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Toxicological Assessment in *Tetrahymena* of Intermediates in Aerobic Microbial Transformation of Toluene and p-Xylene

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The Toxic Release Inventory, issued by the U.S. Environmental Protection Agency chronicles more than 300 chemicals routinely released into the environment by 20,000 industrial facilities. Priority chemicals identified from this inventory include aromatic solvents, such as toluene and xylenes. These aromatic solvents are common environmental pollutants that are currently found in many hazardous waste sites and are a primary concern as aquifer contaminants. As the methodologies of bioremediation move from the flask to the field, one of the concerns raised is the potential hazard of major intermediates in microbial transformation of pollutants. In the case of aromatic solvents, the type and position of substituents on the ring influences the pathway and, thereby, the specific intermediates formed in biotic transformation.

Toluene ($C_6H_5CH_2$) can be aerobically transformed via five pathways. The TOD-pathway (Gerben et al. 1988) is initiated by the simultaneous addition of two atoms of oxygen. This pathway is unique to procaryotes and leads to the formation of 3-methylcatechol (i.e., the ortho-dihydroxy derivative) via a dihydrodiol intermediate. The second major pathway is the TOL-pathway (Worsey and Williams 1975). In this pathway, toluene is converted to benzoic acid and m-cresol prior to transformation to catechol. The remaining pathways also involve the monooxygenation of toluene. In the first of these, the major intermediates are benzyl alcohol, benzaldehyde, methyl benzoate and finally catechol (Rochkind et al. 1986). It has been shown that toluene can be converted to p-cresol prior to conversion to the corresponding alcohol, aldehyde and benzoate derivatives (Whited and Gibson 1991). In another pathway, the primary intermediates are o-cresol and m-cresol which via a second monooxygenase reaction give rise to 3-methylcatechol and 4-methylcatechol, respectively (Kukor and Olson 1991). In the final pathway, toluene is converted to p-cresol prior to formation of 3,4-dihydroxybenzoic acid (Whited and Gibson 1991).

An examination of the literature reveals that in the aerobic biotransformation of toluene and xylenes the same enzyme systems are used, and therefore, similar derivatives are formed. The microbial transformation of p-xylene (C_6H_5-1,4-

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CH_2) via the TOD-pathway leads to the formation of 3,6-dimethylcatechol or 2,5-dimethylphenol (Gibson et al. 1974). Transformation via the TOL-pathway results in the formation of 4-methylbenzyl alcohol which is oxidized to p-tolualdehyde, p-toluic acid and eventually 4-methylcatechol (Worsey and Williams 1975). Para-toluic acid can be transformed to p-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid (Smith 1990).

The purpose of this investigation was to determine the toxicity of aromatic intermediates of aerobic microbial transformation of toluene and p-xylene. Observed toxicities of the *Tetrahymena* population growth impairment assay were related to hydrophobicity and mechanisms of action.

MATERIALS AND METHODS

The chemicals selected for testing form a series of substituted benzenes which have been identified as major intermediates in the aerobic transformation by bacteria of either toluene or p-xylene. Each was purchased from Aldrich Chemical Co., Milwaukee, Wisconsin, USA or MTM Research Chemicals Lancaster Synthesis Inc., Windham, New Hampshire, USA, and had a purity of 95 % or better. Stock solutions were prepared in dimethylsulfoxide (DMSO) at concentrations of 5, 10, 25, or 50 g/L. In all cases, the volume of stock solution added to each flask was not in excess of 0.75 %, DMSO. This concentration does not alter *Tetrahymena* population growth (Schultz and Cajina-Quezada 1982).

The population-growth impairment testing was done in batch using *Tetrahymena pyriformis* (Schultz et al. 1990). Briefly, this 2-d assay uses population density measured spectrophotometrically at 540 mn as its endpoint. Tests were conducted at 27 +/- 1 °C in 250-mL Erlenmeyer flasks containing 50 mL of sterile, pH 7.35 medium. Each flask was inoculated with log-phase culture to attain a starting concentration of ~2,500 cells/ml. Each chemical was tested in a range-finder, followed by testing in duplicate for three or more replicates. Each replicate was a 5 to 10-step concentration series using freshly prepared stock solutions. Only replicates with control values in late log-growth-phase (absorbance from 0.6 to 0.9) were used in the analyses.

The 50 % growth inhibitory concentration (IGC_{50}) and 95 % fiducial limits were determined for each metabolite using Probit Analysis of Statistical Analysis System (SAS) software (SAS Institute Inc. 1989). The dependent variable was the absorbance normalized as percentage of control, and the independent variable was the toxicant concentration in mg/L. The 1-octanol/water partition coefficient value (log K_{ow}) of each chemical was obtained from MEDCHEM CLOGP version 3.53 (Leo and Weininger 1988).

RESULTS AND DISCUSSION

The toxicity and hydrophobicity of the 21 aromatic compounds evaluated in this study are summarized in Table 1. Hydrophobicity measured as log K_{ow} covered three log units from 0.44 for 4-hydroxybenzyl alcohol to 3.44 for p-xylene.

Toxicity as log IGC_{50}^{-1} varied over two orders of magnitude. 4-hydroxybenzyl alcohol was not toxic at saturation. In general, toxicity was correlated with hydrophobicity. The major exception was the catechols, which were more toxic than expected from their log K_{ow} values.

As noted by McFarland (1970), the manifestation of a toxic effect is preceded by two processes. First, the penetration of the toxicant to the molecular site of action, and second, the interaction of the toxicant with the site. Penetration is modeled by hydrophobicity , whereas, interaction is modeled by stereoelectronic parameters. For those industrial organic chemicals exhibiting the narcosis mode of toxic action, log K_{ow} has been a quality molecular descriptor. These toxicants are unreactive, and the interaction of the toxicant with the site of action is null or minimal. However, there are a number of cases where the observed toxicity of chemicals was significantly higher than predicted by narcosis models. These chemicals are most often thought to be reactive and irreversibly bound to macromolecules. Reactivity encompasses a variety of competing electrophilic, redox and free radical processes. The most common type of reactivity appears to be soft electrophilicity.

The aromatic solvents toluene and p-xylene, compounds 1 and 16, respectively, are considered to act as nonpolar narcotics (Könemann 1981). Nonpolar narcosis is a reversible mechanism of action and is considered baseline toxicity. The fact that structure-toxicity relationship for nonpolar narcotics is a simple hydrophobic-dependent model log $IGC_{50}^{-1} = 0.83 (\log K_{ow}) - 2.07$ (Schultz et al. 1990), suggests that the rate-limiting step for this mechanism of action is the ability of the toxicant to reach the site of action.

The addition of oxygen to these monoaromatic compounds and subsequent biotransformation results in the formation of chemicals from a number of chemical classes. The unsubstituted, alkyl-substituted carboxylic acids, compounds 3, and 20, aryl alcohols, compounds 6 and 18, and ester, compound 8, also were considered to elicit their effect via the nonpolar narcotic mechanism of action. Observed toxicities match well with those predicted by the above noted nonpolar narcosis model.

The presence of different chemical classes also resulted in the expression of different mechanisms of toxic action. The chemical class represented most often in this study was the phenols. Chemicals included in this class were compounds 4, 9-13, 15, 17, and 21. Most phenols act as polar narcotics (Schultz et al. 1990). Polar narcosis, like its nonpolar counterpart, is a reversible mechanism of action with a structure-toxicity relationship that is hydrophobic-dependent, log $IGC_{50}^{-1} = 0.58 (\log K_{ow}) - 0.96$ (Schultz et al. 1990). However, polar narcotics are more water soluble and more toxic than nonpolar narcotics. Kamlet et al. (1986) showed that the observed increased toxicity of polar narcotics, when compared with nonpolar narcotics, was due to greater dipolarity and/or hydrogen bond donor acidity of the former.

The aromatic aldehydes, compounds 7 and 19, and the catechols, compounds 2, 5, and 14, are thought to be reactive toxicants (Hermens 1990). As reactive

Table 1. Toxicity and hydrophobicity of major aromatic aerobic biotransformation products of toluene and p-xylene

Chemical	CAS[a] #	IGC_{50} (mg/L)	95% conf. limits	IGC_{50} (mM)	log IGC_{50}^{-1}	log K_{ow}
1. toluene	108-88-3	289.00	249.31-310.52	3.137	-0.4965	2.79
2. 3-methylcatechol	488-17-5	65.17	43.73-86.37	0.525	0.2799	1.46
3. benzoic acid	65-85-0	251.67	197.27-298.89	2.061	-0.3140	1.87
4. m-cresol	108-39-4	121.05	100.70-152.49	1.120	-0.0491	2.12
5. catechol	120-80-9	57.14	39.81-88.37	0.519	0.2848	0.81
6. benzyl alcohol	100-51-6	891.88	561.90-1168.90	8.092	-0.9081	1.10
7. benzaldehyde	100-52-7	166.73	124.71-198.49	1.357	-0.1326	1.45
8. methyl benzoate	93-58-3	233.13	192.25-291.10	1.712	-0.2335	2.11
9. p-cresol	106-44-5	157.00	105.99-200.58	1.452	-0.1619	2.12
10. 4-hydroxybenzyl alcohol	623-05-2	NTAS[b]	---	---	---	0.44
11. 4-hydroxybenzaldehyde	123-08-0	66.18	28.49-87.54	0.542	0.266	1.44
12. methyl 4-hydroxybenzoate	99-76-3	125.34	94.30-157.06	0.824	0.0841	1.98
13. o-cresol	95-48-7	213.15	165.33-275.48	1.971	-0.2948	2.12
14. 4-methylcatechol	452-86-8	53.25	22.95-77.36	.429	0.3675	1.46
15. 3,4-dihydroxybenzoic acid	99-50-3	4709.30	3427.80-5647.30	30.556	-1.4851	1.06
16. p-xylene	106-42-3	88.10	81.67-99.52	.830	0.0812	3.44
17 2,5-dimethylphenol	95-87-4	116.16	96.68-150.87	.951	0.0220	2.77
18. 4-methylbenzyl alcohol	589-18-4	379.80	305.38-456.64	3.109	-0.4926	1.66
19. p-tolualdehyde	104-87-0	137.08	97.64-167.78	1.064	-0.0269	2.01
20. p-toluic acid	99-94-5	213.42	163.16-261.88	1.568	-0.1952	2.53
21. p-hydroxybenzoic acid	99-96-7	967.97	663.37-1420.04	7.008	-0.8456	1.56

[a] Chemical Abstract Service registry number
[b] NTAS - Not Toxic at Saturation

chemicals, they should also exhibit excess toxicity (T_e). T_e is defined as the ratio of predicted toxicity to observed toxicity (Lipnick et al. 1987) with the predicted toxicity calculated from narcosis models. Reactivity is an irreversible, chemical toxicity mode of toxic action. While aromatic aldehydes are considered direct-acting, catechols have the ability to tautomerize and form the reactive quinone moiety (Lipnick et al. 1987). Thus, both groups are, or become a-b unsaturated compounds. Both types of compound are considered to act as soft electrophiles (Hermens 1990). In contrast to what has been observed with fish, aromatic aldehydes in the *Tetrahymena* assay model well as polar narcotics (Schultz et al. 1989). However, as observed in the present study, the toxicity of catechols was not predicted well by either of the above noted narcosis models. In the latter cases, the expected decrease in toxicity, due to a decrease in hydrophobicity, was offset by an increase in reactivity.

In summary, the toxicity of the major aromatic intermediates of aerobic microbial transformation of toluene and p-xylene was evaluated in the static *Tetrahymena pyriformis* population growth assay. The majority of these metabolites elicit narcosis toxicities that are directly related to hydrophobicity. The major exceptions were the ortho-dihydroxy derivatives. The expected decrease in toxicity resulting from a decrease in hydrophobicity was offset by an increase in reactivity due to quinone formation.

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