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Study on the Binding Nature of Acridine Orange to DNA by Means of Flow Dichroism

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The binding nature of acridine orange to DNA was studied by means of equilibrium dialysis and flow dichroism with spectrophotometric analysis. The adsorption isotherm of the acridine orange-DNA system was obtained by means of equilibrium dialysis. From the experiment, the binding constant (K) and the fractional amount of the binding site to the total number of nucleotides (f) were estimated for native- and heat-denatured DNA. Under a shear gradient, the acridine orange-DNA complex showed a negative dichroism in visible region as well as in the ultraviolet region. This implies that acridine orange is oriented on DNA rather perpendicularly to its main axis, like base pairs. The magnitude of the dichroism of the complex at 500 mμ, near the visible absorption peak of acridine orange, was larger than each value of the complex and that of the DNA itself (260 mμ). The transition moment corresponding to the absorption at 500 mμ may be oriented in a more orderly manner and/or more nearly perpendicularly to the main axis of DNA than that corresponding to 260 mμ.

It was found that acridine orange (AO) acted as a sensitizer in the photodynamic inactivation of transforming DNA extracted from *Diplococcus pneumoniae* and that the inactivation was mediated by the dye bound to the DNA.[1] The damage to DNA accompanying the inactivation must resulted from an energy migration system involving the bound dye. In order to clarify the mechanism of the photodynamic action of DNA at a molecular level, physico-chemical studies of the binding of the dye to DNA are required.

The primary interest should be in the binding amount of the dye and the mode of binding. The nature of the acridine dye-DNA complex has been

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reported on by many investigators.[2-8] Lerman[5] proposed an intercalation model; *i.e.*, acridine is inserted into the space between adjacent base pairs of DNA. He also[6] measured the polarized fluorescence and the dichroism of a flowing quinacrine-DNA solution and emphasized the former, which was considered to be strong evidence for his model. This model is supported by some other evidences also, *i.e.*, the reactivities of an acridine and amino groups of bases,[7] the mass per unit length,[9] the sensitized fluorescence,[10] the delayed fluorescence,[11] and the autoradiography.[12] The differential flow dichroism is one of the most sensitive techniques for demonstrating the arrangement of dye on DNA as well as the base-arrangement in a DNA molecule.[13] Nagata *et al.*[14] studied the interaction of polynuclear aromatic hydrocarbons, 4-nitroquinoline 1-oxides, and various dyes with DNA by applying the flow dichroism method developed by Wada and Kozawa.[15] In the present study, we measured the differential flow dichroism of the AO-DNA complex with the absorption spectra in order to obtain basic data for the interpretation of the photodynamic action. In addition to this, the binding constant and the fractional amount of the binding site to the total number of nucleotides were determined from the results of quantitative equilibrium dialysis.

Experimental

DNA. Pneumococcal DNA (R/Sm) was used throughout the present work in relation to the study of bacterial transformation. It was prepared by Marmur's method.[16] Bacteriophage T_2-DNA extracted by the method of Mandel and Hershey[17] was also used in the flow dichroism measurements. Denatured DNA was prepared by heating native DNA in a boiling-water bath for 15 min and by then rapidly chilling it in ice water.[18] Partially-denatured DNA was made by placing a solution of native DNA in a 0.001M phosphate buffer containing 0.0001M EDTA and 0.0025M NaCl (pH 7.0) at temperatures near but below the 'melting' temperature, *i. e.*, 62.5 and 64°C, for 15 min, followed by rapid chilling. The concentration of the DNA was determined from its optical density ($\varepsilon_{258}=6600$).

Dye. AO (Tokyo Chemical Industry) was purified in the following way.[19] It was dissolved in a mixture of water and ethanol (1 : 1), and 0.1N NaOH was added to precipitate it in the form of a free base. The dried precipitate was recrystallized twice from a mixture of water and ethanol (1 : 9). Finally the crystal was dried in a vacuum over P_2O_5. The purified AO base was identified by its melting point. A stock solution was prepared by dissolving a purified AO-base in water and by then neutralizing it with HCl.

Buffer Solution. The buffer solutions used were 0.15M NaCl+0.015M sodium citrate (pH 7.3) and a 0.001M phosphate buffer containing 0.0001M EDTA and 0.0025M NaCl (pH 7.0). The former, called the standard saline-citrate solution (abbreviated as SSC), was used in the study of equilibrium dialysis. The latter, with a low ionic strength, was used in the measurements of the flow dichroism in order to minimize the amount of unbound dye.

AO-DNA Complex. The mixture of AO and DNA was shaken vigorously in a test tube with a stopper and then stored in the dark for several hours to allow it to attain equilibrium. The composition of the mixture or the complex was expressed as the molar ratio of the nucleotide to the added dye, P/D, or of the nucleotide to the bound dye, P/D_b.

Absorption Spectrum. Measurements of the absorption spectra in the visible- and ultraviolet regions were made at room temperature by a Cary-14 spectrophotometer or by a Shimadzu spectrophotometer (Model QR-50). Silica cuvettes with a 1-cm light path were used. For diluted solutions, cuvettes with a 5-cm light path were used, while for concentrated ones a 0.8-cm silica spacer was used to make the light path 0.2 cm.

Equilibrium Dialysis. A Visking cellophane tube (23 A, 20/32) was used. The cellophane tube was washed with distilled water and conditioned by being immersed in SSC for more than 12 hr before use. Dialysis was performed with Erlenmeyer flasks in the dark. The cellophane bag containing 4 ml of the DNA solution was immersed in 20 ml of a dye solution. Equilibrium was attained after 16 to 20 hr of mechanical shaking at 30°C in a water bath. The measurements of the optical density of the inner- and outer solutions were made at room temperature. The adsorption of the dye to the cellophane membrane was estimated to be 15 to 20% of the total amount of dye, but it was presumed

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that such adsorption did not affect the equilibrium between the inner- and outer solutions.

Flow Dichroism. The details of the theory and the apparatus for the measurement of the flow dichroism were reported by Wada and Kozawa.[15]

(a) Apparatus. Measurements were made by a Shimadzu spectrophotometer equipped with an attachment. A transparent rotating cell of fused silica and a polarizer of calcite are included in this attachment. The gap between the outer- and the inner coaxial cylinders is 0.5 mm; therefore, the light-path length is 1.0 mm. The rotation speed of the inner cylinder was measured by a tachometer. The direction of the electric vector of the plane-polarized light chosen was either perpendicular or parallel to the flow axis.

(b) Measurements. The reduced flow dichroism is defined as:

$$\Delta\varepsilon/\varepsilon = \frac{\varepsilon_{//}-\varepsilon_{\perp}}{\varepsilon} = \frac{(-\log I_{//}/I_{\perp})-(-\log I_{//0}/I_{\perp 0})}{(-\log I/I_0)} \times A$$

where $I_{\perp 0}$ and $I_{//0}$ are the intensities of incident polarized lights with the electric vector perpendicular to and parallel to the flow axis respectively, where $I_{\perp}$ and $I_{//}$ are the intensities of transmitted polarized lights with the electric vector perpendicular to and parallel to the flow axis respectively, and where A is a factor depending on the concentration of the solution and on the light path of the cell.

In the present study two sorts of measurements were made: (1) measurements of $\Delta\varepsilon/\varepsilon$ over a range of wavelengths (λ) at a constant velocity gradient (3090 sec^{-1}), and (2) measurements of $\Delta\varepsilon/\varepsilon$ at 260 and 500 mμ at various velocity gradients (G). The extent of the flow dichroism is a measure of the orientation both of a macromolecule itself to the flow axis and of the chromophore in the macromolecule to the molecular axis. The reduced flow dichroism at a perfect orientation, $B(\alpha)$, was estimated from the following relation:

$$\Delta\varepsilon/\varepsilon = A(G/\Theta)\cdot B(\alpha)$$

supposing that DNA is a rigid ellipsoid of evolution.[13] In this equation, G is the velocity gradient, Θ is the rotary diffusion constant, and α is the angle between the long axis of the macromolecule and the plane of the chromophore. Theoretically, $-B(\alpha)$ takes a value from -3.0 to 1.5, with α from 0 to 90 degrees.[15]

Experimental Results

Absorption Spectra. Figures 1 (A) and (B) show two series of absorption spectra; they were obtained by adding native and denatured DNA solutions respectively of various concentrations to an AO solution of a given concentration (0.9×10^{-5}M). It can be seen in Fig. 1(A) that the absorption maximum shifts from 492 to 500 mμ and that the molar extinction coefficient at the wavelength corresponding to the maximum absorption increases with an increase in the concentration of native DNA. In this series, an isosbestic point is observed at 490 mμ. The quantitative estimation of the bound dye in the equilibrium dialysis was made at this wavelength. At low P/D ratios, the shoulder at about 470 mμ is remarkably higher for denatured DNA (Fig. 1 (B)) than for the native DNA. It is gradually lowered, however, with an increase in the P/D ratio, finally attaining the same level as that for native DNA. In the case of denatured DNA, it appears that an isosbestic point is present at 485 mμ, but, contrary to the case for native DNA, the absorption curve of free AO does

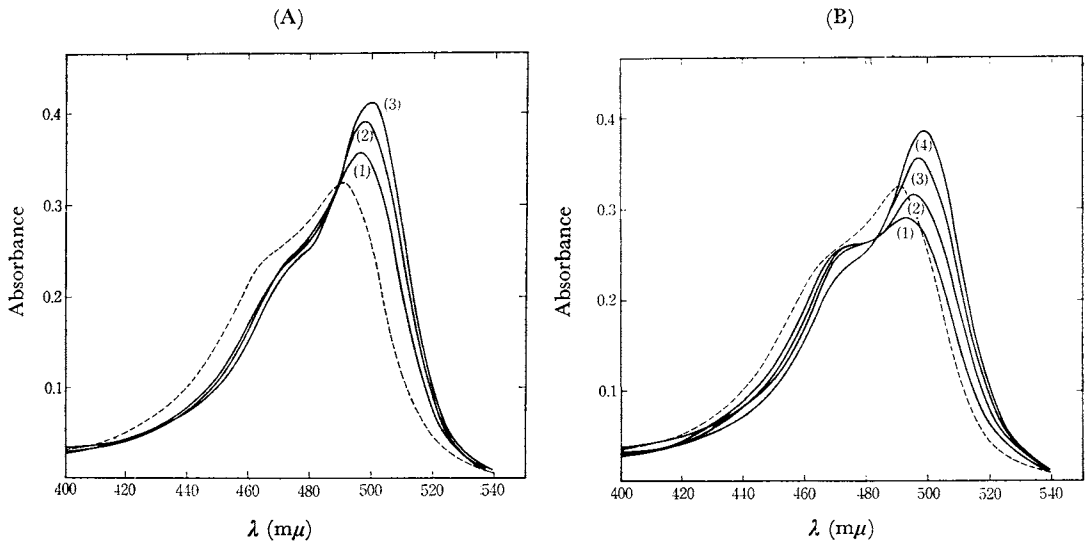

Fig. 1. The change of the absorption spectrum of AO solution by the addition of DNA (*Diplococcus pneumoniae*). The concentration of AO in SSC was 0.9×10^{-5}M. (A) Native DNA: ------, without DNA. ——, (1), (2) and (3) are the spectra in which P/D ratios are 10, 20 and 50, respectively. (B) Denatured DNA: ------, without DNA. ——, (1), (2), (3) and (4) are the spectra in which P/D ratios are 5, 10, 20 and 50, respectively.

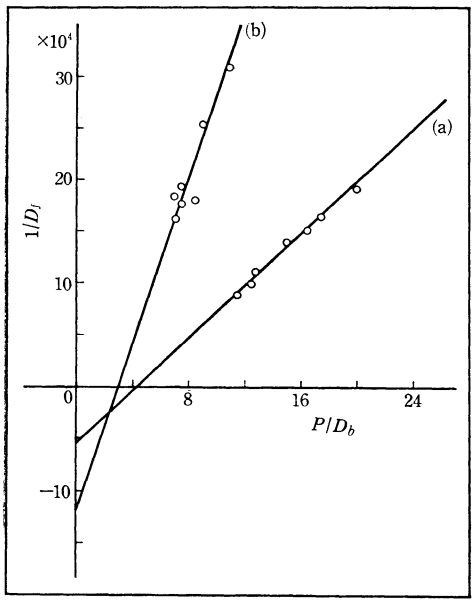

Fig. 2. Adsorption isotherm of AO to DNA in SSC at 30°C. The concentration of DNA (*D. pneumoniae*) was 20 μg/ml or 40 μg/ml (6 or 9×10^{-5}M$_p$).
(a) native DNA; (b) denatured DNA

not pass over this point, as can be seen in Fig. 1 (B). However, since it was shown by equilibrium dialysis that more than 80% of the dye cations were bound to the DNA even at ratios as low as 5, for convenience the extinction coefficient at 485 mμ was used to determine the amount of bound dye.

Equilibrium Dialysis. The adsorption isotherm was obtained in the following way. The binding constant (K) of one AO cation to a site on DNA was assumed not to be variable even if neighboring sites had been occupied by other AO cations. Then, if the fraction (f) to the total amount of nucleotides (P) in DNA is involved in forming a complex with AO, K must be given by $K=D_b/(Pf-D_b)\cdot(D_f)$. In this equation, D_f and D_b are the amounts of the unbound free dye and of the bound dye respectively. They were calculated from the optical density of the outer solution and from the difference in the optical densities of the inner- and outer solutions. P was obtained from the optical density at 258 mμ of the DNA solution. If $1/D_f$ is plotted against P/D_b, the intercept on the ordinate must give K, and that on the abscissa, $1/f$ (see Fig. 2). The values obtained at 30°C are, for the native DNA, $K=5.5\times10^4$ l/mol and $1/f=4.3$, and for the denatured DNA, $K=1.2\times10^5$ l/mol and $1/f=3.0$. Both K and f were larger for denatured DNA than for native DNA. Since the molar concentration of DNA was taken to be sufficiently lower than that of the buffer itself, the difference in the concentration of electrolytes between the inner- and outer solutions was considered to be negligible.

Flow Dichroism of the Complex. The AO-DNA complex showed a negative flow dichroism

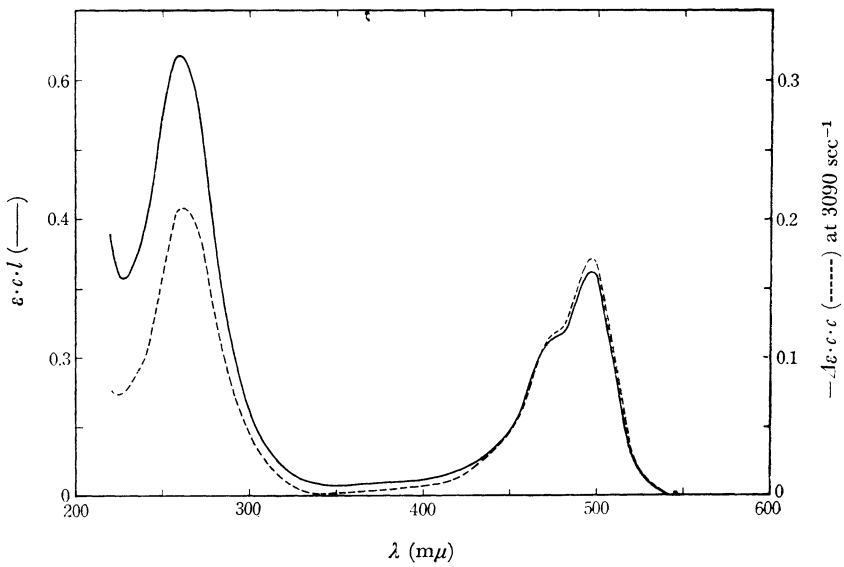

Fig. 3. Absorption spectrum (——) and differential flow dichroic spectrum (------)of AO-DNAcomplex. The concentrations of DNA and AO were 1.5×10^{-3}M$_p$ and 1.87×10^{-4}M, respectively. DNA and AO were dissolved in 0.001M phosphate buffer containing 0.0001M EDTA and 0.0025M NaCl (pH 7.0). Under this condition, more than 99.6% of AO cations are bound to DNA and the ratio of nucleotide to bound dye (P/D_b) is about 8.

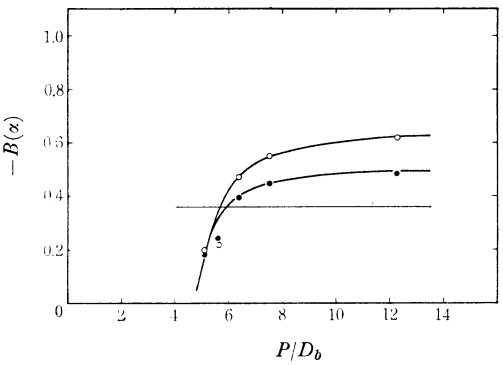

Fig. 4. The differential flow dichroism at varying P/D_b ratios of the complex of AO and native DNA. The concentration of DNA was 4.0×10^{-4} to 1.58×10^{-3}M$_p$ in 0.001M phosphate buffer containing EDTA and NaCl (see the legend of Fig. 3). The complex was prepared through the equilibrium dialysis at 30°C. —○—, complex at 500 mμ; —●—, complex at 260 mμ; ———, DNA (4.9×10^{-4}M$_p$) at 260 mμ.

in the visible region as well as in the ultraviolet region. Figure 3 shows the results of the differential flow dichroism of the complex at a P/D_b ratio of 8 over a range of wavelengths at a constant velocity gradient (3090 sec^{-1}). The dichroism of the complex measured with varying P/D_b ratios at 260 mμ and 500 mμ is shown in Fig. 4. The value of $-B(\alpha)$ of pneumococcal DNA at 260 mμ was 0.36. This is smaller than that of the complex at 260 or 500 mμ above a P/D_b of 6. At a P/D_b of 12, the value at 500 mμ of the complex was 1.7 times that of the DNA at 260 mμ. The lower values of $-B(\alpha)$ in the region of P/D_b below 6 may be due to the formation of intermolecular aggregates of the complex. Contrary to the case of pneumococcal DNA, the value of $-B(\alpha)$ at 260 mμ of the bacteriophage T_2 DNA-AO complex was nearly equal to the value at 500 mμ and also to the value of DNA itself (Preliminary experiment).

The value of $-B(\alpha)$ for DNA is very sensitive to its molecular conformation. For example, the value was diminished by partial denaturation depending on the temperature at which the DNA had been treated. The preparations of partially denatured DNA with different values of $-B(\alpha)$ at 260 mμ were equilibrated with AO at 20°C by dialysis and the flow dichroism of the complexes was measured. As is shown in Table 1, the values of the complexes at 260 mμ were decreased by elevating the treated temperature, as were those of the DNA. The value at 500 mμ of the complex, the DNA of which had been treated at 62.5°C, was larger than that of the corresponding DNA; the same tendency was seen in the complex with the native DNA.

Table 1. The effect of partial denaturation of DNA on the values of $-B(\alpha)$ of DNA (*D. pneumoniae*) and its complex with AO*

	Complex with AO		DNA
	at 500 mμ	at 260 mμ	at 260 mμ
Native DNA	0.66	0.46	0.41
Heated at 62.5°C	0.46	0.27	0.24
Heated at 64.0°C	0.15	0.15	0.15

* The concentration of DNA is 1.18×10^{-3}M$_p$. P/D_b=10.5 to 13.1
The buffer solution is the same as described in the legend of Fig. 3.

Discussion

For the analysis of the equilibrium dialysis and the flow dichroism, the amount of dye bound to DNA should be determined as accurately as possible. However, the concentration of the dye can not be simply determined from the optical density, since the observed spectrum of AO in the presence of DNA is composed of the spectra corresponding to the free monomer, the bound monomer, the free aggregate and the bound aggregate of the dye; that is, it changes depending on the P/D ratio. When various amounts of native- or denatured DNA were added to the AO solution, a series of spectra were obtained. The clear isosbestic point was observed for the native DNA-AO system. For the denatured system, the real isosbestic point was not observed, but the absorption curves passed over one point in the P/D-ratio range from 5 to 50. The extinction coefficients at these wavelengths were used for the calculation of the concentration of AO.

As may be seen in Fig. 1, the absorption spectra of the AO-denatured DNA mixture are very similar to that of the AO-native DNA mixture in the region of high P/D ratios, but it is rather different at low ratios. It is likely that, in a mixture with an extremely high P/D ratio, the dye cations bound to DNA must keep a large distance between them and so there should be very little dye-dye interaction. Therefore, the conformational change of DNA does not produce any spectral modification at such high P/D ratios. On the contray, an interaction between bound dye cations can be expected at low P/D ratios. Actually, this sort of interaction appears to occur more strongly on the denatured DNA-AO complex, since the shoulder band at about 470 mμ in the absorption spectrum is higher for the denatured DNA than for the native DNA, when the spectra for these complexes are measured at the same P/D ratio.

From the f value obtained by the adsorption isotherm, it appears that AO cations can bind to native DNA up to the ratio of 1 to 4.3 nucleotides at most. On the other hand, the ratio was 1 to 3 in the case of denatured DNA. The binding constant, K, for the denatured DNA was about twice as large

as that for the native DNA. Thus it was concluded that a large amount of AO can bind to the denatured DNA than to the native DNA. This suggests that the bases may provide further binding sites since they must come to be exposed to an outer medium by the denaturation, or that the denaturation facilitates, in some way, the adsorption of dye-aggregates to DNA, while the total number of binding sites does not increase.

The f value for the native DNA is in agreement with the saturation value of the "strong binding" obtained for the proflavine-herring sperm DNA system at an ionic strength of 0.1 at 20°C by Peacocke and Skerret.[20] Chambron *et al.*[21] investigated the thermodynamics of the interaction of proflavine and calf thymus DNA. They obtained values of $\log K=5.30$ ($K=2.0\times10^5$) and $1/f=6.1$ at 35°C, and values of $\log K=5.50$ ($K=3.2\times10^5$) and $1/f=4.4$ at 20°C, for the complex with the native DNA at an ionic strength of 0.1. The K values for their system are 4 to 5 times as large as that for the present system, while the f values for their system seem to be a little smaller than that of the present system. This discrepancy may be due to the differences in the experimental conditions and in the methods of estimating the values. They introduced the interaction energy between two bound dye-cations into their calculation, whereas we neglected it.

The principle of the ultraviolet flow dichroism of DNA must also be applicable to AO cations firmly bound to DNA. Since the oscillator dipoles of the π-π^* transition of bases and dye are known to be in the plane of these aromatic molecules, the magnitude of $-B(\alpha)$ gives an index of the orientation of the bases or dye to the long axis of the DNA molecule. The experimental results on the AO-DNA complex may be summarized as follows: (1) The AO-DNA complex gave a negative flow dichroism in the visible as well as in the ultraviolet region.

(2) The complex of pneumococcal DNA and AO gave a larger $-B(\alpha)$ value at 500 mμ than at 260 mμ, while both values are over that of DNA itself.

(3) A reduced dichroism, $(\Delta\varepsilon/\varepsilon)$, was almost constant throughout any visible region at the velocity gradient of 3090 sec^{-1}.

The theoretical value of $-B(\alpha)$ is 1.5 at 90 degrees of α,[15] but the observed values are much smaller. This may be attributed to the coexistence of random coiling parts in the DNA and small fragments, which were made during the extraction process, and/or of a folding structure of DNA like a hairpin with residual proteins.

The dichroism at 500 mμ of the complex must be due to the AO cations bound to DNA, since it was not observed in the absence of DNA. The (1) evidence suggests that the plane of the bound AO is nearly parallel to the plane of the bases in DNA. If AO cations are inserted into the spaces between base pairs parallel to them, the dichroism shown by bound dye could not be over the value for the DNA. However, the experimental result presented in (2) is not consistent with this view. According to the viscosity measurements,[5] DNA becomes longer and straighter by the binding of acridine dyes in solution. Considering that a longer molecule can be oriented more easily and gives a higher value of $-B(\alpha)$,[13] the present result that the $-B(\alpha)$ at 260 mμ of the complex is larger than that of DNA itself is reasonable. One model to explain the $-B(\alpha)_{500\,m\mu} > -B(\alpha)_{260\,m\mu}$ relation in the complex is that AO cations bind selectively to ordered parts rather than to disordered parts of DNA. However, this model is inconsistent with the results of equilibrium dialysis. Another model is as follows: On some loose structure of DNA, the transition moment corresponding to the absorption at 500 mμ may be oriented in a more orderly manner and/or nearly perpendicularly to the main axis of the DNA compared with that corresponding to 260 mμ. If the transition moments of 500 and 260 mμ were in the directions parallel to the long and short axes of AO respectively, the transition moment along the long axis could be oriented more in parallel to the plane of bases, but the other moment would be slightly declined. Actually, in the case of bacteriophage T_2-DNA which is believed to be more intact and to have better chain regularity,[13] the $-B(\alpha)$ at 500 mμ of the complex was nearly equal to the values at 260 mμ of the complex and of the DNA itself.

It can be seen in Fig. 3 that the dichroic spectrum is almost identical with the absorption spectrum. The shoulder band at about 470 mμ is also observed in the dichroic spectrum, and $\Delta\varepsilon/\varepsilon$ is almost constant over the range of wavelengths in the visible region. If the shoulder band in an absorption spectrum is attributed to dye-aggregates, the shoulder in a dichroism at the same wavelength might be also due to the dye-aggregates. However, it would be impossible to insert the dye-aggregates, even if they are dimers, into the spaces between base pairs in the sense of the intercalation model. Thus, the observed shoulder at about 470 mμ in the dichroic spectrum at the P/D_b ratio of 8 may be attributed to the characteristic of the AO monomer, as has been suggested by Zanker.[22]

As has been mentioned earlier, the intercalation model is supported by several pieces of evidence.

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On the other hand, Peacocke *et al.*[23,24] proposed a modified intercalation model based on their spectrophotometric analysis. In this model, when acridine cations are bound by the "process 1" (defined by them), the acridine lies between successive nucleotide bases on the same polynucleotide chain, in a plane approximately parallel to the base planes, in which the cationic-ring nitrogen is as close as possible to a phosphate group of DNA and in which the edges of the acridine rings (positions 1—4 or 6—9) partially overlap the base ring(s) of DNA. The present dichroic measurements show that the plane of the acridine dye is oriented parallel to that of the bases rather than perpendicular. However, by this method we can not distinguish between the intercalation model and the modified one. Although the nature of binding site is still obscure, it is very likely that phosphate groups and the spaces between adjacent base pairs of DNA act as sites of binding with acridine dye.

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