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pH-Stress and Toxicity of Nitrophenols to *Tetrahymena pyriformis*

T. W. Schultz, J. T. Burgan

Department of Comparative Medicine, The University of Tennessee, College of Veterinary Medicine, A205, 2407 River Drive, Knoxville, TN 37996-4543, USA

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Toxicologically, phenols are one of the best-studied groups of industrial organic chemicals. Recent studies on this group of chemicals (Aptula et al. 2002; Cronin et al. 2002; Escher and Schwarzenbach 2002) attempt to model toxic potency and/or predict mode of toxic action of phenols from molecular structure. Earlier, Cronin and Schultz (1996) observed some problems in predicting the toxicity of nitro-substituted phenols, especially those substituted in the *ortho*-position. During the course of routine testing of the toxicity of 2,6-dibromo-4-nitrophenol to the fresh-water ciliate *Tetrahymena pyriformis*, it was observed that selected populations showed premature death during the 40-hr incubation period. Moreover, it was observed that death did not occur at all concentrations, but only at concentrations eliciting growth inhibition between 10-30%. It was hypothesized that this death was pH-related.

Ecotoxicity testing is frequently population-based and typically with unicellular organisms (i.e., algae, bacteria or protozoa). Such population-level toxicity assays commonly evaluate the concentration-response relationship for sublethal effects on reproduction or population growth. Because the technique involves such large numbers of organisms, ecotoxicity evaluations with protista are customarily static in design (Schultz 1997). The use of a static testing technique results in a set of criteria not considered when using a flow-through technique. Such parameters include the potential build up of metabolites from test organism respiration. Such metabolites can interact with the chemical being tested, producing a toxic response different from the one that would have been produced had the excretory products been removed.

Phenols with electron-withdrawing nitro-moieties are weak-to-moderate acids (Dean 1992). They exist in two molecular configurations in aqueous solution. One configuration is the less soluble unionized arrangement, the other the more soluble ionic form. In aqueous systems, the ionization equilibrium can be calculated from the ionization constant, pK_a. For example, at pH 7.0 the equilibrium for 2,4-dinitrophenol (pK_a 4.1) is more than 99% of the compound in the unionized form. Könemann and Musch (1981) and Saarikoski and Viluksela (1981) reported concurrently the effect of ionization on phenolic toxicity to fish. They documented that the toxicity of more acidic phenols decreased as the pH increased.

However, the changes in toxicity were substantially less than would be expected if the phenolate ion only contributed to toxicity. Similar findings were noted for

Correspondence to: T. W. Schultz

Daphnia magna toxicity (Cronin et al. 2000). Barron (1990) noted that although both ionized and non-ionized forms of a weak acid were absorbable, the uptake of the non-ionized form was generally quicker. Escher and Schwarzenbach (2002) give an excellent overview of the effect of pH on phenolic toxicity and note that pH-dependent toxicity is mainly determined by pH-dependent bioaccumulation.

The most often evaluated nitro-substituted phenol is the respiratory decoupler of oxidative phosphorylation (i.e., weak acid respiratory uncoupler) 2,4-dinitrophenol. In earlier investigations using *Tetrahymena*, 2,4-dinitrophenol was noted to affect respiration (Hamburger and Zeuthen 1957) and population density (Conner et al. 1961) in a concentration-response manner. Nilsson (1995) examined *Tetrahymena* population growth kinetics upon exposure to 2,4-dinitrophenol and demonstrated a linear dependence between the concentration of un-dissociated compound and the length of the lag phase prior to ciliate propagation.

In the present study, toxicity, measured as population growth impairment to the freshwater ciliate *T. pyriformis*, was evaluated for a series of nitrophenols. It was hypothesized that pH-related stress would have an effect on population growth-based ecotoxicity to *Tetrahymena* for selected nitrophenols, but, moreover, this stress could be predicted from molecular structure and related to physicochemical properties.

METHODS AND MATERIALS

A group of ten nitro-substituted phenols, selected so as to uniformly cover the range of ionization was evaluated (Table 1). The compounds were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). None were repurified prior to use. Stock solutions of each derivative were prepared by correcting water content of the commercial formulization and dissolving in dimethyl sulfoxide.

Toxicity was assessed as population growth impairment of *Tetrahymena pyriformis* in the 40-hr assay described by Schultz (1997). To summarize, tests were conducted in foam-stoppered 250-mL Erlenmeyer flasks containing 50 mL of proteose-peptone-based medium. Each flask was inoculated initially with approximately 1,200 ± 300 cells per mL and incubated at 27 ± 1°C. At the end of 40 hr, spectrophotometric absorbance at 540 nm was measured and recorded. In order to determine the chemical concentration that covered from no effect to complete inhibition of growth, a range finding test was first done for each phenol. Definitive tests were then repeated three times in duplicate runs with no less than eight levels in the exposure concentration series.

The toxicity data were analyzed by probit analysis using probit analysis of Statistical Analysis System (SAS) software (SAS Inc. 1989) with $n \geq 50$. Chemical concentration was the dependent variable and population growth inhibition (absorbance normalized to control absorbance) was the independent variable. The 50% inhibitory growth concentration (IGC_{50}) was determined by regression analysis (SAS Inc. 1989).

Table 1. Comparison of toxicity and molecular properties of substituted nitrophenols

	Derivative	CAS number [a]	log (IGC_{50}^{-1}) non-neutralized [b]	log (IGC_{50}^{-1}) neutralized [c]	log K_{ow} [d]	pK_a [f]
1.	2,6-dinitrophenol	573-56-8	0.83	0.62	1.38[m]	3.71[m]
2.	2,6-dinitro-4-methylphenol	609-93-8	1.23	1.08	2.29[e]	3.99[e]
3.	2,4-dinitrophenol	51-28-5	1.08	0.97	1.54[m]	4.08[m]
4.	4,6-dinitro-2-methylphenol	534-52-1	1.73	1.48	2.12[m]	4.23[e]
5.	2,5-dinitrophenol	329-71-5	1.04		1.86[m]	5.22[m]
6.	2-chloro-4-nitrophenol	619-08-9	1.59		0.73[m]	5.64[e]
7.	4-chloro-2-nitrophenol	89-64-5	2.12		2.46[m]	6.48[m]
8.	3,5-dinitrophenol*	586-11-8	1.41		2.35[m]	6.73[e]
9.	4-chloro-3-nitrophenol*	610-78-6	0.05		2.46[m]	7.73[e]
10.	3-nitrophenol*	554-84-7	0.30		2.00[m]	8.36[m]

[a] Chemical Abstract Services registry number
[b] non-neutralized medium
[c] neutralized medium
[d] logarithm of 1-octanol/water partition coefficient
[e] = estimated values
[f] ionization constant
[m] = measured
*pK_a value outside the range for respiratory uncouplers

Values for log K_{ow} were secured as either a measured or a computer estimated value from the CLOGP for Windows software (BIOBYTE Corp., Claremont, CA, USA). Measured pK_a values were obtained from Dean (1992). Estimated pK_a values were obtained from Hunter (1988).

RESULTS AND DISCUSSION

Table 1 is a summary of the toxicity and physicochemical data for the nitrophenols considered in this study. For several of the compounds (derivatives-1 - 4), two separate toxicity values were measured. These chemicals elicited premature death of *Tetrahymena* during the 40-hr incubation period. It was observed that death occurred only in concentrations eliciting growth inhibition between 10-30%. It was further detected that death occurred during the interval between 34-39 hr and the time of death was related to toxicant concentration. It was hypothesized that this death was pH-related.

Upon further observation, it was noted that flasks displaying cell death had pH levels < 6.5 at the end of 40 hr. Those flasks displaying no cell death had pH levels > 6.5 at the end of 40 hr. Controls with media and nitro-phenolic toxicant, but without *Tetrahymena* were examined and not found to display significant drops in pH over time. However, the pH of the media did drop slightly (7.4 → 7.2) after sterilization and addition of the toxicant at time zero. This latter observation indicated that the buffering capacity of the medium was compromised upon addition of the more acidic toxicants. By taking pH measurements of the flasks as the *Tetrahymena* began to die, it was determined that the pH conditions in these flasks had dropped to between 6.2 and 6.5 at the onset of death. As reported in the literature (Cameron 1973) and observed during the course of these investigations, the pH of the environment must drop below 5.0 before *Tetrahymena* begin to die from acid-stress. Thus, death occurring at pH levels above 6.0 was unexpected. It is known that *Tetrahymena* will naturally lower the pH of their environment by excreting waste metabolites such as lactic acid (Levy 1973). However, for the duration of this assay, *Tetrahymena* did not produce significant metabolites to lower the pH to a level less than 5.0 (pH of controls at 40 hr = 6.8 - 6.9). Therefore, a drop in pH to levels below 6.5 would not be sufficient to cause the mortality observed in these experiments. There was, however, clearly a pH-related threshold where death began to occur for derivatives 1 - 4.

As noted previously, there was a critical range of toxicant concentration (~ > 10 and < 30% inhibition) in which death consistently was observed. If for example, 3, 6, 10, and 30 mg/L represent concentrations of 2,4-dinitrophenol for 10, 20, 30, and 70% inhibition, respectively, then death was not seen in cultures with 3 or 30 mg/L, but was seen in middle two concentrations. Moreover, death was observed first in the 10 mg/L concentration. Death was not seen in 3 mg/L because the chemical concentration was too low to affect the overall growth of the population (i.e., no toxicant stress). It is hypothesized that in the course of these studies, death was not seen at 30 mg/L because the concentration of chemical impaired

Tetrahymena growth and the concomitant production of acidic respiratory metabolites (i.e., no pH-stress).

In eukaryotic organisms, respiratory uncouplers are considered to act at the mitochondrial level membrane by inhibiting the coupling between the electron-transport chain and phosphorylation reactions without affecting the respiratory chain (McLaughlin and Dilger 1980). Specifically, uncouplers work by transporting hydrogen ions across the otherwise hydrogen ion-impermeable membrane of the mitochondria (Terada 1981). The result of this uncoupling is that the synthesis of ATP from ADP and inorganic phosphate by ATP synthase is prevented without affecting the respiratory chain (Hanstein 1976).

Concerning the flasks that did display death, it was the lowest concentration (6 mg/L) that exhibited the lowest pH ($\approx$ 6.2) at the time of death and at 40 hr. However, it was the highest concentration of chemical (10 mg/L) in which death first began to occur and contained the highest pH ($\approx$ 6.5) at the time of death. Consequently, inverse relationships emerge between toxicant concentration and death or pH. These observations suggest that acid-stress itself was not the cause of death.

Based upon the population growth experiments, *Tetrahymena* survive longer at lower concentrations of toxicant, have a greater number of organisms per unit volume, and a longer period of time during which to produce acidic metabolites; thereby, resulting in a lower pH at the time of death and at 40 hr. Similarly, if *Tetrahymena* died sooner at higher concentrations, they would produce less metabolites (i.e., fewer organisms and less time), resulting in a higher pH at these time/concentration points.

It was observed that upon death, the opaqueness of *Tetrahymena* changes– the cells become whiter. It also was observed that, at the time of death, many of the *Tetrahymena* had changed from a normal pear-shape to a thin, flattened, and more elongated form. These results mean that in dead and dying populations, the same number of cells per aliquot gave reduced absorbance readings.

Although data are limited, this study suggested pH-related death is seen in uncouplers with pK_a values of $\leq$ 5.06. An examination of the pK_a values from Table 1 provides the best possible justification as to why pH-related death was seen. The derivatives exhibiting death have the lowest pK_a values (i.e., were the stronger acids). Thus, populations exposed to these phenols are placed under a greater acid-stress. This acid-stress when combined with the chemical-stress results in population mortality.

In an effort to correct for possible pH-related death, flasks were titrated back to pH of 7.4 (the addition of chemicals and processes of sterilization had reduced the pH to as low as 7.2). The assay was then conducted using a wide range of concentrations. The calculated results are presented in Table 1 as neutralized media values. Overall, there were fewer concentrations exhibiting death at 40 hr, and decreased toxic potency.

The toxic potency values reveal a general trend of increasing toxicity with increasing hydrophobicity. This trend was expected, since hydrophobicity is the physicochemical property describing the ability of the toxicant to diffuse across the membrane to the site of action (Cronin and Schultz 1996). However, the modeling of toxicity for weak acids, like phenols, is made more complex by the fact that toxicity is related to ionization (represented by pK_a) as well as hydrophobicity (Terada 1981). As pointed out by Schultz et al. (1996) for ionic phenols, toxic potency in *T. pyriformis* is related directly to hydrophobicity and inversely related to ionization.

A further examination of Table 1 supports the thesis that the position of substituents on the phenolic ring (i.e., molecular structure) plays an important role in determining properties and toxicity. An examination of the data for the dinitro-derivatives, compounds 1, 3, 5, and 8, reveals that nitro-substitution at the *ortho*-position and more so at the 2- and 6- position results in greater ionization (i.e., lower pK_a), lower hydrophobicity, and lower toxicity. This correlation of toxicity potency with pK_a is reinforced by a comparison of derivatives 2 and 4.

The difference between 2,4-dinitrophenol and 2,5-dinitrophenol when both derivatives contain a nitro group in the *ortho*-position is due to the ability of 2,4-dinitrophenol to undergo resonance or electronic effects through the ring. In other words, in 2,4-dinitrophenol, one of the electron-withdrawing nitro moieties is in the *para*-position to the electron-releasing hydroxyl group. The resulting increased acidity (i.e., lower pK_a) of the 2,4-dinitro-derivative is due to the *para*-substituted nitro group stabilizing the conjugate-based anion by resonance. Consequently, a charge is placed on either one of the oxygen atoms. This charge does not occur with nitro groups at the *meta*-positions because they are asymmetrical to the hydroxyl group and the negative charge cannot be delocalized to the nitro moiety (March 1992).

The toxicity of phenols exhibits at least two distinct partitioning-dependent structure-toxicity relationships (Escher and Schwarzenbach 2002). One relationship represented a narcosis mechanism of action and the other model represented the weak acid respiratory uncouplers. Phenols exhibiting the ionization constant (pK_a) values < 6.63 elicit their response by weak acid respiratory uncoupling (Schultz et al. 1996). Thus, while compounds 1 - 7 have pK_a values that suggest the respiratory uncoupling mechanism of toxic action, compounds 8 - 10 have pK_a values that lie outside the range for respiratory uncouplers, and thus are deemed narcotic (Schultz et al. 1997).

Biological membranes are the primary target organelle for acute toxicity of nitro-phenols (Escher and Schwarzenbach 2002). While toxicity is mainly determined pH-dependent bioaccumulation it is suggest by these results that metabolism-related alterations in the environmental pH also has a significant impact on acute aquatic toxicity, especially in static protocols.

In summary, it is the pH of the test solution that clearly is influencing toxicity, particularly so for the acidic phenols reported here. A pH-related death was

observed in phenols that are more strongly acidic. While acid-stress alone was not sufficient to result in death, the acid-stress when combined with the stress of uncoupling respiration resulted in protozoan mortality. Studies of dinitro-substituted derivatives confirm that the position of substituents on the phenolic ring plays an important role in determining molecular properties, mechanism of action, and subsequently toxic potency.

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