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Effects of Organotin Compounds on the Synthesis of Nucleic Acids and ATP in the Growth Process of *Bacillus subtilis*

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Over the years, organotin compounds have used widely as agricultural chemicals, stabilizers in plastics, and marine antifoulants. However, these compounds, having toxic properties, are known as pollutants. The principal effect of the compounds on the function of the eucaryotic cell ascribes to the inhibition of oxidative phosphorylation caused by damage of the mitochondria membrane (Aldrige 1958, Aldrige and Street 1964, Wulf and Byington 1975, Chain and Griffiths 1977). The aim of this article is to elucidate the effects of organotin compounds on the growth of procaryotic cell. The effects of organotin compounds on intracellular DNA and RNA synthesis were measured in growth process of the synchronous cell. The addition of the organotin compound prolonged the generation time as it did with DNA and RNA. Inactivation of DNA synthesis by the compounds could not be detected from the results of the experiments *in vitro*. On the other hands, the parameter obtained through analysis of the inhibitive degree of ATP synthesis correlated closely with that of the cell growth. It was estimated from these results that the organotin compounds inhibited energy transformation of the cell and thereby induced the prolongation of DNA and RNA synthesis.

MATERIALS AND METHODS

As the sample of organotin compounds, trimethyltin chloride (TMC), triethyltin bromide (TEB), tributyltin chloride (TBC) and triphenyltin chloride (TPC) were used. The strain was *Bacillus subtilis* (IFO 3022).

Culture: Spizien medium was used for the asynchronous culture and consisted of 2.0g $(NH_4)_2SO_4$, 14.0g K_2HPO_4, 6.0g KH_2PO_4, 1.0g sodium citrate·$2H_2O$, 0.2g $MgSO_4 \cdot 7H_2O$, 0.02g $MnCl_2 \cdot 4H_2O$ and 5.0g glucose per 1000 ml of aqueous solution. The medium of composition of the synchronous culture was the same as that described above but contained 0.6g glucose. The concentrations of the organotin compounds in the media are shown in each figure. These media, 100 ml, contained the organotin compound were prepared and sterilized by steam heating at 120°C for 15 min in an autoclave. The precultivation broth, 1.0 ml, grown at 37°C for 6 hours in

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the medium, was inoculated in the same medium, 100 ml, and shaken at 37°C. The cell concentrations of the synchronous cultures were determined by measurement of transmittance at 660 nm, and those of the synchronous cultures were read under a microscope using Petroff-Hauser chamber.

DNA and RNA contents: Intracellular DNA and RNA were fractionated by the method of Schmidt, Thannhauser and Schneider (Schneider 1946). Their concentrations were determined, respectively, by measurement of absorbance at 260 and 270 nm.

ATP contents: The cells were collected by centrifugation, suspended in distilled water, and then burst by the freeze and thawing method. Light densities, a and c, emitted with luciferin-luciferase were measured, respectively, by use of CHEM-GLOW photometer (American Instrument Company) in a system with and without the organotin compound. The values correspond to ATP quantities (Lyman and DeVicenzo 1967). Therefore, the ratios of ATP quantities in both systems were determined by the relative intensity, a/c.

Activity of DNA polymerase 1: The template DNA was previously prepared by the stndard method from calf thymus DNA. Each of the solutiom: dATP, dCTP, dGTP, and dTTP, template DNA solution, organotin compound, and then DNA polymerase 1 were added to phosphate buffer solution (pH 7.4). After incubating at 35°C for 30 min, the enzymatic reaction was terminated by the addition of sodium pyrophosphate and perchloric acid. DNA produced was collected by centrifugation, wash with ethanol, and dissolved in 0.01M NaCl solution. The DNA concentration was determined by the absorbance at 260 nm at 85°C (Ogawa et al. 1990).

RESULTS AND DISCUSSION

The growth curves in the asynchronous culture containing the organotin compound were measured. Figure 1 shows an example of the results. The growth curves were analyzed as follows to evaluate quantitatively the growth inhibition:

$$\ln(C_2/C_1) = k(t_2 - t_1) \qquad (1)$$

where C_1 and C_2 are each cell concentration at culture time, t_1 and t_2. The inhibitive degree, H, is defined as

$$H = 1 - (kg/ko) \qquad (2)$$

where kg and ko are the growth rate constants in a system with and without the organotin compound. It has been shown experimentally for many inhibitors that H may be represented as

$$\log[H/(1 - H)] = n\log G - n\log\phi \qquad (3)$$

where n is the exponent indicating inhibitive ability of the cell growth, G, the concentration of the inhibitor, and ϕ, the concentration of the inhibitor at H = 0.5 (Yanagida 1981). The H-values were calculated applying the data of the growth curves to equation (1) and (2), and were then plotted using the relation of log[H/(1 - H)] vs. logG as shown in Figure 2. The values of n and ϕ were determined, respectively, from the slope of the straight line and G-value at log[H/(1 - H)] = 0, and are listed in Table 1.

The ϕ-values of TMC, TEB and TBC decreased in that order. This

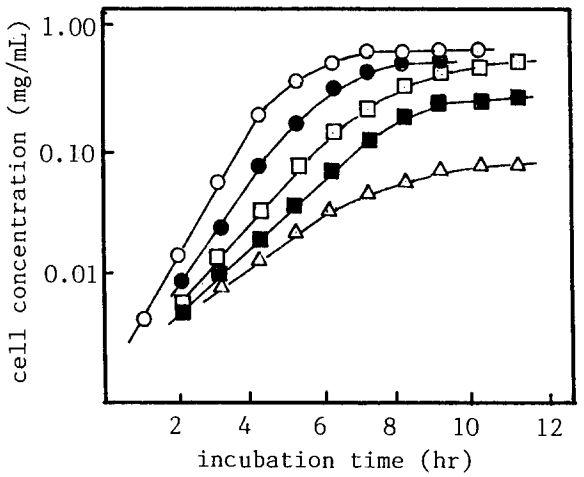

Figure 1. Asynchronous growth curves of the cell in culture with TPC.

temperature: 37°C, ⊙ control, ● 6.0×10^{-6}M, □ 7.5×10^{-6}M, ■ 8.0×10^{-6}M, △ 10.0×10^{-6}M

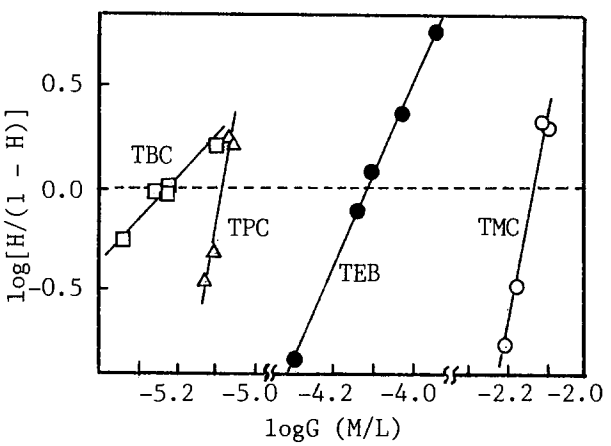

Figure 2. Plot of log[H/(1 - H)] vs. logG

Table 1. n- and ϕ-values

reagent	cell growth		ATP synthesis	
	n	ϕ (M/L)	n	ϕ (M/L)
TMC	10.7	7.16×10^{-3}	11.2	6.3×10^{-3}
TEB	4.41	7.52×10^{-5}	3.82	7.4×10^{-5}
TBC	1.68	6.00×10^{-6}	0.34	6.8×10^{-6}
TPC	12.4	8.32×10^{-6}	10.0	6.6×10^{-6}

can be understood on the fact that (1) the lipophylic compounds, having a higher affinity to cell membranes, are absorbed by the cells in greater quantity, and (2) the rates of many enzymatic reactions correlate positively with hydrophobicity of the substrate (Leo et al. 1971).

The microbes were synchronously cultivated in the media containing the organotin compound. Figure 3 shows an example of the growth curves measured. All organotin compounds used caused prolongation of a cell cycle, but little reduction in the cell division. The compound, TBC, was added to the media at different cell ages. Figure 4 shows the growth curves. The generation time was prolonged in the case added previously, but not in the case added at the later time. Similar phenomena were also observed for the other compounds used. The synthesis of nucleic acids is generally initiated in the initial period of a cell cycle. In the case of *E. coli.*, DNA replication is completed in 40 minutes during a cell cycle of 60 minutes, and thereafter, the cell consituents, such as cell wall are formed (Yanagida 1981). It was suggested, therefore, from the experimental results, that the organotin compounds inhibited DNA synthesis directly or indirectly.

In order to clarify the effects of the organotin compounds on the cell cycle, synchronous cultures were used, and the contents of intracellular DNA and RNA were measured at different ages. Figure 5 shows the results. Cell division requires the doubling of all cell constituents and their partitioning into the daughter cells. The contents of the constituents during the cell cycle increase with the growth of a cell, and then fall at division time. Figure 5 depicts such a phenomenon. The DNA contents decrease little by the addition of organotin compounds, and the RNA contents somewhat. The time taken for the DNA contents to reach a maximum and a minimum correspond, respectively, to the replication period of DNA and the generation time of a cell in Figure 3. Table 2 lists the replication period of DNA, A, of each system, the generation time, B, and A/B. The (A/B)-values were approximately equal, that is, ca. 0.9. In other words, the replication of DNA was prolonged in proportion to the generation time.

The cause of the inhibition was investigated as follows. Amounts of DNA synthesized with DNA polymerase 1 were measured in the presence of the organotin compound. Figure 6 shows the results. The organotin compound did not affect the amount of DNA synthesized, consequently, the activity of DNA polymerase 1. Further, melting temperature of calf thymus DNA, measured by the method reported in the preceding paper (Ogawa et al. 1989), was not changed by the addition of the organotin compound. It was known, therefore, that the organotin compound did not contribute to the stabilization of the double helix of DNA. The double helix of DNA separates into two strands prior to replication and transcription. The above result means that the energy for its separation is not affected by the organotin compounds. It was not substantiated in our experiments that the organotin compounds used inhibited DNA synthesis directly. Therefore, the effect

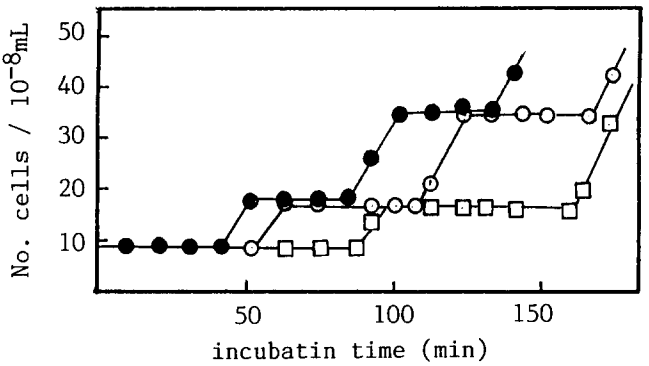

Figure 3. Synchronous growth curves of the cells in culture with TBC.
● control, ⊙ 1.25×10^{-7}M, ⊡ 1.0×10^{-6}M

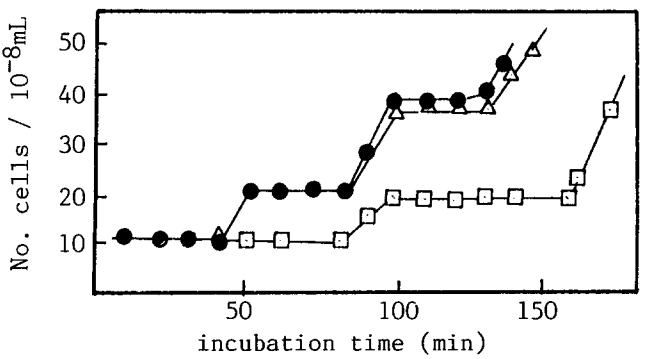

Figure 4. Influence of addition time of TBC (1.0×10^{-6}M) on synchronous growth curves of the cells.
● control, ⊙ the case added previously
△ the cases added at culture time;10, 20 and 30 min

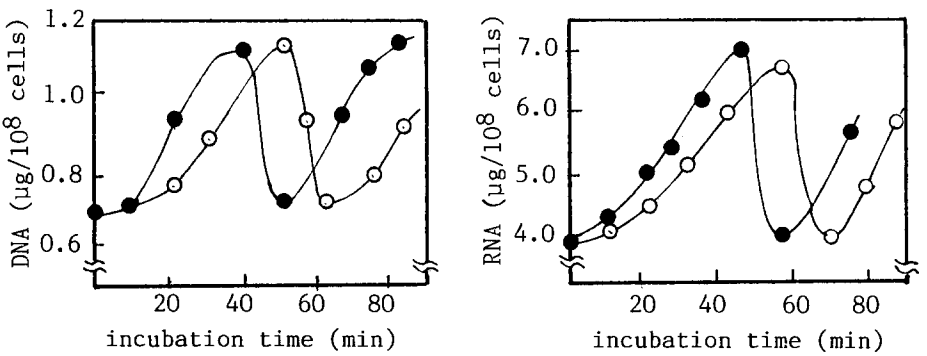

Figure 5. The synthesis of nucleic acids of synchronous cell.
● control, ○ TBC 5.0×10^{-7}M

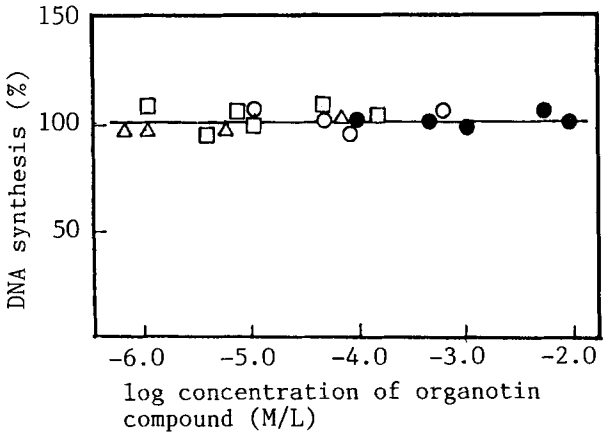

Figure 6. Effect of orgaotin compound on DNA synthesis with DNA polymerase 1.
● TMC, ⊙ TEB, ⊡ TBC, △ TPC

Table 2. A period of the synthesis of nucleic acids in a cell cycle.

reagent	(M/L)	A* (min)	B** (min)	C*** (min)	A/B
control		40	45	45	0.89
TMC	2.0×10^{-4}	50	55	55	0.91
TEB	2.0×10^{-5}	50	55	55	0.91
TBC	2.5×10^{-5}	50	60	55	0.83
TPC	2.5×10^{-6}	65	75	70	0.87

* a completion period of DNA replication, **generation time,
*** a period reaching to maximum contents of RNA

of the organotin compound on ATP contents was investigated as a means of evaluating the other inhibition. The cells were cultivated in a system with and without the organotin compound, treated by the method described previously, and then the relative value, a/c, of the light density emitted with luciferin-luciferase was measured. The inhibitive degree was defined as

$$H = 1 - (a/c) \qquad (4)$$

The application of equation (3) could be confirmed to hold also for the inhibition of the ATP synthesis because a straight line was formed from the plot of $\log[1/(1 - H)]$ vs. logG. The parameter, n and ϕ, on the inhibition of the ATP synthesis were plotted as ordinate and those on the inhibition of the cell growth as abscissa. The results were shown in Figure 7. Each correlation coefficient on n and ϕ were very high, respectively, 0.97 and 0.99. Intracellular ATP is synthesized in the process of energy transformation. Therefore, the good correlation suggests that the inhibition of energy transformation by the organotin compound is a dominant factor of the growth inhibition. The quantity of energy, liberated by the addition of the organotin compound, was measured by use of the differential calorimeter. The result

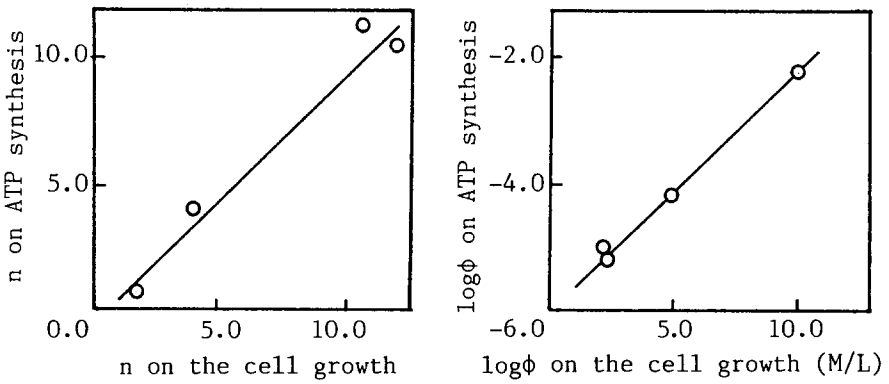

Figure 7. Inhibitive parameters on the ATP synthesis and the cell growth.

of the thermal analysis will be reported in a subsequent paper.

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