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Role of Oxygen Radicals in Induction of DNA Damage by Metabolites of Benzene

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ABSTRACT

Benzene is strongly suspected of being an animal and human carcinogen, but the mechanisms by which benzene induces tumors of lymphoid and hematopoietic organs are unknown. Binding studies *in* rho suggest a very low level of covalent binding to the DNA of bone marrow elements. Since several metabolites of benzene have the potential to undergo autooxidation and thereby generate reactive oxygen intermediates, we have tested the hypothesis that benzene metabolites can induce DNA damage through the generation of oxygen radicals. Hydroquinone (HQ), benzoquinone (BQ), catechol, and 1,2,4-benzenetriol (BT) were first tested for their ability to generate $O_2^{\cdot-}$ at a physiological pH. BT, and to a lesser extent HQ, were autooxidized and produced significant quantities of $O_2^{\cdot-}$. No detectable $O_2^{\cdot-}$ was produced by catechol or BQ. Similarly, BT was very efficient at degrading DNA, and this degradation was inhibited by scavengers of $O_2^{\cdot-}$, H_2O_2 and $\cdot OH$. HQ did not degrade DNA but did induce single- and double-strand breaks. In contrast to the action of BT, the breakage of DNA by HQ was not inhibited by scavengers of reactive oxygen intermediates. The metabolites which did not produce $O_2^{\cdot-}$ (catechol and BQ) did not induce significant breakage of DNA. Taken together, the data support the hypothesis that certain benzene metabolites can induce DNA damage through the production of oxygen radicals; they further suggest that other metabolites may act, via another mechanism, to damage DNA.

INTRODUCTION

Benzene is a major organic product, solvent, and fuel additive; and, accordingly, humans are widely exposed to benzene (1). Exposure of humans and animals to benzene can result in severe depression of the bone marrow; aplastic anemia, leukopenia, leukemia, and lymphoma have also been observed (1, 2). Benzene has been classified as a human carcinogen because of reported increases in the incidence of leukemia in workers exposed to high concentrations (3). Studies in animals have shown that benzene is metabolized in the liver to phenol, polyphenol, and quinone metabolites such as CAT,[2] HQ, BQ, and BT (4). Such metabolites of benzene concentrate in the marrow (5) and can induce many of the toxic effects of benzene (6, 7). Thus, these metabolites are thought to be the principal toxic species of benzene (5–7).

The precise mechanism(s) by which benzene and its metabolites cause cancer are not known. Little if any benzene or its metabolites actually binds to DNA of marrow cells and, indeed, the vast majority of binding is to sulfhydryl compounds, especially cytoskeletal components (8, 9). Since the metabolites HQ and BT can autooxidize and could potentially generate $O_2^{\cdot-}$, these metabolites of benzene might be genotoxic by generating reactive oxygen intermediates or semiquinone radical intermediates (Fig. 1). (10). Several studies support this possibility. Khan *et al.* (11) demonstrated that exposure of mice to benzene induced lipid peroxidation and epinephrine autooxidation, suggesting damage by oxygen radicals, possibly $O_2^{\cdot-}$. The benzene metabolites BQ and BT have been shown to induce single-strand breaks in DNA of cultured cells, although the mechanism by which this occurs remains unexplained (12). In addition, the bladder carcinogen *N*-hydroxy-2-naphthylamine has been shown to induce oxidative DNA damage *in vitro* and *in vivo* (13).

We thus addressed directly the hypothesis that benzene metabolites have the capacity to induce DNA damage through the production of oxygen radicals. We here report that metabolites of benzene can generate oxygen radicals at a physiological pH, that these metabolites can induce strand breaks in DNA, and that reactive oxygen intermediates are responsible, at least in part, for these breaks.

MATERIALS AND METHODS

Chemicals. HQ, BQ, CAT, and benzoate were purchased from Sigma. BT was purchased from Aldrich. SOD was purchased from Calbiochem (3016 units/mg), and catalase was purchased from Worthington (10,563 units/mg). All compounds were dissolved in distilled, chelex (Bio-Rad)-treated, H_2O immediately before use. The replicative, supercoiled form of ϕX-174 DNA was obtained from New England Biolabs. $FeCl_3 \cdot 6H_2O$ was obtained from Fisher.

Quantification of $O_2^{\cdot-}$. $O_2^{\cdot-}$ was quantified by measuring the superoxide dismutase-inhibitable reduction of cytochrome *c* as previously described (14, 15). Specificity of this assay for measuring $O_2^{\cdot-}$ is determined by calculating only that reduction of cytochrome *c* which is inhibitable with SOD. The specificity of SOD for $O_2^{\cdot-}$ has been well established (16).

Assay for DNA Strand Breaks. DNA strand breaks and degradation were measured by the conversion of supercoiled ϕX-174 to the open, relaxed, or linear forms as previously described (17, 18). Briefly, 0.1 μg of ϕX-174 DNA was incubated with and without the indicated concentrations of benzene metabolites and with and without the indicated amounts of scavengers for 60 min. When SOD or catalase was used, an equal amount of bovine albumin was also run as a control for nonspecific competition of protein for radicals. Protein levels above 1 μg began to compete nonspecifically with the DNA (data not shown). All exposures were in distilled water which had been further passed over a 2- x 30-cm column of Chelex 100 (Bio-Rad) and brought back to neutral pH by addition of 1 mM Tris-HCl buffer (pH 7.0). No iron was included because iron induced breaks in the absence of benzene metabolites (data not shown). Following incubation the supercoiled, open circular, and linear forms were separated by electrophoresis through 1% agarose gels as described previously (18). The DNA bands were visualized by staining for 3 min with ethidium bromide (25 μg/ml in distilled H_2O), washing in distilled water for 10 min, and irradiation with UV light at 254 nm. The gels were photographed, and the negatives were scanned with an LKB Ultrascan XL densitometer. The percentage of DNA in each form was calculated by integrating the peaks. ϕX-174 DNA cut with the restriction enzyme *Pst*I at its single recognition site served as a standard for full length linear ϕX-174 DNA.

RESULTS

Generation of $O_2^{\cdot-}$. We first determined the capacity of various benzene metabolites to produce $O_2^{\cdot-}$ at pH 7.4. BT was much more efficient at reducing cytochrome *c* than HQ (Fig. 2). CAT (Fig. 2) and BQ (data not shown) caused no reduction of cytochrome c. The addition of ferric iron enhanced the rate of cytochrome *c* reduction by both BT and HQ (Fig. 2). The rate of $O_2^{\cdot-}$ formation by BT, at a concentration of 1 mM,

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[2] The abbreviations used are: CAT, catechol; HQ, hydroquinone; BQ, benzoquinone; BT, 1,2,4 benzenetriol; SOD, superoxide dismutase; ED_{50}, concentration causing 50% loss of supercoiled DNA.

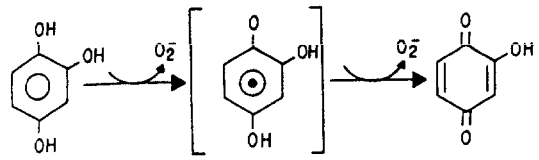

Fig. 1. Autooxidation of 1,2,4-benzenetriol to the 2-hydroxybenzoquinone by passing through a semiquinone radical intermediate. Two molecules of O_2^- are generated for each molecule of 1,2,4-benzenetriol oxidized.

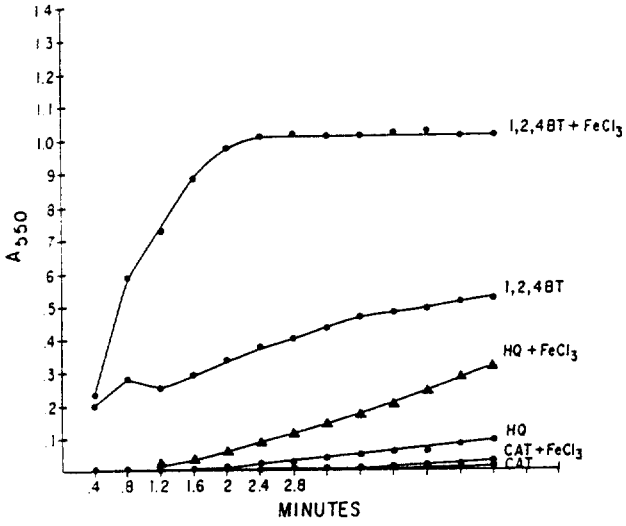

Fig. 2. Reduction of cytochrome *c* by benzene metabolites. Cytochrome *c* was incubated with 1 mM concentrations of benzene metabolites, and the reduction was measured at 550 nM as previously described (14, 15). *Points*, mean of triplicate samples in which the SEM < 10%.

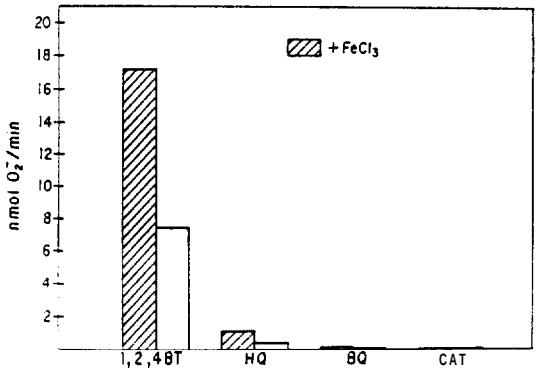

Fig. 3. The rate of O_2^- generated by 1 mM solutions of benzene metabolites. O_2^- production was measured by the SOD-inhibitable reduction of cytochrome *c* as previously described (14,15). Measurements were taken from the linear portion of the curves shown in Fig. 2. *Columns*, mean of triplicate samples in which the SEM < 10%. □, metabolite alone; ▩, metabolite in combination with $FeCl_3$.

7.5 nmol/min in the absence of iron and 17.4 nmol/min in the presence of 10 μM $FeCl_3$ (Fig. 3); much less O_2^- was generated by HQ, even in the presence of iron (Fig. 3). In sum, BT at a concentration of 1 mM can generate sufficient O_2^- to achieve a concentration of O_2^- of over 50 μM in 5 min (1 nmol in 1 ml = 1 μM solution).

Induction of DNA Strand Breaks. We next determined the capacity of the benzene metabolites to damage DNA, as measured by the induction of strand breaks. φX-174 DNA was exposed to 1 mM solutions of BT, HQ, CAT, or BQ. BT caused complete degradation of the DNA (Fig. 4). HQ did not cause the extensive degradation induced by BT but did cause single- and double-strand breaks resulting in the complete breakdown of supercoiled DNA to the open circular and linear form (Fig. 4). BQ and CAT caused very little breakage (Fig. 4).

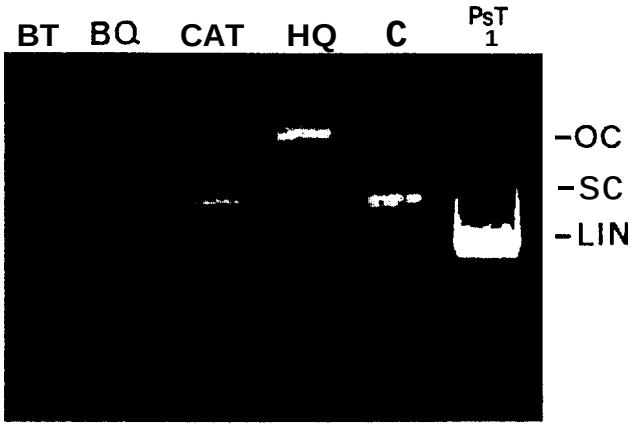

Fig. 4. Breakage and degradation of 9X-174 supercoiled DNA by benzene metabolites. Type I 9X-174 DNA (0.1 μg) was incubated with 1 mM solutions of benzene metabolites for 60 min at 25°C and pH 7.4. The supercoiled (*SC*), open circular (*OC*), and linear (*LIN*) forms were then separated on a 1% agarose gel, stained with ethidium bromide, and visualized with ultraviolet light as previously described (17, 18). *Lane PstI* is control φX-174 DNA digested with the restriction endonuclease *PstI* to yield the linear form.

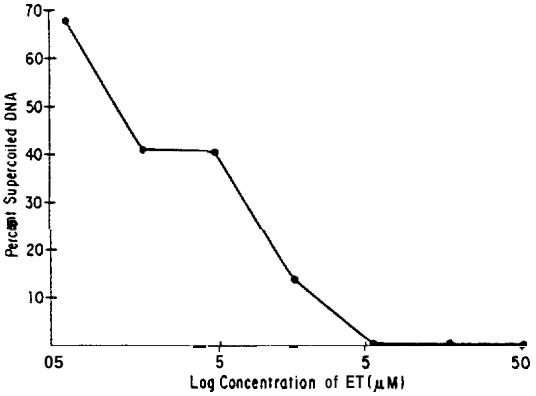

Fig. 5. Effect of concentrations on the breakage of φX-174 DNA by BT. Type I φX-174 DNA (0.1 μg) was incubated with the indicated concentration of BT for 60 min at 25°C and pH 7.4. The percentage of DNA remaining in the supercoiled form was determined as described in "Materials and Methods."

Effect of Dose of BT and HQ on Induction of DNA Strand Breaks. When BT and HQ were tested at multiple concentrations, strand breakage was induced in a dose-dependent manner (Figs. 5 and 6). BT caused complete loss of supercoiled DNA at concentrations as low as 5.5×10^{-6} M. The ED_{50} was 7×10^{-7} M (Fig. 5). HQ was less potent and required higher doses (*i.e.*, 10^{-3} M to 1.1×10^{-4} M) for complete loss of supercoiled DNA (Fig. 6). The ED_{50} for HQ was 10^{-5} M (14-fold higher than BT).

Kinetics of DNA Strand Breaks Induced by HQ and BT. The breakage of DNA by a concentration of 40 μM BQ was very rapid with maximal breakage achieved in less than 2 min (Fig. 7). Breakage by BT at the same concentration was somewhat slower in that 14% of the DNA remained in the supercoiled form at 2 min, and 4% was still in the supercoiled form at 5 min (Fig. 7). After 5 min of exposure to BT, none of the DNA remained in the supercoiled form.

Effects of Scavengers on Induction of DNA Strand Breaks. We next determined what portion of breakage of the DNA could be blocked by inhibition of reactive species of oxygen.

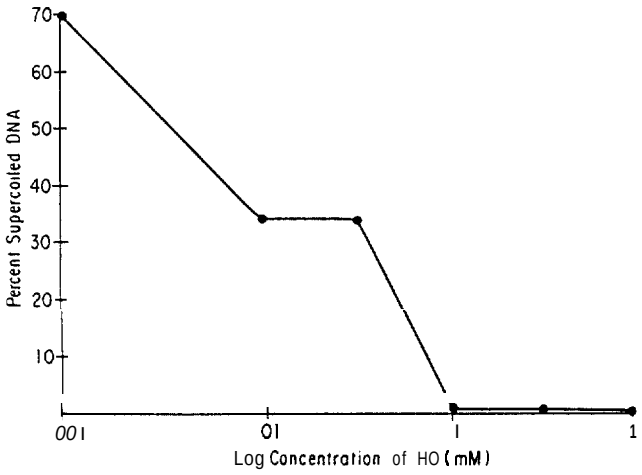

Fig. 6. The effect of concentration of the breakage of OX-174 DNA by HQ. Type 1 OX-174 DNA (0.1 μg) was incubated with the indicated concentration of HQ for 60 min at 25°C and pH 7.4. The percentage of DNA remaining in the supercoiled form was determined in 'Materials and Methods."

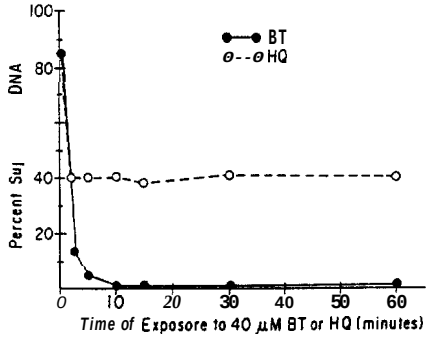

Fig. 7. The effect of time on the breakage of OX-174 DNA by BT and HQ. Type 1 OX-174 DNA (0.1 μg) was incubated with 40 μM concentrations of BT or HQ for the indicated times at 25°C and pH 7.4. The pemntage of DNA in the supercoiled form was determined as described 'Materials and Methods."

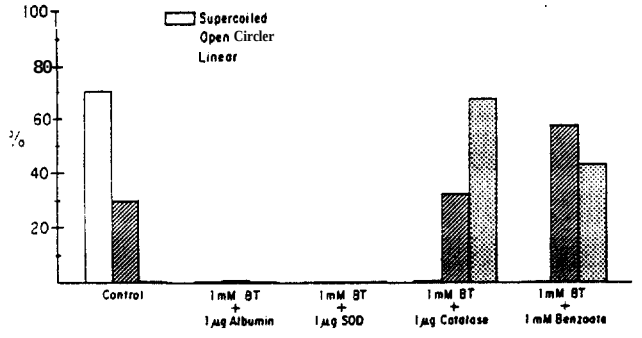

Fig. 8. Effect of scavengers on the degradation of φX1-47 DNA by 1 mM BT. Type 1 OX-174 DNA (0.1 μg) was incubated with 1 mM BT for 30 min at 25°C, pH 7.4, with and without the indicated scavengers. Albumin was added to control for nonspecific competition of protein with the DNA. The amount of DNA in the supercoiled, open circular, and linear forms was determined as described in "Materials and Methods."

We tested the capacity of SOD, catalase, and benzoate, which scavenge O_2^-, H_2O_2, and ·OH, respectively, to inhibit the breakage of DNA by BT and HQ. Benzoate and catalase were potent inhibitors of the breakage of DNA by BT and offered effective protection against the complete degradation of DNA induced by 1 mM concentrations of BT (Fig. 8). SOD did not protect against 1 mM concentrations of BT (Fig. 8), but did offer effective protection against 40 μM concentrations of B (Fig. 9). By contrast, none of the inhibitors offered effecti protection from breakage by HQ (Fig. 10). Albumin, used the same concentrations as the radical scavengers, was inacti in protecting the DNA from breakage (Figs. 8 and 9).

DISCUSSION

These data indicate HQ and BT, at a physiological p generate significant amounts of O_2^- (BT produced a concentr tion of >50 μM O_2^- in 5 min (Fig. 3). BT caused release more O_2^- than HQ, while CAT and BQ did not cause generati of O_2^-. BT, at low concentrations, very rapidly induced brea age of φX-174 supercoiled DNA, and this damage was inhibite by scavengers of O_2^-, H_2O_2, and ·OH (Fig. 8). HQ was le efficient at generating O_2^- and, concomitantly, at producin DNA damage. Furthermore the breakage at DNA by HQ wa not inhibited by scavengers of reactive oxygen intermediate Taken together, these observations suggest that BT, a metab lite of benzene, induced DNA damage, mostly through t generation of oxygen radicals. Another metabolite, HQ, al induced DNA breakage which was possibly through a differe mechanism.

That SOD, catalase, and benzoate all inhibited breakage DNA by BT strongly suggests that BT was acting through t metal-catalyzed Haber-Weiss reaction, in which O_2^- both r

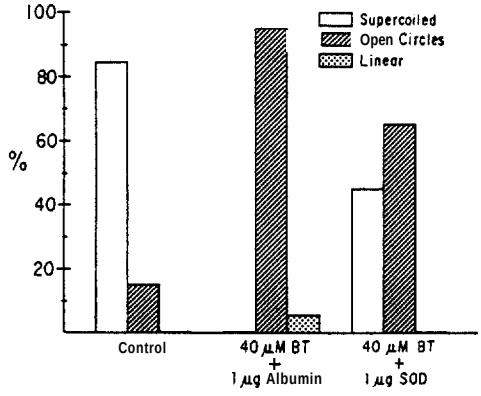

Fig. 9. The effect of SOD on the breakage of φX-174 DNA by 40 μM B Type 1 φX-174 DNA (0.1 μg) was incubated with 40 μM BT for 30 min at 25°C and pH 7.4 with and without 1 μg of SOD. One μg of albumin was included as control for nonspecific competition of protein with the DNA. The amounts o DNA in the supercoiled, open circular. and linear form were determined described in 'Materials and Methods."

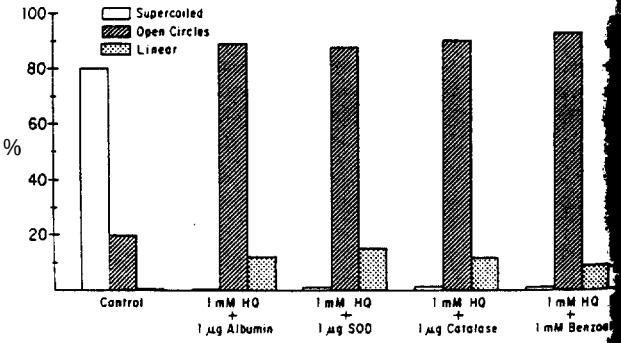

Fig. 10. The effects of scavengers on the breakage of φX-174 DNA by 1 m HQ. Type 1 6X-174 (0.1 μg) was incubated with 1 mM HQ for 30 min at 25 and pH 7.4 with and without the indicted scavengers. Albumin was added as control for n o. d i competition of protein with the DNA. The amount DNA in the supercoiled, open circular, and linear form was determined described in "Materials and Methods."

duces metal ions and dismutates to form H_2O_2 (17). H_2O_2 could then interact with reduced metal ions to form the very reactive hydroxyl radical which breaks DNA without regard to sequence (20, 22). There was not, however, complete inhibition of breakage by these scavengers, especially by SOD. This may be due to the lower turnover rate of SOD for its substrate than catalase. Although the units of activity are not directly comparable, one can determine the turnover rates under ideal conditions. The specific activity of the SOD used was 3016 units/mg. One μg or a total of 3 units was added in the assays. One unit of SOD as calculated under the standard conditions as described by McCord and Fridovitch (15) can scavenge approximately 0.6 nM of O_2^-/min. One unit of catalase is defined as that amount of enzyme which can convert 1 μmol of H_2O_2 to H_2O/min. It was not possible to add more SOD to the higher concentration, because larger amounts of proteins began inhibiting nonspecifically by competing with DNA rather than by their specific scavenger activities. This is evidenced by the fact that albumin began protecting the DNA from breakage when more than 1 μg was added (data not shown). The inability of these scavengers to inhibit more completely conversion of the supercoiled DNA to open circles may be a result of the sensitivity of the assay. Only a single-strand break is required to relax the supercoiled form of ϕX-174 DNA to the open circle form (17, 18), but much more extensive damage is required to convert the DNA to the linear form or completely degrade the DNA (17, 18). High concentrations of BT completely degraded the DNA (Fig. 4), whereas lower concentrations caused conversion to open circles (Fig. 5). The scavengers were very efficient at protecting the DNA from conversion to linear form or complete degradation. Alternatively, the semiquinone radical intermediates formed in the autoxidation process may also interact with the DNA and contribute to the breakage. This could be especially true for HQ which was not inhibited by any of the scavengers tested.

BQ and CAT induced little if any DNA breakage in these studies, which is in accordance with their inability to generate detectable O_2^-. Studies by Pellack-Walker and Blumer conducted on leukemia cells have shown that BT and BQ, but not HQ, induced alkali label sites or DNA breakage in these cells, and that BT induced breakage of DNA at a rate consistent with the rate of its autooxidation and the generation of O_2^- (12). By contrast, BQ induced immediate breakage, even at 4°C. Since BQ is already fully oxidized, it could not produce O_2^-. Furthermore, enzymatic or nonenzymatic intracellular reduction of BQ cannot explain the rapid and potent breakage of DNA in leukemia cells by BQ at 4°C. A recent study suggesting that alterations in chromatin structure alone in the absence of DNA damage can cause enhanced unwinding and elution of DNA under certain alkaline conditions offers a possible explanation of these findings (19). The observation, that BQ binds avidly to cytoskeletal elements and disrupts their assembly (9), suggests that BQ may possibly alter chromatin structure and the elution pattern of the DNA in this manner. This, however, does not trivialize the studies by Pellack-Walker and Blumer because altered chromatin structure may itself pose a significant genetic threat to the host.

Previous studies have demonstrated that polyphenol metabolites of benzene concentrate in the marrow of mice exposed to radiolabeled benzene or its metabolites (5). Very little radioactivity, however, appears in the DNA of marrow cells in animals exposed to ^{14}C-labeled benzene (8). In these latter studies, no control was included for the possible incorporation of ^{14}C via the C-1 pool into normal bases. Since the measurements were taken 22 h after exposure, which allows time for the metabolism of benzene, the rate of cell division and DNA synthesis is high in bone marrow cells, and since metabolic incorporation of ^{14}C has been shown to occur in liver in this time (21), most of the counts observed in the marrow may have been localized in normal bases rather than as DNA adducts. This point requires further investigation. Studies by Gill and Ahmed (23) also reported covalent binding of benzene to macromolecules of bone marrow. These studies also had very low counts (<15 dpm/femur or 86 dpm for 20 pooled femurs in combined nucleic acids not purified DNA) and did not control for the possible metabolic incorporation of a few dpm of ^{14}C via the C-1 pool, and the methods used for the separation of macromolecules were not sufficient to provide purities needed for such low amounts of radioactivity. Given the bioconcentration of the metabolites in the marrow and the low covalent binding to DNA *in* vivo, the data presented here offer an alternative genotoxic mechanism for some of the toxic and carcinogenic actions of benzene.

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