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A Rapid Biochemical Test for Measuring Chemical Toxicity

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Many chemicals undergo multiple transformations in the environment, owing to abiotic and biotic processes. Whether they bioaccumulate or not depends, among other things, on their toxicity to the biota. Therefore, toxicity to organisms affecting the transforming is obviously an importrant factor governing the fate and behaviour of a chemical compound in the natural environment. Numerous bioassay procedures employing fish (SASTRY & AGRAWAL 1979), invertebrates (van DIJK et al. 1977), algae (WONG et al. 1979), protozoan (HONIG et al. 1980) and bacteria (MOWAT 1976, BABICH & STOTZKY 1977) have been developed for the measurement of toxicity. Microbial toxicity tests have the advantages of speed and ease of performance (MOWAT 1976) due to the much shorter life cycles of these organisms and the higher test concentrations which can generally be employed. Such tests are therefore suitable for use as primary biological monitoring systems for screening large number of samples for relative toxicity.

Chemical toxicity to bacteria is normally measured in terms of inhibition of microbial growth rate (NARKIS & ZUR 1979), oxygen uptake (BROUZES et al. 1978) and colony formation on agar plates (ANDERSON & ABDELGHANI 1980). The purpose of this paper was to report the development of a rapid and sensitive biochemical toxicity test based on the resazurin reduction by microbial dehydrogenase.

MATERIALS AND METHODS

Reagents: Resazurin solution was prepared by dissolving one resazurin tablet (5 mg per tablet from BDH) in 50 mL of distilled water using a volumetric flask. The resazurin solution was stored in a brown bottle and was stable under storage conditions for approximately one week at 4°C. Phthalate-HCl buffer (0.05M) was made up by dissolving 1.02 g of potassium biphthalate in 95 mL of distilled water and the pH was adjusted to 3.5 with 6N HCl. Sodium bicarbonate and n-amyl alcohol (solvent) were laboratory reagent grade.

Mixed bacterial cultures were developed by adding 0.1 mL of fresh activated sludge to a 125-mL Erlenmyer flask containing 50 mL of 1/10 strength nutrient broth (DIFCO) fortified with 200 ppm each of glucose and sodium acetate. After overnight growth (approximately 18 h) on a shaker at 21°C, 0.1 mL of the culture was transferred into another flask containing fresh medium. The transfer was

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repeated daily for two weeks so that an active and stabilized mixed culture was established. The cell concentration was adjusted to 0.5 O.D. (650 nm) with fresh medium just prior to the toxicity test.

The effect of toxicant on the mixed bacterial culture was determined by the following scheme:

A. Reagent control : 4 mL medium + 1 mL resazurin solution
B. Cell control : 3 mL medium + 1 mL cell + 1 mL resazurin solution
C. Reaction mixture: 3 mL medium with toxicant + 1 mL cell + 1 mL resazurin solution.

The experiments were conducted in standard 2 x 15 cm test tubes at 21°C. After an exact incubation of 90 min, 10 mL of n-amyl alcohol and 0.1 mL of buffer solution were rapidly added to each tube to stop the reaction. The contents were vigorously mixed for 15 sec on a vortex mixer followd by centrifugation in the same test tube at 1,000 rpm for 5 min. Approximately 8-9 mL of the upper alcohol layer were transferred into a clean test tube containing approximately 2 g of sodium bicarbonate. The contents were gently mixed and the absorbance of the supernatant was read on the spectrophotometer at 610 nm (the maximum absorbance of unreduced resazurin). Toxicity to the mixed culture, expressed as % inhibition, of the test substance was calculated using the following:

$$I = \frac{(A - B) - (A - C)}{(A - B)} \times 100$$

where A = final O.D. of reagent control
B = final O.D. of cell control
C = final O.D. of reaction mixture

The term IC_{50} used in this study refers to the effective concentration of the toxicant causing 50% inhibition of the microbial dehydrogenase activity.

RESULTS AND DISCUSSION

Interaction between toxicant and enzyme frequently results in the inhibiting enzyme activity which could eventually lead to death of the organism (SASTRY & AGRAWAL 1979). Studies of toxicity effects at cellular (enzyme) level have the advantage of being more sensitive than investigations at the population level such as the measurement of LC_{50}. Dehydrogenases are involved in the vital anabolic and catabolic reactions and seem ideal for use in such toxicity studies. The resazurin reduction method (LIU & STRACHAN 1979), after modification, was consequently employed to examine for the toxicity of some arsenical compounds (sodium arsenite, sodium arsenate, and sodium cacodylate) on the dehydrogenase activity of the mixed bacterial culture. Various concentrations (10-10,000 ppm) of these arsenical compounds were incubated with the culture

for the determination of their IC_{50} to microorganisms. The results are presented in Table 1 along with the data of ANDERSON & ABDELGHANI (1980) for comparison. Obviously, the resazurin reduction method offers advantages over the conventional plate count method both in terms of sensitivity and speed. The former was at least ten times more sensitive and also required less time to perform (2 h vs 24 h), an important factor limiting the numbers of sample that a laboratory can process.

Table 1. IC_{50} of some arsenical compounds to the mixed culture

Chemicals	Test Concentration ppm	Agar Plate Count* ppm
Na arsenite	25	270
Na arsenate	100	1300
Na cacodylate	2000	27000

*From Anderson et al. (1980)

The reproducibility of the resazurin test was also examined using potassium cyanide and mercuric chloride as the test toxicants at three concentration levels (0.1, 1 and 5 ppm) over a period of eight days (Table 2). In view of the complexity involved with the response of a biological system to any foreign toxicant, the resazurin test can be considered having good reproducibility (average 10%), an essential criterion for any test to be used in routine monitoring work.

Table 2. % Inhibition of microbial dehydrogenase by KCN and $HgCl_2$

	KCN			$HgCl_2$		
Date	0.1 ppm	1 ppm	5 ppm	0.1 ppm	1 ppm	5 ppm
14/8/80	10	10	15	5	68	90
20/8/80	9	11	16	8	61	90
22/8/80	8	11	18	6	71	90
mean	9	10.7	16.3	6.3	66.7	90
s.d. (r.s.d.)	1(11%)	0.6(6%)	1.5(9%)	1.5(24%)	5.1(8%)	0(0%)

The technique has many different potential applications. For example, the effect of degree of chlorination in the phenol molecule on the chemical toxicity to the microorganisms may be easily examined with this method (Figure 1). In this experiment, phenol, 2-chlorophenol (MCP), 2,6-dichlorophenol (DCP),

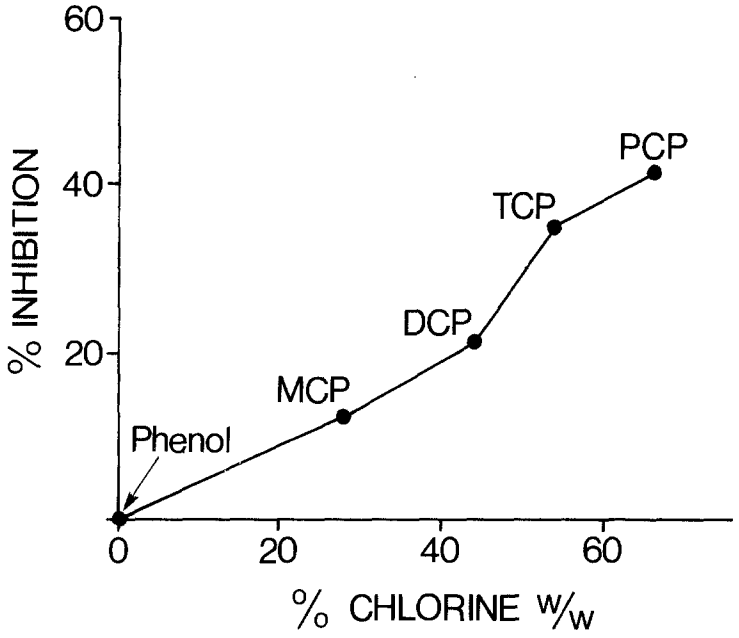

Figure 1. Percent inhibition as a function of extent of phenol chlorination (see text for abbreviations).

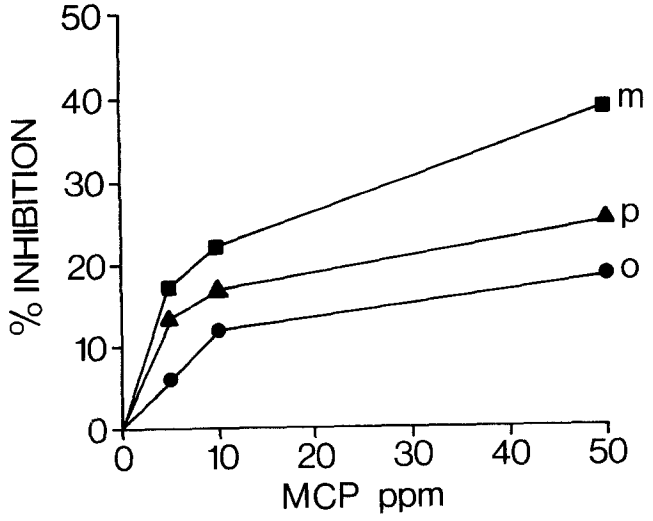

Figure 2. Percent inhibition as a function of monochlorophenols concentration.

2,4,6-trichlorophenol (TCP) and pentachlorophenol (PCP) were assessed for their toxicity at the concentration of 10 ppm level and the results vividly illustrate that the toxicity of chlorophenols to micoorganisms is a function of the degree of chlorination in the phenol nucleus. Thus the resazurin test can readily be used in structure/toxicity studies where rigid control and accuracy are needed to obtain comparative data.

Information concerning structure/toxicity is essential for predicting the environmental impact of a toxic contaminant in the ecosystem (KAISER 1980). In order to demonstrate the versatility of the resazurin test for use in such a study, three monochlorophenols (ortho, meta and para) were assessed for their toxicity to the mixed culture at concentrations of 5, 10 and 50 ppm. Their effect on inhibition is shown in Figure 2. At the 5 ppm level, o-MCP was found to be half as toxic as p-MCP and one third as toxic as m-MCP to the culture. The results agree with the data of KOBAYASHI et al. (1979) who report that p-MCP was about 1.8 times more toxic than o-MCP to the goldfish. Moreover, m-MCP has been reported as being more toxic than its ortho and para isomers to rat (WINDHOLZ et al. 1976). As steric, electronic and hydrophobic effects of the substituents could all affect a molecule's toxicity, it is difficult here to speculate why m-MCP is more toxic than its other isomers to the biota. Obviously there is still room for further improvement of the resazurin test and work is being conducted to refine this test for both sensitivity and simplicity.

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