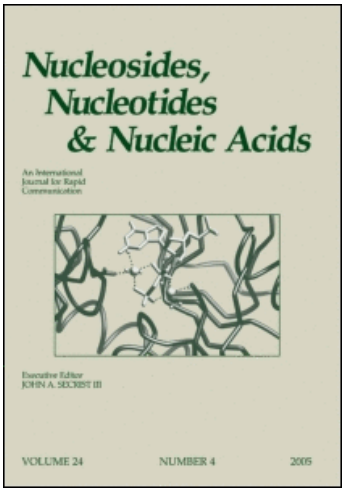


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# Change of the Higher Order Structure in a Giant DNA Induced by 4', 6-Diamidino-2-phenylindole as a Minor Groove Binder and Ethidium Bromide as an Intercalator

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# CHANGE OF THE HIGHER ORDER STRUCTURE IN A GIANT DNA INDUCED BY 4',6-DIAMIDINO-2-PHENYLINDOLE AS A MINOR GROOVE BINDER AND ETHIDIUM BROMIDE AS AN INTERCALATOR

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**Abstract**: From a quantitative study of the contour length and the persistent length of DNA molecules with fluorescence microscopy, it is shown that 4',6-diamidino-2-phenylindole (DAPI) and ethidium bromide (EB) change the higher order structure of DNA in a different manner.



## INTRODUCTION

There have been numerous studies on the interaction of drugs with DNA molecules, in relation to the targets such as the development of anti-cancer drug and regulation of the gene expression.[1-2] As for the detailed structural change of DNA induced by drugs, several spectroscopic methods have been actively applied, including NMR, CD and fluorescence spectroscopy.[3-7] Among them, X-ray diffractometry has been shown to be quite useful to obtain the three dimensional structure of DNA molecules in their crystal forms.[8] In spite of the accumulated knowledge on the detailed structure of DNA molecules, it is rather surprising that studies on the change of their higher order structure induced by different kinds of drugs have been quite few. This may be due to the lack of suitable methodology to study the higher order structure of giant DNA molecules in an aqueous environment.

About a decade ago, Yanagida *et al.* found that individual DNA molecules can be visualized with fluorescence microscopy.[9] They showed very interesting pictures of

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This paper is dedicated to Prof. M. Ikehara.

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DNA molecules exhibiting randomly fluctuating Brownian motion, although the resolution limit was rather low, ca. 0.2-0.3 μm. They described that DAPI was a quite useful fluorescence dye to visualize the DNA molecules and that DAPI showed negligible effect on the structure of DNA. Contrary to this, from a study with fluorescence microscopy, we recently found that fluorescence dyes, including DAPI, exhibit marked effect on the higher order structure of DNA.[10] As an extension of our previous study, in the present article we would like to show the result of quantitative measurement on the higher order structure of DNA induced by drugs with different binding modes. We have chosen DAPI and EB as different types of interacting drugs on DNA.

## RESULTS AND DISCUSSION

### 1. Association Constant of DAPI to DNA

Before the description of the change of DNA images with fluorescence microscopy, we would like to discuss the binding equilibrium of the fluorescence dye to DNA. Barcellona, *et al.*, reported that the binding of DAPI to DNA proceeds in two steps.[7] They have measured the intensity change of the fluorescence at 442 nm by changing the DAPI concentration at a fixed DNA concentration. Then, they have analyzed the change of the fluorescence intensity using a standard Scatchard plot. Although the Scatchard plot was not a straight line but a highly curved line, they draw two limiting straight lines on the graph. From the slope of the limiting straight lines, they evaluated the equilibrium constant $K_1 = 3.1\times10^7\ M^{-1}$ with the number of binding site per nucleotide $n_1 = 0.03$ for the first step equilibrium, and $K_2 = 2.9\times10^5\ M^{-1}$ with $n_2 = 0.39$ for the second step equilibrium, respectively.

We have examined the analysis by Barcellona, *et al.* [1] and found that the equilibrium constants reported by them are rather unreliable. It is well known that a multi-step equilibrium should not be analyzed by use of the Scatchard plot.[11] Instead of this, the equilibrium should be analyzed using a "nonlinear least-square" treatment with the following equations,

$$\frac{1}{r_1} = \frac{1}{n_1K_1}\cdot\frac{1}{c} + \frac{1}{n_1}, \quad \frac{1}{r_2} = \frac{1}{n_2K_2}\cdot\frac{1}{c} + \frac{1}{n_2}$$

where c is the concentration of free dye, $r_1$ and $r_2$ are the numbers of dye molecules bound to DNA per nucleotide with the first and second step equilibrium, respectively. We have measured the change of the fluorescence intensity depending on the DNA concentration at a fixed dye concentration. In order to obtain the equilibrium constants from the fluorescence intensity, we have assumed the linear relationship between the

fluorescence intensity, F, and the total binding number, $r_1 + r_2$. From a theoretical consideration of the effect of the difference of the fluorescence intensity between first and second step bindings, we have confirmed that the above mentioned assumption causes only negligible error in the equilibrium constants. Fig. 1 shows the experimental result on the fluorescence intensity together with the curve with nonlinear least-square fitting. From a nonlinear least-square analysis, we have obtained the equilibrium parameters: $K_1 = 1.4\times10^9\ M^{-1}$, $n_1 = 0.010$, $K_2 = 4.3\times10^5\ M^{-1}$, $n_2 = 0.18$. It is well known that a minor groove binding drug prefers to bind an AT rich region.[5-8, 12] It was suggested that one DAPI molecule binds to DNA with four AT pairs.[8] If we notice that the AT content is about 65 % in the T4-DNA molecule,[13] the probability for the successive arrangement of four AT pairs, $(AT)_4$ becomes $(0.65)^4$ per four pairs of nucleotides. It is, therefore, expected that the binding site with four AT pairs per one pair of nucleotides is $(1/4) \times (0.65)^4 = 0.044$. The limiting number of the binding site, n, per nucleotide becomes thus 0.022. Taking into consideration the effect of different ordering of A and T bases along the $(AT)_4$ structure on the binding strength of DAPI,[5] the estimated value, $n_1 = 0.010$, seems rather reasonable. It is, thus, expected that the first step binding is attributed to the minor groove binding with an AT rich region. As for the second step of binding, the number of binding site per nucleotides, $n_2$, is 0.18. This means that one DAPI molecule binds per four base pairs, suggesting that the binding nature is electrostatic and the maximum number of binding is determined by the steric size effect of DAPI molecules. As for the equilibrium of binding of EB to DNA, the binding nature seems to be one step within the experimental error.[3] Thus, we would like to adapt the equilibrium constant $K_1 = 8.1\times10^6\ M^{-1}$ and $n_1 = 0.16$ from the data by Douthart, *et al.*[3] in the following discussion.



2. Contour Length of DNA with Dye Concentration

Fig.2 (a) and (b) show the photographs of stretched T4-DNA molecules fixed on the glass surface, stained with DAPI and EB, respectively. Here, the surface of the cover glass was positively charged after treatment with the agent of silanization. The stretched structure was obtained with the operation of an one-directional slip of the cover glass. Fig. 3 shows the distribution of the contour length of the stretched T4-DNA molecules. As the stretched DNA molecules are not genuine straight lines, the maximum length in the distribution of Fig. 3 is expected to correspond to the actual contour length. Based on the X-ray crystallographic data on the average distance, 3.3 Å, between the base pairs,[11] the contour length of uncomplexed T4-DNA is estimated to be 55 μm, which is quite near to 57μm as the maximum value in Fig. 3 a). This indicates that DAPI has no apparent effect on the contour length of DNA up to a concentration of [dye]/[nucleotide]

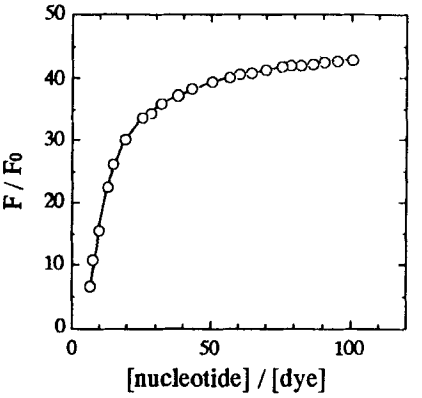


FIG. 1 Change of the fluorescence intensity of DAPI depending on the concentration of T4 DNA. Open circles are the experimental data and the solid line is the curve obtained by nonlinear least-square fitting. $F_0$ indicates the fluorescence intensity of DAPI in the absent of DNA molecules and F indicates the fluorescence intensity of the DNA—DAPI complex. The concentration of DAPI was kept constant at 2 μM.



= 10. This is reasonable because DAPI binds to DNA from the lateral side as a minor groove binder.[8] In contrast to this, the stretched length of T4-DNA complexed with EB was rather large (Fig. 3 (b)). The contour length is, thus, estimated to be 74 μm from the maximum value in the length distribution. Based on the binding constant of $8.1\times10^6$ $M^{-1}$,[3] it is expected that one EB molecule intercalates to DNA per about four base pairs in this dye concentration. It is, thus, calculated that one intercalator expands the longitudinal length of DNA by ca. 3Å,[14] corresponding well to the estimated value by Douthart, *et al.*[3]

## 3. Persistent Length of DNA Molecules

Next, let us discuss the effect of DAPI and EB on the persistent length of DNA. Persistent length is defined as in the following equationa,[13]

$$\lambda = 3R_g^2 / L,$$

where $R_g$ is the radius of gyration and L is the contour length of DNA molecule. Since we observed the two-dimensional picture of the three dimensional obstacle, $R_g$ is given from the following relationship:[15]

$$R_g=\{3/2(R_l^2 + R_s^2)\}^{1/2}$$

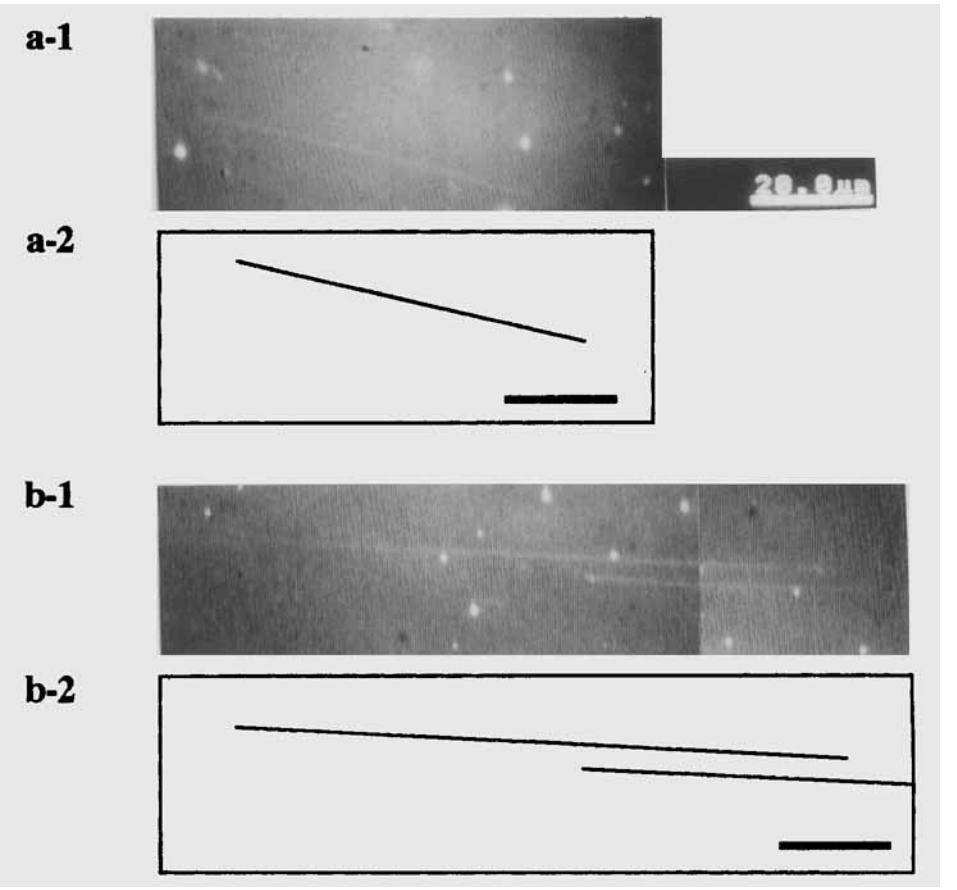


FIG. 2 Stretched T4-DNA molecules fixed on a glass surface observed with fluorescence microscopy. The surface of the over glass was positively charged after the treatment with the silanization agent (see text). [dye]/[nucleotide] = 10.
Scale bar is 20 μm.
a-1) DNA molecules complexed with DAPI, and b-1) DNA molecules complexed with EB. a-2) and b-2) are the schematic drawings of a-1) and b-1), respectively.

where $R_l$ and $R_s$ are the lengths of the long and short principal axes, respectively, obtained from two-dimensional images of the DNA molecules. Persistent length is considered as a quantitative measure of the degree of the bending of the double-helical DNA. Based on the data on the binding equilibrium with DAPI and EB discussed in section 1, we would like to discuss the effect of the dyes on the persistent length. Fig. 4 shows the relationship of the persistent length with the bind-dye concentration, which is expressed as the ratio of attached dye molecules per nucleotide, $[dye]_b/[nucleotide]$.

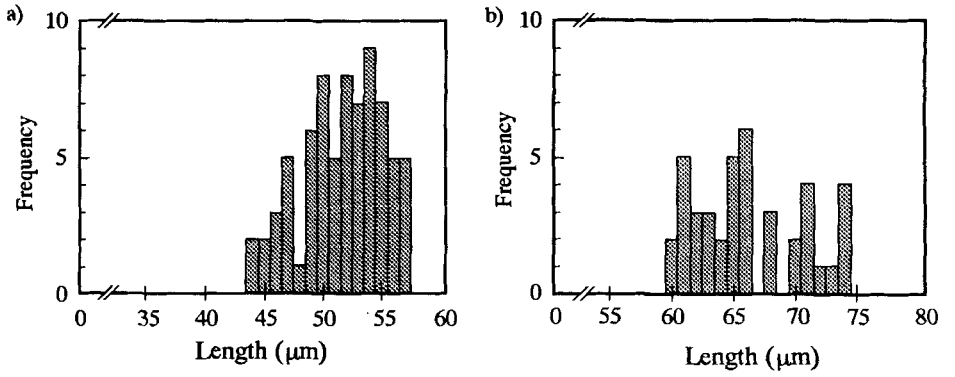


FIG. 3 Length distribution of the contour length of the stretched T4-DNA molecules fixed on the glass surface. [dye]/[nucleotide] = 10.
a) DNA-DAPI complex, and b) DNA-EB complex.
About 50 molecules were measured for each experiment.



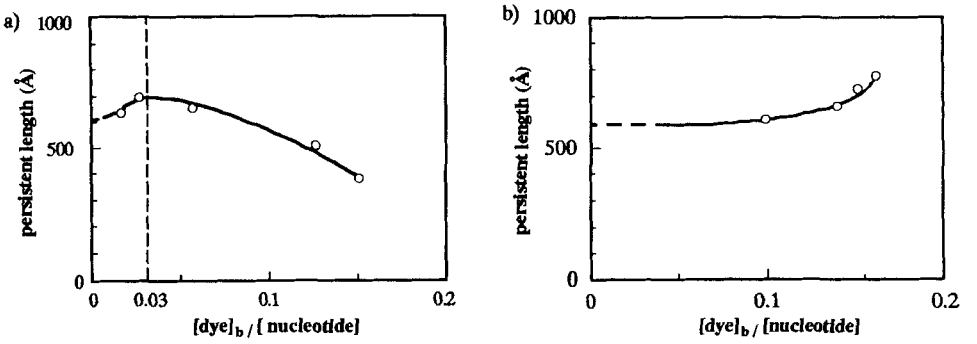


Fig. 4 Change of the persistent length depending on the number of the attached dye molecules per nucleotide, $[dye]_b/[nucleotide]$. $[dye]_b$ indicates the concentration of dye bound to DNA molecules in the aqueous sample. a) DAPI b) EB

Here, $[dye]_b$ indicates the concentration of dye bound to DNA molecules. From an extrapolation of the lower limit of the dye concentration in the figure, the persistent length in the absence of the drug is evaluated to be 600 Å.[10] It is clear that DAPI has a biphasic effect on the persistent length, the persistent length becomes longer up to the concentration, $[dye]_b/[nucleotide] = 0.03$, where the first step binding predominates on the second step binding ($r_1 > r_2$). On the other hand, the persistent length becomes shorter when $[dye]_b/[nucleotide]$ exceeds 0.03 ($r_1 < r_2$). Such an experimental trend may be explained as follows. When the DAPI concentration is low, in other words, when DAPI binds to DNA only with the first step equilibrium, the persistent length becomes

larger due to the expanding effect of DAPI which has the effect on DNA as a kind of supporter along the lateral site with the rather strong minor-groove binding. After the electrostatic attraction, which results in a decrease of the effective negative charge of the saturation of the first step equilibrium, DAPI molecules interact with DNA through phosphate groups along the DNA strand. Owing to this shielding effect on the negative charge, DNA molecule tends to bend; in other words, the persistent length decreases. Contrary to the effect of DAPI on the persistent length, EB shows a monotonous change as in Fig. 4 (b). This result corresponds well to the established knowledge that intercalator tends to expand the contour length.[3]

Fig. 5 shows a schematic representation of the difference of the effect between DAPI and EB, for the conditions that the number of binding dye per nucleotide exceeds 0.1. In the present article, we have shown that fluorescence microscopy is useful to obtain information on the higher order structure of giant DNA molecules. Although the resolution is rather low with fluorescence microscopy, one can obtain quantitative information such as the persistent length and the contour length through statistical analysis of the fluorescence images.



## EXPERIMENTAL PART

T4-DNA, 166 kbps, was purchased from Nippon Gene. All the other chemicals including fluorescence dyes, DAPI and EB were commercially available from Wako Pure Chemical Industries Ltd. We used 2-mercaptoethanol (2-ME) as an anti-oxidant. The DNA molecules were diluted with TBE buffer solution (45 mM Tris, 45 mM borate, 1 mM EDTA) containing 4 % (v/v) 2-ME and a fluorescent dye. The final concentration of DNA in nucleotide was 0.6 $\mu$M. A Nikon TMD microscope equipped with a 100$_\times$ oil-immersed objective lens was used. The samples were illuminated with 365 nm UV for the DNA-DAPI complex, and 520 nm for the DNA-EB complex. The fluorescence images were recorded on video tapes using a high sensitive SIT TV camera and an image processor, Argus 10 (Hamamatsu Photonics). The conformational data of DNA molecules recorded on video tapes were analyzed by use of a personal computer.

Special care was taken to clean the glass surface of the bottom and cover glasses. Glass plates soaked in hydrogen peroxide were washed with water and then with ethanol. Finally, the glass plates were dried at 150 ℃ for 2 hours. In order to observe fully stretched DNA fixed on the glass surface, the surface of the glass was treated with *N*-trimethoxysilylpropyl-*N,N,N*-trimethylammonium chloride to modify the glass surface to be positively charged.

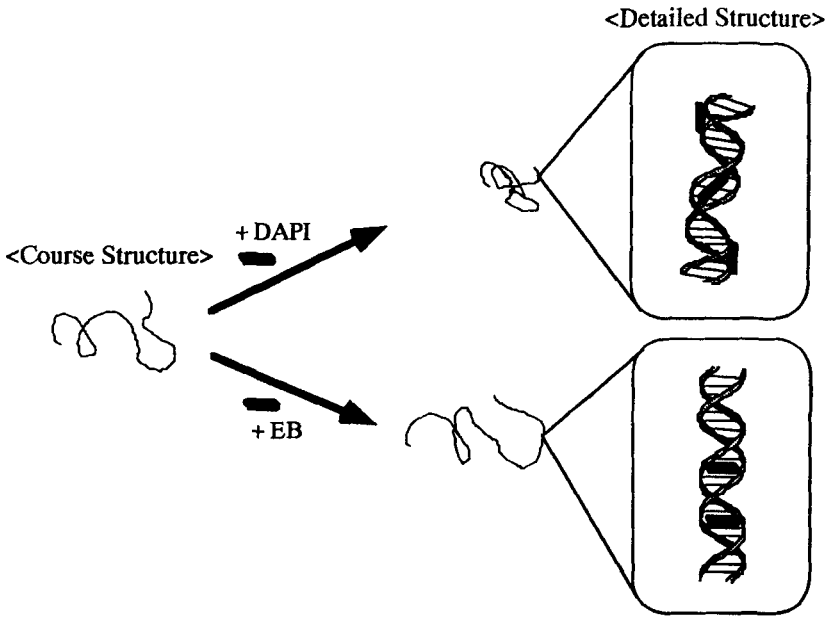


Fig. 5 Schematic representation of the opposite effect of DAPI and EB on the higher order structure of DNA for the case, $[dye]_b/[nucleotide] > 0.1$.



In order to evaluate the equilibrium constant for the binding of DAPI to DNA, fluorescence intensity was measured qualitatively by changing the DNA concentration at a fixed DAPI concentration. Fluorescence spectra were recorded on a Hitachi 650-40 fluorescence spectrophotometer equipped with a thermostatted cell holder regulated at 24 ℃. Quartz cuvettes with 1-cm light path were used. Excitation was carried at 365 nm with 10 nm band width and the emission was observed at 454 nm.

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