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Chemosensory and Electrophysiological Responses in Toxicity Assessment: Investigations with a Ciliated Protozoan

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The development of rapid, reliable, sensitive and cost effective screening methods is essential for the hazard assessment of environmental chemicals. The need to reduce vertebrate animal testing for ethical reasons is recognized. Trace levels of aquatic pollutants which do not directly impair survival of animals have been observed to limit their feeding, distribution, orientation, migration or reproductive behavior.

Chemoreception plays an important role in mediating behavior of aquatic animals. The ability to select favorable conditions is of obvious importance to survival. Although ciliated protozoa are often described as 'swimming neurons or receptors', few attempts have been made to utilize their sensory characteristics as sublethal endpoints in ecotoxicological risk assessment.

Tetrahymena thermophila, a ubiquitous freshwater, ciliated protozoan, exhibits chemosensory responses to various stimuli. It plays an important ecological role in microbial based food chains, regeneration of nutrients and regulation of bacterial populations in the aquatic environment. Due to the ease with which it can be cultivated axenically and the comprehensive knowledge available on its biochemistry and physiology, Tetrahymena has gained recognition in toxicity testing.

In this study a quantitative method for the measurement of negative chemosensory response is presented. The effects of five aniline derivatives on the chemosensory response (i.e., repulsion) of Tetrahymena thermophila were determined. The relationship between these results and perturbation of the membrane potential, determined with a fluorescent dye, was examined. In addition, growth impairment, a common cytotoxic endpoint was determined for these five industrial chemicals.

MATERIALS AND METHODS

The test chemicals, obtained from Riedel de Haën, Seelze, were of the highest purity available (aniline: >99.5%, 2,4-dinitroaniline: 97%, 4-nitroaniline: >99%, 3-hydroxyaniline: 99%, 4-hydroxyaniline: 98%). Chemicals were chosen for their ecotoxicological relevance: industrial chemicals having a high exposure to the environment.

Tetrahymena thermophila B III, kindly supplied by Dr. Tiedke, Münster, was grown axenically without shaking at 28^0C in defined medium (Rasmussen and Modeweg-Hansen, 1973).

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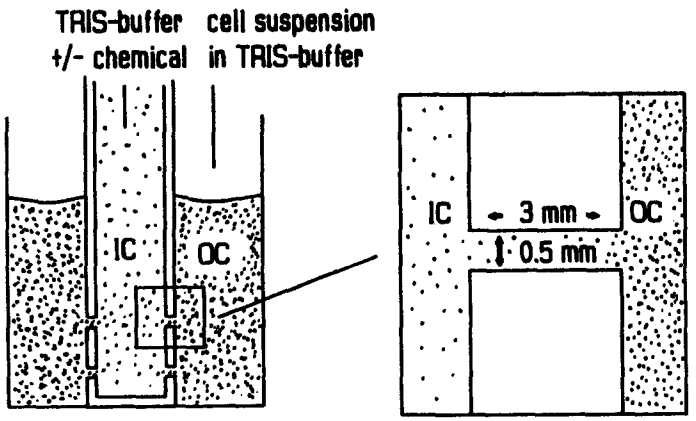

Figure 1. Cross section of the chemosensory assay chamber. OC outer compartment; IC inner compartment.

The chemosensory assay was performed using the apparatus designed by Leick (1983, scheme of one assay unit see Fig. 1). It comprises two compartments: an outer chamber , containing the cell suspension, and the inner tube filled with buffer and various concentrations of the chemical to be tested. The two compartments are joined by 16 capillaries (0.5 mm in diameter and 3 mm in length). Within these capillaries a stable gradient from the inner to the outer compartment is upheld during the test period (for 2,4-dinitroaniline a diffusion of less than 3% through the capillaries was measured). This assay is designed so that the inner chamber functions as a trap. The cells have to swim in a horizontal direction through the capillaries and tend to swim upwards once they reach the inner compartment.

Cells were grown to late logarithmic phase, harvested at 500 x g, washed and re-suspended in 10 mM Tris/HCl, pH 7.4 (control solution) at a cell concentration of 8 x 10^5/mL. Two mL aliquots of cells were transferred to the outer chamber and left for 1hr after centrifugation to recover and adapt to the buffer. The inner compartment, into which the cells migrate, was filled with control solution with/without various concentrations of the chemical to be tested. Substances were dissolved directly in the buffer with the exception of 2,4-dinitroaniline, where DMSO was used as solvent up to a maximum test concentration of 0.025% (no observed effect up to 0.1%). In the case of 4-hydroxyaniline, the control solution was supplemented with 1mg/mL ascorbic acid to prevent oxidation. Since in addition, this chemical increases swimming speed in low concentrations (data not shown), isobutyl-methyl-xanthine (IBMX, 2mM), a well known phosphodiesterase inhibitor, was added to both compartments. IBMX itself effectively increases the swimming speed of the cells and can thereby compensate for this effect of the chemical: speeding of cells in the chemical gradient of the capillaries can lead to an accumulation in the inner chamber, which would falsify the results (Hellung-Larsen et al. 1986).

The inner compartment was placed in the outer chamber. The experiment was run for 90 min after which the concentration of cells in the inner chamber is about 3 x 10^5/ml. The number of cells in the plexiglass assay tubes was determined electronically (Coulter Counter, Luton, UK). All measurements were in triplicate and repeated on three different days. The decrease in cell migration into the inner

chamber serves as a measure for repulsion due to the presence of toxicants. A linear regression analysis (least squares) was made between the log_{10} dose of the chemicals and the % decrease in migration. From this best fit, dose response curves were established. Threshold values were determined by extrapolating the regression line.

The fluorescence dye, rhodamin 6G (R6G), was purchased from Sigma Chemie, Deisenhofen. Fluorescence was measured with a Perkin Elmer (Conneticut, USA) 650-40 fluorescence spectrophotometer at 23^0C (excitation wavelength: 520 nm, emission wavelength: 550 nm and 5nm slit widths on both excitation and emission monochromators). Cells from the late logarithmic growth phase were washed twice with control solution and resuspended in the same buffer ($1x10^6$ cells/mL). Two μL of a stock solution of R6G (dissolved in DMSO) were added to 2 mL cell suspension in a cuvette to a test concentration of 0.7 μM). This suspension was allowed to equilibrate under gentle stirring with a magnetic stirrer for 20 min at 23 ^0C, by which time a constant fluorescence signal was obtained.

A given volume of the aniline derivatives, dissolved in DMSO, was added to the cuvette and the fluorescence intensity was measured. The final concentration of DMSO never exceeded 0.2%. Up to this concentration no effect was observed on the fluorescence activity. The change in the fluorescence intensity was determined 5 min after the addition of the chemicals. Since all these aromatic substances showed quenching effects on the fluorescence signal, a control containing only the fluorescence dye was measured for each concentration of the chemicals in parallel, from which a correction factor was derived. After subtraction of the background fluorescence of the cells (<10%), which remained unaltered by the addition of the anilines, and correction by this factor, the change in the fluorescence intensity was defined as $dF = (F-F_0)/F_0$, where F_0 and F represent the fluorescence signal before and after addition of the chemical. The effects were determined from two experiments on different days.

The growth assay was performed in 200 mL Erlenmeyer flasks each containing 20 mL defined medium and various concentrations of the chemicals to be tested. In the case of 2,4-dinitroaniline DMSO was used as a solvent to final concentration of 0.25% at which no effect was observed. The medium was supplemented with 1 mg/mL ascorbic acid to prevent oxidation of the hydroxyanilines. Organisms from the stationary phase were added to an initial concentration of 20,000 cells/mL. Growth kinetics were followed by measuring the optical density of the suspension at 530 nm (Lange Digitalphotometer, Berlin, Germany) over a period of 48 hr. A calibration curve relating the optical density to the cell concentration was established using electronic cell counting. All values are the average of at least three experiments, performed in duplicate. From the reduction of the proliferation rate, dose response curves were obtained: regression lines, by the method of least squares, were determined from the probit transformed percentage of growth rate impairment and the log_{10} concentration of the respective chemicals. The NOEC-values represent the highest values at which no effect was observed.

RESULTS AND DISCUSSION

All aniline derivatives can cause irreversible physiological damage to Tetrahymena at high concentration leading ultimately to cell death. Far below this concentration range (40 to 10,000 times) an avoiding reaction of these animals can be observed. This relative sensitivity of chemoreception indicates that the organisms can recognize

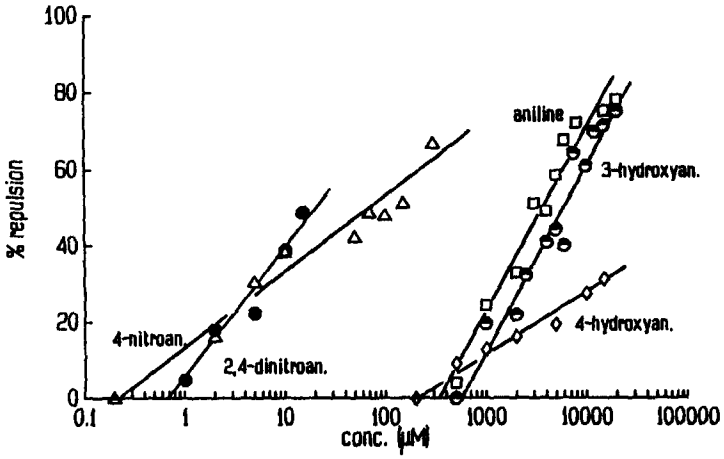

Figure 2. The magnitude of the negative chemosensory response (repulsion) to the five aniline derivatives.

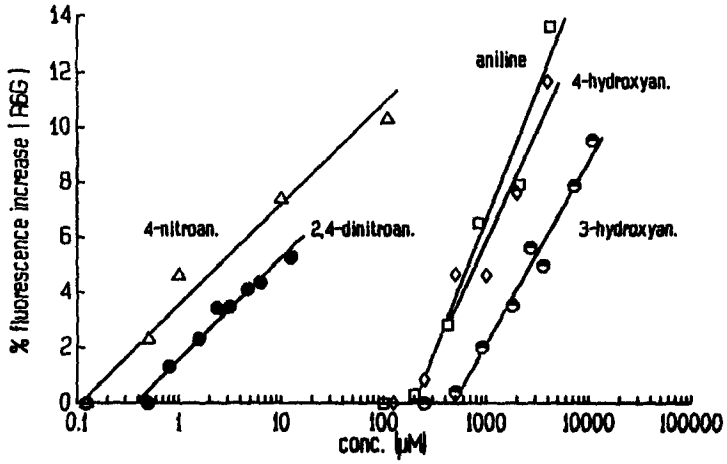

Figure 3. The Fluorescence increase (dF) of 0.7 μM Rhodamine 6G in Tetrahymena suspensions versus the concentrations of the anilines.

harmful substances at much lower concentrations than those leading to damage. The ecological function of this rejective behavior may be to assist these animals in avoiding suboptimal environmental conditions.

Fig. 2 illustrates the concentration dependent negative chemosensory response of Tetrahymena towards all five chemicals. Despite different slopes, repulsion increases linearly with the logarithm (log) of the concentration of the compounds tested. Threshold values were determined by extrapolating this relationship and range from 0.2 μM to 500 μM for the five aniline derivatives. The anilines with nitro-groups

elicit a response at a significantly lower threshold level than the hydroxyanilines and aniline itself, between which no marked differences in threshold concentration is observed.

Alterations in the membrane potential of Tetrahymena after addition of the anilines were monitored by measuring the fluorescence intensity of R6G. A concentration dependent quench of the R6G fluorescence signal (cell-free dye solution) was observed for each of the five compounds. The fluorescence intensity, corrected for the respective quench, also increases linearly as a function of the log concentration in each case (Fig. 3). An increase in the R6G fluorescence signal implies that depolarization occurs in response to the stimulus (Aiuchi et al. 1980). Each compound has a respective threshold concentration at which the fluorescence intensity starts to increase. Table 1 shows that the threshold concentrations required to elicit fluorescence increase and repulsion in Tetrahymena are practically identical for the five compounds tested, compare also Fig. 2 and Fig. 3.

Table 1. Thresholds (in μmoles/L) for the five aniline derivatives in all three test systems.

	Repulsion	Fluorescence	Growth impairment
3-hydroxyaniline	500	450	500
aniline	340	200	80
4-hydroxyaniline	200	200	50
2,4-dinitroaniline	0.7	0.5	10
4-nitroaniline	0.2	0.125	20

For salts and hydrophobic stimuli, this increase in fluorescence signal is strictly correlated with a negative chemosensory response (Aiuchi et al. 1980; Tanabe et al. 1980). It is known that ciliate motile behavior is finely controlled by the membrane potential: graded shifts in membrane potentials induce graded transitions in ciliary motor responses (Machemer and Sugino 1989). A depolarization reduces the forward locomotion or reverses the ciliary beat. The ciliary membrane includes voltage-sensitive calcium channels, and ionic calcium is an intracellular messenger of the electromotor coupling process. Electric membrane signals are rapidly exploited for the generation of motor responses (Machemer 1986). Both fluorescence increase and chemosensory response illustrate this direct connection: the R6G fluorescence signal increases (depolarization) shortly after the addition of the chemicals ; cells having a normal swimming speed of 0.2 mm/sec have only about 15 sec to react to the chemical gradient in the horizontal capillaries (Fig. 1), before becoming trapped in the inner chamber.

The impairment of the growth rate reflects a wide range of different cytotoxic interactions. This toxic endpoint was measured and the no observed effect concentrations (NOEC) were compared to the threshold values obtained with the chemosensory and fluorescence assays. Above the NOEC a dose dependent reduction of cell proliferation in exponentially growing cultures was observed. Sigmoidal dose reponse curves were obtained for all chemicals tested.

No significant correlation ($p>0.05$) exists between the thresholds for growth inhibition (NOEC) and those for the chemosensory response and fluorescence increase (Table 1). The log_{10} concentrations were taken for this regression analysis due to

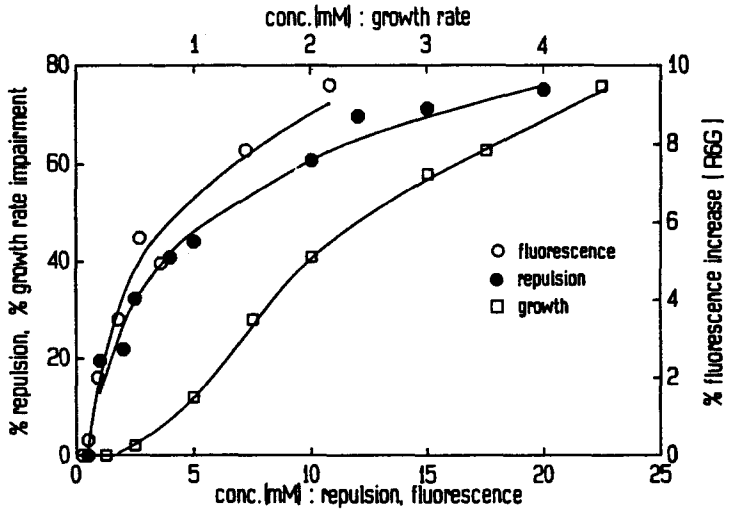

Figure 4. shows the dose dependent increase in R6G fluorescence, repulsion and growth rate impairment for 3-hydroxyaniline in a linear scale.

the wide range of values involved. The impairment of the cell growth rate is only slightly more or equally sensitive in three of five cases, whereas the thresholds for chemosensory and fluorescence assays are one to two orders of magnitude more sensitive towards the two nitroanilines. Furthermore the sigmoidal dose response kinetics obtained for all substances from the growth assay are in contrast to the typical L-shaped curves obtained for the chemicals in the fluorescence and chemosensory assays, indicating a different underlying mechanism of interaction with the compounds (Shirazi and Lowrie 1988). As an example Fig. 4 shows the dose response curves of all three test systems for 3-hydroxyaniline.

Whereas the growth rate reflects the sum of a variety of sublethal cytotoxic effects, the translation of different external stimuli into the appropriate behavioral responses occurs in ciliates via the cell membrane. Despite the existence of specific receptors in Tetrahymena (Csaba 1985; Köhidai et al. 1986; O'Neill et al. 1988), effects of hydrophobic compounds are mainly attributed to aspecific receptor binding (Franks and Lieb 1990) or accumulation of the molecules in the lipid phase of the biomembrane and thereby affecting the membrane potential (Ataka et al. 1978). Hydrophobic compounds with polar groups (i.e., alcohols and so-called odorants) have been observed to perturb the surface and intramembrane diffusion potential of Tetrahymena (Tanabe et al. 1980), both reflected in the membrane potential. Differences in the polarity of substances may account for the extent of these perturbations. Anilines are described as weakly polar narcotics (Veith and Broderius 1990), having a surface active effect and are almost uncharged at physiological pH (Elliott and Haydon 1989). The strong electron attracting property of the nitrogroups leads to higher polarity of the aromatic ring (high σ-Hammett values, Hansch and Leo 1979) and could explain the high sensitivity of the threshold levels for these anilines. From quantitative structure activity relationships (QSAR) studies an excess toxicity has been reported for nitro substituents containing aromates and was attributed to electrophilicity (Hermens et al. 1985; Roberts 1987; Schultz et al. 1991), i.e., the ability

to undergo nucleophilic substitution reactions with macromolecules. This chemical reactivity may not only play an important role for the toxic effects of the respective aniline derivatives, but may also account for the differences in sensitivity between the growth rate and chemosensory / fluorescence assays, carried out in media with distinct formulation (defined medium, Tris/HCl buffer,respectively).

The combination of the common cytotoxic endpoint growth with a behavioral characteristic (avoiding reaction) offers the possibility to implement a complementary set of sublethal endpoints in ecotoxicological risk assessment.

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