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EFFECTS OF TOLUENE ON THE METABOLISM, DISPOSITION AND HEMOPOIETIC TOXICITY OF [^{3}H]BENZENE

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Abstract—The administration of [^{3}H]benzene to mice resulted in the decreased incorporation of ^{59}Fe into red cells and the accumulation of benzene and its metabolites in bone marrow and other tissues. Toluene protected against the benzene-induced depression of red cell ^{59}Fe uptake and reduced the levels of benzene metabolites in bone marrow without affecting the level of benzene in this tissue. The results of this study suggest that toluene exerted its protective effect by inhibiting benzene metabolism and that a metabolite of benzene probably mediates the observed hemopietic toxicity of benzene.

Benzene, the most extensively used aromatic hydrocarbon in chemical industry, produces serious blood dyscrasias and is metabolized in man and in several species of laboratory animals to phenols and glucuronide and ethereal sulfate conjugates of phenols [1]. It is generally accepted that the liver is the major site of benzene metabolism. Although there are many reports in the literature concerning benzene metabolism and toxicity, whether benzene itself or a metabolite of benzene is the toxic agent is at this time unclear. Ikeda *et al.* [2] suggested that benzene itself may produce hemopoietic toxicity, while many other investigators [3–5] have proposed that a metabolite of benzene mediates the hematotoxic response. Hirokawa and Nomiyama [3] and Nomiyama [4] observed that rats whose livers oxidized benzene less rapidly *in vitro* were less susceptible to benzene poisoning. Abramova and Gadaskina [5] determined that the administration of several antioxidants decreased benzene metabolism and protected against the development of leukopenia. Because benzene toxicity is expressed in bone marrow it is of particular interest that we have identified metabolites of benzene in bone marrow.

Benzene characteristically causes decreases in the production of erythrocytes, leukocytes and/or thrombocytes by bone marrow. Although cell-counting techniques are frequently used to determine benzene toxicity, multiple doses of benzene are usually required and benzene metabolism is frequently complete before the effects on bone marrow cell production can be observed. The uptake of ^{59}Fe into erythrocytes is commonly used as a measure of erythropoiesis by many investigators [6–10], and we have adapted this technique to provide a rapid, sensitive method for evaluating the effects of benzene on the hemopoietic system of the mouse [11, 12].

To relate these effects on erythrocyte ^{59}Fe incorporation to benzene metabolism, it was necessary to describe benzene metabolism in the mouse despite the fact that related reports on benzene metabolism in the rat and rabbit have appeared previously [1]. Since toluene, a structurally related hydrocarbon frequently found in mixtures with benzene, has been shown to inhibit benzene metabolism in the rat *in vivo* [2], we used it as a tool to evaluate the effects of altering the metabolic disposition of benzene on iron utilization.

Thus, it became possible to study the relationship between the metabolism of [^{3}H]benzene and its effect on red cell ^{59}Fe incorporation in the same mouse within a few days after giving a single dose of the benzene.

MATERIALS AND METHODS

Animals used in these experiments were male Swiss albino (ICR) mice (25–35 g) fed Purina Lab Chow *ad lib.* Injections of generally labeled [^{3}H]benzene (radiochromatographically pure from New England Nuclear Corp., Boston, Mass.) or toluene were made s.c. using olive oil as a diluent. After injection, animals were kept in metabolism cages and their urine was collected at regular intervals. Urine samples were diluted to 25 ml, and 0.5-ml aliquots were added to 10 ml of Benson's counting solution [13] and counted in a liquid scintillation spectrometer. The total radioactivity present in the sample was reported as the per cent of administered dose or as benzene equivalents (μmoles).

The expression of urinary radioactivity as benzene equivalents (μmoles) involves a correction factor of 1/6 for the loss of one tritium due to hydroxylation. This correction can be justified, since virtually all (95 per cent) of the urinary metabolites were recovered as free and conjugated [^{3}H]phenol whereas only about 5 per cent were conjugates of [^{3}H]catechol.

In order to separate urinary [^{3}H]benzene metabolites, urine samples were applied to DEAE Sephadex A-25 columns and eluted with a linear pyridine acetate gradient (0.05 to 1.5 M). This treatment separated urinary radioactivity into four components.

Peak I displayed the following properties: (1) none of the radioactivity in peak I was extractable from neutral, acidic or basic solution into ether; (2) peak I emerged from the DEAE-Sephadex column at a point identical to that at which an authentic sample

of ^3H_2O was recovered; and (3) when distilled and collected as ten successive constant boiling fractions (100°), the specific radioactivity of each fraction was the same. Using this procedure, 70 per cent of the radioactivity in peak I codistilled with water. Thus, these data suggest that about 70 per cent of the radioactivity in peak I was ^3H_2O whereas the remainder has not yet been identified.

The second component from DEAE columns was extracted with 2 vol. of ethyl ether and the extract was concentrated. Samples were applied to Quanta/Gram precoated Silica gel thin-layer chromatography plates (Quantum Industries) and chromatographed along with a standard containing phenol, pyrocatechol, quinol and 1,2,4-trihydroxybenzene. The two solvent systems employed were benzene–dioxane–acetic acid (19:5:1) and benzene–methanol–water–ammonia (17:3:0.1:0.1). Radioactive phenols present in peak II were detected by scraping appropriate sections of the plate into counting solution and counting in a liquid scintillation spectrometer.

The third component in the DEAE column effluent was concentrated 50–100× and the sample was hydrolyzed by incubation overnight with β-glucuronidase (0.94 mg/ml) (type B-1 from Bovine Liver, Sigma Chemical Co., St. Louis, Mo.) in sodium acetate buffer (0.06 M, pH 4.5). Glucuronidase-hydrolyzable metabolites were estimated by the radioactivity extractable into 2 vol. of ethyl ether after incubation. The phenols released by β-glucuronidase were identified as phenol and catechol by using the thin-layer chromatographic methods described above. Component IV and chemically synthesized sodium phenylsulfate [14] when chromatographed on cellulose thin-layer plates (Eastman Chromagram Sheet, Eastman Kodak Co., Rochester, N.Y.) were found to have the same R_f values in the following solvent systems: (a) butanol–ammonia–water (10:1:1), R_f 0.61; (b) propanol–ammonia (7:3), R_f 0.79; and (c) butanol–acetic acid–water (4:1:2), R_f 0.70. After the acidification of peak IV to pH 1 with H_2SO_4 and subsequent heating at 90–95° for 15 min, greater than 90 per cent of the radioactive sample was extractable into 2 vol. ethyl ether. The phenols produced by acid hydrolysis were identified as phenol and catechol by using the thin-layer chromatographic methods described above.

For experiments *in vitro* mice were sacrificed by cervical dislocation and their livers perfused with ice-cold physiological saline, gall bladders were removed and the livers were homogenized in 0.1 M potassium phosphate, 0.15 N KCl buffer, pH 6.5. Microsomes were isolated as previously described [15].

Samples containing microsomes (1.0 to 1.5 mg protein/ml), an NADPH-generating system [5 mM $MgCl_2$, 2 mM NADP, 0.022 i.u. IDH, 8 mM isocitric acid (Sigma Chemical Co., St. Louis, Mo.)] and [^{3}H]benzene (0.12 to 1.39 mM) in a total volume of 5 ml were incubated at 25° for 20 min in screw-capped bottles. The reaction was stopped with trichloroacetic acid (5% final concentration). Assays for [^{3}H]benzene metabolism were performed by measuring [^{3}H]phenol formation as previously described [16].

Binding spectra were measured by the method of Schenkman *et al.* [17] and protein concentration was determined by the procedure of Lowry *et al.* [18].

Respiratory elimination of [^{3}H]benzene was studied using an enclosed metabolism chamber which permitted the collection of respiratory gases (Nuclear Associates, Inc., Westbury, N.Y.). Air continuously drawn through the chamber was bubbled through two towers each containing 100 ml toluene. The contents of the two towers were periodically sampled and counted and the results were described as the per cent of administered dose in the expired air. The first tower contained 95 per cent of the recovered benzene and the second, 5 per cent. The recovery of the exhaled [^{3}H]benzene was assumed to be complete, since a third tower placed in the series collected no detectable radioactivity.

[^{3}H]benzene and [^{3}H]benzene metabolites were determined in the liver, spleen, epididymal fat pads, blood and bone marrow of mice. The tissues were weighed, added immediately to 20 ml toluene, homogenized in a Waring blender and centrifuged to separate the toluene and tissue water layers. A 10-ml aliquot of the toluene supernatant (containing [^{3}H]benzene) was added to 0.4 ml Permafluor II (Packard Instrument Co., Downers Grove, Ill.) and counted in a liquid scintillation spectrometer.

The femoral bone marrow from each mouse was removed by inserting the needle of a 1-ml syringe into one end of the bone and forcing water through the bone cavity. This procedure was repeated twice, the combined washings were extracted with 20 ml toluene, and the amount of [^{3}H]benzene present was determined by scintillation counting. Since wet weights of the individual bone marrow samples could not be directly obtained because of the manipulations required to extract the radioactivity, an average dry weight for mouse femoral bone marrow was determined by collecting individual bone marrow washings from the femurs of nineteen mice and evaporating them to dryness. The mean dry weight was 6.64 ± 1.01 mg/mouse and the wet weight was calculated to be 22.1 mg/mouse for two femurs assuming a water content of 70 per cent [19].

Free phenols were separated from unchanged benzene in toluene extracts of tissue homogenates by back extraction with base whereas conjugated phenols were determined in the supernatants of the tissue homogenates after toluene extraction. In each case radioactivity was measured using a counting fluid appropriate for aqueous samples [20]. Toluene-extracted whole blood was counted in a similar fashion to determine water-soluble metabolites of benzene except that it was necessary to add an internal standard to correct for color quenching. Using the methods detailed above, the metabolites of benzene not extracted into toluene were found to be glucuronide and ethereal sulfate conjugates of [^{3}H]phenol (95 per cent) and [^{3}H]catechol (5 per cent).

^{59}Fe utilization was evaluated as previously described [11, 12]. Mice were given ^{59}Fe (0.5 μCi, 20–40 ng iron) in the form of ferrous citrate (New England Nuclear) intraperitoneally 48 hr after benzene administration and bled 24 hr later. Blood (0.2 ml) was counted in a scintillation well counter and the percentage of ^{59}Fe taken up by the erythrocytes was calculated assuming a blood volume of 6 per cent body weight.

Tests of significance were performed by Student's *t*-test.

Table 1. Effects of toluene on benzene metabolism and on red cell ^{59}Fe uptake in the mouse

Treatment	[^{3}H]benzene metabolism*		^{59}Fe utilization
	% Administered dose (mean ± S. D.)	Benzene equivalents† (μmoles)	% ^{59}Fe utilization (mean ± S. D.)
Benzene (880 mg/kg)	22.6 ± 5.7 (17)‡	72.6	4.9 ± 3.4§ (19)
Benzene + toluene	9.9 ± 3.9 (17)	33.2	9.9 ± 4.5‖ (17)
Toluene (1720 mg/kg)			15.8 ± 5.5 (19)
Control			18.4 ± 6.2 (22)
Benzene (440 mg/kg)	35.8 ± 1.1 (2)¶	60.1	15.7 ± 4.8§ (21)
Benzene + toluene	10.9 ± 7.5 (2)¶	18.3	22.0 ± 5.9‖ (28)
Toluene (1720 mg/kg)			23.3 ± 4.6 (25)
Control			24.2 ± 4.7 (27)

* Benzene metabolism and ^{59}Fe utilization expressed as mean value/mouse/day.
† Calculated as μmoles equivalents of [^{3}H]benzene metabolism by a 30 g mouse.
‡ Numbers in parentheses, except where specifically noted, indicate the number of animals.
§ Significantly different from both control and toluene groups, $P < 0.05$.
‖ Significantly different from group receiving benzene alone, $P < 0.05$.
¶ Two groups of animals, three animals/group.

RESULTS

Effects of toluene on total benzene metabolism in vivo and on benzene-induced depression of ^{59}Fe uptake into red cells. When [^{3}H]benzene was administered to mice at two dose levels, 440 and 880 mg/kg, the quantity of benzene metabolites (benzene equivalents) that appeared in the urine was greater after the higher dose than after the lower dose despite the fact that the recovery after the higher dose represents a smaller percentage of the administered dose (Table 1). Co-administration of toluene (1720 mg/kg) with either dose of benzene resulted in a reduction in the quantity of benzene metabolites in the urine to 30 and 46 per cent of the control values for the respective low and high doses. In both cases, the recovery expressed as the per cent of administered dose was reduced by toluene to about 10 per cent. In view of the effects of benzene on ^{59}Fe utilization described below, the quantity of benzene metabolites in urine appears to be a more useful measure for evaluating the relationship between benzene metabolism and toxicity.

It has been shown that benzene inhibits the incorporation of ^{59}Fe into developing erythrocytes in a dose-dependent manner and that such inhibition becomes maximal 48 hr after the administration of a single dose of benzene [12]. Table 1 shows that as toluene decreased the excretion of [^{3}H]benzene metabolites it also counteracted benzene-induced reduction of red cell ^{59}Fe uptake. While toluene itself did not affect radio-iron uptake, it completely prevented the depression in ^{59}Fe uptake produced by 440 mg/kg of benzene and partially offset the greater depression induced by 880 mg/kg of benzene. Thus, toluene inhibited benzene metabolism and protected against benzene-induced depression of ^{59}Fe utilization by red cells.

[^{3}H]benzene metabolism in the mouse. The following experiments were conducted to determine whether the protection afforded by toluene could be related to toluene-induced changes in the metabolic disposition of benzene. Since benzene metabolism has not previously been studied in the mouse, baseline data were collected on the rate of benzene metabolism in mice *in vivo* as a function of the dose of benzene. Mice were given olive oil solutions of [^{3}H]benzene (0.5 to 10 ml/kg), and the urinary excretion of radioactivity was monitored for 24 hr after injection. Little [^{3}H]benzene (< 0.001 per cent of the administered dose) was found in the urine upon extraction with toluene. For each dose, greater than 90 per cent of the urinary radioactivity was identified as free [^{3}H]phenol and glucuronide and ethereal sulfate conjugates of [^{3}H]phenol and [^{3}H]catechol. The data are expressed as total metabolites in Fig. 1. The double-reciprocal plot is linear and the y-intercept suggests that the maximum rate of [^{3}H]benzene metabolism in the mouse is about 2.0 m-moles/24 hr. The elimin-

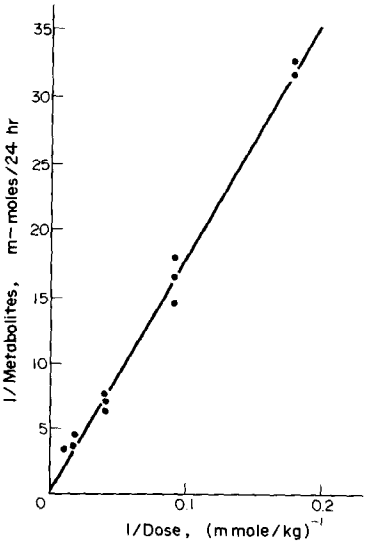

Fig. 1. Urinary excretion of [^{3}H]benzene metabolites as a function of dose. Each value was obtained from the analysis of pooled urine from six or more animals. Least squares analysis of the data yielded a linear regression coefficient of $r = 0.995$.

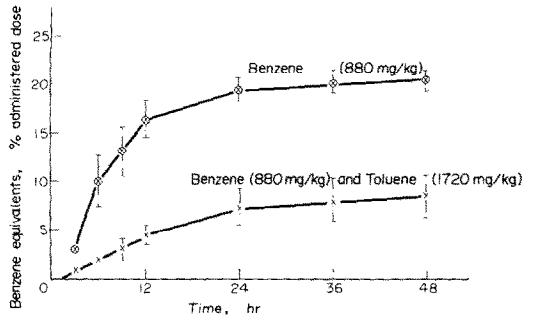

Fig. 2. Effects of toluene on the excretion of [^{3}H]benzene metabolites in the mouse. Symbols represent urinary radioactivity expressed as the per cent of administered dose after [^{3}H]benzene (880 mg/kg) (⊗) and [^{3}H]benzene (880 mg/kg) with toluene (1720 mg/kg) (×). Each point represents the mean of data obtained from fifteen or more animals.

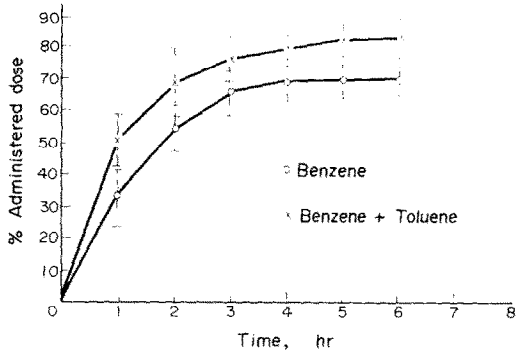

Fig. 3. Effects of toluene on the respiratory elimination of unchanged [^{3}H]benzene in the mouse. Symbols represent respiratory excretion of radioactivity expressed as the per cent of administered dose at the indicated time intervals after injection of [^{3}H]benzene (880 mg/kg) (○), or [^{3}H]benzene (880 mg/kg) with toluene (1720 mg/kg) (×). Each point represents the mean of data obtained from six or more animals.

ation of benzene metabolites was mediated almost entirely by the kidneys, since less than 0.1 per cent of the administered dose was excreted in the faeces as metabolites of benzene. Additionally, no [^{3}H]phenol was detected in the respired air from mice given [^{3}H]benzene.

The time course of urinary excretion of labeled metabolites after a single dose of [^{3}H]benzene (upper curve) or [^{3}H]benzene with toluene (lower curve) is shown in Fig. 2. Toluene co-administration reduced the excretion of labeled metabolites (7.7 vs 18.6 per cent of the administered dose) without altering the time required to complete metabolite elimination. Although no additional excretion was observed beyond the 24-hr period, most of the metabolites were recovered in the urine within the first 12 hr.

When urine samples from both [^{3}H]benzene- and [^{3}H]benzene plus toluene-treated animals were analyzed for benzene metabolites, it was found that toluene altered neither the proportion of [^{3}H]benzene metabolized to phenol (90 per cent) or catechol (10 per cent) nor the proportion of these phenolic metabolites conjugated with glucuronic acid and sulfuric acid. Toluene did cause the excretion of smaller amounts of free [^{3}H]phenol and glucuronide and ethereal sulfate conjugates of [^{3}H]phenol and [^{3}H]-catechol.

Respiratory elimination of [^{3}H]benzene. Figure 3 demonstrates the time course of respiratory elimination of unchanged [^{3}H]benzene in mice given [^{3}H]benzene (880 mg/kg) (lower curve) or [^{3}H]benzene (880 mg/kg) with toluene (1720 mg/kg) (upper curve). Table 2, which summarizes the effects of toluene on the elimination of [^{3}H]benzene, shows that, while toluene markedly reduced the urinary metabolites of benzene, it produced a compensatory increase in the pulmonary excretion of unchanged benzene, so that total recovery of administered benzene was 93 per cent for both groups of animals. Thus, the amount of unchanged benzene excreted through the lungs increased from 71.5 per cent in the absence of toluene to 85.0 per cent when toluene was given with the benzene. In contrast to the urinary excretion of radioactivity which required 24 hr for its completion (see Fig. 1), respiratory excretion of benzene was complete within 4–6 hr (see Fig. 3). These results strongly suggest that toluene probably increased the respiratory excretion of unchanged benzene by blocking its metabolism.

Effects of toluene on [^{3}H]benzene metabolism in vitro. We have previously demonstrated that benzene reacts with hepatic microsomal cytochrome P-450 to yield a type I spectral change [16]. The fact that toluene also yielded a type I binding spectrum with a maximum at 388 nm and a minimum at 418 nm suggested the possibility that toluene might compete with benzene as a substrate for the microsomal mixed-function oxidase system. The inhibition of [^{3}H]benzene metabolism by toluene is shown in Fig.

Table 2. Effects of toluene on respiratory and urinary elimination of [^{3}H]benzene and its metabolites in the mouse*

	% Administered dose recovered in 24 hr	
	[^{3}H]benzene (880 mg/kg) (mean ± S. D.)	[^{3}H]benzene (880 mg/kg) + toluene (1720 mg/kg) (mean ± S. D.)
Exhaled [^{3}H]benzene	71.5 ± 6.6	85.0 ± 5.5†
Urinary [^{3}H]benzene equivalents	20.1 ± 4.5	7.9 ± 2.7†
Total recovery	92.5 ± 5.2	92.8 ± 4.5

* This table summarizes urinary excretion data presented in Fig. 2 and pulmonary excretion data presented in Fig. 3; N = 6.

† Significantly different from group receiving only benzene, $P < 0.01$.

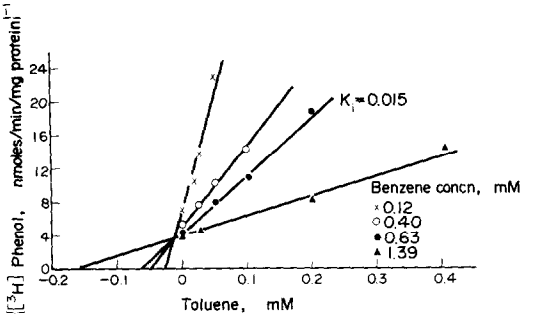

Fig. 4. Effects of toluene on [^{3}H]benzene metabolism in mouse liver microsomes. Symbols represent data obtained when different concentrations of [^{3}H]benzene (×, 0.12 mM; ○, 0.40 mM; ●, 0.63 mM; and ▲, 1.39 mM) were incubated with varying concentrations of toluene and mouse liver microsomes from untreated animals. Details of microsome preparation, incubation and assay procedures are described or referenced in Materials and Methods. Data represent the mean of two experiments.

4 as a Dixon plot [21]. The intersection of the lines in a single point (above the abscissa to the left of the ordinate) indicates that toluene is a competitive inhibitor of benzene metabolism *in vitro*. From the point of intersection of the lines it was determined that the inhibitor constant for toluene, K_i, is 0.015 mM. In these studies, the K_m for benzene was 0.18 mM and the V_{max} was 0.33 nmole phenol formed/minute/mg of microsomal protein.

Effects of toluene on the distribution of [^{3}H]benzene and its metabolites. Although the data presented above are consistent with the postulate that toluene counteracted the effect of benzene on radio-iron uptake by erythrocytes because it inhibited the formation of a toxic metabolite, an alternative explanation might be that toluene acted by changing the distribution of benzene in the mouse. Therefore, the following studies were performed to determine the levels of benzene and its metabolites in the organs of the mouse given benzene alone or with toluene.

Concentrations of [^{3}H]benzene in various tissues of mice given [^{3}H]benzene (880 mg/kg) with or without toluene (1720 mg/kg) are shown in Fig. 5. Values are reported as mean nmoles [^{3}H]benzene per g of tissue wet wt or per ml of blood. The absorption of a subcutaneous dose of [^{3}H]benzene alone is rapid, since peak tissue levels were attained between 15 min and 2 hr after injection. The peak concentration of [^{3}H]benzene in liver was nearly the same as that for blood (800 nmoles/ml), whereas peak levels in spleen were only half this amount. On a wet weight basis a greater amount of benzene accumulated in fat (8000 nmoles/g) and bone marrow (2000 nmoles/g). Co-administration of toluene slightly delayed the accumulation of [^{3}H]benzene in all tissues, but in no case were the peak levels attained significantly less than those determined for mice given [^{3}H]benzene alone. Additionally, the total amount of [^{3}H]benzene in the respective tissue over the entire 8-hr period as measured by the area underneath the curves is

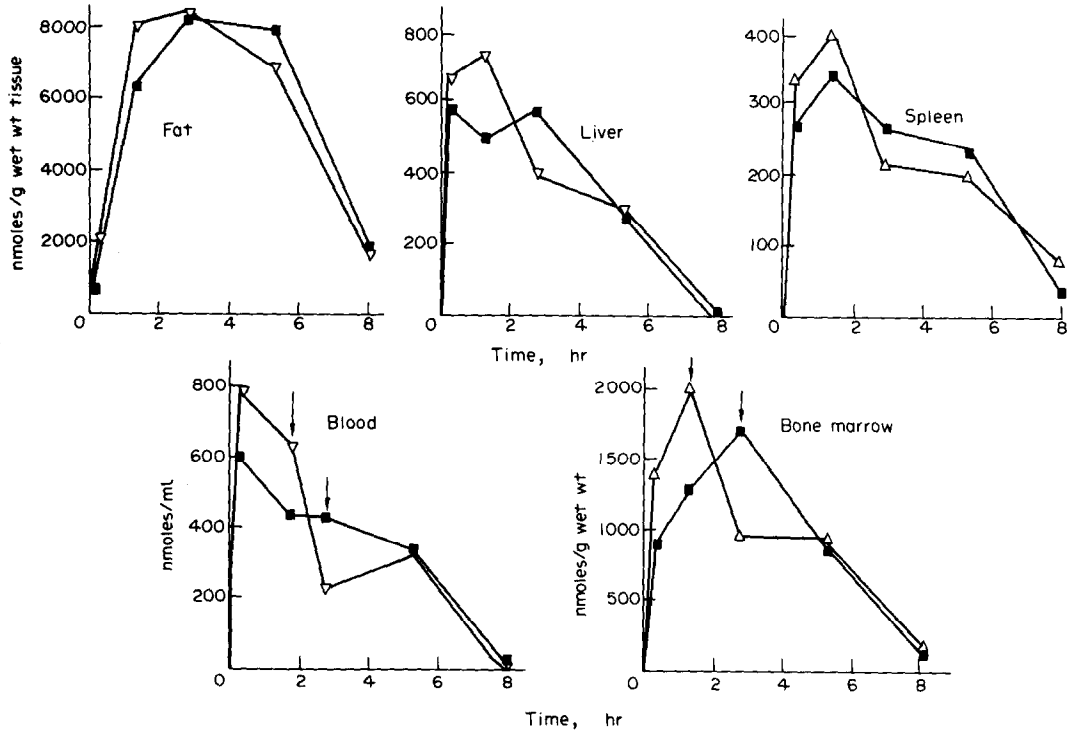

Fig. 5. Effect of toluene on the accumulation of [^{3}H]benzene in mouse tissues. Mice (N = 6) were injected s.c. with [^{3}H]benzene (880 mg/kg) (▽), or with [^{3}H]benzene plus toluene (1720 mg/kg) (■); and concentrations of [^{3}H]benzene in tissues at various time intervals were determined. The experimental procedure is described in Materials and Methods. The data are expressed as mean nmoles [^{3}H]benzene per g wet weight tissue or per ml blood. Standard deviations ranged from 15 to 40 per cent of mean values. Significantly different points are indicated by arrows, $P < 0.05$.

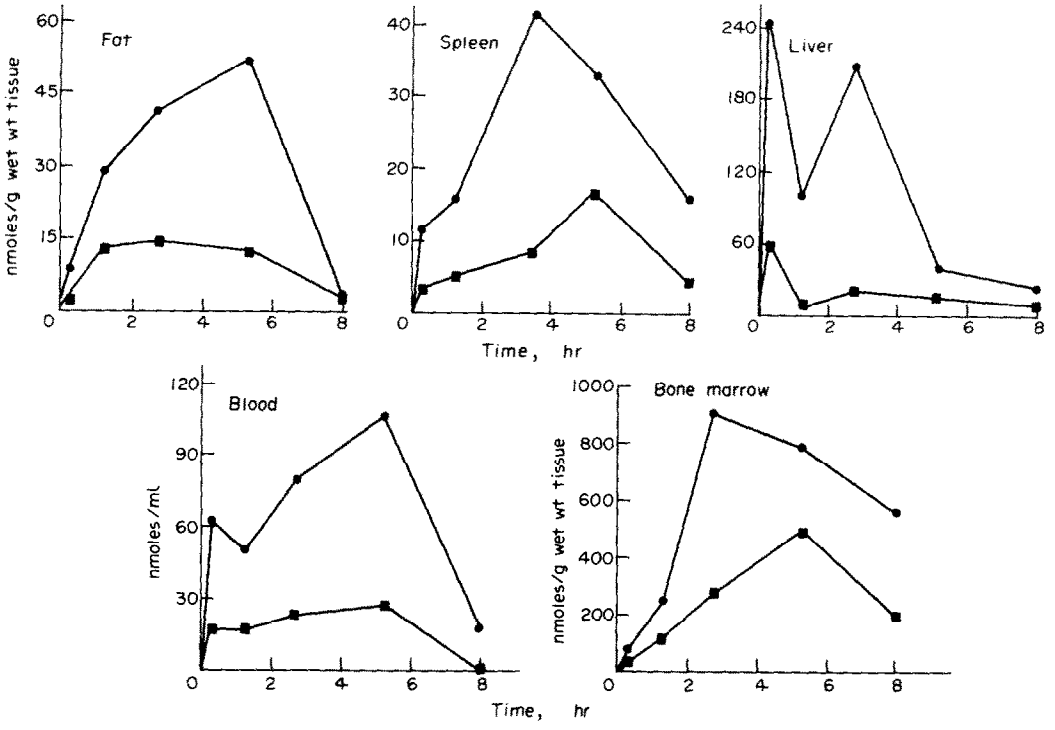

Fig. 6. Effect of toluene on the accumulation of [^{3}H]benzene metabolites in mouse tissues. Mice (N = 6) were injected s.c. with [^{3}H]benzene (880 mg/kg) (●), or with [^{3}H]benzene plus toluene (1720 mg/kg) (■), and concentrations of [^{3}H]benzene metabolites in tissues at various time intervals were determined. The experimental procedure is described in Materials and Methods. The data are expressed as mean nmoles [^{3}H]benzene metabolites per g wet weight tissue or per ml blood. Standard deviations ranged from 15 to 40 per cent of mean values. Significantly fewer ($P < 0.05$) metabolites were present at each time period in each tissue when toluene was given with [^{3}H]benzene.

nearly the same for both benzene and benzene with toluene treatments. Thus, co-administration of toluene did not alter the total amount of [^{3}H]benzene appearing in fat, liver, spleen, blood and bone marrow of mice for the 8-hr period after injection.

Figure 6 depicts the concentration of [^{3}H]benzene metabolites in tissues of mice given [^{3}H]benzene (880 mg/kg) with or without toluene (1720 mg/kg). Benzene metabolites were detected in all tissues 15 min after injection, and with the exception of the liver, peak levels of [^{3}H]benzene metabolites were found between 3 hr and 5 hr after injection. Co-administration of toluene did not delay the appearance of benzene metabolites in tissues but did markedly reduce the concentration of metabolites found in each tissue for all time intervals. If the tissue exposure to benzene metabolites over the 8-hr period monitored is reflected in the area under the curves shown in Fig. 6, it is clear that bone marrow exposure exceeds that of all other tissues. It is of particular interest that amounts of bone marrow metabolites were nine times that found in blood and six times that found in liver, the organ best known to metabolize benzene.

When metabolite fractions from liver and bone marrow were analyzed, it was found that they consisted predominantly of glucuronide and ethereal sulfate conjugates of [^{3}H]phenol. Approximately 10 per

Table 3. Administration and distribution of [^{3}H]benzene metabolites in blood and bone marrow*

Metabolites	Blood concn† (nmoles/ml; mean ± S. D.)		Marrow concn (nmoles/g; mean ± S. D.)	
	T = 15 min	T = 30 min	T = 15 min	T = 30 min
[^{3}H]phenol	66.9 ± 15.9	30.2 ± 11.2	19.4 ± 8.4	12.0 ± 3.6
[^{3}H]phenyl-glucuronide‡	60.6 ± 16.4	57.0 ± 25.2	14.0 ± 4.6	17.8 ± 2.9
[^{3}H]phenyl-sulfate§	50.1 ± 12.2	84.2 ± 13.8	8.1 ± 2.9	13.9 ± 7.5

* Mice (N = 5) were injected i.p. with [^{3}H]phenol (12.5 mg/kg), [^{3}H]phenylglucuronide (15.3 mg/kg) or [^{3}H]phenylsulfate (9.9 mg/kg). Concentrations of benzene metabolites in blood and bone marrow were not detectable 3 hr after injection.

† Blood concentrations were greater than respective marrow concentrations, $P < 0.05$.

‡ Primarily [^{3}H]phenylglucuronide (< 5% [^{3}H]catechol glucuronide).

§ Primarily [^{3}H]phenylsulfate (< 5% [^{3}H]catechol sulfate).

cent of the radioactivity found in these tissues consisted of free [^{3}H]phenol and glucuronide and ethereal sulfate conjugates of [^{3}H]catechol.

In order to determine if the high concentration of benzene metabolites in bone marrow was due to uptake of metabolites from blood, [^{3}H]phenol and glucuronide and ethereal sulfate conjugates of [^{3}H]phenol were isolated from the urine of mice given [^{3}H]benzene, injected i.p., and blood and marrow levels of radioactivity were determined. Table 3 shows the blood and bone marrow concentrations (nmoles/ml or nmoles/g) of benzene metabolites 15 and 30 min after the administration of the indicated doses. For each metabolite at each time period the blood concentration exceeded the respective marrow concentration. Thus, under these conditions, benzene metabolites were not taken up from blood and concentrated in bone marrow.

DISCUSSION

The observations made in this study, that toluene reduced the level of urinary metabolites of benzene and also reduced the benzene-induced inhibition of erythrocyte ^{59}Fe uptake, suggest that the metabolism of benzene is closely related to its toxicity. More direct support comes from the observation that toluene markedly reduced bone marrow concentrations of benzene metabolites but had no significant effect on those of benzene. Thus, it seems likely that toluene protects against the benzene-induced inhibition of erythrocyte ^{59}Fe uptake by reducing the level of benzene metabolites in the bone marrow. We identified labeled phenol and its ethereal sulfate and glucuronide conjugates as well as the analogous conjugates of catechol as benzene metabolites in bone marrow. Although direct evidence that a metabolite of benzene may produce the observed toxicity is lacking, previous studies have shown that catechol [22] and hydroquinone [23] depress bone marrow activity. Benzene oxide, an intermediate through which benzene metabolism is thought to proceed [24], may also play a role in the production of toxicity.

In the 8-hr period after the administration of [^{3}H]benzene to mice, femoral bone marrow accumulated four times more benzene metabolites than the liver. Equally remarkable was the fact that these metabolites were still present in bone marrow at greater than 60 per cent of their peak levels 8 hr after benzene administration, a time at which metabolite levels in fat, spleen, liver and blood had been reduced to very low or undetectable levels. It seems possible that the unusual persistence of such high levels of metabolites in bone marrow is related to the characteristic toxicity benzene exerts on hematopoietic functions. Whether the liver or the bone marrow is the source of these metabolites cannot be clearly determined from these studies; however, the results of these experiments showed that total metabolite concentrations in marrow exceeded those in blood while no evidence for facilitated or active transport of either ^{3}H-labeled phenol, phenylglucuronide, phenylsulfate or the catechol analogs was found.

The state of the bone marrow can be evaluated using a strictly functional approach such as ^{59}Fe utilization, a morphological approach such as histological examination of marrow aspirates, or by counting circulating cell levels. The incorporation of ^{59}Fe into circulating erythrocytes, the method used in the present study to measure benzene toxicity, is an adaptation of a method widely used in clinical medicine to evaluate bone marrow function [6–10] and should be placed in perspective with other methods used for this purpose. Isotope incorporation into the developing blood precursor cells is clearly the most sensitive means of assaying bone marrow function because such methods can detect even short-term discontinuities in the continuous stream of developing blood cells. Discontinuities produced by single doses of bone marrow poisons (unless these are relatively massive doses) are usually too short-lived to produce any significant decreases in the total circulating pool of either platelets, leukocytes or red blood cells. Direct histological examination of the bone marrow, on the other hand, is the most difficult to assess in a quantitative manner. In the present experiments, toluene reduced the bone marrow levels of benzene metabolites and decreased the benzene-induced inhibition of erythrocyte ^{59}Fe uptake. It remains to be determined whether toluene can also reduce the leukopenia and the histological changes in bone marrow induced by more prolonged administration of benzene.

Previous studies aimed at defining the relationship between benzene metabolism and its toxicity have generally focused on the liver as the presumed source of the toxic metabolite(s). Thus, protection against benzene-induced leukopenia has been associated both with stimulation of hepatic benzene metabolism by phenobarbital [2] and with its inhibition by aminotriazole [3, 4]. These apparently contradictory results might be reconciled if the levels of benzene metabolites in the bone marrow were known. The present study emphasizes the importance of examining the bone marrow itself for the metabolites of benzene if we assume that the metabolism of benzene determines its toxicity.

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