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Toxicity of Aromatic Aerobic Biotransformation Products of Toluene to Hela Cells

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Petroleum contamination of groundwater is widely recognized as a serious environmental problem. Toluene (methylbenzene) occurs naturally in crude oil and is commonly found as a contaminant in the subsurface as a result of waste disposal and storage activities. Biological transformation and volatilization are the major removal mechanisms for toluene contamination of soils and groundwater. Biological transformation may be expedited through the introduction of additional oxygen, alternate electron acceptors, and/or nutrients. Toluene can be aerobically transformed via five pathways, with initial hydroxylation at the *ortho* (Shields et al. 1989), *meta* (Kaphammer et al. 1991), or *para* (Whited and Gibson 1991) positions, on the methyl group (Worsey and Williams 1975), or with deoxygenation at the 2,3 positions (Gibson et al. 1979). The major biotransformation products include catechol (CAT), 3-methylcatechol (3MC), 4-methylcatechol(4MC), *o*-cresol (OC), *m*-cresol (MC), *p*-cresol (PC), benzaldehyde (BZ), and methyl benzoate (MB). One of the concerns raised is the potential hazard of major biotransformation products (Aust et al. 1994).

The purpose of this investigation was to test the cytotoxicity of these aromatic intermediates of aerobic microbial transformation of toluene in Hela cells. Inhibition of cell growth, protein synthesis, and neutral red uptake were used in the present study as parameters of cytotoxicity induced by these transformation products.

MATERIALS AND METHODS

The chemicals selected for testing were purchased from Aldrich Chemical Co. (Milwaukee, WI) and had purity of 95% or better. Culture medium, protein reagent and neutral red reagent were obtained from Sigma Chemical Company (St. Louis, MO). Stock solutions were prepared in dimethyl sulfoxide (DMSO), and diluted in Eagle's minimum essential medium. The final DMSO concentration in the medium was less than 0.05%, which did not alter cell growth as compared to cultures without DMSO.

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Hela cells were originally obtained from the American Type Culture Collection (Rockville, MD). The cells were maintained as a monolayer and routinely passed in Eagle's minimum essential medium supplemented with L-glutamine (0.292 g/L) and 10% heat-inactivated calf serum. The cells were grown at 37°C in an atmosphere of 5% CO_2-95% air and 95-100% humidity. After 24 hr of exposure with different chemicals, cytotoxicity was determined by measuring three endpoints.

Trypan blue exclusion was used to evaluate cell growth. Cells (5×10^5) were inoculated into 60 mm tissue culture dishes with 5 ml medium and incubated for 24 hr. The medium was exchanged with the fresh medium containing 0.05% DMSO (control) or with the fresh medium containing varying concentrations of chemicals (3MC, 4MC and CAT, 0.5 to 50 µg/ml; OC, MC and PC, 50 to 1,000 µg/ml; MB and BZ, 500 to 2,000 µg/ml). The growth-inhibitory ratio [Y] for each concentration of test chemical was calculated using the equation Y(%)=(C-T)/(C-Co) x 100 where T is the cell count for each concentration of chemical after 24 hr exposure, C is the mean cell count for the control, and Co is the mean cell count at the start of chemical treatment (Susa et al. 1992). The cells were trypsinized and counted in a hemacytometer. The ID_{50} value (the concentration of the test compound required to induce a 50% inhibition in cell growth) served as an index of toxicity of these chemicals.

The protein content was used to evaluate the total mass of cells. To determine the protein content of Hela cells, the cells were seeded at 5×10^5 cells per 60 mm glass petri dish with 5 ml of the medium and incubated for 24 hr, after which the medium was removed and the cells were refed with fresh medium unamended or amended with varied concentrations of test chemical. After 24 hr of incubation, the cells were trypsinized and counted. Known quantities of cells were then washed three times in PBS (pH=7.4) and homogenized. The protein content was determined by protein measurement using Peterson's modification of the micro Lowry protein assay kit (Sigma Chemical Company, St. Louis, MO). Serial dilutions of bovine serum albumin were used for the protein standards.

Uptake of neutral red was used to evaluate cell viability (Nemes et al. 1979). Individual wells of 96-well microtiter plates were inoculated with 0.2 ml of medium containing 1×10^3 Hela cells. The plates were incubated at 37°C for 24 hr. Old medium was removed and the cells were refed with the fresh medium containing 0.05% DMSO (control) or with fresh medium containing varying concentrations of chemical. After 24 hr of exposure to test agents, the medium with chemical was removed and 0.33% neutral red solution was added in an amount equal to 10% of the culture medium volume. The cell cultures were returned to the incubator for 3 hr. At the end of the incubation period, the medium

was carefully removed and the cells quickly rinsed with a fixative (4% formaldehyde-1% $CaCl_2$) and then 0.2 ml of a mixture of 1% acetic acid-50% ethanol was added to each well to extract the dye. After 20 min at room temperature and rapid agitation on a microtitre plate shaker, the plates were transferred to a microplate reader equipped with a 540 nm filter to measure absorbance of the extracted dye (Borenfreund and Puerner 1985). Results are expressed as a percentage of the optical density determined with extracts from control cultures at 540 nm.

All experiments were performed four times. ID_{50}, EC_{50} (the concentration of the test compound required to induce a 50% inhibition in protein synthesis), NI_{50} (the concentration of the test compound required to induce a 50% inhibition in neutral red uptake), and 95% confidence units were calculated using pharmacologic calculations with computer programs (Tallarida and Murray 1986). The data are presented graphically as the means ± SD. The differences between the mean values for the data were evaluated by the Student t-test and $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Contamination from leaks, spills, and other unplanned releases of gasoline has become a major environmental problem. In recent years it has been recognized that biodegradation of environmental contaminants such as toluene is the most significant determination of eventual fate and impact (Aust et al. 1994). Biodegradation of toluene by bacteria under aerobic conditions has been studied for many years. Five pathways have been demonstrated for the aerobic metabolism of toluene (Gibson and Subramanian 1984). It has been shown that a variety of different microorganisms are able to metabolize toluene. In some cases even complete mineralization has been found, whereas in others only biotransformation reactions took place, producing more or less toxic metabolites (Gorontzy et al. 1994).

In vitro cytotoxicity assays are considered useful for detecting pollution in the environment and representing an important achievement in the environmental assessment of toxicants. Hela cell culture techniques can be performed easily and faster than *in vivo* techniques. Cell growth, protein content, and neutral red uptake are often used to measure cell viability and cellular proliferation. Our data showed that the concentrations of each biotransformation product causing a midpoint value, as compared to control value, for each cytotoxicity test varied with the specific assay. The concentrations of 3MC, 4MC, CAT, MC, OC, PC, BZ and MB causing a 50% inhibition in cell growth were 0.011, 0.039, 0.125, 2.841, 1.101, 2.529, 6.731 and 7.251 mM, respectively, causing a 50% decrease in protein content were 0.031, 0.049, 0.330, 4.623, 1.833, 3.056, 10.770 and 11.066

mM, respectively, and causing a 50% reduction in the absorbance of neutral red were 0.046, 0.088, 0.336, 2.937, 1.046, 1.658, 5.084 and 5.109 mM, respectively. CAT is about 11 times less toxic than 3MC in ID_{50}. 4MC is about 3.5 times less toxic than 3MC in ID_{50}, and MC, OC and PC are about 22 times, 9 times and 20 times less toxic than CAT in ID_{50}, respectively.

Cells exposed to differing concentrations of these transformation products produced dose-related inhibition of growth, protein synthesis, and neutral red uptake as indicated by ID_{50}, EC_{50} and NI_{50} values for 3-methylcatechol, 4-methylcatechol, catechol, *o*-cresol, *p*-cresol, *m*-cresol, benzaldehyde, and methyl benzoate (Table 1). The neutral red uptake inhibition data in cultured Hela cells correlated very well with cell growth and protein determination values. A linear correlation coefficient r^2 of 0.96 was obtained between the ID_{50} and the NI_{50} values for these biotransformation products. The relationships between the ID_{50} and ED_{50} values (r^2=0.99), and the NI_{50} and EC_{50} values (r^2=0.98) were also linear. The NI_{50} values of these compounds were compared with the ID_{50} and the ED_{50} values. Cell growth ratio seems more sensitive than protein determination in the greater cytotoxicities (NI_{50} < 100µg/ml). Neutral red uptake seems more efficiency in intermediate (NI_{50} < 300µg/ml) and the least toxic biotransformation products (NI_{50} > 500µg/glml) than protein determination in Hela cells. Neutral red uptake assay is an acute toxicity assay that provides information on the general, overall, toxic potential of a test agent. This assay is based on the incorporation of the neutral red into lysosomes of viable cells after incubation of the cell culture with test chemicals. The advantages of this method are the economical and rapid assay as well as the preciseness of measurement. Figure 1 demonstrates the types of dose-response cytotoxicity curves that can be generated with neutral red uptake and shows the relative cytotoxicities to Hela cells of these biotransformation products. 3MC was most toxic and MB was the least toxic. The sequence of potency for them was 3MC > 4MC > CAT > OC > PC > MC > BZ > MB.

Hydrophobicity was measured as octanol/water partition coefficient (Log K_{ow}) and the toxicity as Log NI_{50}^{-1} values for test agents (Table 2). K_{ow} of a chemical refers to the ratio of its solubility in octanol and in water. The octanol/water partition coefficients of these toluene transformation products were obtained from the publication of Schultz et al. (1996). In general, toxicity is correlated with hydrophobicity. The order of toxicity in our results did not appear to be a function of the degree of hydrophobicity. Log K_{ow} values of CAT, 4MC and 3MC are 0.81, 1.46 and 1.46. Their Log NI_{50}^{-1} values are 0.474, 1.056 and 1.337. These values seem more toxic than those expected from their Log $_{Kow}$ values. Catechols are thought to be reactive toxicants. They have the ability to tautomerize and form the reactive quinone moiety, and can exhibit excess toxicity (Lipnick et al. 1987).

Table 1. ID_{50}, EC_{50}, and NI_{50} values and 95% confidence limits obtained for Hela cells following 24 hr treatment with biotransformation products of toluene. Values are expressed in μg/ml.

Chemicals	ID_{50} (cell growth ratio)	EC_{50} (protein content)	NI_{50} (neutral red uptake)
3-methylcatechol (3MC)	1.40 (0.88-2.23)	[a]3.86 (3.19-4.66)	[a,b]5.76 (3.10-10.69)
4-methylcatechol (4MC)	4.80 (4.18-5.52)	6.05 (3.78-9.68)	[a,b]10.92 (8.21-14.51)
catechol (CAT)	13.79 (11.25-16.89)	[a]36.35 (21.16-62.45)	[a]37.04 (20.83-65.84)
m-cresol (MC)	307.19 (264.55-356.71)	[a]499.93 (461.67-541.46)	[b]317.63 (149.63-674.26)
o-cresol (OC)	119.04 (73.16-193.68)	[a]198.25 (146.36-268.54)	[b]113.08 (60.41-211.68)
p-cresol (PC)	273.45 (194.67-384.09)	330.46 (252.32-432.80)	[a,b]179.33 (79.23-405.88)
benzaldehyde (BZ)	714.37 (631.74-807.82)	[a]1142.99 (989.99-1319.62)	[b]619.73 (574.78-668.19)
methyl benzoate (MB)	987.19 (605.14-1610.43)	[a]1506.58 (1349.27-1682.22)	[a,b]683.30 (466.46-1000.91)

[a] significant differences with ID_{50}
[b] significant differences with EC_{50}

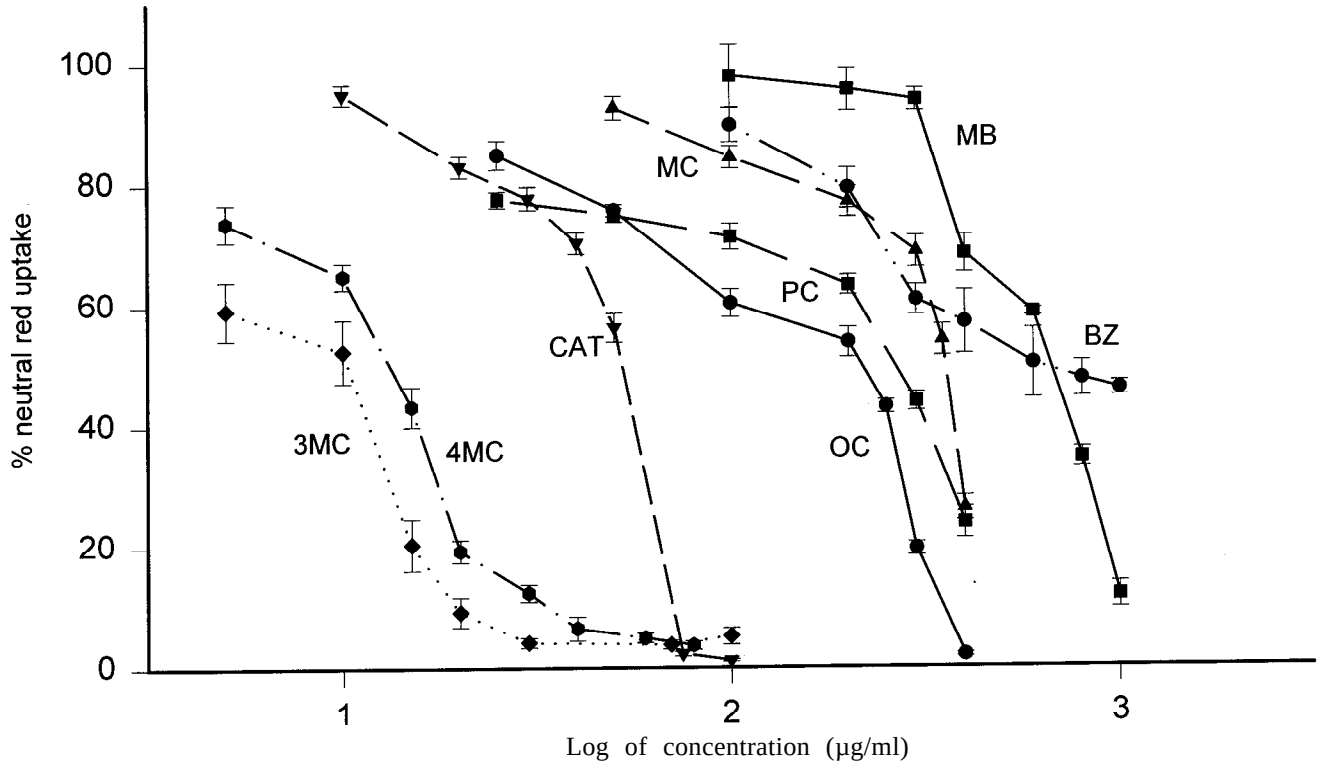

Figure 1. Effects of 24 hr treatment with various concentrations of 3-methylcatechol (3MC), 4-methylcatechol (4MC), catechol (CAT), *o*-cresol (OC), *m*-cresol (MC), *p*-cresol (PC), benzaldehyde (BZ) and methyl benzoate (MB) on neutral red uptake of Hela cells. Each point represents the mean of 4 independent determinations (±SD).

Table 2. Toxicity and hydrophobicity of eight aerobic biotransformation products of toluene.

Chemicals	Log NI_{50}^{-1}	Log K_{ow}
3-methylcatechol(3MC)	1.337	1.46
4-methylcatechol(4MC)	1.056	1.46
catechol (CAT)	0.474	0.81
m-cresol (MC)	-2.502	2.12
o-cresol (OC)	-2.053	2.12
p-cresol (PC)	-2.254	2.12
benzaldehyde (BZ)	-2.792	1.45
methyl benzoate (MB)	-2.835	2.11

These compounds are considered to act as soft electrophiles (Hermens 1990). *m*-cresol, *o*-cresol and *p*-cresol, like most phenols, may act as polar narcotics and use a reversible mechanism of action with a structure-toxicity relationship that is hydrophobic-dependent. Methyl benzoate is considered to elicit its effect via the nonpolar narcotic mechanism of action.

In summary, the toxicity of the major aromatic intermediates of aerobic transformation products of toluene was evaluated in Hela cells. They can elicit different types of cytotoxicity. The results of the study indicate a direct relationship between the exposure concentration of these intermediates and observed cytotoxic effects. These data can serve as a basis for development of toxicity tests in animals and in ecosystems.

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