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Influence of Chromium Compounds on Microbial Growth and Nucleic Acid Synthesis

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The wastewaters of the dyeing and the tanning industry contain often various chromium compounds, e.g. $K_2Cr_2O_7$ and $CrCl_3$, with a large quantity of organic substances. Biolobical treatments have generally been employed in these industrial factories for the biodegradation of organic substances. Many of chromium compounds inhibit in such a case the growth of microbes, and thereby induce a lowering of the purification efficiency in the treatments. The toxicity of the chromium compounds have been studied regarding mutagenicity and carcinogenicity from the medical view point (Flessel 1979, Tkeshelashvili et al. 1980). This is also of interest from the view point of wastewater biological treatments. The inhibitive effects of the compounds on the cell growth and the respiration in activated sludge have been reported in detail, but mechanisms have not been sufficiently elucidated (Kaneko and Nanbu 1973, Kaneko 1973). Therefore, the influence of $K_2Cr_2O_7$ and $CrCl_3$ on the cell growth and on the nucleic acid content was measured. Both compounds were the inhibitors of DNA synthesis. These action resulted in increased generation time and a decrease in cell devision. A greater effect was produced by $K_2Cr_2O_7$ than by $CrCl_3$. The inhibitive factors of DNA synthesis may be grouped into two categories: (1) the interaction between the chromium compounds and DNA, (2) the inhibition of the DNA polymerase activity by the chromium compounds. The former (1), estimated by the measurements of the melting temperature of DNA and the equilibrium dialysis, were not responsible for inhibition of DNA synthesis. The function of the latter (2) will be mentioned in a subsequent paper.
Chromium compounds and dyes coexist often in the wastewaters of the dyeing industries. The growth inhibitions of the mixed solution were measured. A lowering of the inhibition occured in the $K_2Cr_2O_7$-basic dye solution by the formation of the ionic complex with $Cr_2O_7^{2-}$ and the dye cation.

MATERIALS AND METHODS

$CrCl_3$ and $K_2Cr_2O_7$ were used as the samples of chromium compounds. The strain used was *Bacillus subtilis*. The strain, the DNA sample, the culture condition, the determination of the RNA and DNA con-

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tents, and the measurement of the melting temperature of DNA were the same as these in the previous paper (Ogawa et al. 1988, 1989). 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% $MgSO_4 \cdot 7H_2O$, 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 0.0072% glucose as well. The cell population was synchronized by the stationary phase method. The cultures were shaken at 37°C. The cell concentration of the asynchronous culture were determined by measurement of transmittance at 660 nm, and those of the synchronous cultures were read under a microscope using Thoma's hemacytometer.

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

Melting temperature (Tm): It was measured in order to elucidate the influence of the chromium compound on stabilizing of DNA helix. Commercial calf thymus DNA was added with the chromium compound to 0.01 M NaCl solution. The melting curves of the DNA solution, the absorbance plotted at 260 nm as ordinate and the temperature as abscissa, were obtained by measurement with a spectrophotometer. Tm was determined grafically in the way that it is temperature at midpoint of the absorbance increase.

Equilibrium dialysis: Calf thymus DNA was dissolved together with the chromium compound in 0.005 M phosphate buffer (6.9), and the solution was controlled at ionic strength of 0.01 by addition of NaCl solution. The solutions of 4.93 mg DNA / mL (5 mL) were sealed in dialysis bags (diameter 6 mm, pore size 24 Å, Union Carbride). The bags were immersed in 50 mL test tubes containing the Cr-solutions (30 mL) of various concentrations. After keeping in the thermostat at 25°C for 48 hours, Cr concentrations of both the bags inner and outer solution were determined by colorimetric method using diphenylcarbazide and the atomic absorption method.

RESULTS AND DISCUSSION

Growth inhibition

Asynchronous culture: The microbes were cultured in the medium containing $K_2Cr_2O_7$. The experiment using $CrCl_3$ proved impossible owing to the compounds low solubility. The growth curves of the cells in the system with $K_2Cr_2O_7$ are shown in Fig. 1. The growth rate of the cells in the log phase and the cell concentration in the stationary phase decreased with increasing concentration of $K_2Cr_2O_7$. The biological treatments in the factories are operated continuously in order that cells in the log phase are kept at high concentration. Thus, the influence of $K_2Cr_2O_7$ on the growth rate in the log phase was investigated as follows.

The growth rate constant, k, in the phase can be expressed as equation (1), setting the cell concentration C_1 and C_2, respectively, at cuture time t_1 and t_2:

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

The degree of the growth inhibition, H, is defines as

$$H = 1 - (k_i/k_o) \quad (2)$$

where k_i and k_o are the growth rate constants in the systems with and without $K_2Cr_2O_7$ respectively. It has been shown experimentally for many kinds of inhibitive substances that the degree of inhibition may be represented as:

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

where n is the exponent indicating inhibitive ability, G, the concentration of the inhibitive substance, and ϕ, the concentration of the inhibitive substance at H = 0.5 (Yanagida 1981). The values of H 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 Fig. 2.

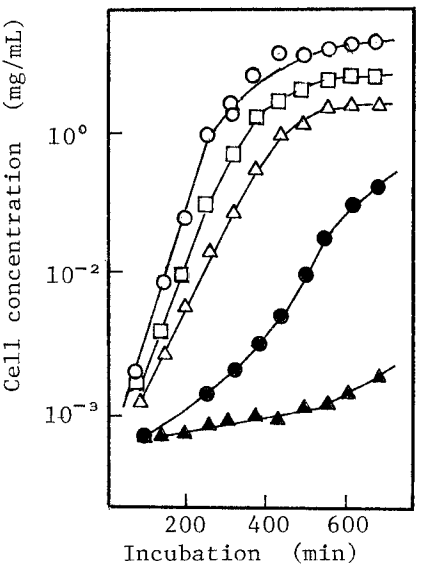


Fig. 1 Asynchronous growth curves of the cells in culture containing $K_2Cr_2O_7$
$K_2Cr_2O_7$ concentration (mol/l):
○ 0, □ 1.92×10^{-5}, △ 3.85×10^{-5},
● 4.81×10^{-5}, ▲ 6.73×10^{-5}

The plots deviated from the straight line at concentrations below about 3×10^{-5} mol/l, that is, 3.1 ppm Cr(VI) owing to lowering of inhibitive ability. Such behaviors have been observed on other metallic ions. For example, Fe^{3+} in dilute solution accelerates the growth of microbes (Kaneko and Nanbu 1973). Applying a linear relationship to equation (3), n = 2.67 and $\phi = 4.38\times10^{-5}$ mol/l were obtained. The values of n was higher and ϕ was mid-range compared with those of many basic dyes in the previous paper (Ogawa et al. 1988).

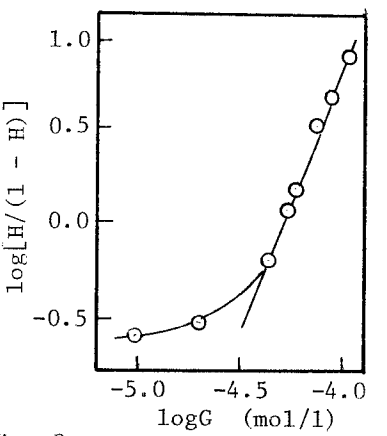
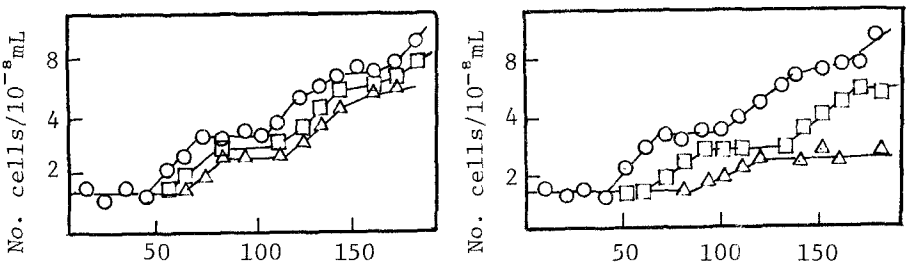


Fig. 2 Relationship between $\log[H/(1-H)]$ and logG

Synchronous culture: The strain was grown synchronously in the medium containing the chromium compound. $CrCl_3$ was more soluble in this medium than in the asynchronous medium, and therefore the experiment using it was possible. The growth curves are shown in Fig. 3. The addition of the chromium compound resulted in an increase of generation time and a decrease in cell division. The effect of $K_2Cr_2O_7$ was much greater than that of $CrCl_3$.

The influences of the chromium compounds on the nucleic acid contents of cells of various age were measured. The results are shown



$CrCl_3$ concentration (mol/l)
○ 0, □ 5.0×10^{-5}, △ 4.5×10^{-4}

$K_2Cr_2O_7$ concentration (mol/l):
○ 0, □ 4.0×10^{-5}, △ 8.0×10^{-5}

Fig. 3 Synchronous growth curves of the cells in culture containing the chromium compound.

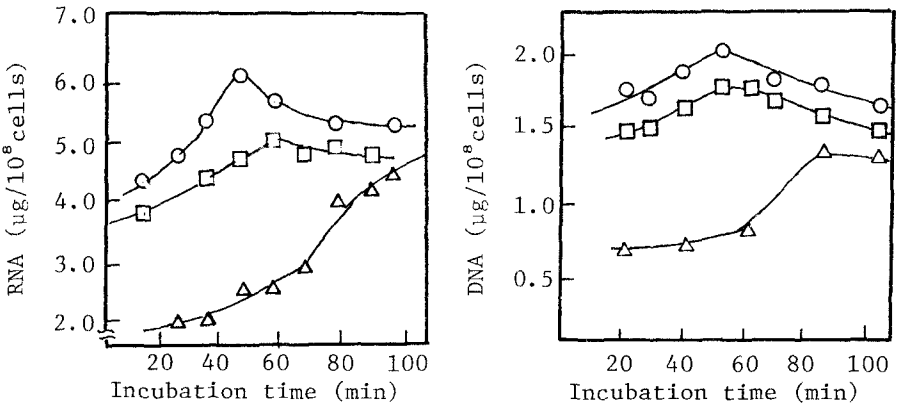


Fig. 4 The nucleic acid contents of synchronous cells.
○ control, □ $CrCl_3$ 5.35×10^{-5}mol/l, △ $K_2Cr_2O_7$ 3.00×10^{-5}mol/l

in Fig. 4. The chromium compounds induced not only the retardation of the initiation of nucleic acid synthesis, but the decrease of nucleic acid content. The effect by $K_2Cr_2O_7$ was much higher than it by $CrCl_3$. As shown by comparing Fig. 3 and 4, the nucleic acids were largely synthesized in the interphase of the cell growth.

In order to know the relationship between the cell age and growth inhibition, $K_2Cr_2O_7$ was added to the medium at different incubation time, after which the growth curves were measured. The results are shown in Fig. 5. The influences of $K_2Cr_2O_7$ on the decrease of the cell devision and the increase of the generation time were remarkable when it was added to young cells. The nucleic

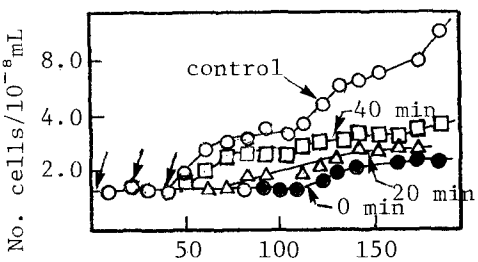


Fig. 5 Effect of the inhibition of synchronous cell growth by addition of $K_2Cr_2O_7$ during the cell cycle.
$K_2Cr_2O_7$ 10^{-4} mol/l,
Arrows indicate times of the addition.

acid synthesis is active generally in the early life of bacteria. It is thus also evident from the results that the growth inhibition is due to inhibition of nucleic acid synthesis.

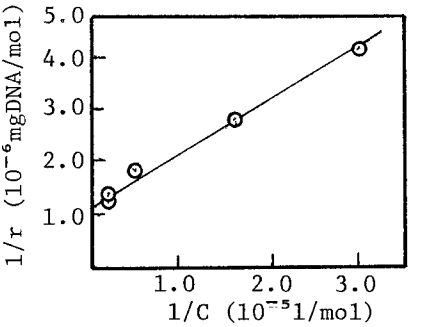


Fig. 6 Plot of 1/r against 1/C for the DNA-$CrCl_3$ system. temperature 25°C ionic strength 0.01

The interaction between the chromium compound and DNA.
The equilibrium dialysis in the DNA - $CrCl_3$ solution and the influences of $CrCl_3$ and $K_2Cr_2O_7$ on the melting temperature of DNA were mreasured respectively to obtain information about the interaction. The quantitative analysis of equilibrium dialysis in the DNA - $K_2Cr_2O_7$ solution was impossible because the binding was minimal. The results of dialysis in the DNA - $CrCl_3$ solution were applied to the equation of Klotz (4):

$$1/r = [1/(SK)](1/C) + 1/S \qquad (4)$$

where r and S are the number of bound $CrCl_3$ and the maximum number of binding site per DNA, respectively, C is the concentration of free $CrCl_3$, and K is the constant. A plot of 1/r against 1/C was found to be linear as shown in Fig. 6. Applying it to equation (4), S = 8.3×10^{-7} mol/mgDNA was obtained. Several basic dyes used in the previous paper (Ogawa et al. 1988) were measured similarly for comparison with $CrCl_3$; their values were lower than that of $CrCl_3$. This is assumed to be due to steric hindrance of the basic dye.
The effects of the chromium compounds on the melting temperature of DNA are shown in Fig. 7. The temperature, Tm, rose and then dropped with increasing $CrCl_3$ concentration. Such behavior has also been observed on the metallic ion (Eichorn and Shin 1968), and can be explained as follows.
The double helix of DNA is stabilized by the hydrogen bond between the bases and is labilized by a mutual repulsion between the negatively charged phosphate.
With addition of Cr^{3+}, the double helix of DNA is stabilized owing to neutralization of the negatively charged phosphate, and thereby the melting temperature rises. With increased addition of Cr^{3+}, the double helix is labilized owing to a weaking of the hydrogen bond between the bases, and thereby the melting temperature drops. On the other hand, the temperature was little affected by the addition of $K_2Cr_2O_7$. This is consistent with

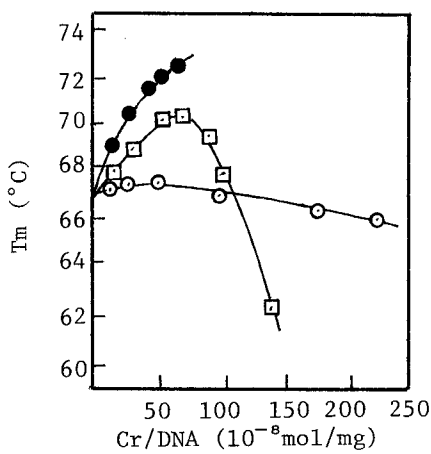


Fig. 7 Variation in Tm with Cr/DNA, the amount of Cr per mg of calf thymus DNA in 0.01 M NaCl solution.
pH 7.0, Scan at 260 nm, Temperature rises 1°C/min.
○ $CrCl_3$, □ $K_2Cr_2O_7$
(Ref. ● Basic Orange 14)

result of the dialysis that $Cr_2O_7^{2-}$ bound little to DNA. The inhibitive functions by the chromium compounds are summerized as follows. $K_2Cr_2O_7$ inhibits strongly the DNA synthesis despite little interaction with DNA. It is thus noted as one factor that the enzymes relating to the DNA synthesis would be inactivated. The authors verified the inactivation of DNA polymerase. The result will be reported in a subsequent paper. $CrCl_3$ contributes in the limited concentration range to stabilization of the DNA helix, but not appreciably to the inhibition of cell growth and DNA synthesis. This differs from the previous obtained results, where the inhibition of DNA synthesis by basic dyes demonstrated significantly a correlation with stabilization of the DNA helix (Ogawa et al. 1989). It may be noted as a reason that the stabililizing degree was different in both the basic dyes and $CrCl_3$. The dye contribute to stabilizing not only by neutralization of the negatively charged phosphate, but also by intercalation. On the other hand, $CrCl_3$ should be contributed to weakening of the hydrogen bond between the bases. The difference in the stability was also substantiated from the fact in Fig. 7, where the rising ratio of the melting temperature by the basic dye, Basic Orange 14, was higher than that by $CrCl_3$.

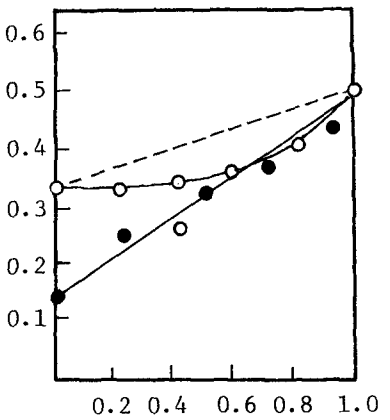
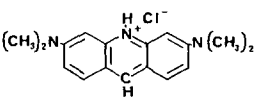
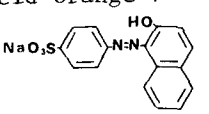


Fig. 8 Relationship between inhibitive degree and molar fraction of $K_2Cr_2O_7$ in the $K_2Cr_2O_7$-dye system.
total concentration: 4.1×10^{-5} mol/l
○ Basic Orange 14



● Acid Orange 7



The growth inhibition in the $K_2Cr_2O_7$ - dye solution.
The mediums containing both $K_2Cr_2O_7$ and the dye were prepared as the model water in the dyeing factories. The microbes were cultivated asynchronously in the medium, and the degrees of growth inhibition, H, were determined by analysis the growth curve. The plot of H against the molar fraction of $K_2Cr_2O_7$ in both components of $K_2Cr_2O_7$ / dye are shown in Fig. 8. The plot in the system of $K_2Cr_2O_7$/ Basic Orange 14 deviated below the straight line, inducing a lowering of the inhibition. The complex formation by the mixing of both components was ascertained by mrasurement of visible spectrum. Such a lowering of inhibition had also been observed in the system of the basic dye / the acid dye (Ogawa et al. 1978). On the other hand, the linear relationship was maintained in the system of $K_2Cr_2O_7$/ Acid Orange 7, where complex formation was impossible owing to a mutual repulsion between $Cr_2O_7^{2-}$ and the dye anion. It is suggested from these results, on the means lowering

the inhibition by $K_2Cr_2O_7$, the complex formation by the cationoid reagents are effective.

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