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Relationship between the Oxidation Potential of Benzene Metabolites and Their Inhibitory Effect on DNA Synthesis in L5178YS Cells

PEGGY PELLACK-WALKER, J. KEN WALKER, HELEN H. EVANS, AND JEFFREY L. BLUMER

Departments of Pharmacology (P.P.-W., J.L.B.), Pediatrics (J.L.B.), and Radiology (H.H.E.), Case Western Reserve University, and the Division of Pediatric Pharmacology and Critical Care (J.L.B.), Rainbow Babies and Childrens Hospital, Cleveland, Ohio 44106; and Eltech Systems Corporation, Research and Development Center (J.K.W.), Fairport Harbor, Ohio 44077

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SUMMARY

The effects of benzene and its metabolites on the rate of DNA synthesis were measured in the mouse lymphoma cell line, L5178YS. The direct toxicity of benzene could be distinguished from that of its metabolites since bioactivation of benzene in L5178YS cells was not observed. Cells were exposed to benzene, phenol, catechol, hydroquinone, *p*-benzoquinone, or 1,2,4-benzenetriol over the range of 1.0×10^{-7} to 1.0×10^{-2} M for 30 min, and the rate of DNA synthesis was measured at various times after chemical washout. Cell viability and protein synthesis were determined by trypan blue dye exclusion and [^{3} H]leucine incorporation, respectively. Effects were designated as "DNA specific" when DNA synthesis was inhibited in the absence of discernible effects on cell membrane integrity *and* protein synthesis. Concentrations of benzene as high as 1 mM had no effect on DNA synthesis. Comparison of the effects at the maximum nontoxic dose for each compound showed that catechol and hydroquinone were the most effective, inhibiting DNA synthesis by 65%. Phenol, benzoquinone, and benzenetriol inhibited DNA synthesis by approximately 40%. Maximum inhibition was observed 60 min after metabolite washout in each case. Benzoquinone was the most potent inhibitor of DNA synthesis, followed by hydroquinone, benzenetriol, catechol, and phenol with ED_{50} values of 5×10^{-6}, 1×10^{-5}, 1.8×10^{-4}, 2.5×10^{-4}, and 8.0×10^{-4}, respectively. Cyclic voltammetric experiments were performed on the hydroxylated metabolites of benzene to assess the possible involvement of a redox-type mechanism in their inhibition of DNA synthesis. The ease of oxidation of these metabolites correlated with their ED_{50} values for inhibition of DNA synthesis ($r = 0.997$). This suggests that oxidation of phenol or one of its metabolites may be necessary for production of the species involved in inhibition of DNA synthesis.

INTRODUCTION

Benzene, a known leukemogen, is among the most widely distributed environmental pollutants. Repeated human and animal exposure results in bone marrow toxicity which may be manifested as leukopenia, aplastic anemia, and, in the most serious human cases, leukemia (1). Unfortunately, the mechanism responsible for the myelotoxic effect of benzene is unknown. Existing evidence suggests that a benzene metabolite, rather than benzene itself, may be responsible for these myelotoxic effects (2, 3).

Possible routes of benzene bioactivation are shown in Fig. 1. The initial step involves the monooxygenase-catalyzed conversion of benzene into benzeneoxide (4). Benzene oxide may spontaneously rearrange or be enzymatically hydrolyzed (via epoxide hydrolase) to phenol, the major metabolite of benzene detected *in vivo* (5, 6). Phenol can undergo further cytochrome P-450-mediated hydroxylation to hydroquinone and/or catechol (7). Similarly, catechol may be oxidized further to 1,2,4-benzenetriol. All of these hydroxylated metabolites have been detected in mammalian systems following benzene exposure (5, 6). In addition, it is also possible for these hydroxylated metabolites to be oxidized to their corresponding quinones and semiquinone radicals, generating reactive oxygen species in the process (8).

Although epoxides are believed to be toxic and carcinogenic derivatives of aromatic hydrocarbons, most evidence suggests that benzene oxide itself is not the ultimate toxic metabolite of benzene. Using a rat liver microsomal system, Tunek *et al.* (9) showed that addition of phenol, but not benzene oxide, resulted in covalent binding to microsomal proteins, similar to that seen with benzene itself. Further evidence implicating a phenol

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metabolite was provided by Rickert *et al.* (10), who reported that, following exposure of rats to benzene, the hydroxylated metabolites of phenol, hydroquinone, and catechol were retained selectively in bone marrow, the target organ for benzene toxicity.

In an attempt to identify the proximate toxin responsible for benzene-induced myelotoxicity, we examined cell viability, DNA synthesis, and protein synthesis following exposure of the mouse lymphoma cell line, LY-S,[1] to benzene and its metabolites. The "DNA-specific" effects of these metabolites were compared with their oxidation behavior as determined by cyclic voltammetry in order to ascertain the role of a redox-type mechanism in the inhibitory effects exerted on DNA synthesis.

MATERIALS AND METHODS

Chemicals and Reagents

The benzene metabolites and other chemicals tested in Painter's assay (11) (except streptonigrin) were purchased from Aldrich Chemical Company (Milwaukee, WI), were the highest grade available, and were used without further purification. Streptonigrin was kindly supplied by the Natural Products Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). All solvents used were glass distilled and were obtained from Burdick and Jackson Laboratory, Inc. (Muskegon, Mi). [*methyl*-^{3}H]Thymidine (specific activity 2 Ci/mmol) was obtained from New England Nuclear (Boston, MA). [2-^{14}C]Thymidine (specific activity 56 mCi/mol) and L-[4,5-^{3}H]Leucine (specific activity 50 Ci/mmol) were purchased from ICN Pharmaceuticals, Inc. (Irvine, CA). Scintiverse II, liquid scintillation cocktail, was obtained from Fisher Scientific Company (Pittsburgh, PA).

[1] The abbreviations used are: LY-S, L5178YS cell line; MNNG, 1-methyl-3-nitro-1-nitrosoguanidine; SCE, saturated calomel reference electrode.

Cell Culture

The mouse lymphoma cell line, LY-S, was used to assess benzene toxicity in this study. This cell line was originally derived from a thymic tumor induced in the DBA/2 mouse by 3-methylcholanthrene (12). An X-ray-sensitive strain, LY-S, isolated from the original X-ray-resistant strain by Alexander (13), was used for all experiments described below.

LY-S cells were propagated as suspension cultures in growth media consisting of Fischer's medium supplemented with 10% horse serum, 2.4 mM glutamine, 100 units/ml of penicillin, and 183 μM streptomycin. To ensure optimal growth, cells were maintained at a density between 5×10^3 and 7×10^5 cells/ml in a humidified 5% CO_2 atmosphere at 37°.

Determination of Benzene Metabolism in Vitro

To ascertain the bioactivation of benzene, the procedure of Tunek *et al.* (9) was modified. Microsomes isolated from phenobarbital-induced rabbits were kindly provided by Dr. J. Mieyal of the Department of Pharmacology, Case Western Reserve University. The 2.0-ml reaction mixture contained 1.0 mM NADPH and 3.0 mg of microsomal protein. Following a 30-min incubation with 0.028 M benzene, the reaction was terminated by addition of 4.0 ml of cold ethyl acetate, and extractions were carried out as described by Tunek *et al.* (9). The combined organic phases were evaporated to dryness using a rotary evaporator and redissolved in 200 μl of methanol/water (75:25 v/v) for analysis via high presure liquid chromatography.

To determine whether LY-S cells were capable of metabolizing benzene, the presence of benzene metabolites was examined following

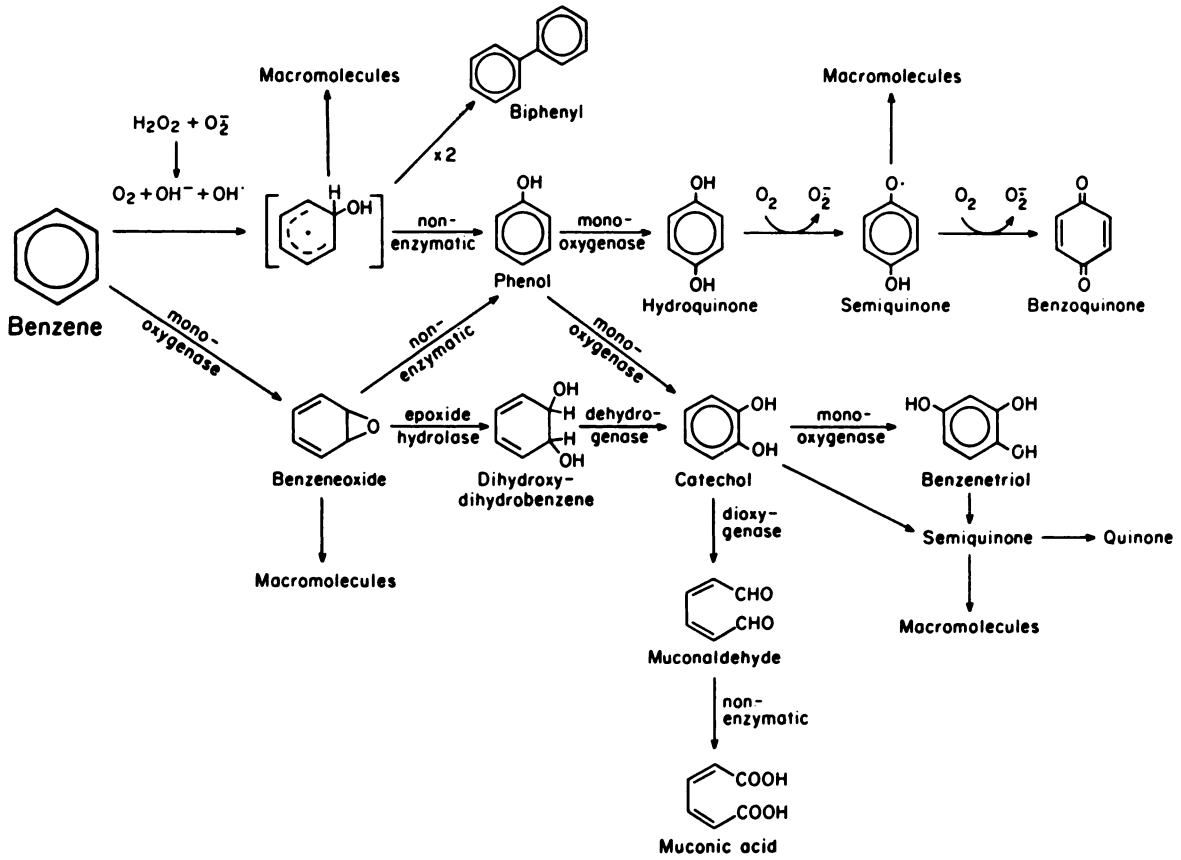

FIG. 1. *Possible pathways for bioactivation of benzene in vivo*

treatment of LY-S cells with benzene. Reaction mixtures and incubation procedures were identical to that described above except that microsomal protein was omitted, and the incubation was carried out for up to 2.0 hr.

Analysis of Benzene Metabolites

Separation of the metabolites of benzene using high pressure liquid chromatography was performed according to the procedure of Greenlee *et al.* (14). All chromatography was performed using a Varian model 5060 liquid chromatograph with a MicroPak-SPC 18 reverse phase column, 30.0 cm × 4.0 mm. Benzene and its metabolites eluted within 14 min of injection using a methanol/water linear gradient ranging from 10–90% methanol at a flow rate of 1.5 ml/min. The solvents were acidified with formic acid (200 μl/liter) and degassed by bubbling with N_2 for a short time. Benzene and its metabolites were measured at 254 and 280 nm, respectively, with a Varian UV 100 variable wavelength detector. With these procedures as little as 9 nmol of hydroquinone or 10 nmol of either phenol or benzenetriol or 25 nmol of catechol produced *in situ* could be detected following extraction from LY-S cells.

Cell Viability

For the purposes of this study, benzene toxicity will be defined in terms of the ability of benzene and/or its metabolites to inhibit DNA synthesis in LY-S cells. Since cell death would also appear to inhibit DNA synthesis, only viable cells were used to assess the effect of benzene and its metabolites on DNA synthesis. Effects were identified as "DNA specific" when DNA synthesis was inhibited but, (a) membrane integrity was ≥90% as assessed by trypan blue dye exclusion, and (b) [^{3}H]leucine incorporation into acid-precipitable protein was ≥90% of control.

Trypan blue dye exclusion. Following chemical exposure and washout, cells were suspended in growth media and diluted 1:1 with a 1% solution of trypan blue dye in phosphate-buffered saline. Cells were examined microscopically for their viability.

Protein synthesis. Approximately 30 min after chemical washout, a 2.0-ml aliquot of control and treated cells was incubated with [^{3}H] leucine (10 μCi/ml). The cells were pelleted by centrifugation, resuspended in 1.5 ml of ice cold 0.15 M sodium chloride/0.015 M sodium citrate, filtered, and counted as described below for assaying [^{3}H] thymidine incorporation.

Inhibition of DNA Synthesis

To assess the effect of benzene and its metabolites on DNA synthesis, a modification of the DNA synthesis inhibition assay described by Painter (11) was employed. This assay, used to estimate the DNA damaging potential of various xenobiotic compounds, measures [^{3}H] thymidine incorporation into DNA at various times after treatment and removal of a presumptive mutagen or carcinogen.

Cells were prelabeled during an 18-h incubation with [2-^{14}C]thymidine (0.02 mCi/ml). Immediately before chemical treatment, they were washed to remove unincorporated [^{14}C]thymidine and resuspended in growth medium at a density of 2×10^5 cells/ml. Incubation with the desired concentrations of benzene, phenol, hydroquinone, benzoquinone, catechol, or benzenetriol was performed for 30 min. Control cells were treated with an equivalent volume of absolute ethanol, the carrier solvent. To terminate the exposure, the cells were pelleted, washed twice, and resuspended in Fischer's media plus horse serum. Immediately, and at 30, 60, and 90 min after chemical washout, a 2.0-ml aliquot of control and treated cells was pulsed with [*methyl*-^{3}H]thymidine (20 μCi/ml) for 10 min at 37°. The pulse was terminated by pelleting the cells, removing the radioactive medium, and resuspending the cells in ice cold 0.15 M sodium chloride/0.015 M sodium citrate. The cells, numbering $\approx 8 \times 10^4$, were then filtered through Gelman type A/E glass fiber filters pretreated with cold 0.6 M perchloric acid. The filters were washed extensively with cold perchloric acid followed by 95% ethanol. Then they were dried and suspended in scintillation fluid, and the radioactivity was quantified in a Beckman LS-7500 liquid scintillation counter. Counting efficiencies for ^{3}H and ^{14}C routinely were 50% and 75%, respectively. The rate of DNA synthesis, expressed as the percentage of control [^{3}H]thymidine incorporated, was calculated as follows:

$$\frac{\dfrac{^3\text{H cpm treated cells}}{^{14}\text{C cpm treated cells}}}{\dfrac{^3\text{H cpm control cells}}{^{14}\text{C cpm control cells}}} \times 100 = \% \text{ of control incorporation}$$

Determination of ED_{50}

The concentration of each metabolite required to inhibit [^{3}H]thymidine incorporation into the DNA of LY-S cells by 50%, i.e., ED_{50}, was calculated according to the method of Litchfield and Wilcoxon (15). Since the apparent rate of DNA synthesis would be influenced by an overall toxic effect of the metabolites, only nontoxic concentrations of metabolites were used to determine the ED_{50} values.

Electrochemical Determination of Redox Behavior

Since the hydroxylated metabolites of benzene can be oxidized to cytotoxic semiquinones and quinones, the redox behavior of these metabolites was studied (8). Cyclic voltammetric experiments were carried out using a Princeton Applied Research model 173 potentiostat, model 175 universal programmer, and a Hewlett-Packard model 136-A X-Y recorder. Measurements were made in an IBM electrochemical cell at 25° using platinum working and counter electrodes and an SCE. The electrolyte was a 0.1 M phosphate buffer, pH 7. Solutions were deaerated with N_2. Redox potentials were calculated according to the equation:

$$E_{1/2} = (E_C + E_A)/2$$

where E_C and E_A are the potentials for cathodic and anodic waves, respectively.

RESULTS

Benzene metabolism. To distinguish the direct toxicity of benzene from that of its metabolites, we demonstrated initially that LY-S cells were not capable of metabolizing benzene. The major metabolite observed in the presence of microsomes was phenol, which represents 54% of the total metabolites detected. Hydroquinone and catechol were also formed, representing 7 and 39%, respectively, of the total metabolites detected. In contrast, up to 2 hr of incubation of LY-S cells with 0.028 M benzene yielded no detectable metabolites. The inability of LY-S cells to metabolize benzene enabled the toxicity of benzene to be evaluated without interference from its metabolites.

Cell viability. Since the observed rate of DNA synthesis might be influenced by an overall cytotoxic effect of benzene, its metabolites, or both, it was important to use nontoxic doses of chemical when examining their effect on DNA synthesis. As shown in Table 1, concentrations of benzene, phenol, and catechol as high as 1.0 mM had no effect on protein synthesis. In contrast, inhibitory effects on protein synthesis were observed after incubation with 0.1 mM benzenetriol or 0.06 mM hydroquinone. Benzoquinone proved to be the most potent inhibitor of protein synthesis; doses as low as 6.0 μM suppressed protein synthesis by approximately 35%.

Determination of cell viability based upon trypan blue dye exclusion yielded results similar to those obtained with protein synthesis. Treatment with 1.0 mM benzene, phenol, or catechol had no effect on membrane integrity. Only the most potent metabolites in inhibiting protein

TABLE 1

Effect of benzene and metabolites on L5178YS cell viability

Cells were exposed to benzene or its metabolites over the concentration ranges indicated and aliquots at each dose were taken for the determination of cell viability. Trypan blue dye exclusion and [^{3}H]leucine incorporation were performed as described under "Materials and Methods."

	Experimental dose range	Maximum dose yielding 90% viability according to trypan blue dye exclusion	Maximum dose yielding 90% viability according to protein synthesis
	M	*M*	*M*
Benzene	1×10^{-3}–1×10^{-2}	1×10^{-2}	1×10^{-2}
Phenol	1×10^{-4}–1×10^{-2}	7×10^{-3}	7×10^{-3}
Catechol	1×10^{-6}–1×10^{-2}	1×10^{-2}	1×10^{-3}
Hydroquinone	1×10^{-7}–1×10^{-4}	4×10^{-5}	4×10^{-5}
p-Benzoquinone	4×10^{-7}–4×10^{-5}	4×10^{-6}	4×10^{-6}
1,2,4-Benzenetriol	5×10^{-6}–5×10^{-4}	6×10^{-5}	5×10^{-5}

synthesis, i.e., benzoquinone, hydroquinone, and benzenetriol, affected membrane integrity at low concentrations.

Using the criteria adopted herein for ascertaining cell viability, i.e., maintenance of membrane integrity and rate of protein synthesis at ≥90% of control levels, these results suggest that expression of benzene cytotoxicity requires metabolism of the parent compound to one or more toxic species. Of the benzene metabolites studied, benzoquinone was the most potent cellular toxin, followed, in decreasing order of potency, by hydroquinone, benzenetriol, and catechol. Treatment of LY-S cells with phenol or benzene did not result in significant toxicity.

DNA synthesis inhibition. In order to confirm the DNA synthesis inhibition assay in our cell line, the effects of several standard mutagens on [^{3}H]thymidine incorporation were examined.

The response of LY-S cells to the mutagens streptonigrin and MNNG is shown in Fig. 2. Streptonigrin inhibited DNA synthesis by 40% within 90 min after chemical washout. A 60% inhibition was observed within 120 min after removal of 2.0 μM MNNG. Similar results were reported by Painter in his original HeLa cell assay system (16). In contrast, cycloheximide, an agent that inhibits protein synthesis but does not damage DNA, did not appear to affect the rate of DNA synthesis in LY-S cells. HeLa cells are reported to respond in a similar fashion (11).

To determine the effect of benzene and its metabolites on DNA synthesis, [^{3}H]thymidine incorporation was determined in LY-S cells at various times after a 30-min chemical exposure. Fig. 3 shows the results obtained with the highest nontoxic dose of catechol, hydroquinone, benzenetriol, and benzoquinone, and 1.0 mM benzene and phenol. Although concentrations of benzene and phenol greater than 1.0 mM were nontoxic, higher doses were not thoroughly examined in the DNA synthesis inhibition assay since it is unlikely that such high concentrations would be found in biologic systems.

Benzene had no effect on DNA synthesis; [^{3}H]thymidine incorporation remained at ≥90% of control levels at all times tested. Hydroquinone and catechol were the most efficacious metabolites, inhibiting DNA synthesis by ≈65%, followed by benzenetriol and benzoquinone and phenol which decreased [^{3}H]thymidine incorporation by ≈40%.

Time-dependent inhibition of DNA synthesis. The rate of DNA synthesis was determined at various time intervals following a 30-min exposure of LY-S cells to benzene and its metabolites. As shown in Fig. 3, the maximum inhibitory effect was observed 60 min after chemical washout with all metabolites tested. Beyond 60 min, a recovery toward control levels of DNA synthesis was observed with all metabolites, except catechol and benzoquinone. When the dose of catechol was increased further, an irreversible inhibition of DNA synthesis was observed at ≥1.0 mM. In contrast, low doses of benzoquinone (4.0 μM) were sufficient to inhibit [^{3}H]thymidine incorporation irreversibly.

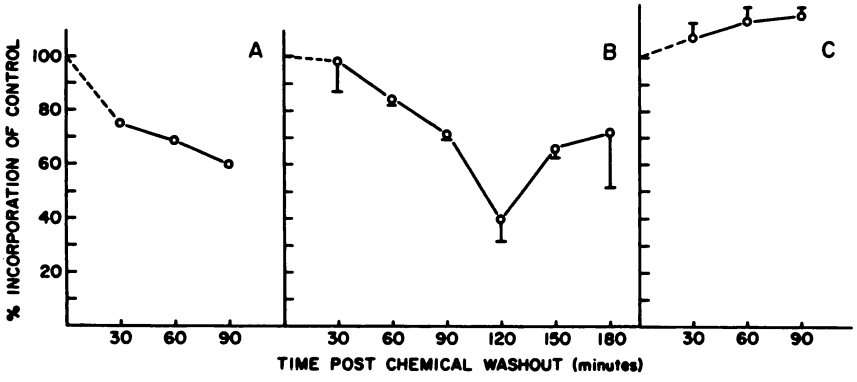

FIG. 2. *Effect of known mutagenic and nonmutagenic agents on DNA synthesis in LY-S cells*

Following a 30-min exposure to A) 0.1 μM streptonigrin, B) 2.0 μM MNNG, or C) 0.23 mM cycloheximide, the chemical was washed out and cells were subjected to a 10-min pulse with [^{3}H]thymidine as described under "Materials and Methods." Results shown here are with nontoxic doses of chemicals, according to trypan blue dye exclusion and protein synthesis. Results represent the mean ± standard error of at least three experiments with MNNG and cycloheximide and one experiment with streptonigrin.

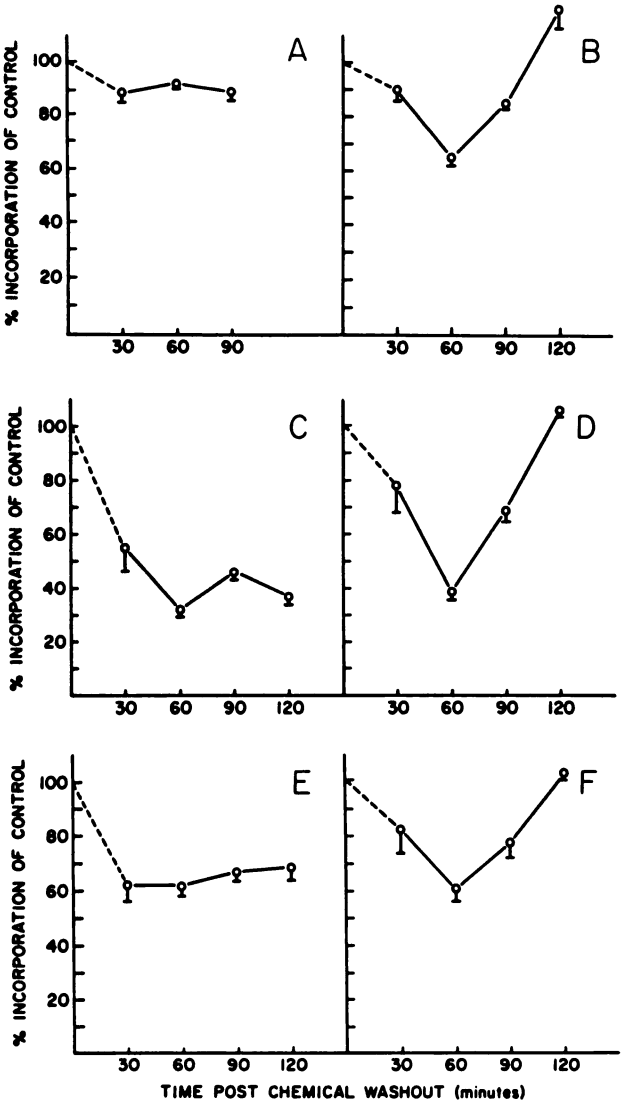

FIG. 3. *Effect of benzene and its metabolites on [^{3}H]thymidine incorporation in LY-S cells*

Following a 30-min exposure of LY-S cells to A) 1.0 mM benzene, B) 1.0 mM phenol, C) 1.0 mM catechol, D) 40.0 μM hydroquinone, E) 4.0 μM benzoquinone, and F) 50 μM benzenetriol, cells were washed, resuspended in media, and pulsed with [^{3}H]thymidine at the times indicated. Shown here are the results obtained with the highest nontoxic dose of all chemicals, except benzene and phenol, which were not tested at doses >1.0 mM. Results represent the mean ± standard error of at least four experiments with each compound.

Dose-dependent inhibition of DNA synthesis. Fig. 4 depicts the effects of various concentrations of benzene metabolites on [^{3}H]thymidine incorporation 60 min after chemical washout. The ED_{50} values for each metabolite are reported in Table 2. Although the overall cellular toxicity associated with benzoquinone treatment masked the DNA-specific effects and prevented rigorous determination of an ED_{50}, it was obviously the most potent inhibitor of [^{3}H]thymidine incorporation in the series.

Oxidation-reduction potentials. To assess the possible involvement of a redox-type mechanism in the inhibition of DNA synthesis by phenol, catechol, hydroquinone, and benzenetriol, the electrochemical oxidation of these metabolites was studied by cyclic voltammetry. Both hydroquinone and catechol exhibited two oxidation waves. The first wave, observed at lower anodic potential, is nearly reversible and has been attributed to the two-electron oxidation to the respective quinone (17). For hydroquinone, the half-wave potential for the first oxidation wave occurs at 0.05 V versus SCE, which is in close agreement with 0.04 V versus SCE determined by Meites (18) with an Hg electrode.

As reported in Table 2, the first oxidation wave for catechol occurs at higher anodic potential, 0.16 V versus SCE. The difference between the half-wave potentials of catechol and hydroquinone, 0.11 V, is consistent with the results of Peover (19) and of Eggins and Chambers (20), which predict a difference in potential of 0.17 V. The voltammogram of benzenetriol again displayed two oxidation waves. Unlike hydroquinone and catechol, the first oxidation wave of benezenetriol was quasi-reversible with an $E_{1/4}$ of 0.13 V.

The electrochemical behavior of phenol is significantly different from that of the other benzene metabolites studied in that only one oxidation wave is observed in the potential range examined. The oxidation wave occurs at much higher potential than the first oxidation wave of hydroquinone, catechol, and benzenetriol and, in fact, is similar in shape and position to the second wave exhibited by these metabolites. The half-wave potential for this oxidation is approximately 0.35 V versus SCE.

As shown in Fig. 5, the ease of oxidation of the hydroxylated metabolites of benzene correlates with their ED_{50} values for inhibition of DNA synthesis with a correlation coefficient of 0.997. Phenol, the most difficult metabolite to oxidize, was the least potent inhibitor of DNA synthesis. The inhibitory effect of phenol, catechol, benzenetriol, and hydroquinone paralleled their ease of oxidation.

DISCUSSION

The metabolism of benzene both *in vivo* (2, 5, 6, 8, 10) and *in vitro* (4, 7, 8, 9) has been investigated thoroughly and shown to be extensive. The plethora of potential metabolites derived from this simple hydrocarbon might explain why the mechanism responsible for benzene-induced myelotoxicity has proved so evasive. We have developed an approach aimed at defining the proximate toxin responsible for the array of bone marrow toxicities associated with benzene exposure.

Our experimental system, the LY-S cell, was chosen both because of its lymphoid origin (12, 13) and because, during initial screening, it proved incapable of catalyzing benzene hydroxylation. Such a system should permit the toxic effects of benzene to be distinguished from those of its metabolites. Most previous investigations have been performed using whole animals (2) or microsomal systems which are competent in benzene hydroxylation (4, 7, 9, 21). Even those groups examining the effects of benzene and its metabolites on cell cycle kinetics (22) and sister chromatid exchange (3, 22) have employed human lymphocytes which are known to be able to hydroxylate other aromatic hydrocarbons (23). Thus, it would be difficult to distinguish between the effects of

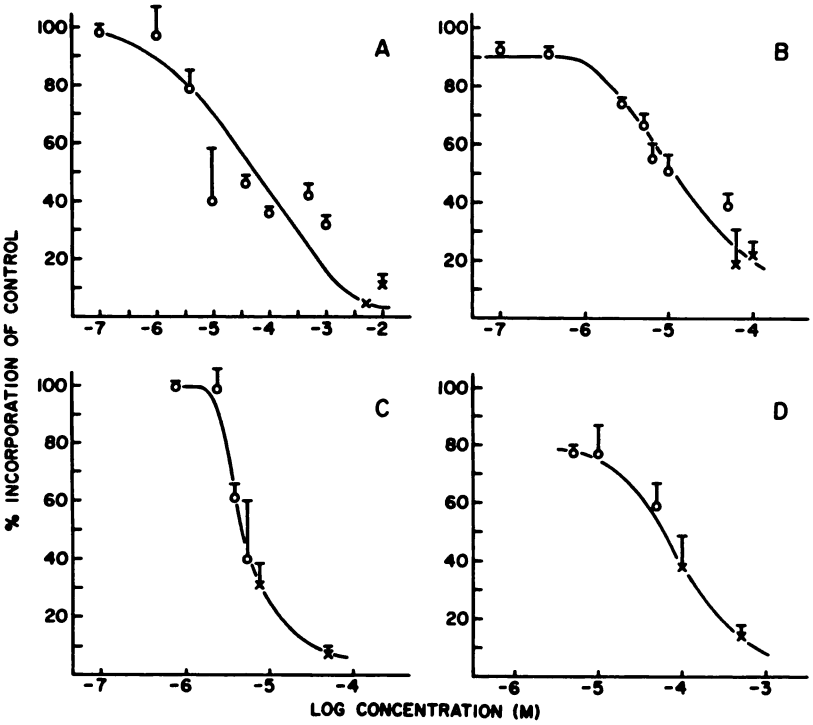

FIG. 4. *Dose-dependent effect of benzene metabolites on the rate of DNA synthesis in LY-S cells*

Following a 30-min treatment with various doses of A) catechol, B) hydroquinone, C) benzoquinone, and D) benzenetriol, cells were washed, resuspended in media, and pulsed with [^{3}H]thymidine as detailed under "Materials and Methods." Shown here are the results obtained 60 min after chemical washout, the time at which maximum inhibition of DNA synthesis was observed. ×, concentrations of metabolite yielding ≤90% viability according to trypan blue dye exclusion or [^{3}H]leucine incorporation; O, nontoxic doses of chemical. Results represent the mean ± standard error of at least four experiments with each compound.

TABLE 2

Relationship between oxidation potentials of benzene metabolites and their effect on DNA synthesis

Metabolite	ED_{50}	$E_{1/2}$
	M	*V*
Benzoquinone	$\approx 5 \times 10^{-6}$ [a]	[b]
Hydroquinone	1.0×10^{-5}	0.05
Benzenetriol	1.8×10^{-4}	0.13
Catechol	2.5×10^{-4}	0.16
Phenol	0.8×10^{-3}	0.35

[a] The overall cellular toxicity associated with benzoquinone treatment masked the DNA specific effects and prevented rigorous determination of an ED_{50}. The value reported is estimated from the data in Fig. 5.

[b] Since benzoquinone exists in a fully oxidized form, an oxidation potential is not presented.

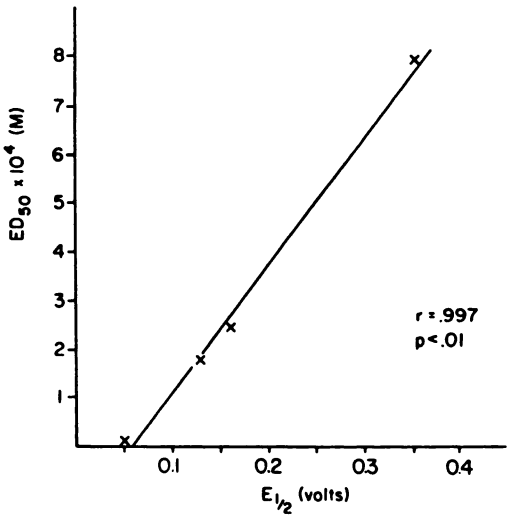

FIG. 5. *Relationship between the half-wave potential ($E_{1/2}$) and the ED_{50} for inhibition of DNA synthesis by the hydroxylated metabolites of benzene*

benzene alone and those with one or more of its metabolites when using these other experimental systems.

Of consummate interest in our laboratory is the pathophysiology of leukemia. Benzene has been chosen as a model chemical because of its specific association with leukemia in humans (1). Since DNA modification seems to be a prerequisite for chemical carcinogenesis, it was anticipated that the inhibition of DNA synthesis by benzene or its metabolites might provide insight regarding the mechanism of benzene-induced myelotoxicity and, ultimately, leukemia. In order to evaluate a number of chemicals simultaneously and under similar conditions, the DNA synthesis inhibition assay of Painter (11) was selected. Painter (11, 16) has demonstrated that an inhibition of [^{3}H]thymidine incorporation following treatment with a given chemical agent reflects the DNA-damaging potential of that chemical.

While this assay appeared sufficiently discriminating for our purpose, it is quite possible that an inhibition of DNA synthesis observed following treatment with a chemical may be due to an overall toxic effect of the chemical and is not an indication of the chemical's specific effect on DNA. For this reason, DNA synthesis inhibition was evaluated only when certain adopted criteria for cell viability were fulfilled. These included trypan blue dye exclusion and [^{3}H]leucine incorporation to ≥90% of control values. This is in contrast to previous studies by Morimoto and Wolff (3) examining mitotic indices in human lymphocytes and Erexson *et al.* (22), who examined the cell cycle kinetics of human T-lymphocytes. In neither study was cell viability considered among the evaluative criteria. Thus, the effects seen at metabolite concentrations in excess of those observed to

cause <90% viability in our system (Table 1) might simply reflect cytotoxicity rather than DNA-specific activity.

The results of the present study show a specific dose-dependent inhibition of DNA synthesis following exposure to catechol, hydroquinone, benzenetriol, and benzoquinone (Fig. 4). The ED_{50} for each of these metabolites was strongly correlated ($r = 0.997$; $p < 0.01$) with the respective oxidation potentials of the various compounds (Fig. 5), suggesting that oxidation of a phenol metabolite may be a necessary prerequisite to benzene-induced DNA damage. Semiquinones generated during the oxidation of these hydroxylated metabolites might directly damage DNA via alkylation (24). Alternatively, reactive oxygen species such as hydrogen peroxide, superoxide, and hydroxyl radicals, generated during the redox cycling of the semiquinone with its reduced or oxidized counterpart, might be damaging to DNA (25).

Greenlee *et al.* (8) have proposed that benzene toxicity may be mediated by superoxide radicals, semiquinone, and/or quinone oxidation products resulting from the autooxidation of hydroquinone and 1,2,4-benzenetriol. Smart *et al.* (21) examined the role of benzoquinone, the oxidized form of hydroquinone, in binding to microsomal protein by manipulating its redox state with the enzyme NAD(P):(acceptor) oxidoreductase (EC 1.6.99). This enzyme, which reduces quinones through a direct two-electron transfer to give the corresponding hydroquinone, inhibited the covalent binding of benzoquinone to microsomal proteins. The inhibition was reversible with the addition of dicoumarol, an inhibitor of NAD(P):(acceptor) oxidoreductase activity. These results provide evidence suggesting that quinone or semiquinone metabolites of benzene may be the reactive intermediates responsible for benzene-associated toxicity.

The present study has provided evidence to implicate benzoquinone as the benzene metabolite with the greatest DNA-damaging potential. Low doses of benzoquinone, which had no effect on cell viability, resulted in an apparently irreversible inhibition of DNA synthesis (Fig. 3). While a similar effect was observed with catechol (Fig. 3), it occurred only at concentrations which were ≥250 times greater than that of benzoquinone. The nonreversible inhibition of DNA synthesis following treatment with nontoxic concentrations of benzoquinone may represent nonrepairable DNA damage. Perpetuation of this altered DNA might have significant implications for the process of carcinogenesis.

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Send reprint requests to: Dr. Jeffrey L. Blumer, Department of Pediatrics, Rainbow Babies and Childrens Hospital, 2101 Adelbert Road, Cleveland, OH 44106.