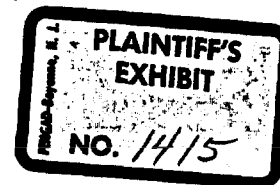


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Multiple Activation Pathways of Benzene Leading to Products with Varying Genotoxic Characteristics

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Benzene and 13 potential metabolites were investigated for genotoxicity in *Salmonella typhimurium* and V79 Chinese hamster cells. In the presence of NADPH-fortified hepatic postmitochondrial fraction (S9 mix), benzene reverted his⁺ *S. typhimurium* strains. The effect was strongest in strain TA1535. Among the potential metabolites, only the *trans*-1,2-dihydrodiol, in the presence of S9 mix, and the diol epoxides, in the presence and absence of S9 mix, proved mutagenic in this strain. The anti-diol epoxide was more potent than the *syn*-diastereomer. Both enantiomers of the *anti*-diastereomer showed similar activities. S9 mix did not appreciably affect the mutagenicity of the anti-diol epoxide. However, detoxification was observed when purified rat liver dihydrodiol dehydrogenase (EC 1.3.1.20) was used at concentrations comparable to that present in the liver. The (1*S*)-*anti*-diol epoxide was a much better substrate than the (1*R*)-enantiomer, as was true also for (1*S*)-*versus* (1*R*)-*trans*-1,2-dihydrodiol. The anti-diol epoxide reverted all six strains of *S. typhimurium* used and induced all four genotoxic effects studied in V79 cells (sister chromatid exchange > acquisition of 6-thioguanine resistance, acquisition of ouabain resistance, micronuclei). However, other potential benzene metabolites showed genotoxic effects in V79 cells, as well: sister chromatid exchange was induced by the *syn*-diol epoxide, 1,2,4-trihydroxybenzene, hydroquinone, catechol, and 1,2,3-trihydroxybenzene. Elevated frequencies of micronucleated cells were observed after treatment with hydroquinone, 1,2,4-trihydroxybenzene, catechol, phenol, 1,2,3-trihydroxybenzene, and quinone. Mutations to 6-thioguanine resistance were induced by quinone, hydroquinone, 1,2,4-trihydroxybenzene, catechol, and the *trans*-1,2-dihydrodiol. By far the most prominent effect in the whole study was the potent induction of gene mutations by quinone and hydroquinone. Quinone produced an approximately 100-fold increase in the frequency 6-thioguanine-resistant cells even at a concentration of 1 μ M. This effect was surprising, since benzoquinone induced almost no mutations in *S. typhimurium*. In the V79 cells, it did not induce mutations to ouabain resistance and sister chromatid exchange, and it induced micronuclei with moderate efficiency only. This unique and narrow spectrum of genotoxic activities differs from the broad spectrum observed with the *anti*-diol epoxide, suggesting qualitative differences in their interaction with genetic material.

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Introduction

Substantial evidence has accumulated on the important role mutations play in chemical carcinogenesis. This is true in particular for a number of carcinogenic polycyclic aromatic hydrocarbons (1). Epoxides, especially vicinal diol epoxides, appear to constitute their major mutagenic metabolites (1,2). Such epoxides are carcinogenic (1), transform cells in culture (3,4), and induce a wide spec-

trum of mutations (1,2,5), including the reversion of auxotrophic bacteria, the acquisition of resistance toward toxic chemicals, and the activation of oncogenes.

Unlike the case with polycyclic aromatic hydrocarbons, the evidence for a role for mutations is weak with the carcinogen benzene. Heritable functional changes (termed gene mutations in the present study) were usually not observed in bacteria and mammalian cells treated with benzene or benzene metabolites (6). However, chromosomal damage was detected by means of cytogenetic techniques (6-13). Interestingly, the metabolites that showed this genotoxic activity were not epoxides, but were polyhydroxylated metabolites (catechol, hydroquinone, 1,2,4-trihydroxybenzene) and quinones (9-13).

These findings suggest that benzene and polycyclic aromatic hydrocarbons differ in the chemical nature as well

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as in the biological activities of their active metabolites. Nevertheless, the following points should prevent us from overemphasizing these differences: a) The doses of benzene required for carcinogenesis are higher by several orders of magnitude than those required with polycyclic aromatic hydrocarbons. Very minor metabolites and/or metabolites of relatively weak biological activity could therefore be responsible for benzene carcinogenesis; b) only very few biochemical and toxicological studies have been conducted on benzene oxides, dihydrodiols, and diol epoxides, the structural analogues of the critical metabolites of polycyclic aromatic hydrocarbons; and c) in short-term tests with benzene, its high volatility may pose a problem, rendering standard methods insensitive.

In the past decade, we have studied intensively the mutagenicity of metabolites of polycyclic aromatic hydrocarbons and their enzymatic control (2). We included benzene metabolites sporadically in these studies. Benzene and *trans*-benzene-1,2-dihydrodiol showed mutagenic activity in *S. typhimurium* when a hepatic enzyme system was present. Systematic mutagenicity studies were initiated when benzene-1,2-diol 3,4-oxides became synthetically available (14). Knowing the clastogenic activity of polyhydroxylated metabolites, it was of particular interest to examine whether multiple activation pathways exist for benzene and whether the genotoxic metabolites differ qualitatively in their effects. This required that several genotoxic effects could be determined in the same target cell system, since otherwise differences in responses could be interpreted as the result of trivial differences between cells, e.g., in activation, detoxification, or repair. Chinese hamster V79 cells were a particularly suitable experimental model, since one could study at the same time the inductions of gene mutations (to ouabain and 6-thioguanine resistance), sister chromatid exchange (SCE), and micronuclei.

The structures of the compounds studied are shown in Figure 1. All compounds, except duroquinone (which was used for mechanistic reasons), the diol epoxides (which have not yet been available as standards in any metabolic studies) and 1,2,3-trihydroxybenzene (an oxidation-sensitive isomer of 1,2,4-trihydroxybenzene) are known metabolites of benzene, observed at least in certain *in vitro* systems. Muconaldehyde, a reactive metabolite of benzene that was recently detected (15), was not available for this study. Also not investigated were the conjugated benzene metabolites and muconic acid.

Mutagenicity in *Salmonella typhimurium*

Unlike other reports in the literature (16-20), we observed a positive response of benzene in *S. typhimurium*. The major methodological difference was that in the earlier studies the conventional plate-incorporation and preincubation protocols were used, whereas we exposed the bacteria to benzene vapor in desiccators. This technique allows much longer exposure periods than is possible for highly volatile compounds with conventional protocols.

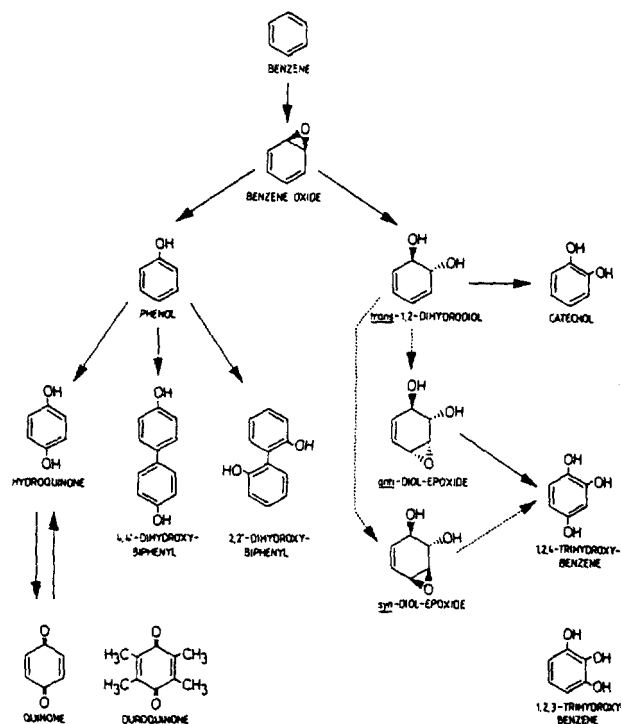


FIGURE 1. Structures and metabolic relations of the investigated compounds. Full and dashed arrows indicate established and hypothetical metabolic pathways, respectively. Enantiomeric aspects are not implied in the structures. Some benzene metabolites may be formed on additional routes, e.g., the catechol possibly through further hydroxylation of the phenol. The *syn*-diol epoxide is the diastereomer in which the allylic hydroxyl group and the oxirane moiety are on the same face of the benzene ring, whereas these groups are on opposite faces in the *anti*-diol epoxide.

Mutagenicity of benzene was observed only in the presence, but not in the absence, of NADPH-fortified post-mitochondrial fraction (S9 mix) from rat and mouse liver homogenate (Fig. 2). The most responsive strain was TA1535. A 2-fold increase in the number of mutants above control was observed even at a benzene concentration as low as 10 ppm. However, further increases in the concentration had only a modest effect. The maximal mutant number was about 3-fold the values for the control plates. Similar effects were then seen over a wide concentration range, with the limitation that details of the concentration-response curve may have been overshadowed by the unfavorable ratio of effect and variation. For this same reason, it was impossible to detect quantitative differences in effect between the various metabolizing systems used. It would be very difficult, therefore, to elucidate the activation mechanism by studying the effect of modulations of the metabolizing system.

Benzene oxide, tested only in the absence of liver S9 mix, did not show mutagenicity in a study conducted in our laboratory (21). A positive result in strain TA100 was reported for benzene oxide when high concentrations (≥ 7.1 mM) were used in a preincubation assay (22). How-

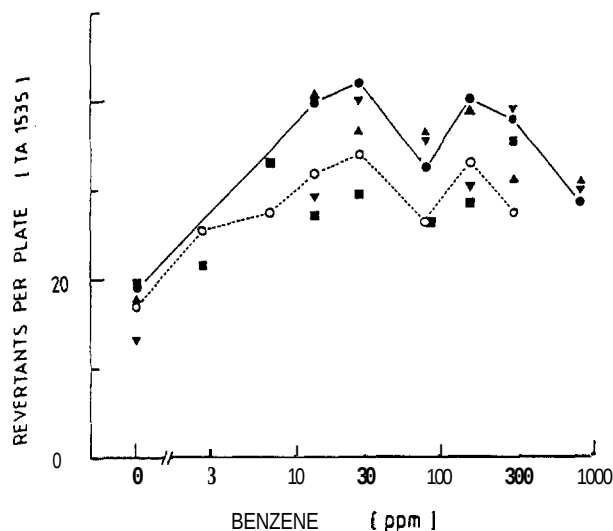


FIGURE 2. Mutagenicity of benzene in *Salmonella typhimurium* TA1535 in the presence of mouse and rat liver postmitochondrial supernatant (S9) preparation. Agar plates, containing bacteria (about 10^8 colony forming units) and the liver preparation in an agar overlay, were replaced for 18 hr in a desiccator with benzene. After further incubation for 2 days in the dark in the absence of benzene at 37°C, the colonies were counted. Each type of symbol represents a separate set of experiments, the individual symbol represents the mean number of colonies from two plates. Hepatic S9 fractions were prepared from male Sprague-Dawley rats (closed symbols) or female C57BL mice (open symbols) that had received a single IP injection of Aroclor 1254, 500 mg/kg body weight, 6 days previously. Each incubation contained S9 fraction equivalent to 50 mg (♂) or 100 mg liver (other symbols) and the following cofactor systems: 2 μ mole NADP⁺ and 2.5 μ mole glucose 6-phosphate (*), or 20 μ mole NAD⁺, 10 μ mole NADP⁺, and 5 μ mole glucose 6-phosphate (other symbols). The parameters of the assay not specifically mentioned were as described previously (30).

ever, the positive result essentially depended on misinterpretation of the data, involving the use of an inappropriate method for the correction for cytotoxicity (23). Except for benzene oxide and the syn-diol epoxide, all benzene derivatives were investigated for mutagenicity in the presence and absence of rat liver S9 mix (Table 1). In TA1535, the strain showing the strongest response for bioactivated benzene, only *trans*-benzene-1,2-dihydrodiol (Fig. 3) and the diol epoxides (Fig. 4) proved positive. Mutagenicity of the *trans*-1,2-dihydrodiol required the presence of a hepatic metabolizing system (Fig. 3). Cytosolic dihydrodiol dehydrogenase converts the *trans*-1,2-dihydrodiol to catechol (24-26), otherwise virtually nothing is known about the metabolic fate of the *trans*-1,2-dihydrodiol. The catechol was not mutagenic either in the presence or in the absence of liver S9 mix (Fig. 3) and, therefore, could not account for the S9 mix-mediated mutagenicity of the *trans*-1,2-dihydrodiol. Moreover, the cytosol was not required for the activation of the dihydrodiol, as shown in experiments where the postmitochondrial fraction was substituted by a microsomal preparation (data not shown).

The kinds of reactions that may occur in such a system appear to be very limited. In fact, the only precedent is

the metabolism of dihydrodiols derived from polycyclic aromatic hydrocarbons to diol epoxides (1,2). The idea that diol epoxides are the mutagenic metabolites formed from the *trans*-1,2-dihydrodiol is further supported by the response spectra in the series of bacterial strains used. For the *trans*-1,2-dihydrodiol and the diol epoxides (as well as for benzene) the order of responsiveness was TA1535 > TA100 > TA98 (with the other strains the data are incomplete). Two diastereomers of the diol epoxides are possible, depending on which face of the *trans*-1,2-dihydrodiol the epoxide group is introduced. In strain TA1535, bioactivated *trans*-1,2-dihydrodiol, syn-diol epoxide, and anti-diol epoxide induced about 12, 20, and 800 revertants per μ mole of test compound. In a first approximation, 60 and 1.5% of the *trans*-dihydrodiol would therefore have to be converted to the syn-diol and anti-diol epoxide, respectively, if this were the only active metabolite. It is therefore reasonable to suspect that at least some anti-diol epoxide is formed.

trans-Dihydrodiols and diol epoxides are chiral molecules. For the anti-diol epoxides, we synthesized the individual enantiomers separately. In all six bacterial strains used, the mutagenic potencies were virtually identical for both enantiomers (data not shown).

Whereas in strain TA1535 only benzene, the *trans*-1,2-dihydrodiol, and the diol epoxides showed mutagenic effects, positive results were observed for additional benzene derivatives in other strains (Table 1). 1,2,3-Trihydroxybenzene, whose metabolic formation has not yet been observed, showed relatively potent effects in several strains. 1,2,4-Trihydroxybenzene, a minor metabolite of benzene, was mutagenic in strain TA104 in the presence of liver S9 mix. With hydroquinone, quinone, and catechol, small increases, in the range of the detection limit, in the number of mutants were frequently observed. While we believe in the reality of these effects, their usefulness in the elucidation of the activation mechanisms of benzene may be negligible.

Benzene Dihydrodiols and Diol Epoxides As Substrates of Dihydrodiol Dehydrogenase

Many metabolites of polycyclic aromatic hydrocarbons do not appear to be carcinogenic, although they are potent mutagens in *S. typhimurium* (2). One of the additional prerequisites for appreciable carcinogenicity is resistance to detoxification (2). (1*R*)- as well as (1*S*)-anti-benzene-diol epoxide showed similar mutagenic activity in the presence and absence of rat liver S9 mix (data not shown). However, in bacterial mutagenicity assays the S9 enzymes are present in strongly diluted form, as compared to the liver. In the experiments with the benzene metabolites, including the diol epoxides, we normally used S9 fraction equivalent to 17 mg liver in an incubation volume of 620 μ L. In a previous mutagenicity study we showed that diol epoxides derived from polycyclic aromatic hydrocarbons may be detoxified in mutagenicity assays by purified rat liver dihydrodiol dehydrogenase (27).

Table 1. Mutagenicity of benzene and potential metabolites in *Salmonella typhimurium*.^a

Compound	S9	Dose range tested.		Response ^c					
		$\mu\text{g}/\text{plate}$	LD ₅₀ ^b $\mu\text{g}/\text{plate}$	TA97	TA98	TA100	TA102	TA104	TA1535
Benzene	-	3-1000 ppm ^d	NT	NT	NT	-	NT	NT	-
	+	3-1000 ppm ^d	NT	NT	-	-	NT	-	+
Benzene oxide ^e	-	20-4000	NT	NT	-	-	NT	NT	-
Phenol	-	100-5000	3000	-	-	-	-	-	-
	+	20-5000	1800	-	-	-	-	-	-
Hydroquinone	-	1.25-40	> 40	-	-	-	-	-	-
	+	50-1000	> 1000	-	-	-	-	-	-
Quinone	-	0.125-4	5	-	-	-	-	-	-
	+	10-200	70	-	-	-	-	-	-
2,2'-Dihydroxybiphenyl	-	20-1000	300	-	-	-	-	-	-
	+	20-1000	300	-	-	-	-	-	-
4,4'-Dihydroxybiphenyl	-	20-1000	1100	-	-	-	-	-	-
	+	20-1000	> 1000	-	-	-	-	-	-
(±)- <i>trans</i> -1,2-Dihydrodiol	-	10-3000	NT	-	-	-	NT	NT	-
	+	3.15-3000	NT	NT	-	+	NT	NT	+
Catechol	-	50-1000	380	-	-	-	-	-	-
	+	50-5000	4000	-	-	-	-	-	-
(±)- <i>syn</i> -Diol epoxide	-	10-315	NT	NT	-	-	NT	NT	+
(1 <i>R</i>)- <i>anti</i> -Diol epoxide	-	10-320	NT	-	-	+	+	+	++
(1 <i>S</i>)- <i>anti</i> -Diol epoxide	-	10-320	NT	-	-	+	+	+	++
1,2,4-Trihydroxybenzene	-	2.5-40	> 40	-	-	-	-	-	-
	+	1-2000	2200	-	-	-	-	+	-
1,2,3-Trihydroxybenzene	-	6.25-200	> 200	+	-	+	+	+	-
	+	1.25-500	400	+	-	+	+	+	-

^aThe test compound, bacteria and NADPH-fortified postmitochondrial fraction (S9) from liver homogenate of Aroclor 1254-treated rats (or buffer), were preincubated for 20 min at 37°C and then added to minimal agar plates. After incubation for 3 days, the colonies were counted. Except for the preincubation and the amount of S9 fraction—in the present study the equivalent of 17 mg liver was used per plate—details of the protocol were as described in (4). For technical reasons, a modified protocol was used for benzene (exposure in desiccators). For historical reasons, no preincubation was made with benzene oxide and *trans*-1,2-dihydrodiol, and for the S9-mediated experiments with the latter compound, the equivalent of 50 mg liver was used.

Toxicity was determined using *his*⁺ mutants as an internal standard (31).

(+) The colony number at the optimal dose exceeded the value of the solvent controls by a factor of >20; (+) 2- to 20-fold (TA1535, TA98) or 1.5- to 10-fold (other strains), increase in colony number at optimal dose; (-) at all dose levels tested, the numbers of colonies were less than the 2-fold (TA1535, TA98) or 1.5-fold (other strains) of the solvent control values. In some cases (*), our data suggest the existence of a weak effect below this criterion. NT, not tested.

^bExposure in desiccators; see Fig. 2.

^cData from a previous study (21). Bacteria and test compound were added to plates without a preincubation. Toxicity, in form of dilution of the *his*⁻ background lawn, was observed at doses >2000 μg .

^dRacemic anti-diol epoxide, at a dose of 1000 μg per plate (which is more than that used with the individual enantiomers), led to a 2-fold increase in the number of mutants above the value of the controls.

With the same protocol, 0.3 units of dihydrodiol dehydrogenase, which is the amount of enzyme present in 120 mg liver, reduced the mutagenicity of *anti*-benzene-diol epoxide in *S. typhimurium* TA1535 by 17% (incubation volume = 620 μL); 0.6 units (equivalent to 240 mg liver) led to a reduction of 35%, but a further increase in the amount of enzyme to 2.4 units (equivalent to 960 mg liver) showed no additional protective effect. The products formed by dihydrodiol dehydrogenase from anti-diol epoxide have not yet been quantitatively identified; however, formation of 1,2,4-trihydroxybenzene (a nonmutagen in strain TA1535) was observed.

Kinetic analysis in biochemical assays (Table 2) shows that the racemic anti-diol and syn-diol epoxides are relatively good substrates. Their V_{max}/K_m values were 5 and 18%, respectively, of the value for *trans*-1,2-dihydrodiol, which is a good substrate. Dihydrodiol dehydrogenase may therefore detoxify benzene-diol epoxides by two mechanisms, sequestration of the metabolic precursor and inactivation of the already formed diol epoxides. However, it is noteworthy that only one enantiomer of

trans-benzene-1,2-dihydrodiol and one enantiomer of the anti-diol epoxide [in both cases the (1*S*) enantiomer] are good substrates. Unfortunately, it appears that the (1*R*)-*trans*-1,2-dihydrodiol is preferentially formed in the liver (24). Nevertheless, Billings observed the formation of catechol from benzene in isolated hepatocytes via the dihydrodiol pathway (26).

Genotoxicity in Chinese Hamster V79 Cells

The results from the genotoxicity experiments in V79 cells are summarized in Table 3 (gene mutations to 6-thioguanine resistance), Table 4 (induction of micronuclei), and Table 5 (induction of SCE). Usually the compounds were tested up to their cytotoxic limits. (These limits varied with the end point. For the detection of gene mutations, a substantial fraction of the cell population has to survive for many generations, whereas initial growth retardation is acceptable. Long-term survival is dispensable for the cytogenetic assays, but two rounds

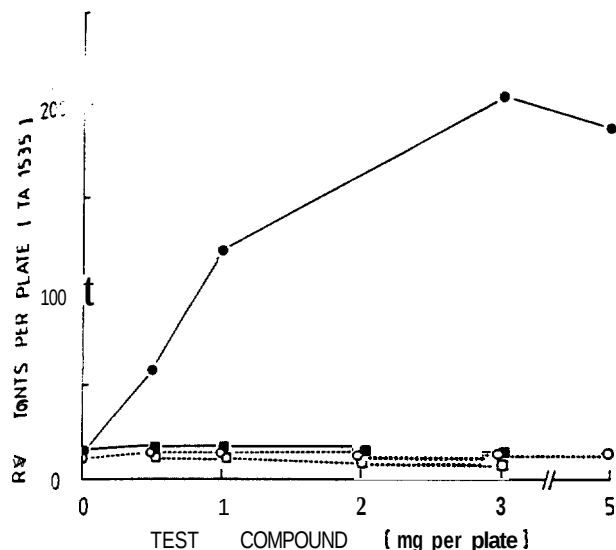


FIGURE 3. Mutagenicity of *trans*-benzene-1,2-dihydrodiol (●, ○) and catechol (■, □) in *Salmonella typhimurium* TA1535 in the presence (closed symbols) and absence (open symbols) of NADPH-fortified post-mitochondrial supernatant (S9) fraction from liver homogenate of Aroclor 1254-treated male Sprague-Dawley rats. The plate-incorporation protocol was used and each plate received S9 equivalent to 100 mg liver tissue. Details of the assays were as described previously (4).

of replication are required for the detection of SCE and at least one cell division is necessary for the formation of micronuclei. In the SCE assay, but not in the micronucleus assay, cells fulfilling these requirements can be distinguished from other cells, permitting scoring at higher levels of cytotoxicity.)

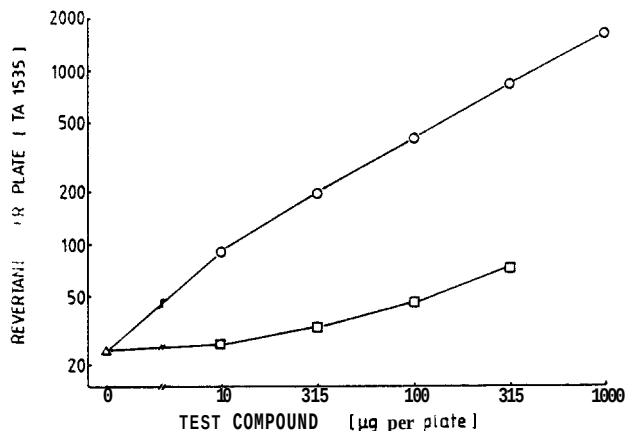


FIGURE 4. Mutagenicity of anti-benzene-diol epoxide (○) and *syn*-benzene-diol epoxide (□) in *Salmonella typhimurium* TA1535. The test compound (dissolved in 10 μ L dimethylsulfoxide:triethylamine, 1000:1), 100 μ L of the bacterial resuspension (1.04×10^8 colony forming units) and 500 μ L buffer (150 mM KCl, 10 mM sodium phosphate buffer, pH 7.4) were incubated for 20 min at 37°C. Soft agar was then added and the mixture poured onto minimal agar plates (27). After incubation for 3 days at 37°C in the dark, the colonies were counted. Values are means from two plates, the average standard deviation being 10.7% of the mean.

Table 2. Benzene dihydrodiols and diol epoxides as substrates of rat liver dihydrodiol dehydrogenase.^a

Compound	V_{max}/K_m , mL/min/mg protein
<i>trans</i> -1,2-Dihydrodiol	
Racemic	28
(1 <i>S</i>)-Enantiomer	60
(1 <i>R</i>)-Enantiomer	0.12
anti-Diol epoxide	
Racemic	0.14
(1 <i>S</i>)-Enantiomer	0.25
(1 <i>R</i>)-Enantiomer	0.014
<i>syn</i> -Diol epoxide	
Racemic	0.52

^aThe enzyme was purified to apparent homogeneity from rat liver (25). Activity was determined spectrophotometrically at 37°C by following NADPH production. The assay mixture contained NADP⁺ (2.3 mM), the substrate and Tris-HCl buffer (50 mM, pH 9.0).

First investigated was anti-diol epoxide, the putative principal active metabolite of benzene in the Ames test. Racemic anti-diol epoxide induced SCE, mutations to 6-thioguanine resistance, as well as micronuclei. Significant effects were observed at concentrations of 10, 50, and 250 μ M, respectively. A fourth end point, induction of mutations to ouabain resistance, was studied using the individual enantiomers of the anti-diol epoxide. Both enantiomers showed significant, but weak effects, as they did with regard to 6-thioguanine resistance (data not shown). The (1*R*) enantiomer was slightly more active than the (1*S*) enantiomer in respect to 6-thioguanine resistance, whereas the reverse was true with respect to ouabain resistance. The *syn*-diol epoxide was studied for induction of SCE and micronuclei. Ten times higher concentrations than of the anti-diol epoxide were required for the equal effect on SCE, whereas an induction of micronuclei was not observed even at very high concentration of the *syn*-diol epoxide.

Quinone was the most cytotoxic of all benzene derivatives tested. Much to our surprise, it was a potent inducer of gene mutations to 6-thioguanine resistance. Exposure at a concentration of 1 μ M led to a 100-fold increase in the mutation frequency above the control value (from 4 to 400 $\times 10^{-6}$). At the most effective concentration (1.7 μ M) it induced 700 mutants per 10^6 cells, which is about 10 times the effect of the anti-diol epoxide at the optimal concentration (400 μ M). Therefore, if effect and exposure concentration are taken into account, the quinone was about 2000 times more potent than the anti-diol epoxide. The mutagenic activity of quinone (410 mutations/ 10^6 cells/ μ M) was similar to those of some bay-region diol epoxides derived from polycyclic aromatic hydrocarbons. Under comparable, but not identical conditions, the anti- and *syn*-chrysene-1,2-diol 3,4-oxides and the anti- and *syn*-9-hydroxychrysene-1,2-diol 3,4-oxides induced 75, 9, 130, and 18 mutations/ 10^6 cells/ μ M, respectively (4). However, in contrast to diol epoxides derived from polycyclic aromatic hydrocarbons and benzene, quinone did not induce mutations to ouabain resistance. The ratio of the number of induced mutations, resistant to 6-thioguanine

Table 3. Induction of gene mutations (6-thioguanine resistance) in V79 cells by benzene derivatives.^a

Compound	Optimal/maximal concentration, μM	Mutation frequency above background $\times 10^6$	Potency ^b (induced mutation frequency $\times 10^6/\mu\text{M}$)
Phenol	4000	< 10	< 0.003
Hydroquinone	3.5	400	110
Quinone	1.7	700	410
2,2'-Dihydroxybiphenyl	1000	< 10	< 0.01
4,4'-Dihydroxybiphenyl	1000	< 10	< 0.01
<i>trans</i> -1,2-Dihydrodiol	8000	10	0.0013
Catechol	25	43	1.7
<i>anti</i> -Diol epoxide	400	74	0.19
1,2,4-Trihydroxybenzene	10	42	4.2
Duroquinone	250	< 10	< 0.04

^aMutagenicity was determined as described (32). Briefly, a total of 1.5×10^6 cells and 30 mL medium were put into 15-cm Petri dishes. After 18 hr, the test compound was added for 24 hr. The frequency of mutants resistant to 6-thioguanine (7 $\mu\text{g/mL}$) was determined after an expression period of 6 days. Each compound was tested at least at 5 concentrations, up to the limits of toxicity. Column 2 shows the highest concentration at which sufficient cells survived for appropriate determination of the mutation frequency. For all mutagens, this was the concentration leading to the strongest increase in the mutation frequency. This value, minus the mean mutation frequency of the solvent controls of the respective experiment, are shown in column 3. The mutation frequency of the 37 solvent control cultures of this study varied from 0.0 to 32.0×10^{-6} with a median of 8.7×10^{-6} . All positive test results were confirmed in repeat experiments. For negative test results the detection limit is shown.

^bThe effect (column 3) was divided by the concentration (column 2). Note that this measure of mutagenic activity does not take into account the stability of the test compound, its distribution between cells and medium, and potential nonlinearities in the concentration-response curves.

Table 4. Induction of micronuclei in V19 cells by benzene derivatives.^a

Compound	Optimal/maximal concentration, μM	Frequency of micronucleated cells $\times 10^3$ above background ^b
Phenol	4000	35
Hydroquinone	17.5 ^c	88
Quinone	6.7 ^c	27
<i>trans</i> -1,2-Dihydrodiol	4000	3
Catechol	25 ^c	35
<i>anti</i> -Diol epoxide	250	21
<i>syn</i> -Diol epoxide	1000	1
1,2,4-Trihydroxybenzene	25	52
1,2,3-Trihydroxybenzene	50 ^c	32
Duroquinone	50	30

^aInduction of micronuclei was studied as described (33). Briefly, a total of 2×10^5 cells and 4 mL medium were put into 6-cm Petri dishes. On the next day, the test compound was added, whereby at least 5 concentrations were used. 24 hr later, the cells were harvested, treated with hypotonic KCl-solution and fixed. After staining with Giemsa, 2000 cells were scored per data point for micronuclei. In control cultures, the frequency of micro-nucleated cells was usually approximately 10×10^{-3} . Column 2 shows the concentration which gave the strongest effect or, with the inactive compounds (*trans*-1,2-dihydrodiol and *syn*-diol epoxide), the highest concentration used.

^bExcept for *trans*-1,2-dihydrodiol and *syn*-diol epoxide, all values are considered outside accidental variation.

^cHigher concentrations were used; however, the frequency of the micronucleated cells declined toward the value of the solvent controls. This phenomenon is attributed to the inhibition of cell proliferation.

and ouabain, respectively, was 3 for the positive control, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, 4 for (1*S*)-*anti*-benzene-diol epoxide, about 20 for (1*R*)-*anti*-benzene-diol epoxide and > 100 for quinone. Furthermore, quinone did not induce a significant increase in SCE, and the frequency of micronucleated cells was increased by a factor of only three above spontaneous levels. Thus, quinone showed a much narrower spectrum of genotoxic activity in V79 cells than did the *anti*-diol epoxide.

Hydroquinone was similar to quinone with regard to the induction of 6-thioguanine resistance, except that about three times higher concentrations were required.

Table 5. Induction of sister chromatid exchange (SCE) in V79 cells by benzene derivatives.^a

Compound	Optimal/maximal concentration, μM	Number of SCE per cell above background
Phenol	1000	2.2
Hydroquinone	20	3.4 ^c
Quinone	8	2.0
<i>trans</i> -1,2-Dihydrodiol	4000	2.0
Catechol	12.5	5.8 ^c
<i>anti</i> -Diol epoxide	100	15.5 ^c
<i>syn</i> -Diol epoxide	1000	10.6 ^c
1,2,4-Trihydroxybenzene	10	7.3 ^c
1,2,3-Trihydroxybenzene	25	3.2 ^c

^aInduction of SCE was studied as described (33). Into 6-cm Petri dishes were given at the times shown in parentheses: 4 mL medium and 2×10^5 cells (0 hr), the test compound (24 hr), 10 μM 5-bromo-2'-deoxyuridine (25 hr), and 0.2 $\mu\text{g/mL}$ colcemid (47 hr). Cells were harvested (51 hr), treated with hypotonic XCl, fixed, and stained using the Hoechst 33258/UV/Giemsa procedure. A total of 30 metaphases was scored for SCE per data point. With all compounds, including those without a significant effect, the number of SCEs was highest at the highest concentration with differential staining. The number of SCEs (mean $\pm$ SD) in the control cultures of this study varied from 6.2 ± 2.9 to 8.4 ± 2.6 . The t-test was used for statistical analysis.

^bStatistically significant, $p > 0.05$.

^cStatistically significant, $p > 0.01$.

However, hydroquinone was substantially more effective than quinone in the induction of micronuclei. It was a better inducer of SCE as well.

Catechol induced all three genotoxic effects studied. The main difference it had from its isomer, hydroquinone, was its low efficiency in the induction of 6-thioguanine-resistant mutants. In its relative activities in the induction of gene mutations, SCE, and micronuclei, it was more similar to the *anti*-diol epoxide than to hydroquinone and quinone. In addition, it arrested cells in the first mitosis (data not shown). This was the reason it could be studied only at relatively low concentrations in the SCE assay.

Phenol and the *trans*-1,2-dihydrodiol showed very low

cytotoxicity. Genotoxic effects were observed only at very high concentrations. Phenol was a weak inducer of micronuclei, while the *trans*-1,2-dihydrodiol led to a slight increase in the frequency of 6-thioguanine-resistant cells. It is not known whether these effects were caused by the test compound itself, metabolites, or impurities. A very small amount of metabolites or impurity may be sufficient, since the mutagenic activity of the *trans*-1,2-dihydrodiol and the micronucleus-inducing activity of the phenol were less than 1% of the activities of the potential metabolites, catechol and anti-diol epoxide, and hydroquinone, respectively.

The minor benzene metabolites 2,2'-dihydroxybiphenyl, 4,4'-dihydroxybiphenyl, and 1,2,4-trihydroxybenzene did not show any particularly marked effects. 1,2,4-Trihydroxybenzene induced gene mutations and SCE and micronuclei, the activities being similar to those of catechol. The dihydroxybiphenyls were much less cytotoxic than the monocyclic compounds substituted with two or three phenolic hydroxyl groups, and in contrast to these compounds they did not induce gene mutations.

1,2,3-Trihydroxybenzene was studied for induction of SCE and of micronuclei. In both systems, it showed positive responses, which however were weaker than those of its isomer 1,2,4-trihydroxybenzene.

Duroquinone differs from quinone in that it does not conjugate with glutathione (28). However, in hepatocytes, it oxidized glutathione, suggesting that it underwent redox cycling with formation of reactive oxygen (28). Duroquinone was much less cytotoxic in V79 cells than was quinone. It did not induce gene mutations with a detection limit that was 10,009 times below the activity of quinone; however, it increased the frequency of micronucleated cells.

Conclusions

It has been demonstrated in the present study that metabolites of benzene can induce heritable functional changes (gene mutations) in bacterial and mammalian cells. Moreover, the results of the bacterial mutagenicity experiments provide circumstantial evidence for the idea that a diol epoxide is formed from benzene and accounts for its mutagenicity. The anti-diol epoxide showed a broad spectrum of genotoxic activities in mammalian and bacterial cells and was resistant to metabolic detoxification, at least as far as the systems operative in the S9 mix were concerned. Partial detoxification may occur in tissues with high activity of dihydrodiol dehydrogenase, as is the case in rat liver. However, the (1*R*) enantiomer of the *trans*-diol epoxide is a relatively poor substrate. This is the enantiomer that is expected to be formed preferentially due to stereoselective metabolism of benzene to the (1*R*)-*trans*-1,2-dihydrodiol (24).

After application of benzene to animals, a high proportion of the dose can be recovered in the form of unmetabolized benzene, phenol, hydroquinone, catechol, and their conjugates. It is unlikely that these metabolites are formed from a diol epoxide. In contrast, 1,2,4-trihydroxy-

benzene is both a metabolite of benzene and a product formed from the anti-diol epoxide by dihydrodiol dehydrogenase. However, alternative pathways for its formation, not involving a diol epoxide intermediate, are conceivable.

While it is obvious that diol epoxides are not major metabolites in the animal as a whole, the situation may be different in an individual tissue. For example, in isolated hepatocytes from phenobarbital-treated rats, *trans*-1,2-dihydrodiol and catechol, which appeared to be formed exclusively via the *trans*-1,2-dihydrodiol, made up 26% of the metabolites from benzene (26). This is much more than the contribution of catechol, 1,2,4-trihydroxybenzene, and their conjugates to the recovered metabolites in animal studies. Moreover, there is no rationale for the concept that carcinogenesis is evoked by a major metabolite. This is true in particular for benzene, which is less potent by many orders of magnitude than other carcinogens. Hence, the available data on metabolism argue neither for nor against a possible role for diol epoxides in benzene carcinogenesis. Clarification could be sought in metabolism and carcinogenicity studies with authentic diol epoxides and analytical studies on benzene using authentic diol epoxide metabolites and nucleoside adducts as standards.

The diol epoxides were not the only mutagenic benzene derivatives in this study. Even more striking and impressive were the high activities of quinone and hydroquinone in the mutation to 6-thioguanine resistance. Quinone showed a narrow spectrum of genotoxic activities. The lack of any appreciable activity in *S. typhimurium* may be attributed to toxicokinetic differences from V79 cells. However, such speculations cannot explain the lack of induction of ouabain resistance and SCE in the V79 cells. Resistance to 6-thioguanine usually involves failure of synthesis, or defect in function, of the enzyme hypoxanthine phosphoribosyl transferase. Such purely destructive effects can be achieved by numerous types of mutations, including base substitutions, frameshifts, and large deletions. The lack of induction of ouabain resistance (usually involving base substitution) and the weakness of the cytogenetic effects point to an unknown, unusual, and very selective mechanism for the quinone mutagenicity.

Another aspect of the mechanism involves the active species. Quinones are electrophiles and may undergo addition reactions with nucleophiles. Furthermore, quinones may be reduced to semiquinone free radicals, e.g., through the action of one electron reductases, such as cytochrome P-450 reductase, an enzyme present in V79 cells (I. Gemperlein, F. Oesch, and H. R. Glatt, unpublished result). The semiquinone could then react with the target molecule directly or via the formation of reactive oxygen species. Duroquinone cannot undergo addition reactions, but may undergo redox cycling in cells with formation of active oxygen (28). The lack of mutagenicity of duroquinone argues therefore against a role for active oxygen in the mutagenicity of quinone. Further support for this notion is provided in an accompanying paper, dealing with the mechanism of quinone mutagenicity (29).

The potent mutagenicity of hydroquinone is especially

noteworthy, as this compound can be directly isolated from the tissues and urine of animals treated with benzene, whereas the evidence for the formation of quinone is only indirect. Hydroquinone and its conjugates form the second major group of benzene metabolites, next to phenol and its conjugates. Since the chemical reactivity of hydroquinone is low, one may suspect that it is metabolically activated in the V79 cells. Identification of the involved enzyme(s) should be of the greatest interest, as hydroquinone is available in body fluids and tissues of benzene-treated animals. Hydroquinone was nearly as effective as quinone in the induction of 6-thioguanine resistance, but slightly higher doses were required. However, hydroquinone differed in its genotoxicity from quinone in that it induced micronuclei with a much higher efficiency, and in that in addition it induced SCE. Therefore, it does not appear likely that all the effects of quinone and all the effects of hydroquinone are induced by a single chemical species.

A further benzene metabolite for which mutagenic activity was observed is catechol. Like the hydroquinone, it induced SCE as well as micronuclei and 6-thioguanine resistance. However, its effectiveness in the induction of 6-thioguanine resistance was substantially weaker.

Several additional benzene metabolites showed genotoxic effects. These activities, however, were weak, at least when the potential contribution of the compounds in the benzene metabolism is taken into account. We would therefore not rank them among the prime candidates for the carcinogenic metabolite(s) of benzene if genotoxicity is the essential mechanism. In this respect, preferential attention should be given to quinone, hydroquinone (with semiquinone as a potential active species), the anti-diol epoxide, catechol, and perhaps muconaldehyde (15), which was not included in this study. Considering the qualitative differences in genotoxicity and postulating the requirement of several distinct mutations, it is possible that different metabolites are involved at different stages in benzene-induced carcinogenesis.

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REFERENCES

- Conney, A. H. Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons. *Cancer Res.* 42: 4875-4912 (1982).
- Glatt, H. R., and Oesch, F. Structural and metabolic parameters governing the mutagenicity of polycyclic aromatic hydrocarbons. In: *Chemical Mutagens: Principles and Methods for Their Detection*, Vol. 10 (F. J. de Serres, Ed.), Plenum, New York, 1986, pp. 73-127.
- Heidelberger, C., Freeman, A. E., Pienta, R. J., Sivak, A., Bertram, J. S., Castro, B. C., Dunkel, V. C., Francis, M. W., Kakunaga, T., Little, J. B., and Schechtman, L. M. Cell transformation by chemical agents: a review and analysis of the literature. *Mutat. Res.* 114: 283-385 (1983).
- Glatt, H. R., Seidel, A., Bochnitschek, W., Marquardt, H., Marquardt, H., Hodgson, R. M., Grover, P. L., and Oesch, F. Mutagenic and cell-transforming activities of triol-epoxides as compared to other chrysene metabolites. *Cancer Res.* 44: 4556-4565 (1984).
- Marshall, J. C., Voudsen, K. H., and Phillips, D. H. Activation of c-Ha-ras-1 proto-oncogene by *in vitro* modification with a chemical carcinogen, benzo(a)pyrene diol-epoxide. *Nature* 310: 586-588 (1984).
- Dean, B. J. Recent findings on the genetic toxicology of benzene, toluene, xylenes and phenols. *Mutat. Res.* 154: 153-181 (1986).
- Tice, R., Costa, D., and Drew, R. Cytogenetic effects of inhaled benzene in murine bone marrow: induction of sister chromatid exchanges, chromosomal aberration, and cellular proliferation inhibition in DBA/2 mice. *Proc. Natl. Acad. Sci. (U.S.A.)* 77: 2148-2152 (1980).
- Siou, G., Conan, L., and el Haitem, M. Evaluation of the clastogenic action of benzene by oral administration with 2 cytogenetic techniques in mouse and Chinese hamster. *Mutat. Res.* 90: 273-278 (1981).
- Morimoto, K., and Wolff, S. Increase of sister chromatid exchange and perturbations of cell division kinetics in human lymphocytes by benzene metabolites. *Cancer Res.* 40: 1189-1193 (1980).
- Tunek, A., Hogstedt, B., and Olofsson, T. Mechanism of benzene toxicity, effects of benzene and benzene metabolites on bone marrow cellularity, number of granulopoietic stem cells and frequency of micronuclei in mice. *Chem.-Biol. Interact.* 39: 129-138 (1982).
- Morimoto, K. Induction of sister chromatid exchanges and cell division delays in human lymphocytes by microsomal activation of benzene. *Cancer Res.* 43: 1330-1334 (1983).
- Morimoto, K., Wolff, S., and Koizumi, A. Induction of sister chromatid exchanges in human lymphocytes by microsomal activation of benzene metabolites. *Mutat. Res.* 119: 355-360 (1983).
- Erexson, G. L., Wilmer, J. L., and Kligerman, A. D. Sister chromatid exchange induction in human lymphocytes exposed to benzene and its metabolites *in vitro*. *Cancer Res.* 45: 2471-2477 (1985).
- Aleksejczyk, R. A., Berchtold, G. A., and Braun, A. G. Benzene, diol epoxides. *J. Am. Chem. Soc.* 107: 2554-2555 (1985).
- Latrino, L., Goldstein, B. D., and Witz, G. Formation of muconaldehyde, an open-ring metabolite of benzene, in mouse liver microsomes: an additional pathway for toxic metabolites. *Proc. Natl. Acad. Sci. (U.S.A.)* 83: 8356-8360 (1986).
- Lebowitz, H., Brusick, D., Matheson, D., Jagannath, D. R., Reed, M., Goode, S., and Roy, G. Commonly used fuels and solvents evaluated in a battery of short-term bioassays. *Environ. Mutagen.* 1: 172-173 (1979).
- Bartsch, H., Malaveille, C., Camus, A. M., Martel-Planche, G., Brun, G., Hautefeuille, A., Sabadie, N., Barbin, A., Kuroki, T., Drevon, C., Piccoli, C., and Montesano, R. Validation and comparative studies on 180 chemicals with *S. typhimurium* strains and V79 Chinese hamster cells in the presence of various metabolizing systems. *Mutat. Res.* 76: 1-50 (1980).
- Shimizu, M., Yasui, Y., and Matsumoto, N. Structural specificity of aromatic compounds with special reference to mutagenic activity in *Salmonella typhimurium*: a series of chloro- or fluoro-nitrobenzene derivatives. *Mutat. Res.* 116: 217-238 (1983).
- DeFlora, S., Zanicchi, P., Camoirano, A., Bennicelli, C., and Badolati, C. S. Genotoxic activity and potency of 135 compounds in the Ames reversion test and in a bacterial DNA-repair test. *Mutat. Res.* 133: 161-198 (1984).
- Ashby, J., de Serres, F. J., Draper, M., Ishidate, M., Jr., Margolin, B. H., Matter, B. E., and Shelby, M. D. Evaluation of Short-Term Tests for Carcinogens: Report on the International Programs on Chemical Safety Collaborative Study on *In Vitro* Assays. Elsevier, Amsterdam, 1985.
- Jung, R., Beermann, D., Glatt, H. R., and Oesch, F. Mutagenicity of structurally closely related oxiranes: derivatives of benzene and its hydrogenated congeners. *Mutat. Res.* 81: 11-19 (1981).
- Kinoshita, T., Santella, R., Pulkrabek, P., and Jeffrey, A. Benzene oxide: genetic toxicity. *Mutat. Res.* 91: 99-102 (1981).
- Green, M. H. L., and Muriel, W. J. Mutagen testing using trp⁺ reversion in *Escherichia coli*. *Mutat. Res.* 38: 3-32 (1976).
- Jerina, D. M., Ziffer, H., and Daly, J. W. The role of the arene oxide-oxepin system in the metabolism of aromatic systems: IV. Stereochemical considerations of dihydrodiol formation and dehy-

- drogenation. *J. Am. Chem. Soc.* 92: 1056-1061 (1970).
25. Vogel, K., Bentley, P., Platt, K.-L., and Oesch, F. Rat liver cytoplasmic dihydrodiol dehydrogenase: purification to apparent homogeneity and properties. *J. Biol. Chem.* 255: 9621-9625 (1980).
26. Billings, R. E. Mechanisms of catechol formation from aromatic compounds in isolated hepatocytes. *Drug Metab. Dispos.* 13: 287-290 (1985).
27. Glatt, H. R., Cooper, C. S., Grover, P. L., Sims, P., Bentley P., Merles, M., Waechter, F., Vogel, K., Guenther, T. M., and Oesch, F. Inactivation of a diol-epoxide by dihydrodiol dehydrogenase, but not by two epoxide hydrolases. *Science* 215: 1507-1509 (1982).
28. Rossi, I., Moore, G. A., Orrenius, S., and O'Brien, P. J. Quinone toxicity in hepatocytes without oxidative stress. *Arch. Biochem. Biophys.* 251: 25-35 (1986).
29. Ludewig, G., Dogra, S., and Glatt, H. R. Genotoxicity of 1,4-benzoquinone and 1,4-naphthoquinone in relation to effects on glutathione and NADP(H) levels in V79 cells. *Environ. Health Perspect.* 82: m-228 (1989).
30. Oesch, F., Protic-Sabljic, M., Friedberg, T., Klinisch, H.-J., and Glatt, H. R. Vinylidenechloride: changes in drug-metabolizing enzymes, mutagenicity and relation to its targets for carcinogenesis. *Carcinogenesis* 4: 1031-1038 (1983).
31. Glatt, H. R., Shtelzer, S., Sheradsky, T., Blum, J., and Oesch, F. Mutagenicity of N-substituted phenanthrene 9,10-oxides in *Salmonella typhimurium* and Chinese hamster V79 cells. *Environ. Mutagen.* 8: 829-837 (1986).
32. Glatt, H. R., and Oesch, F. Activation of N-acetoxy- and N-hydroxy-2-acetylaminofluorene to mutagenic and cytotoxic metabolites by V79 Chinese hamster cells. *Mutat. Res.* 149: 265-269 (1985).
33. Ludewig, G., Dogra, S., Seidel, A., Oesch, F., and Glatt, H. R. Quinones derived from polycyclic aromatic hydrocarbons: induction of diverse mutagenic and genotoxic effects in mammalian cells. In: *Proceedings of the 11th International Symposium on Polynuclear Aromatic Hydrocarbons* (M. Cooke and A. J. Dennis, Eds.), Battelle, Columbus, OH, in press.