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Effects of Monochlorophenols and *p*-Chloroaniline on Nucleic Acid Synthesis in Microbial Growth Process

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Lots of haroaromatics are used to produce herbicides, microbiocides, dyestuffs, plasticizers, and so forth. In general, these compounds, having low biodegradative and remarkable toxic properties, are known as pollutants. In order to elucidate the effects of the chlorine substituents on their toxicities, it is preferable to assay relevant simple compounds at first. The subjects have been studied on the dehydrogenase activity of Bacillus sp. (Liu et al. 1982), the elongation of millet roots (Wang 1985) and the biodegradation of phenols (Beltrame 1988). However, the effects of such compounds on the synthesis of intracellular components in microbial growth have not been revealed. The effects of chlorophenols and p-chloroaniline on the synthesis of nucleic acids in the growth of Bacillus subtilis were reported in this paper.

MATERIALS AND METHODS

Phenol, aniline and their monochlorides were used as the inhibitor. o- and m-Chloroaniline could not be used owing to their lack of solubility in water. The strain, the culture condition, the contents determination of intracellular DNA and RNA, and the determination of the inactivation of DNA polymerase 1 were the same as these in the previous papers (Ogawa et al. 1988, 1990), where were reported the growth inhibition of the microbe by basic dyes. The outlines are as follows.

Culture: Spizien medium was used for the asynchronous culture and consisted of 0.2% $(NH_4)_2SO_4$, 1.4% K_2HPO_4, 0.6% KH_2PO_4, 0.1% sodium citrate·$2H_2O$, 0.02% $MgSO_4$, 0.0002% $FeCl_3 \cdot 6H_2O$, 0.0002% $MnCl_2 \cdot 4H_2O$ and 0.5% glucose. The medium composition of the synchronous culture was the same as that described above but contained 0.06% glucose as well. The cell population was synchronized by the stationary phase method. The cultures were shaken at 37°C. The cell concentrations of the asynchronous cultures were determined by measurement of transmittance at 660 nm, and those of the synchronous cultures were read under a microscope using Petroff-Hausser chamber.
Partition coefficient: The octanol solutions (10 mL) ranging in concentration from 10^{-4} M of the chloro-compound to 10^{-3} M were prepared, added to distilled water (20 mL), and then shaken for 48 hr

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at 37°C. The concentrations of the chloro-compounds in the octanol and the water phase were measured spectrophotometrically. The partition coefficient, P, is defined by equation (1).

$$\log C_A = \log P + \log C_B \quad (1)$$

where C_A and C_B are each concentration of the chloro-compound in the octanol and the water phase. In accordance with this relation, the P-values of the chloro-compounds were calculated by the least-squares method from the intercept of the log C_A vs. log C_B plots. The results were listed in Table 1.

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

Amount of DNA synthesized with DNA polymerase 1: The template DNA was previously prepared by the standard method from calf thymus DNA solution. Each solution of dATP, dCTP, dGTP and dTTP, the template DNA solution, the chloro-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, washed with ethanol, and dissolved in 0.01 M NaCl solution. The DNA concentration was determined by the absorbance at 260 nm at 85°C.

RESULTS AND DISCUSSION

The growth curves in the asynchronous culture containing the chloro-compounds were measured. Figure 1 shows two examples of the results. The mean growth rate in the logarithmic phase decreased with increasing concentration of the chloro-compounds. The rate constant, k, can be expressed as

$$\ln(C_2/C_1) = k(t_2 - t_1) \quad (2)$$

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

$$H = 1 - (k_g/k_o) \quad (3)$$

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

$$\log[H/(1 - H)] = n\log G - n\log F \quad (4)$$

where n is the exponent indicating inhibitive ability, G, the concentration of the inhibitor, and F, the concentration of the inhibitor at H = 0.5, that is, 50% inhibition (Yanagida 1981). H-values were calculated applying the data of the growth curves to equation (2) and (3), and were then plotted using the relation of $\log[H/(1 - H)]$ vs. logG as shown in Figure 2. The values of n and F were determined, respectively, from the slope of the straight line and the G-value at $\log[H/(1 - H) = 0$, and were listed in Table 1.

The chlorine substituents caused the F-values of the phenols and the anilines to decrease. In order to illustrate how the lipophilicity of these compounds affect the microbial growth inhibition, the relation of the n-values and the F-values to the partition coefficients, P, were shown in Figure 3. The P-values have often been adopted as a parameter for representing the lipophilicity of many compounds (Beltrame et al. 1988). The relation between

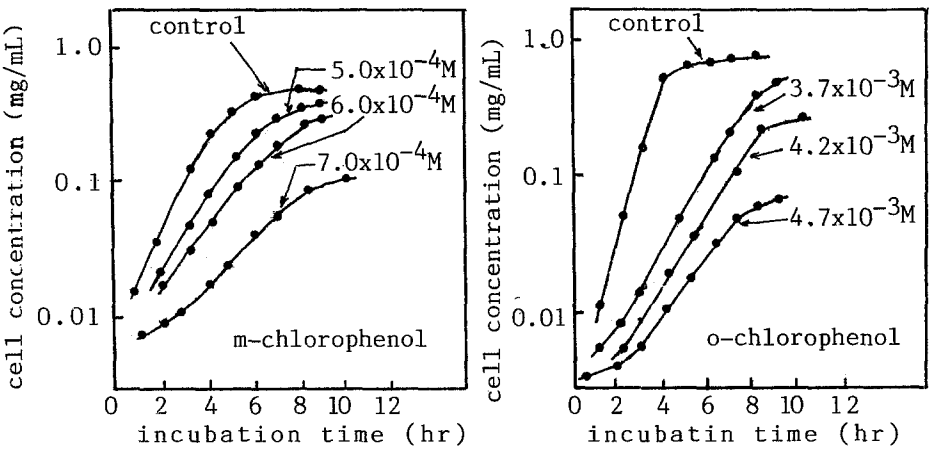

Figure 1. Asynchronous growth curves of the cells in culture with the chloro-compound.
temperature: 37°C

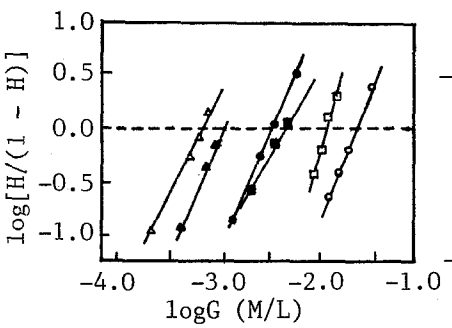

Table 1. n- and F-values

aniline	0.98	3.68	2.14×10^{-2}
p-chloroaniline	1.83	2.33	3.02×10^{-3}
phenol	1.54	4.50	1.05×10^{-2}
o-chlorophenol	2.15	1.80	3.70×10^{-3}
m-chlorophenol	2.61	1.98	6.47×10^{-4}
p-chlorophenol	2.44	2.08	8.32×10^{-4}

Figure 2. Plot of log[H/(1 - H] vs. n
aniline, p-chloroaniline, phenol, o-chlorophenol, m-chlorophenol, p-chlorophenol

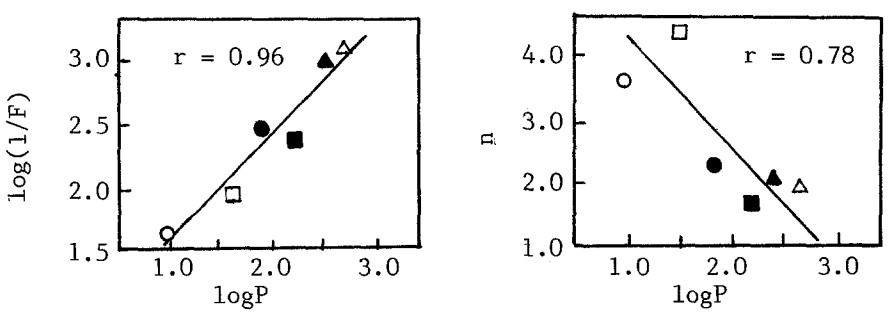

Figure 3. Relation between inhibitive parameter and logP.
r: correlation coefficient
aniline, p-chloroaniline, phenol, o-chlorophenol, m-chlorophenol, p-chlorophenol

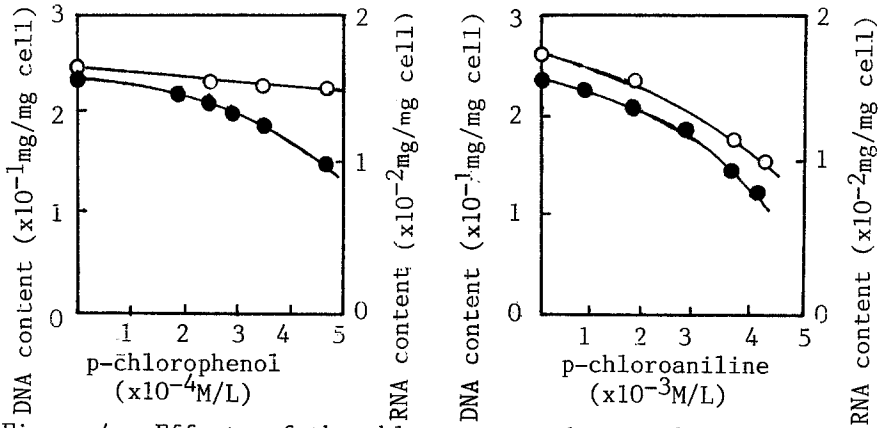

Figure 4. Effects of the chloro-compound on nucleic acid synthesis.
-O- DNA content -●- RNA content

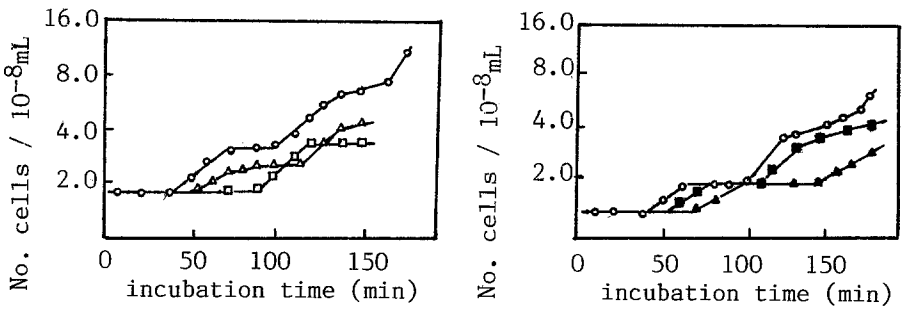

Figure 5. Synchronous growth curves of the cells in culture with the chloro-compounds.
-O- control -□- aniline 2.5x10^{-2}M/L -■- phenol 1.1x10^{-2}M/L
-△- p-chloroaniline 3.0x10^{-3}M/L -▲- o-chlorophenol 3.7x10^{-3}M/L

log(1/F) and logP can be understood through the fact that (1) the lipophilic compounds, having a higher affinity to cell membrances, are absorbed to the cells in more quantity, and (2) the rates of many enzymatic reactions correlate positively with hydrophobicity of the substrates (Leo et al. 1971). On the other hand, the authors have been unable to find a reasonable account on the relationship between n and logP in Figure 3.

The intracellular DNA and RNA contents in the logarithmic phase were measured. Figure 4 shows two examples of the results. The DNA contents slightly decreased as the concentrations of the chloro-compounds excepting p-chloroaniline increased. The inhibition of the RNA synthesis by the chloro-compounds was high in comparision with that of the DNA synthesis.

The microbes were synchronously cultured in medium containing the chloro-compound. Figure 5 shows the examples of the growth curves measured. For the culture with p-chloroaniline, the degree of cell division was reduced, as in the case with the basic dyes which strongly inhibited the DNA synthesis (Ogawa et al. 1988). By every

chloro-compounds used, the generation time were prolonged. Therefore, it is understood that the growth inhibition by the compounds excepting p-chloroaniline is attributed not to the reduction in cell division degree, but to prololongation of the generation time.

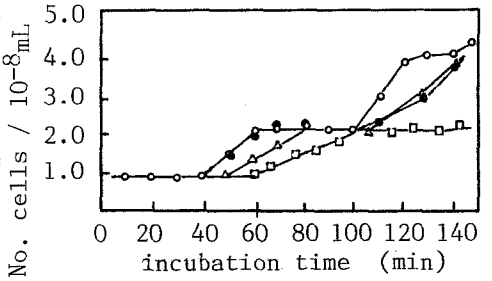

Figure 6. Effects of the inhibition of the inhibition of synchronous cell growth by addition of o-chlorophenol (3.7×10^{-3} M/L) during the cell.
—○— no addition,
time of addition: □ 0 min, △ 15 min, ● 20 min,

o-Chlorophenol was added to the medium at different cell age. Figure 6 shows the growth curves. In the case of the compound added in the initial period of the cell cycle, the generation time was prolonged, while the addition of the compounds in the later period did not prolong the generation time. Similar phenomena was also observed for the other compounds used. In general, the nucleic acid of microbes is synthesized in the initial period of the cell cycle. In the case of E. col., the DNA replication is completed in 40 minutes after the begining of the cell cycle for 60 minutes, and thereafter, the cell constituents such as the cell wall are formed (Yanagida 1981). This suggests that the prolonged generation time, observed when the compounds are added in the initial period of the cell cycle, is caused by the inhibition of the synthesis of the nucleic acid.

p-Chloroaniline inhibited strongly as previously described not only the synthesis of RNA but one of DNA. In order to clarify the inhibition in the cell cycle, the contents of intracellular DNA and RNA in the growth process of the synchronous cells were measured. Figure 7 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 a cell cycle increase with the growth of a cell, and then fall at the division time. Figure 7 depicts such a phenomenon. In the figure the DNA contents gradually decreased. This is caused by incomplete synchronization, that is, the dispersion in the division time of the cell population, as shown by the growth curves in Figure 5. The time taken for the DNA contents to reach a maximum and to reach a minimum correspond, respectively, to the replication period of DNA and the generation time of a cell. Table 2 lists the generation time, A, of each system, the replication period of DNA, B, and the period from the completion of the DNA replication to the cell division, A-B. The effects of p-chloroaniline on the prolongation of the DNA replication period and the generation time were higher than that of aniline. Both compounds were not significantly different in period from the completion of the replication to the cell division. This implies that the generation time prolonged by p-chloroaniline is attributed to the prolonged DNA replication period. The DNA contents of each system increased also with the incubation time as shown in Figure 7. The time, C, taken for the RNA contents to reach a maximum was

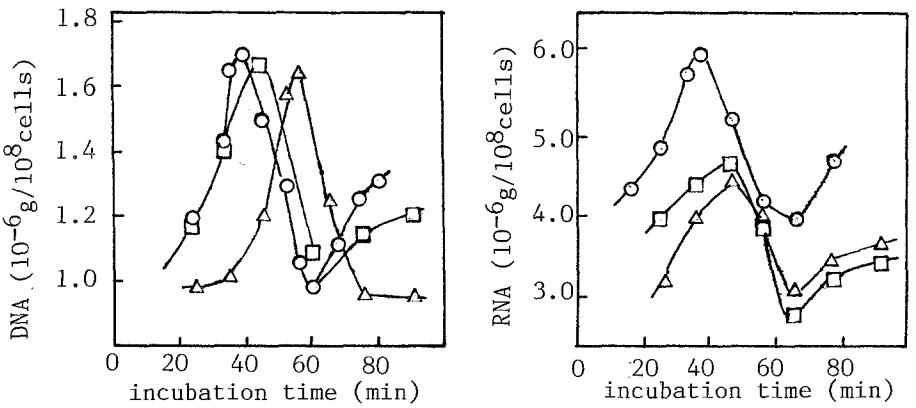

Figure 7. The synthesis of nucleic acid of synchronized cell.

—○— control
—□— aniline 2.1×10^{-2} M/L
—△— p-chloroaniline 3.1×10^{-3} M/L

Table 2. Effects of the anilines on the synthesis of DNA and RNA in the cell cycle.

reagent	concentration	A(min)	B(min)	A-B(min)	C(min)
control		50	40	10	35
aniline	2.1×10^{-2}M/L	57	42	15	45
p-chloroaniline	3.1×10^{-3}M/L	65	53	12	45

listed in Table 2. As understood from the results in Figure 7 and table 2, aniline and p-chloroaniline caused the synthetic rates and the contents of RNA to decrease.

In order to elucidate the factors on the inhibition of the DNA synthesis by p-chloroaniline, the stabilization of the DNA double helix and the inhibition of the enzyme in the DNA synthesis were examined. The melting temperature of DNA and the UV spectroscopy of DNA were not changed by the addition of p-chloroaniline. It was known, therefore, that p-chloroaniline did not contribute to the satbilization of the DNA double helix. The amount, a, of DNA synthesized with DNA polymerase 1 in the presence of anilines and that, b, in the absence of anilines, respectively, were measured. Figure 8 shows the results. The DNA yields on the ordinate indicate the values of (a/b)x100 (%). The DNA yield in the presence of p-chloroaniline was about 70% at F-value (logF = -2.53). On the other hand, the DNA yield in the presence of aniline was about 95% at F-value (logF = -1.66). This means that the difference of the growth inhibition between the anilines depends on the inactivation of the DNA polymerase 1.

The results obtained may be summarized as follows. (1) The growth inhibition shown by the F-values was proportional to hydrophobicity of the compound used. (2) The inhibition of the RNA synthesis by the compound used was more strong than one of DNA, and was increased by the chlorine substituent. (3) p-chloroaniline inhibi-

ted strongly the DNA synthesis, and prolonged the replication period of DNA. This was caused by the inactivation of DNA polymerase 1.

The microbial growth inhibition by polychloroaromatics and the factors in the inhibition of the RNA and ATP synthesie will be reported in the next paper.

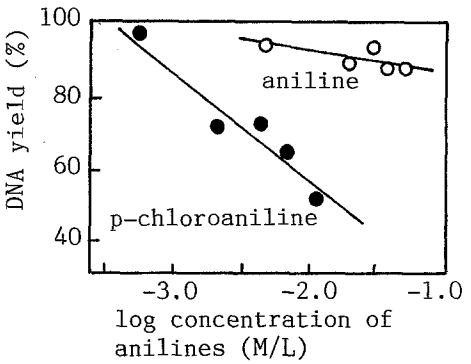

Figure 8. Effects of anilines on DNA synthesis by DNA polymerase 1.

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