, but marked structural changes reported by Kaminski *et al.* [27] argue for functional alterations of this organelle. The growth of several rapidly dividing tissues is inhibited by benzene [28,29], perhaps by interference with normal aerobic metabolism. In addition, incorporation of [^{59}Fe] into hemoglobin occurs via ferrochelatase, a mitochondrial enzyme, and Lee *et al.* [17] have shown that iron utilization is decreased in proportion to the benzene dose. It is uncertain, however, if these actions are related to benzene-induced aplastic anemia or leukemia.

In conclusion, we have shown that ^{14}C from benzene and it metabolites binds covalently to nucleic acids in the hematopoietic cells of mice. This provides strong support for the role of reactive metabolites in the production of previously reported chromosome abnormalities and leukemia by benzene [1–4]. Mitochondria are irreversibly labeled by ^{14}C from benzene and its metabolites, which correlates with previously described morphological and func-

tional abnormalities of this organelle. The role of covalent binding to mitochondria in toxicology has not been well studied, and its importance in the hematologic effects of benzene remains to be investigated.

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