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Toxicity Assessment of Chlorobenzenes Using Bacteria

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Chlorinated benzenes (CB) are composed of twelve chemical species: One mono-, three di-, three tri-, three tetra-, one penta- and one hexachlorobenzene. They are not only important intermediates for manufacturing many kinds of chemcals, but are also extensively used and have a variety of applications such as solvents, heat-transfer fluids, pesticides, and toilet deodorants (FISHBEIN 1979). As a result of the demand for these chemicals, 329 millions pounds of monochlorobenzene were produced in 1976 in the U.S.A. (FISHBEIN 1979). Because of their lipophylic character and slow degradation in the environment (JAN & MALNERSIC 1980), chlorinated benzenes have properties quite similar to those of chlorinated biphenyls (JAN 1980). Thus, CB have been found in fish (NIIMI 1979, JAN & MALNERSIC 1980), seeds (JAN 1980), birds (GILBERTSON 1974, HALLETT et al. 1982), human adipose tissue (MORITA & CHI 1978), and in Great Lakes water and sediment (OLIVER & NICOL 1982).

Although much data are available regarding the distribution of CB in the total environment, there is a paucity of information concerning the toxicity of such compounds towards biota. One explanation for the lack of information may be due to CB's low toxicity (KONEMANN 1980). In an attempt to better understand the effect of CB on biota in a more quantitative terms, the toxicity of eleven chlorinated benzenes on microorganisms was examined in this study using a resazurin DMSO system, which allows the assessment of CB's toxicity at high concentrations.

MATERIALS AND METHODS

Chemicals: Monochlorobenzene (MCB), o-dichlorobenzene (o-DCB). m-dichlorobenzene (m-DCB), p-dichlorobenzene (p-DCB), 1,2,3-trichlorobenzene (1,2,3-TCB), 1,2,4-trichlorobenzene (1,2,4-TCB), 1,3,5-trichlorobenzene (1,3,5-TCB), 1,2,3,4-tetrachlorobenzene (1,2,3,4-TRCB), 1,2,3,5-tetrachlorobenzene (1,2,3,5-TRCB), 1,2,4,5-tetrachlorobenzene (1,2,4,5-TRCB) and pentachlorobenzene (penta-CB) were obtaind from Supelco Inc., Bellefonte, PA. 16823. All chlorobenzenes were dissolved in dimethyl sulfoxide (DMSO) and made to volume in a 10-mL volumetric flask. Stock solutions were normally prepared at the concentrations of 10-50 mg mL^{-1}.

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 for approximately one week at 4°C. Phthalate-HCl buffer (0.05 M) was made up by dissolving 1.02 g of potassium biphthalate in 90 mL of distilled water and the pH was adjusted to 3.0 with 6N HCl (final volume = 100 mL). Sodium bicarbonate and n-amyl alcohol (solvent) were laboratory reagent grade.

Medium: The growth medium (GM) for culturing the test bacterium containing the following components in the amount of g L^{-1}: KH_2PO_4, 1.64; K_2HPO_4, 2.64; glucose, 0.20; sodium acetate, 0.20; nutrient broth, 1.60 and distilled water, 1 L. The medium was sterilized by autoclaving at 121°C for 15 min and the same medium was also employed in all toxicity testing procedures.

Bacteria: A Bacillus sp., (TL81), originally isolated from activated sludge (LIU et al. 1982) was employed as the test organism. The cells were grown for 18 h in 50 mL of GM medium contained in a 125-mL Erlenmyer flask on a rotary shaker at room temperature (21°C). The bacterial growth achieved was consistently 0.75 ± 0.02 O.D. (625 nm) and therefore the culutre was directly used without adjustment for the cell density.

Procedures: The effect of chlorinated benzenes on the bacterial culture (TL81) was determined by the following scheme:

A.	Reagent control:	3.75 mL GM + 250 µL DMSO + 1 mL resazurin
B.	Cell control:	2.75 mL GM + 250 µL DMSO + 1 mL cell + 1 mL resazurin
C.	Reaction mixture:	2.75 mL GM + (250 - Y µL DMSO) + 1 mL cell + 1 mL resazurin (Y = µL CB's stock solution)

The term EC, used in this study, referes to the effective concentration (mg L^{-1}) of CB causing X% stimulation or inhibition of the microbial dehydrogenase activity.

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

The experiments were conducted in standard 2 x 15 cm test tubes at room temperature (21°C). The resazurin solution was always added last and the contents were quickly mixed and placed in the dark. After an exact incubation of 30 min, 10 mL of n-amyl alcohol and 1 mL of phthalate-HCl buffer solution were rapidly added to each tube to stop the reaction. The contents were vigorously mixed for 10 sec on a vortex mixer, followed by centrifugation in the same test tube at 1,000 rpm for 5 min. The upper solvent layer was

Table 1. % Stimulation (+) and inhibition (-) of microbial dehydrogenase activity by CB as measured by the resazurin method.*

Compounds	CB Concentrations (mg L^{-1})												
	20	50	80	100	120	150	200	300	350	400	450	500	1000
MCB	+4.9			+8.2				+13.1	+10.6	-26.1		-79.8	
o-DCB	+5.7			+17.9	+17.4	-27.4	-89.9						
m-DCB	+9.6			+13.2	-20.9	-77.1	-90.9						
p-DCB	+6.3			+13.3				+5.3					-35.0
1,2,3-TCB	+10.1	+14.2	-74.1	-74.6									
1,2,4-TCB	+11.0	-20.3	-51.2	-51.4									
1,3,5-TCB	+8.3			+12.0								+10.3	+8.0
1,2,3,4-TRCB	+9.3			+14.7								+19.3	+15.3
1,2,3,5-TRCB	+10.3			+6.3								+6.3	+5.0
1,2,4,5-TRCB	+8.1			+1.7					-19.0	-22.3		-25.0	
penta-CB	+5.7			+5.7			+5.0					+6.3	

* Average of 9 experiments from different dates.

then transferred by pasteur pipette 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 a spectrophotometer at 610 nm (the maximum absorbance of unreduced resazurin).

RESULTS AND DISCUSSIONS

Studies of toxicity effects at cellular (enzyme) level have the advantage of being more sensitive than investigations at the population level such as the measuremnt of LD_{50}. Dehydrogenases are actively involved in the vital anabolic and catabolic processes of all living organisms. Consequently they are considered ideal for studying the interactions between toxicant and biota at the cellular level. The advantage of using this approach in toxicity assessment is clearly evident in Table 1. Perhaps, the most surprising finding, in the present investigation on CB's toxicity, is the ability of all the 11 chlorobenzenes to stimulate the mirobial dehydrogenases activity (5-19%), implying

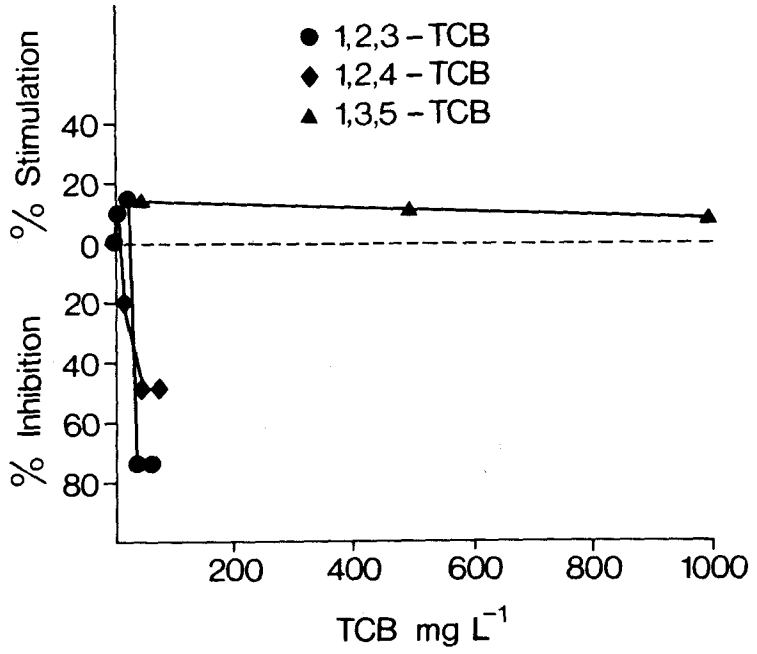

Figure 1. Effect of trichlorobenzenes on microbial dehydrogenase activity.

the perturbation of the bacterial cellular components or function by CB. Such cellular disturbance by toxicant could not easily be observed in toxicity study at the population level. Thus, hexachlorobenzene has been concluded to have a negligible toxicity effect on fish based on LD_{50} data (KONEMANN 1980).

The death of an organism, as brought about by the toxicant, could be considered as the ultimate grand expression of the disruption of various physiological processes within the organism, ie, cellular disruption occurring much earlier than the observed death. Thus toxicity measurement at the population level has little value in assessing a toxicant's chronic or sublethal effect (JOSEPHSON 1980). Based on the concept of toxicity assessment at population level, such as LD_{50} or EC_{50} measurement, one could easily draw the conclusion from Table 1 that CB are a class of chemicals with low biotoxicity, since only 5 CB (MCB, o-DCB, m-DCB, 1,2,3-TCB and 1,2,4-TCB) could yield EC_{50} values. However, the true value of the resazurin method, perhaps, lies on its capability to detect a chemical's sublethal or chronic effect (such as the changes of enzymes activity). Stimulation or

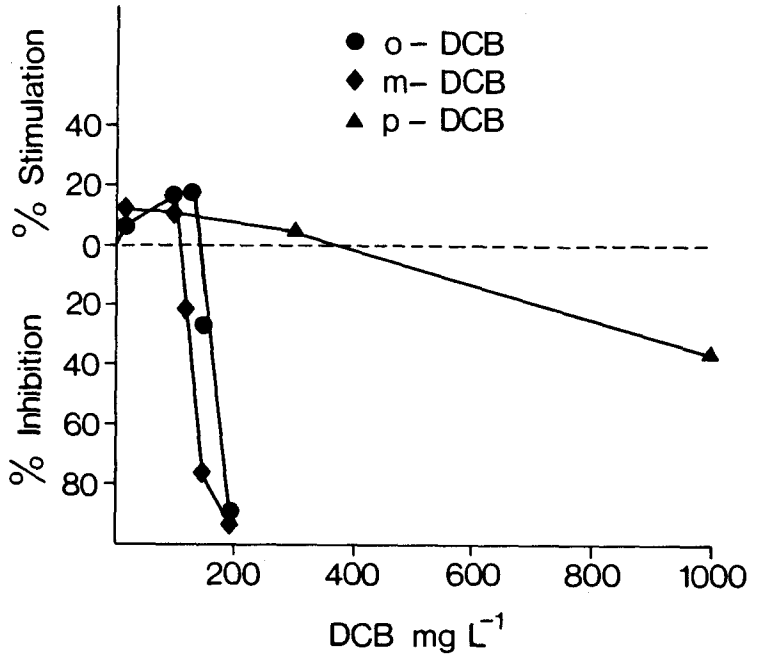

Figure 2. Effect of dichlorobenzenes on microbial dehydrogenase activity.

inhibition of the dehydrogenase activity by toxicants is harmful to a living organism, as this may produce deleterious effect on the organism by interferring with its energy metabolism (GUPTA & SASTRY 1981). Various toxicants such as mercury and cadmium (GUPTA & SASTRY 1981) have been shown capable of stimulating the succinic dehydrogenase activity in fish and pigeon. The elevation in the dehydrogenase activity by heavy metals is probably due to the binding of the metals to an activating site (EICHHORN 1975). CB are highly lipophilic in nature (KONEMANN & van LEEUWEN 1980), thus it can be conceived that these chemicals would tend to bioconcentrate in the phospholipid fraction of cell membranes where most of dehydrogenases are located.

In recent years, research on SAR (structure-activity relationship has gained momentum and some good progress has been achieved on toxicity prediction, based on the toxicant's physicochemical, topological, geometrical and electronic parameters, (SOUTHWORTH et al. 1978, SHULTZ et al. 1980). The demand for the SAR approach in toxicity assessment is understandable, as judged from the estimated 70,000 new chemicals annually synthesized in the United States (JOHNSON 1982). However, the study of quantitative structure-toxicity relationship is an extremely complex endeavor which is clearly demonstrated in Figure 1. According to the data of KONEMANN and van LEEUWEN (1980), the 1,2,3-TCB and the 1,3,5-TCB have an identical log n-octanol/H_2O partition coefficient of 4.20. Thus, theoretically, they both should exhibit the same degree or trend of toxicity. However, the results in Figure 1 indicate that this was not as anticipated. Instead, the 1,3,5-TCB exerted only a stimulating effect on microbial dehydrogenase activity, while the 1,2,3-isomer showed both stimulating and inhibitory effects. Similar variations in toxicity among the isomers were also observed for the dichlorobenzenes (Figure 2). The p-DCB was found to be significantly less toxic than the ortho- and meta isomers. The results of the present study imply that molecular geometry and electronic properties could be very important in eliciting a toxicant's biological activity. Further, since analogues often behave independently, more systematical studies including the use of as complete a set of isomers as possible in the experiment, need to be done to understand the complex interactions between toxicant and biota in the total environment.

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