Bull. Environ. Contam. Toxicol. (1995) 54:662–667

Environmental Contamination and Toxicology

Developmental Hazard Assessment with FETAX: Aerobic Metabolites in Bacterial Transformation of Naphthalene

T. W. Schultz,[1] D. A. Dawson[2]

[1]College of Veterinary Medicine, The University of Tennessee, P.O. Box 1071, Knoxville, Tennessee 37901-1071, USA
[2]Department of Biology and Toxicology, Ashland University, Ashland, Ohio 44805, USA

Received: 20 May 1994/Accepted: 10 September 1994

The underlying principle of bioremediation is the capability of microorganisms to biodegrade pollutants. When a contaminated site is biotreated, it is usually assumed that the disappearance of the pollutant means a reduction in the toxic effects of the contaminants. However, pollutants can undergo partial biodegradation or biotransformation. Microbial-mediated transformations play a critical role in the toxic effects of pollutants, as any alteration in structure can result in a change in physicochemical properties which influence toxicity. Therefore, a relevant question is; what is the toxicity of accumulative metabolites relative to the parent chemical?

One class of chemicals that consistently appears at Superfund hazard waste sites is aromatic hydrocarbons. Studies of the aerobic bacterial metabolism of representative compounds, including benzene, naphthalene, and phenanthrene, have revealed similar oxidative pathways. Bacterial degradation of these aromatic hydrocarbons was initiated by the addition of two molecules of oxygen via a dioxygenase enzyme, with the resulting intermediate being converted to a catechol-like compound (Smith 1990). From a biotransformation standpoint, one of the more thoroughly studied aromatic hydrocarbons has been naphthalene. Cerniglia (1984) has identified five major intermediates, 1,2-dihydroxynaphthalene, salicylaldehyde, salicylic acid, gentisic acid and catechol in the aerobic bacterial degradation of naphthalene.

In vitro test systems such as the Frog Embryo Teratogenesis Assay - *Xenopus* (FETAX) provide a time- and resource-effective means for assessing developmental toxicity on a preliminary basis. FETAX (Dumont et al. 1983) is a 96-hr static-renewal system that uses early embryos of the frog *Xenopus laevis*.

The purpose of this investigation was to determine the developmental hazard, using FETAX, of exposure to the model aromatic hydrocarbon, naphthalene, and it's known major aerobic metabolites from bacterial transformation.

MATERIALS AND METHODS

The major intermediates of naphthalene biotransformation selected for evaluation in these investigations included naphthalene as well as 1,2-dihydroxynaphthalene, 2-hydroxybenzaldehyde (salicylaldehyde), 2-hydroxybenzoic acid (salicylic acid),

Correspondence to: T. W. Schultz

2,5-dihydroxybenzoic acid (gentisic acid) and 1,2-dihydroxybenzene (catechol). All chemicals were purchased from Aldrich Chemical Co., Milwaukee, Wisconsin, USA and tested without repurification.

Breeding and embryo selection were conducted under standard conditions (ASTM 1991). Briefly, adult male and female *Xenopus laevis* frogs were injected with 400-800 and 1000 international units of human chorionic gonadotropin, respectively, to induce amplexus and ovulation. Spawning took place in a false-bottom breeding chamber containing FETAX composition. Normally developing mid-to-late blastula stage embryos were selected with the aid of a dissecting microscope, following removal of the jelly coat with a 2% (w/v) cysteine solution at pH 8.1.

Embryos in groups of 25 were randomly placed in 60 mm Pyrex Petri dishes containing 10 mL of 8 to 10 concentrations of a graded concentration series for each test chemical, dissolved in FETAX solution. Embryos placed in FETAX solution without test chemical served as controls. Test dishes were incubated at 23 +/- 1°C. Tests were 96-hr static-renewal. At 24-, 48- and 72-hr of exposure, dead embryos were removed and test medium replaced. At 96-hr, surviving embryos were fixed in 1% glutaraldehyde in 0.1M cacodylate buffer (pH 7.3). After exposure, the number of dead embryos were determined for each dish. Fixed embryos were evaluated for gross abnormalities with the aid of a dissecting microscope and the number of surviving malformed embryos determined.

Each chemical was tested 3 to 5 times and endpoint data from these tests combined. Endpoints determined included LC_{50} (mortality), EC_{50} (malformation), and Mortality/Malformation Index (MMI) values (MMI = LC_{50}/EC_{50}). Both the LC_{50} and EC_{50} values were determined using the trimmed Spearman-Karber test (Hamilton et al. 1977). The log K_{ow} values were obtained from MEDCHEM CLOGP version 3.53 (Leo and Weininger 1988) either as a calculated or, when available, measured values. The pK_a values were determined using the computer-assisted version of the Perrin method (Hunter 1988). In addition, the molecular orbital quantum chemical generated three-dimensional (3-D) stereoelectronic parameter, the difference in highest-occupied-molecular-orbital (HOMO) and lowest-unoccupied-molecular-orbital (LUMO) energies, the HOMO-LUMO gaps were determined. For HOMO and LUMO calculation, the SMILES string (Anderson et al. 1987) was converted to 3-D coordinates via CONCORD version 2.9.1. A procedure using the MAXMIN2 module of SYBYL version 5.41C was then used to determine the minimum energy conformation of each of the chemicals. These molecular mechanical conformations were used as the starting points for minimization using the PM3 module in the MOPAC6 quantum chemistry program.

RESULTS AND DISCUSSION

The embryo lethality and malformation data for the six compounds examined are reported in Table 1. Except for naphthalene, all of the tested chemicals produced linear concentration-response relationships for both embryo lethality and embryo malformation.

Computations of hydrophobicity, ionization, reactivity, and toxicities are presented in Table 2. A comparison of log LC_{50}^{-1} data revealed 1,2-dihydroxynaphthalene to be the most lethal. Similarly, an examination of log EC_{50}^{-1} data showed it to produce malformations at the lowest relative

Table 1. *Xenopus* embryo lethality and malformation endpoints for naphthalene metabolites

Compound	CAS number	Molecular weight	LC_{50} (mg/L)[a]	EC_{50} (mg/L)[a]
1. naphthalene	91-20-3	128.17	NES[b]	NES[b]
2. 1,2-dihydroxy-naphthalene[c]	574-00-5	160.17	0.50 (0.48 - 0.52)	0.29 (0.28 - 0.30)
3. salicyl-aldehyde	90-02-8	122.12	10.8 (10.7 - 11.1)	5.5 (5.1 - 6.8)
4. salicylic acid	69-72-7	138.12	1067.0 (1030.3 - 1105.1)	476.4 (414.6 - 547.3)
5. gentisic acid	490-79-9	154.12	10839.5 (10277.1 - 11432.7)	1531.8 (1379.0 - 1701.6)
6. catechol	120-80-9	110.11	13.3 (12.6 -14.1)	2.7 (2.5 - 2.9)

[a] 95% confidence interval in parentheses.
[b] NES - no effects at saturation.
[c] Dissolved in 0.001% dimethyl sulfoxide.

Table 2. Molecular properties and toxicities of naphthalene metabolites

Compound	log K_{ow}	pK_a	ΔE	log LC_{50}^{-1} (mM)	log EC_{50}^{-1} (mM)	MMI[a]
1. naphthalene	3.32	---	-8.43	---	---	---
2. 1,2-dihydroxy-naphthalene	1.98	---	-4.82	2.51	2.74	1.7
3. salicyl-aldehyde	2.07	8.25	-9.05	1.05	1.35	2.0
4. salicyl-acid	2.19	2.98	-9.01	-0.89	-0.54	2.2
5. gentisic acid	1.69	2.85	-8.48	-1.85	-1.00	7.1
6. catechol	0.81	9.62	-8.68	0.92	1.61	5.0

[a] MMI - mortality/malformation index (LC_{50}/EC_{50}).

concentrations. Both gentisic acid and catechol had significant MMI values (≥ 5.0), suggesting they are potential developmental hazards.

Bacterial-mediated transformations play a pivotal role in both the fate and toxicity of chemicals. Moreover, the structural positions and types of substituents and, thereby, physicochemical properties influence not only the mechanism and ease of degradation, but also toxicity. Factors regulating transport and stereoelectronic interactions that result in a toxic response are, for the most part, independent. Hydrophobicity is widely used to model transport. Toxic effects, which are due to transport alone, result in reversible physiological alterations which are physical in nature. In such cases, toxicity is related to external concentration and is a function of the 1-octanol/water partition coefficient (log K_{ow}). Toxic effects, which are

due to reactivity, result in irreversible physiological alterations that are chemical in nature. In these cases, toxicity appears to be related to charge- or orbital-controlled stereoelectronic interactions. One parameter often considered to model reactivity is the difference in energies (Zhou and Parr 1990) of the highest occupied and lowest unoccupied molecular orbitals, HOMO and LUMO, respectively. For many chemicals, toxicity can be thought of as a combination of both hydrophobicity and reactivity (McFarland 1970).

The majority of industrial organic chemicals, including naphthalene, elicit their acute toxic effects via narcosis (Bradbury and Lipnick 1990). Narcosis is the reversible state of arrested cytoplasmic activity (Veith and Broderius 1990). Such changes are predicted well by log K_{OW}.

Bioreactivity can be thought of as the ability of a toxicant to have a positive stereoelectronic interaction with a biological system. Bioreactivity is correlated with molecular stability that is quantitated by the HOMO-LUMO gap (Zhou and Parr 1990). Chemical bioreactive interactions are important in two ways. First, the parent molecule may interact directly with biological systems to cause bioreactive toxicity. Second, molecules may be metabolically activated or transformed to bioreactive intermediates. In fact, reactivity of chemicals in metabolic processes can be thought of as a measure of bioreactivity. Activation can take either of two forms: heavy atom exchange/bond rearrangements, or non-heavy atom exchanges.

While the interaction of bioreactive toxicants with macromolecules often involves a substitution process such as alkylation, or conjugation at electron-rich macromolecular sites (i.e., carboxylate, amino, hydroxyl or sulfhydryl moieties), other bioreactive interactions take the form of redox cycling processes caused by toxicants that form radical anion metabolites. The rate of redox cycling (i.e., reactivity in electron transport systems) appears to be related to the one-electron reduction potential of the toxicant. In this process, the toxicant catalytically oxidizes coenzymes of flavoenzymes and reduces molecular oxygen to highly toxic superoxide radical anions. This results in oxidative stress.

As noted earlier, oxidation of naphthalene forms the 1,2-dihydroxy derivative. This structural alteration sharply reduces hydrophobicity but increases reactivity, as well as both mortality and malformation.

Subsequent degradation results in various monoaromatic species that generally showed a step-wise reduction in hydrophobicity, as well as, mortality and malformation, but an increase in reactivity. The exception is with catechol. This monoaromatic species, like 1,2-dihydroxy naphthalene, is an ortho dihydroxy-derivative. Such chemicals are considered bioreactive. In these cases, the expected decrease in toxicity, due to a decrease in hydrophobicity, was offset by an increase in bioreactivity. Specifically, catechol and catechol-like compounds have the ability to tautomerize and form the reactive α-β unsaturated quinone moiety (Lipnick et al. 1987). The enhanced toxicity of catechols has been attributed to quinone formed via autooxidation (Schlosser and Kalf 1989).

The molecular basis for quinone toxicity has been reviewed by O'Brien (1991). Historically, quinone toxicity has been attributed to alkylation. However, other studies have attributed quinone toxicity to oxidative stress due to redox cycling and oxygen activation.

Benzaldehydes, such as salicylaldehyde, are also α-β unsaturated soft

electrophiles (Hermens 1990). Aldehydes are reported to act as bioreactive toxicants by forming Schiff's bases with nucleophiles in proteins (Lipnick 1991). Salicylaldehyde is more hydrophobic but less reactive than its 1,2-dihydroxy counterpart (compare compounds 3 and 6). Moreover, it is less lethal but more developmentally toxic than catechol. Salicylaldehyde is only slightly less hydrophobic and about as reactive as its 1-carboxyl-2-hydroxyl counterpart, salicylic acid (compare compounds 3 and 4). However, it is significantly more lethal and developmentally toxic. This may be explained by bioavailability due to ionization. Unionized chemicals more readily penetrate cellular membranes than do ionized species. Therefore, a greater external concentration of a particularly ionized compound is needed to attain the same internal concentration as obtained for a unionized compound.

The benzoic acid derivatives, compounds 4 and 5, probably act as simple narcotics. However, as noted earlier, interpretation of the toxic responses of these chemicals is complicated by the fact that toxicity can be affected by ionization.

In summary, this investigation has shown that aerobic microbial metabolism of the model aromatic hydrocarbon, naphthalene, may produce metabolites that pose potentially significant developmental harm. Research designed to evaluate the efficacy of bioremediation in toxic site clean-up must consider, therefore, an integrated plan determining the: 1) major metabolites produced, 2) the accumulation of such metabolites, 3) the toxicity of the metabolites, and 4) the organisms likely to be exposed to such breakdown products. The FETAX system may be especially useful for rapid determination of potential developmental effects from metabolites of this biodegradation, both alone and in mixtures (Dawson 1994).

Acknowledgments. This study was supported in part by The University of Tennessee Waste Management Research and Education Institute.

REFERENCES

American Society for Testing and Materials (1991) E 1439 - 91 standard guide for conducting the frog embryo teratogenesis assay-*Xenopus* (Fetax). In: "Biological Effects and Fate, E47.01 - Aquatic Toxicology." American Society for Testing and Materials, Philadelphia

Anderson E, Veith GD, Weininger D (1987) SMILES: A line notation and computerized interpreter for chemical structure. USEPA Environmental Research Brief EPA/600/M-87 021

Bradbury SP, Lipnick RL (1990) Introduction: Structural properties for determining mechanisms of toxic action. Environ Health Perspect 87:181-182

Cerniglia CE (1984) Microbial metabolism of polyaromatic hydrocarbons. Adv Appl Microbial 30:31-17

Dawson DA (1994) Chemical mixture toxicity assessment using an alternative-species model: application, opportunities, and perspectives. In: Yang R.S.H. (ed) Toxicology of Chemical Mixtures: from Real-life Examples to Mechanisms of Action. Academic Press, pp 539-563

Dumont JN, Schultz TW, Buchanan MV, Kao GL (1983) Frog embryo teratogenesis assay: *Xenopus* (FETAX) - A short-term assay applicable to complex environmental mixtures. In: Waters MD, Sandhu SS, Lewtas J, Claxton L, Chernoff N, Nesnow S (ed) Short-term bioassays in the analysis of complex environmental mixtures III. Plenum Publishing, New York, pp 393-405

Hamilton MA, Russo RC, Thurston RV (1977) Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. Environ Sci Technol 11:714-719, Correction 12:417 (1978)
Hermens JLM (1990) Electrophiles and acute toxicity to fish. Environ Health Perspect 87:219-225
Hunter RS (1988) Computer calculation of pK_a. In: Turner JE, England MW, Schultz TW, Kwaak NJ (eds) QSAR 88 Proceedings 3rd international workshop on quantitative structure-activity relationships (QSAR) in environmental toxicology, [CONF-880520-9DE88013180] NTIS, Springfield VA, pp 33-42
Leo A, Weininger D (1988) Medchem software release 3.53, Pomona Medical Chemistry Project, Pomona College, Claremont, California
Lipnick RL (1991) Molecular mechanism of action and quantitative structure-activity relationships in environmental toxicology. In: Silipo C, Vittoria A (eds) QSAR: Rational Approaches to the Design of Bioactive Compounds, Elsevier Science Publishers B.V., Amsterdam, pp 495-500
Lipnick RL, Watson KR, Strausz AK (1987) A QSAR study of the acute toxicity of some industrial organic chemicals to goldfish. Narcosis, electrophiles and proelectrophile mechanisms. Xenobiotica 17:1011-1025
McFarland JW (1970) On the parabolic relationship between drug potency and hydrophobicity. J Med Chem 13:1192-1196
O'Brien PJ (1991) Molecular mechanisms of quinone cytotoxicity. Chem-Biol Interact 80:1-41
Schlosser MJ, Kalf GF (1989) Metabolic activation of hydroquinone by macrophage peroxidase. Chem-Biol Interact 72:191-207
Smith MR (1990) The biodegradation of aromatic hydrocarbons by bacteria. Biodegrad 1:191-206
Veith GD, Broderius SJ (1990) Rules for distinguishing toxicants that cause type I and type II narcosis syndromes. Environ Health Perspect 87:207-211
Zhou Z, Parr RG (1990) Activation hardness: New index for describing the orientation of electrophilic aromatic substitution. J Am Chem Soc 112:5720-5724