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Biochemical Responses of Bacteria after Short Exposure to Alkyltins

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Organotin compounds rank fourth in the industrial production of organometallics but little is known about their effect and fate in the aquatic environments (Hodge et al. 1979). In the United States alone, over two million pounds of alkyltin compounds were produced in 1976 (Laughlin and French 1980) and the trend of using organotins in the products of our daily life continues to rise, due to the increasing demand for plastics and anti-fouling paints. In general, the dialkyltin derivatives are primarily used as heat stabilizers for PVC plastics and as catalytic agents in the production of urethane foam. The trialkyl and triaryl tin derivatives are used mainly as biocides in various agricultural, paper, and paint industries (Piver 1973).

Like other organometallics, organotin compounds are slowly subjected to various abiotic and biotic degradation processes in the environment (Ridley et al. 1977; Soderquist 1978; Chau et al. 1980). Many organotin compounds, including their degradation products, have been recently detected in sea water, coastal sediment, rivers and Great Lakes (Hodge et al. 1979; Hallas and Cooney 1981; Maquire et al. 1982), thus implying their possible wide distribution in the total environment. Generally speaking, inorganic tin compounds are not as toxic as the organic derivatives. The toxicity of organotin compounds varies considerably according to the number and nature of the organic groups with tri- and tetra-alkyltins being the most toxic forms (Wong et al. 1982). The toxicity of organotin compounds to human beings and mammals has been extensively investigated, with much less toxicity data on other biota (Piver 1973). To our knowledge there is no study of of the alkyltins' toxicity to bacteria at the enzyme level. The present study describes some biochemical responses of a mixed bacterial culture after short exposure to various alkyltin compounds and the possible impact of these chemicals on microbial populations in aquatic environment.

MATERIALS AND METHODS

The growth medium (GM) for culturing the test bacterial culture contained the following components in the amount of g/L: K_2HPO_4,

1.32; KH_2PO_4, 0.82; glucose, 0.2; sodium acetate, 0.2; nutrient broth, 1.0; yeast extract, 1.0 and distilled water, 1L. The medium was sterilized by autoclaving at 121°C for 15 min and the same medium was also employed in all toxicity testing procedures.

The mixed bacterial culture was obtained by inoculating 1 mL of fresh activated sludge into 50 mL of GM medium contained in a 125-mL Erlenmyer flask on a rotary shaker at room temperature (21°C) for 18 hr. The culture was transferred twice at the beginning of the week prior to toxicity testing to ensure a fresh young culture. The cells grew very rapidly, therefore a very small inoculum was used in the transfers (0.2 mL culture into 50 mL GM in the morning, or 50 µL in the evening). This would yield an uniform culture with approximately the same metabolic activity when used the next morning (after the night transfer) or in the late afternoon (after the morning transfer). Thus toxicity testing of alkyltins could be carried out twice a day. The culture was used directly in the experiments without adjustment for cell concentration.

The butyltin compounds were dissolved in methanol to give stock solutions of 10 mg/mL with the exception of n-butyltin hydroxide oxide at 5 mg/mL. BTTC (n-butyltin trichloride), BTDC (di-n-butyltin dichloride), TBTC (tri-n-butyltin chloride), TTBT (tetra-n-butyltin), BTHO (n-butyltin hydroxide oxide), and TBTO bis(tri-n-butyltin) oxide were obtained from Ventron Corp., Danver, MA 01923.

The resazurin reduction procedure for dehydrogenase activity (Liu and Thomson 1983) and oxygen uptake measurement were used to assess the biochemical responses of the mixed bacterial culture to butyltin compounds. The reaction mixture for the resazurin test contained the following : 1 mL cells, 2750 µL GM, (250-*X*) µL methanol, *X* µL butyltin stock solution, and 1 mL resazurin solution. Total volume of the reaction mixture was 5 mL. The cell control was made up of 1 mL cells, 2750 µL GM, 250 µL methanol and 1 mL resazurin solution. The reagent control contained only 3750 µL GM, 250 µL methanol and 1 mL resazurin solution.

The test mixtures were incubated for 30 min in a water bath at 24°C and the reaction was stopped by the addition of 10 mL of n-amyl alcohol (solvent) and 1 mL of phthalate-HCl buffer (pH 3.0). The samples were then centrifuged at 1,000 rpm for 5 min and approximately 8-9 mL of the upper solvent layer were transferred to a clean test tube containing ca. 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). The term IC, used in this study, refers to the specific concentration (mg/mL) of n-butyltin compound causing *X*% stimulation or inhibition of the microbial dehydrogenase activity or of the oxygen uptake rate. The IC_{50} values for n-alkyltin compounds were graphically

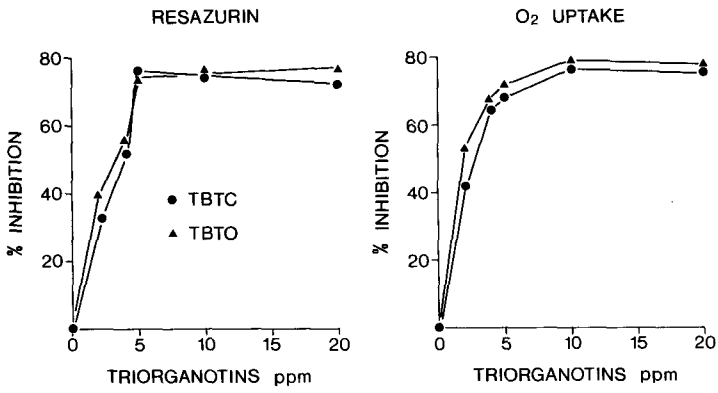

Figure 1. Effect of tri-n-butyltins on the dehydrogenase activity and oxygen consumption of the mixed bacterial culture.

determined by plotting % inhibition against concentrations.

Oxygen uptake measurements were performed in a Gilson differential respirometer. The composition of the assaying preparation, unless otherwise stated, consisted of 1 mL of cells, 2750 µL GM, (250-*X*) µL methanol, *X* µL butyltin stock solution (in the side arm of the Warburg flask), 1 mL of distilled water and 0.2 mL of 20% KOH (in the center well of the Warburg flask for CO_2 absorption). The experiments were carried out at 24°C with a steady shaking rate of 105 strokes/min. Readings were taken every 5 to 10 min for 100 min. The % inhibition for each concentration of the test chemical was calculated using the last reading from the test flask against the control flask without n-alkyltin addition.

RESULTS AND DISCUSSION

Dehydrogenases are actively involved in the vital anabolic and catabolic processes of all living organisms. For instance, the coenzyme NADPH (nicotinamide adenine dinucleotide phosphate) which is required in most biosynthesis processes, is produced via dehydrogenase mediated reactions. Metabolism of organotin compounds by biota has been demonstrated to be NADPH dependent (Kimmel et al. 1977), thus the resazurin reduction procedure (Liu and Thomson 1983), which is based on the quantitative measurement of the interaction between a toxicant and the microbial dehydrogenase activity, seems to offer an ideal tool for probing the biochemical response of microorganism to organotin compounds at the cellular level. On the other hand, oxygen consumption is an established method which could provide useful information of a toxicant's sublethal effects, because energy processes, such as oxygen uptake, in a living organism may serve as an indicator of its overall physiological state (Sigmon 1979). The advantage of using the

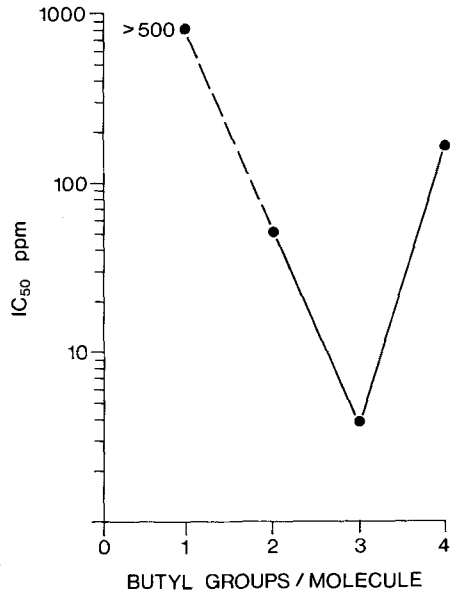

Figure 2. Effect of the degree of alkylation on the biocidal potential of organotins.

integrated approach comprising both the resazurin reduction and oxygen uptake measurement in the assessment of tributyltins toxicity is clearly evident in Figure 1. The striking similarity of the dose-response curves for TBTC and TBTO obtained by the resazurin reduction and oxygen uptake measurement would strongly suggest that triorganotin compounds are capable of interrupting the processes of energy metabolism in microorganism. Since TBTC is extremely lipophilic (Seinen et al. 1981), it can be conceived that the trialkyltin compounds would tend to be concentrated in the phospholipid fraction of the bacterial cell membranes where most of the vital dehydrogenases are located. Thus TBTC or TBTO could effectively exert their inhibitory influence on the bacterial dehydrogenase activity.

The results in Figure 1 also support previous observations that for the toxicity of organotin compounds, the nature of the anionic group is only of secondary importance (Seinen et al. 1981). The IC_{50} values for TBTC (IC_{50} = 3.8 ppm) and TBTO (IC_{50} = 3.4 ppm), as determined by the resazurin procedure, were almost identical to the mixed bacterial culture. The oxygen uptake measurement was slightly more sensitive than the resazurin reduction, but yielded a remarkably similar trend of IC_{50} values for TBTC (IC_{50} = 2.4 ppm) and TBTO (IC_{50} = 1.9 ppm) to the bacterial culture. The above

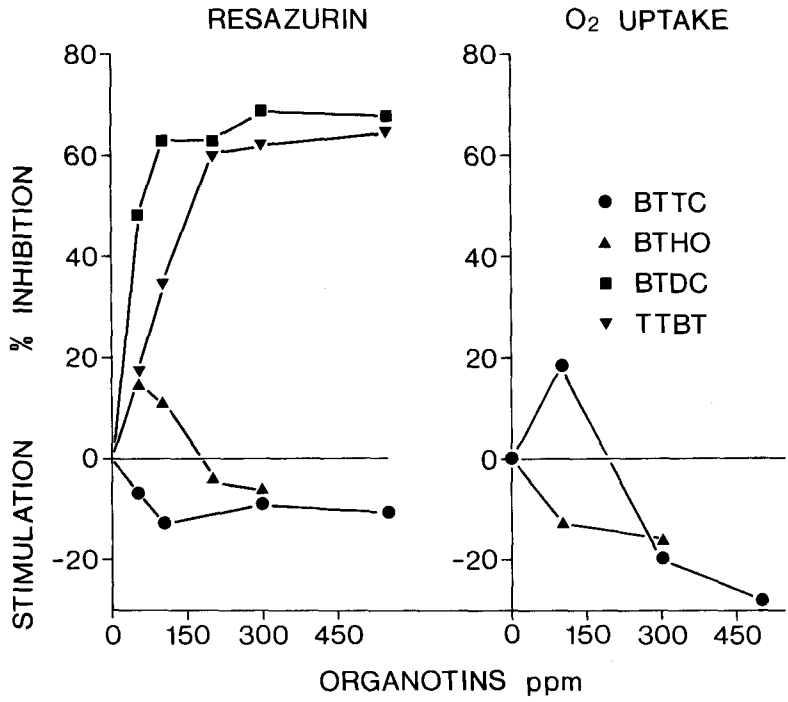

Figure 3. Effect of mono-, di-, and tetra-n-butyltins on the dehydrogenase activity and oxygen consumption of the mixed bacterial culture.

would indicate that the anion chloride or oxide bears little influence, if any, on tributyltins toxicity.

Conversely, the degree of alkylation of the organotin compounds has a profound effect on their biological activity against the bacterial culture. The data in Figures 2 and 3 indicate that progressive introduction of n-butyl groups at the tin atom in the n-butyltin compounds studied results in a linear increase in toxicity. The biocidal activity of n-butyltin compounds reaches the maximum with three alkyl groups attached to the tin atom. Further addition of alkyl group tends to produce a sharp drop in biocidal activity. Thus the tetra-n-butyltin (IC_{50} = 160 ppm) was approximately 47 times less toxic than the tri-n-butyltin chloride (IC_{50} = 3.8 ppm). Even the less alkylated di-n-butyltin dichloride (IC_{50} = 48 ppm) had a biocidal potential three times greater than the tetra-n-butyltin. The monobutyltin compounds had no apparent acute toxicity (IC_{50} > 300-500 ppm) to the mixed bacterial culture.

IC_{50} values are useful for quick comparison of chemical's acute

toxicities. However, from the ecotoxicological view point, they are of little scientific merit, because such IC_{50} determinations do not provide any information on a toxicant's chronic or sublethal effects (Dagani 1983). Thus, the true value of the resazurin reduction method and oxygen uptake measurement probably lies on their ability to detect a chemical's sublethal effects. The results in Figure 3 indicate that the mono-n-butyltins BTTC and BTHO are biologically active, due to their ability to stimulate or inhibit the dehydrogenase activity and oxygen consumption. Stimulation or inhibition of the dehydrogenase activity by toxicant is harmful to a living organism, as this produce deleterious effect on the organism by interferring with its energy metabolism.

The exact mechanism of organotins' biotoxicity to microorganism is unclear. Gram negative bacteria, in general, are more resistant to the toxicity of trialkyltins than are gram positive bacteria by virtue of their complex outer membrane structure (Blair et al. 1982). Dialkyltin and trialkyltin compounds are known to be capable of uncoupling the oxidative phosphorylation as well as of inhibiting the mitochondrial respiration by preventing the oxidation of α-keto acids (Piver 1973). These uncoupling and inhibitory effects of organotin compounds may disturb the intracellular energy metabolism of a living microorganism. Bacteria play an important role in the removal of contaminants from the environment. However, recent study suggested that the capacity to degrade tributyltin compounds is not widespread among bacteria (Blair et al. 1982). Thus a systematic control and monitoring the use of organotin compounds are essential to safeguard the public health and environmental quality.

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