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Inactivation of DNA Polymerase 1, α-Amylase and Protease by Basic Dyes and Inhibition of Microbial Growth

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Various organic substances as well as the dyes and the chromium compounds are contained in the waste dye liquor. As one of the methods of removing such organic materials, the activated sludge method has been frequently used. The microbial growth may be inhibited by the dyes and the chromium compounds in the process, and the inhibition induces a lowering of purification ability. The influences of these compounds on the microbial growth and DNA synthesis were reported as follows in the preceding paper. 1) The basic dyes inhibited strongly the microbial growth and DNA synthesis (Ogawa et al. 1988). One of the causes of the inhibitions might be stabilization of the double helix of DNA by intercalating the dye (Ogawa et al. 1989a). 2) The inhibitions of the microbial growth and DNA synthesis by $K_2Cr_2O_7$ were strong, despite no contribution to stabilization of the DNA helix. On the other hand, those by $CrCl_3$ were weak, despite a positive contribution to stabilization of the DNA helix.
Thus, for the purpose of proving other inhibitive factors than stabilization of the DNA helix, the inactivation of DNA polymerase 1, α-amylase and protease by the basic dyes, and the inactivation of DNA polymerase 1 by the chromium compounds were examined, respectively. The following informations were obtained as the results. 1) The inactivation of DNA polymerase 1 by Crystal Violet, a basic dye, was slightly weaker than that by $K_2Cr_2O_7$, and that by $CrCl_3$ was much weaker. 2) Activities of α-amylase and protease were competitively inhibited by the basic dyes. 3) The inhibition of DNA synthesis by Crystal Violet was due to the inactivation of DNA polymerase 1 and stabilization of the DNA helix. On the other hand, it by $K_2Cr_2O_7$ was due to the inactivation of DNA polymerase 1, and not to stabilization.

MATERIALS AND METHODS

The dyes shown below were used. The enzymes used were available commercially; DNA polymerase 1 from *E. coli*. lysogen, α-amylase from *Bacillus subtilis*, and protease from *Bacillus polymyxa*. The substrate for the equilibrium dialysis and the template DNA for determination of the polymerase activity were prepared from calf thymus DNA.

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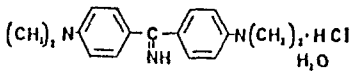
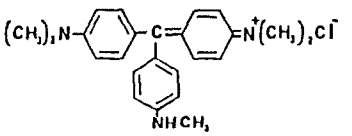
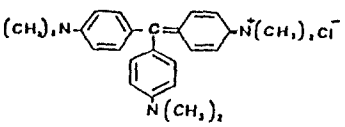


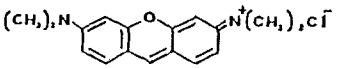
Auramine O (AO)



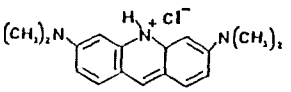
Methyl Violet (MV)

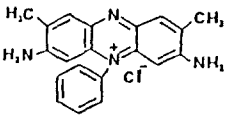


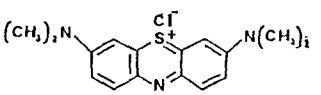
Crystal Violet (CV)



Pyronine G (PG)







Acrydine Orange NS (AN) Safranine T (ST) Methylene Blue (MB)

Figure 1. Dye structures

Equilibrium dialysis: Calf thymus DNA (5 mg) was dissolved in 0.01M NaCl solution (5 mL), and were sealed in the dialysis bag (6 mm in diameter with 24Å pore size; Union Carbide). The bags were immersed in 50 mL test tubes containing 0.01M NaCl solution (30 mL) dissolved in various amounts of the basic dye. After keeping at 25°C for 48 hours, the dye concentrations of bags inner and outer solutions were determined colorimetrically.

Activity determination of DNA polymerase 1 (Aposian and Kornberg 1962): The template DNA was previous prepared by the standard method from 1.0 mg/mL calf thymus DNA solution. Then, 1.0M each solutions (0.05 mL) of dATP, dCTP, dGTP and dTTP, the template DNA solution (0.05 mL) and the basic dye or chromium compound solution (0.85 mL) were added to 1.0M phosphate buffer solution, pH 7.4, so that total of 1.5 mL mixed solution was prepared. Then, 0.1 unit DNA polymerase 1 (0.05 mL) was added to the solution. The mixture was incubated at 30°C for 30 minutes, and the enzymatic reaction was terminated by the addition of 0.2M sodium pyrophosphate solution (1.0 mL) and 1.0M perchloric acid solution (2.5 mL). Thus DNA produced was collected by centrifugation, washed with ethanol, and dissolved in 0.01M NaCl solution (0.5 mL). The concentration of DNA in the solution was determined by the absorbance at 260 nm at 85°C. In the usual experiment, the concentration of synthesized DNA is determined by measuring the radioactivity of DNA using [α-^{32}P]dATP. In the present experiment, the amount of synthesized DNA was sufficient to measure by UV spectrometry.

Activity determination of α-amylase (Fuwa 1954): Varied concentrations (2 mL) of α-amylose was added to 5.0×10^{-4}M basic dye solution (1.0 mL) and 1.0M phosphate buffer, pH 7.4 (1.0 mL), and the mixture was incubated at 30°C in an incubator. The enzymatic reaction was initiated by adding 2.5×10^{-2}mg/L α-amylase solution (1.0 mL) to the incubating mixture. After a certain interval, the

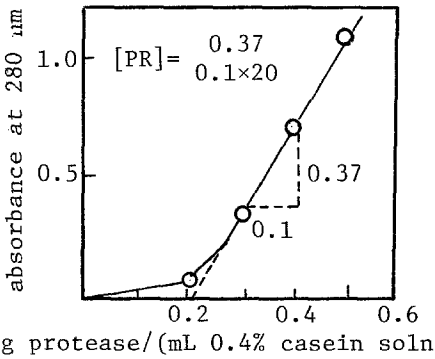


Figure 2. Standard activity curve of protease.

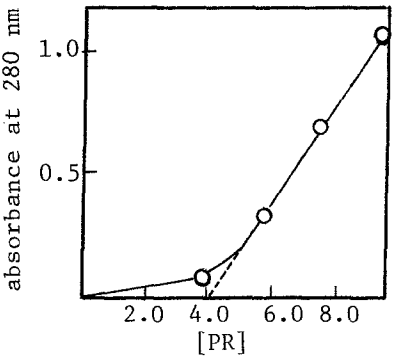


Figure 3. Standard curve of casein digestion.

enzymatic reaction was stopped by the addition of 1.0N acetic acid solution (5 mL). The reaction mixture (1.0 mL) was taken out, and 1/3000 N J_2 solution (5 mL) was added, and the absorbance at 650 nm was measured. The only dyes, that did not absorb light at 650 nm, were used for the determination. The control experiment was similarly carried out on the solution added successively amylose, acetic acid, α-amylase and J_2. Thus, the undigestion concentration of amylose was determined from the difference of the both absorbance.

Activity determination of protease (Kunitz 1946): A standard activity curve of casein digestion by protease was prepared as follows. The protease solution (1.0 mL) of various concentration and the 1.0% casein solution (0.5 mL) in 0.1M phosphaste buffer were added to centrifuge tubes. The mixtures were incubated at 35°C for 20 minutes, and the reactions were terminated by the addition of 5.0% trichloroacetic acid solution (3 mL). After centrifugation at 3,000 rpm, the absorbances of supernatant solutions at 280 nm were measured. The control experiment was similarly carried out on the solution added successively casein, trichloroacetic acid and protease. The results are shown in Figure 2. One protease unit, [PR], was defined as the activity increasing the absorbance 1.00 per a minute. Thus, the specific activity of the specimen of protease used was obtained from the standard activity curve as 0.185. Using this value, Figure 2 was converted to Figure 3. The standard curve obtained was used for the kinetic analysis of enzymatic reaction.

RESULTS AND DICCUSSION

Equilibrium dialysis: The results of dialysis in the DNA - the dye system were applied to the equation (1)of Klotz:

$$1/r = [1/(DK)](1/C) + 1/D \qquad (1)$$

where r and D are the number of the bound dye and the maximum number of binding site per DNA, respectively; C is the concentration of the free dye, and K is the constant. A plot of 1/r against 1/C was found to be linear as shown in Figure 4. The values of D and K for each dyes were obtained from the intercept on the ordinate and the slope of the curve. The standard free change ($-\Delta G_o$) was obtained from the equation (2):

$$-\Delta G_o = RT\ln K \qquad (2)$$

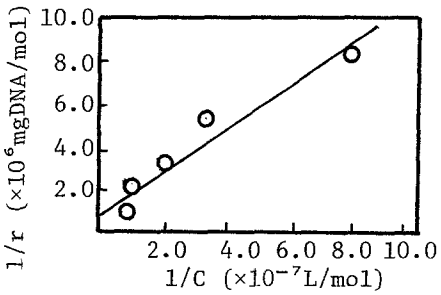


Figure 4. Plots of 1/r against 1/C for the DNA - dye system. dye: Crystal Violet

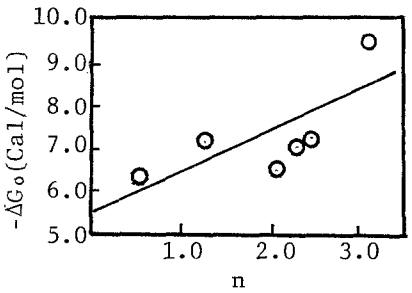


Figure 5. Relationship between n and $-\Delta G_0$.

Table 1. Values of D, K, $-\Delta G_0$ and n.

basic dye		D (mol/mgDNA)	K	$-\Delta G_0$(Cal/mol)	n
Auramine O	(AO)	3.69×10^{-7}	5.26×10^{4}	6.43	0.6
Methyl Violet	(MV)	5.66×10^{-7}	2.12×10^{5}	7.26	1.3
Crystal Violet	(CV)	7.65×10^{-7}	1.34×10^{7}	9.73	3.1
PyronineG	(PG)	1.48×10^{-7}	2.21×10^{4}	5.19	2.5
Acridine Orange NS	(AN)	13.87×10^{-7}	6.56×10^{4}	6.56	2.1
Safranine T	(ST)	4.83×10^{-7}	1.76×10^{5}	7.15	2.3
Methylene Blue	(MB)	5.24×10^{-7}	2.33×10^{5}	7.31	2.5

where R and T are, respectively, the gas constant and the absolute temperature. These values of the dyes used are shown in Table 1. In order to elucidate the relation between these values and the inhibitive parameter of the microbial growth, n, that was obtained by analysis of the growth curve in the incubation with the dye (Ogawa et al. 1988), was shown together in the table. Correlation of n and $-\Delta G_0$ plot was found as shown in Figure 5. Thus, one of the inhibitory factors of the microbial growth is suggested to be stabilization of the bond between the dye and DNA. The fact coincides with the finding that the more the dye contributes to stabilizing the double helix of DNA, the stronger growth inhibition is expressed (Ogawa et al. 1989a).

Inactivation of DNA polymerase 1: Amounts of DNA synthesis by DNA polymerase were determined in the presence of such inhibitor as Crystal Violet, $K_2Cr_2O_7$ and $CrCl_3$. Amounts of DNA synthesis with and without the inhibitor were compared, and the results were shown in Figure6. Among these inhibitors, inactivation by $K_2Cr_2O_7$ was the strongest. In the preceding paper, it was reported that $K_2Cr_2O_7$ did not contribute to stabilizing the double helix of DNA, and logarithm of the inhibitor concentration (mol/L) reducing the rate constant of the microbial growth by one half, logϕ, was -4.38 (Ogawa et al. 1989b). The ratio of DNA synthesis as shown in Figure 6 was 50% in the same concentration. Thus, $K_2Cr_2O_7$ inhibited the microbial growth by the same ratio of the DNA synthesis. The result suggests that the inhibition of the activity of DNA polymerase 1 suppresses the microbial growth strongly. Degree of inhibition of DNA synthesis by Crystal Violet was weaker than that of $K_2Cr_2O_7$, and the ratio of DNA synthesis at ϕ value of Crystal Violet, log ϕ

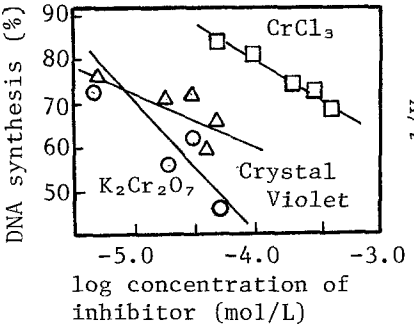


Figure 6. Effect of inhibitor on DNA synthesis.

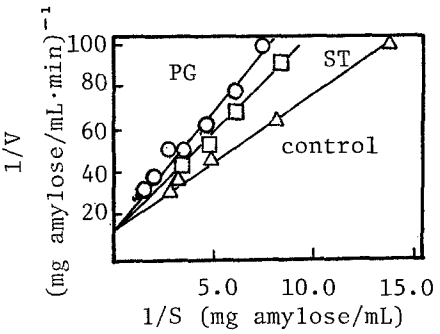


Figure 7. Lineweaver-Burk plots of α-amylase.
dye conc.: 1.0×10^{-4}mol/L

= -5.38, was about 80%. The result suggests that the stabilization of the double helix of DNA as well as the inhibition of DNA polymerase 1 by Crystal Violet might be related to the inhibition of the microbial growth. The inhibition of DNA synthesis by $CrCl_3$ was weak as in the growth inhibition.

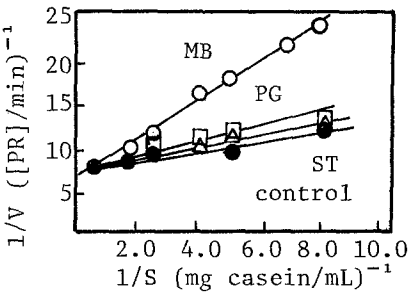


Figure 8. Lineweaver-Burk plots of protease.
dye conc.: 5.0×10^{-6}mol/L

Inactivation of α-amylase and protease: Other factors participating in the inhibition of the microbial growth may be inactivation of metabolic enzyme systems. As the first step of the approach, the digestion velocity, V, of amylose by α-amylase and casein by protease, respectively, were measured in the various concentration, S, of the substrates. The results of Lineweaver-Burk plots were shown in Figure 7 and 8. Curves obtained with or without the dye showed different slopes, but the intercept on the 1/V axis was the same. Therefore, the both enzymatic activities are concluded to be inhibited competitively by the dyes, according to the following equation:

$$\underset{\text{enzyme}}{E} + \underset{\text{substrate}}{S} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \underset{\text{complex}}{ES} \xrightarrow{k_{+2}} E + \underset{\text{product}}{P}$$

$$E + \underset{\text{inhibitor}}{I} \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} \underset{\text{complex}}{EI}$$

Inhibitor constants, $k_i = k_{-3}/k_{+3}$, were calculated for each dyes from the intercept and slope of the curves by a usual analytical procedure. Obtained values were plotted against the inhibitive parameter for the microbial growth, n, as shown in Figure 9 and 10. In the enzymatic systems of α-amylase and protease, k_i is smaller, by the definition, as the equilibrium of the complex formation between the enzyme and the dye is larger. Therefore, the results shown in the figures indicate that stronger inhibitory dyes for the microbial growth inhibit more enzymatic acti-

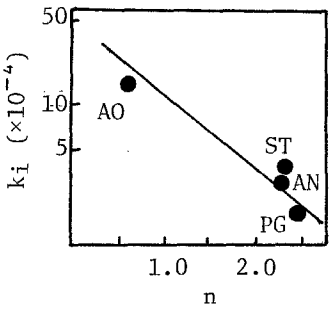


Figure 9. Relationship between n and k_i in the system of α-amylase and the dye.

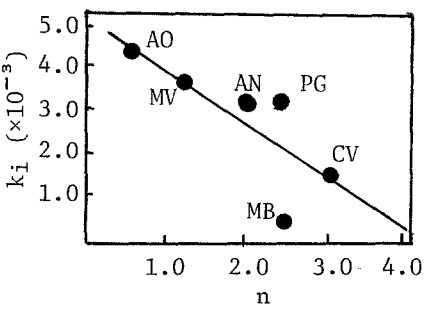


Figure 10. Relationship between n and k_i in the system of protease and the dye.

vity by the complex formation of the enzyme with the dye.

Results obtained may be summarized as follows: 1) the inhibitive factors of DNA synthesis by the basic dyes were the stabilization of the double helix of DNA and the inactivation of DNA polymerase 1, 2) the microbial growth inhibition were correlated with the inactivation of DNA polymerase 1, α-amylase and protease.

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