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Growth Inhibition of *Bacillus subtilis* upon Interaction between Basic Dyes and DNA

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Wastewater in the process of dyeing is not always purified effectively by biological treatment such as the activated sludge method, since dyes, bichromates, etc. contained in the water are toxic to microbes (Hashimoto et al. 1972). In preference to employment of the treatment, therefore, how the components of the water influence growth and physiological activities of microbes should have to be evaluated. It was reported in the previous paper that growth inhibition of cells by dyes results from a lowering of nucleic acid synthesis (Ogawa et al. 1988). In this paper, adsorption isotherms of basic dyes to cells, effects of dyes on melting temperature of DNA and mutagenicity of dyes were measured respectively, and the correlation between these values and cell growth inhibition was investgated. It was found from the results that stabilization of DNA double helix was related closely to cell growth inhibition.

MATERIALS AND METHODS

The dyes shown below were used. *Bacillus subtilis* (IFO 3022) was supplied by Institute for Fermentation, Osaka.

Adsorption equilibrium: Microbial suspensions containing a dye were adjusted to be at pH 7.0 and shaken in a thermostat at 37°C for 30 minutes. After centrifuging, a dye concentration in the solution phase was measured using the spectrophotometer.

Melting temperature: Commercial calf thymus DNA was added with a dye 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. Quartz cuvettes, stoppered during melting, were placed in a cuvetted block eqipped with thermostat. The melting temperatures of DNA samples, Tm, were determined graphically in the way that it is temperature at midpoint of the absorbance increase (Mary and Lawrence 1985).

Mutagenicity: Using both strains of *Bacillus subtilis* H 17 rec^+ and M 45 rec^-, mutagenicity of the dyes was tested. The streak method (Shirasu et al. 1976) was unfeasible to quantitative evaluation because of small difference in length of the growth inhibition zone

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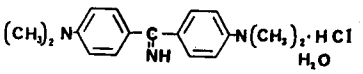
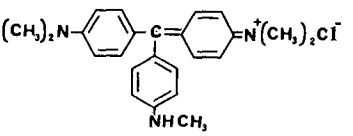
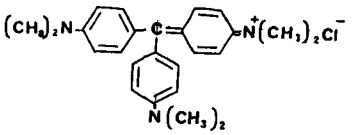


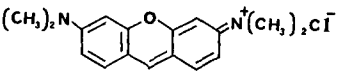
Auramine O (A O)



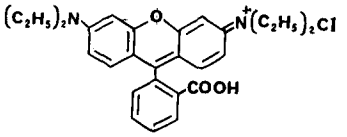
Methyl Violet (M V)



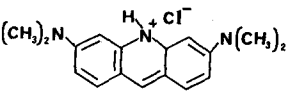
Crystal Violet (C V)



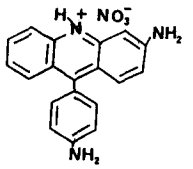
Pyronine G (P G)



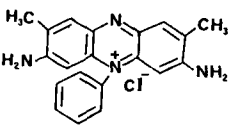
Rhodamine B (R B)



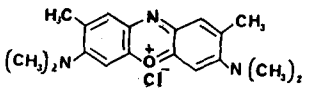
Acridine Orange NS (A N)



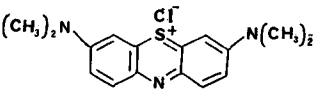
Phosphine E (P E)



Safranine T (S T)



Capri Blue (C B)



Methylene Blue (M B)

of both strains. Thus each strain was cultivated in the presence of a dye at 37°C for 8 hours using liquid medium. Each cell suspension collected at a definite volume was cultivated on agar in petri plate, and mutagenicity was evaluated in comparision of both colony counts.

RESULTS AND DISCUSSION

A result of adsorption equilibrium is shown in Figure 1. The pH of the isoelectric point of the cells measured by potentiometric titration was 6.3. Accordingly in the solution at pH 7.0, dye cations are adsorbed ionically at specific sites of the cells, to which Langumuir's equation (1) is considered to be application.

$$[D]_\phi = \alpha[S]_\phi[D]_\sigma/(1 + \alpha[D]_\sigma) \quad (1)$$

where $[D]_\phi$ and $[D]_\sigma$ are the dye concentration in the cell phase and the solution phase respectively, $[S]_\phi$ is the saturated dye concentration in the cell phase, and α is a constant. Equation (1) is rewritten as follows:

$$1/[D]_\phi = 1/(\alpha[S]_\phi[D]_\sigma) + 1/[S]_\phi \quad (2)$$

When the results of isothermal adsorption may be fitted to Langu-

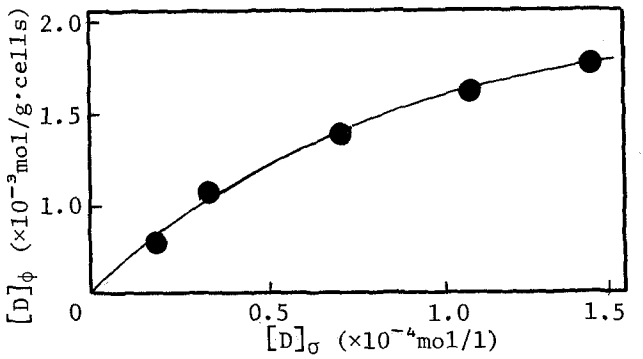


Figure 1. Adsorption isotherm of Crystal Violet to cells. pH 7.0 Temo. 37°C

muir's equation, it is seen from equation (2) that a plot of $1/[D]_\phi$ as ordinate against $1/[D]_\sigma$ as abscissa should be a straight line. When $1/[D]_\sigma$ is zero, i.e. when $[D]_\sigma = \infty$, $1/[D]_\phi = 1/[S]_\phi$, thus the intercept of the line on ordinate gives $1/[S]_\phi$. Futhermore, from a slope of the line α is obtainable. Under the condition applied to Langumuir's equation, the adsorptive affinity, $-\Delta\mu_o$, can be generally expressed by the equation of Gilbert-Rideal (3). The equation (3) has been frequently available in the field of physical chemistry of dyeing to evaluate the dyeing property (Vickerstaff 1954).

$$-\Delta\mu_o = 2RT\ln\theta/(1-\theta) - 2RT\ln[D]_\sigma \quad (3)$$

where θ is $[D]_\phi/[S]_\phi$, R the gas constant, and T the absolute Temperature. On the other hand, basic dyes are adsorbed to activated sludge accordingly to Freundlich's equation (Nakaoka et al. 1973).

$$[D]_\phi = K[D]_\sigma^\gamma \quad (4)$$

where K is a constant, and γ a fractional power. Equation (4) can be rewritten as follow:

$$\ln[D]_\phi = \ln K + \gamma\ln[D]_\sigma \quad (5)$$

When the results of adsorption are applicable to Freundlich's equation, a plot of $\ln[D]_\phi$ as ordinate against $\ln[D]_\sigma$ as abscissa should be a straight line, from which K and γ are obtainable. In either case applied to equation (2) and (5), the experimental data gave linear plots. On the straight line obtained by the least-squared method, $-\Delta\mu_o$, K and correlation coefficient, r, were calculated,respectively. They are shown in Table 1. Since every values of r are rather high, the isothermal adsorption of the dyes is proved to be applicable to both equation, particularly Freundlich's equation.

The relationship between the adsorptive abilities of dyes and the parameter indicating inhibitive abilities for cell growth, n, is shown in Figure 2. The values of n were cited from the previous paper (Ogawa et al. 1988). The correlation indicated that K based on Freundlich's equation was slightly higher than $-\Delta\mu_o$ based on Langumuir's equation. Since either of them is proportional to n, adsorption of dyes is known to be a primitive factor of growth inhibition.

It was reported in the previous paper that dyes were inhibitors of nucleic acid synthesis. It may be noted concerning the factors

Table 1. Adsorption constants of dyes to cells

dye		AO	CV	PG	RB	AN	PE	ST	MB
Langumuir's	$-\Delta\mu_o$(Cal/mol)	6.18	7.32	7.08	6.86	6.84	6.77	7.50	7.09
equation	$r(\times 10^{-1})$	7.22	9.54	8.32	9.94	9.45	8.55	9.56	9.61
Freundlich's	K	1.44	19.3	10.8	2.86	22.4	18.9	9.37	17.8
equation	$r(\times 10^{-1})$	7.90	9.68	9.57	9.66	9.94	9.70	9.35	9.88

that dyes inhibit DNA synthesis by stabilizing the double helix and by inhibiting enzymatic activities. The function of the former on growth inhibition of cells was researched in this paper. One of the latter will be mentioned in a subsequent paper.

From microbes cultivated in the systems with and without dyes, DNA was isolated respectively by the method of Schmidt, Thannhauser and Schneider. DNA of the former indicated the higher melting temperature, Tm, than that of the latter. However, in the process of isolating DNA, a part of dyes desorbed and therefore Tm corresponding to the incubation condition was not assayable. Thus, using calf thymus DNA, changes of Tm upon addition of dyes were measured as indirect means. One of the results is shown in Figure 3. The values of Tm increased with the dye concentration.

The same phenomenon was noted also with acridine dyes such as proflavine, and it has been concluded that dyes intercalate between the base pairs of DNA stabilizing the double helix (Gersch and Jordan 1965). From this point of view, it can be considered that the dyes stronger in contribution to increase Tm of DNA inhibit separation of two strands further, which would be a factor of retarding growth of the cells. A relationship between the increment of Tm depending on the dye concentration per DNA, ΔT, and n is shown in Figure 4. The correlation coefficient was 0.965 in assumption that linear relationship was kept. It was known from the high value that such interaction between DNA and dye is related closely to growth inhibition.

Some intercalators e.g. acridine orange and proflavine are known to show mutagenicity (Joyce et al. 1975). If mutagenicity of the dye used is related to intercalation, it may be anticipated that mutagenicity hold a correlation also with growth inhibition. Thus, using strains of *Bacillus subtilis* H 17 rec^+ and M 45 rec^-, mutagenicity of dyes was tested. One example of the results is shown in Figure 5. A degree of mutagenicity, R, was expressed in equation (5).

$$R = (A - B)/A \qquad (5)$$

where A and B represent respectively a dye concentration when each cell counts of rec^+ and rec^- strains are reduced to half of that without a dye concentration. A relationship between R and n is shown in Figure 6. Although being rather fluctuated, R increased with n. The correlation coefficient calculated by assuming the existence of a linear relationship was 0.787, which was lower than that between Tm and n, indicating that there were specificity of dyes not always corresponding with intercalation. For example, such mutagenicity occured stronger with Crystal Violet and weaker with Acridine Orange NS.

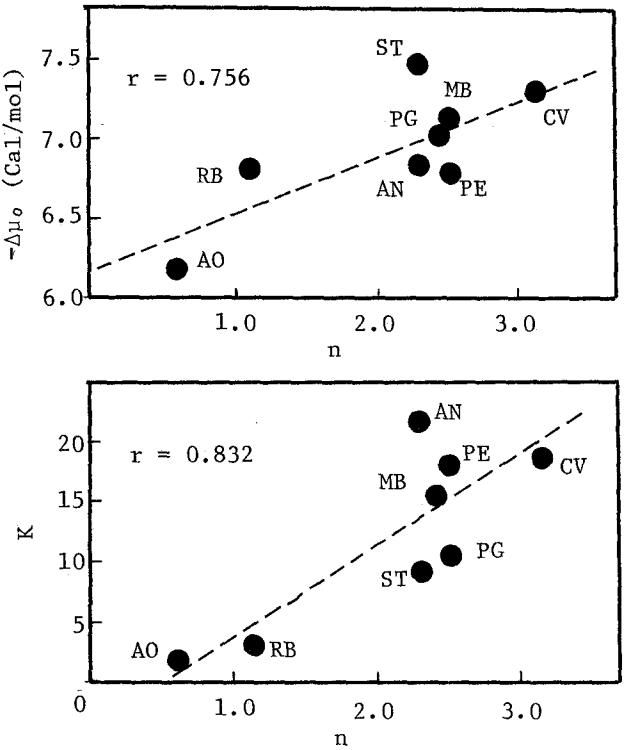


Figure 2. Relations between adsorption of dyes to cells and growth inhibition.

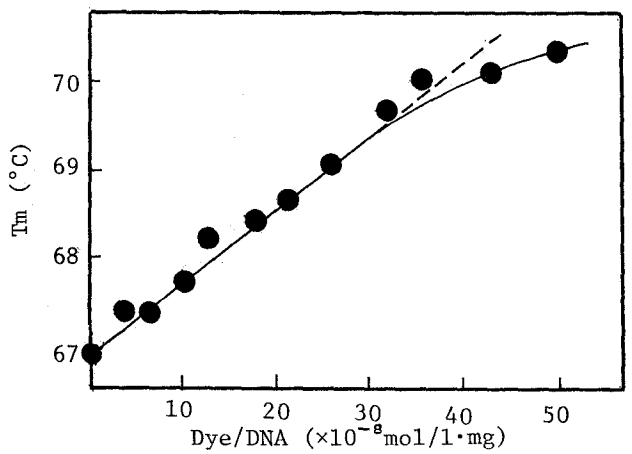


Figure 3. Changes of Tm with concentration of Methylene Blue per mg DNA.
pH 7.5, Scan at 260 nm, Temperature rises 1°C/min.

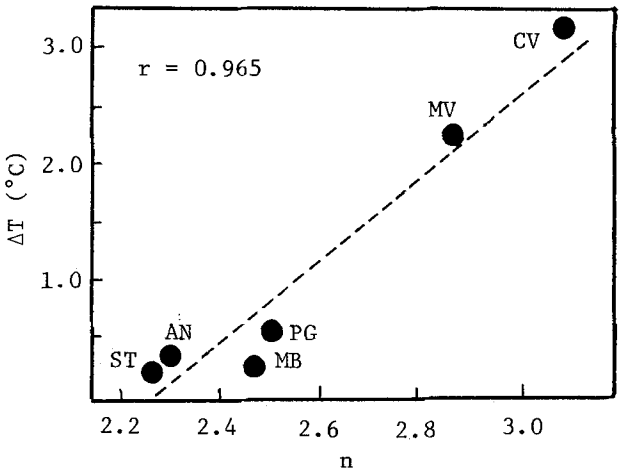


Figure 4. Plot of ΔT vs. n
ΔT: Incerment of Tm per dye concentration / DNA
($\times 10^{-8}$mol/l·mg)

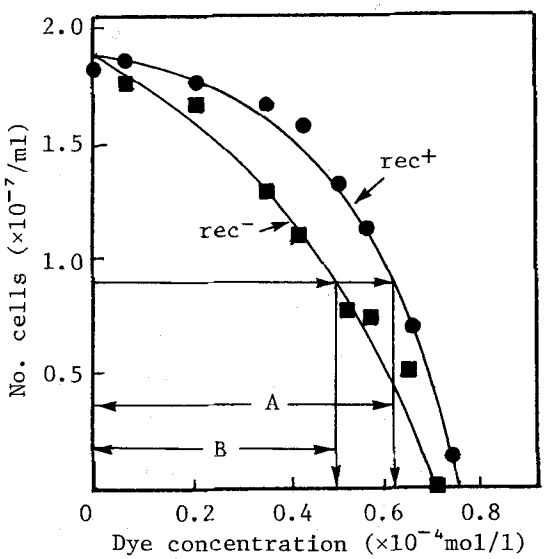


Figure 5. Relation between number of cells and the concentration of Acridine Orange NS.
Incubation time 8hr, Temp. 37°C

To protect cells from the inhibition is important in the treatments of the wastewater containing basic dyes. Adding the acid dye the inhibition by the basic dye disappeared to produce the complex (Ogawa et al. 1978). The lowering of the inhibition was also observed in the system of the basic dye-$K_2Cr_2O_7$, and in such a case the affinity of the basic dye for DNA decreased with the production of the complex. The details will be reported in the subsequent

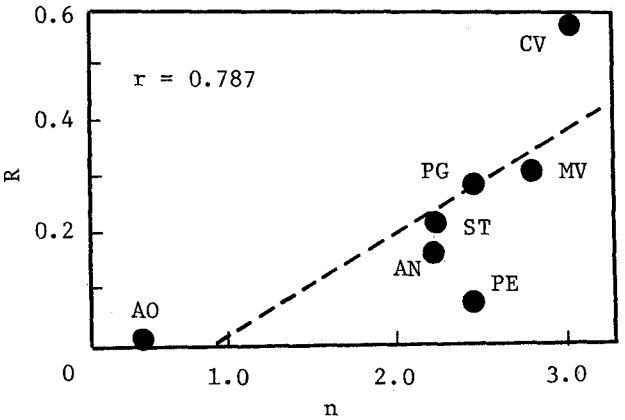


Figure 6. Plot of R vs. n

paper. It is suggested from these results that the addition of negative charged compounds, e. g. polyelectrolytes, wil likely be useful to prevent such a inhibition in the practical treatment.

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