Studies of the DNA–Acridine Complexes. I. Fluorescence Depolarization

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The motion of the DNA–acridine complexes in solution was studied by the fluorescence depolarization method. Five derivatives of 3,6-diaminoacridine were used in order to investigate the effect of amino substituion on the depolarization results. A linear relation between the reciprocal polarization ($1/P$) and T/η was obtained by varying the temperature of a native DNA–acridine solution. The limiting polarization (P_0) and the mean rotational relaxation time (ρ_h) of the complex were evaluated from these data. The ρ_h value of the complex (30—50 nsec) was much larger than the value of free acridine in water, but it was much too small to correspond to the rotation of the whole DNA molecule. Further, it was found that the values of P_0 and ρ_h for 3,6-bis(diethylamino)-acridine are much smaller than those for other acridines. This may result from some steric hindrance of the binding by bulky diethylamino groups.

The interaction of aminoacridines and their derivatives with DNA has been extensively studied by various techniques. Interest in this topic has been stimulated by the biological activity of the acridines and by their structural similarity to carcinogens, such as polycyclic hydrocarbons and benzacridines. At present, it is generally accepted that acridine molecules bind to DNA to form two types of complexes; one (Complex I) results from the acridine-acridine interaction on the outside of the DNA helix at low values of the molar ratio of nucleotides to acridine molecules (N/A),[1,2] while the other (Complex II) occurs at high N/A values, where the acridine-nucleotide interaction is involved.[3] Lerman[4,5] proposed that, in Complex II, acridine molecules are intercalated in a sandwich-like way between adjacent base pairs in the double-stranded helix. Evidence in support of this model includes the data of viscosity,[4] the chemical reactivity of bound acridines,[6] polarized fluorescence,[5] sensitized fluorescence,[7] *etc.* On the other hand, Peacocke and his co-workes[8,9] proposed a modified intercalation model in order to interpret the effects of denaturation and the ionic strength on the interaction of acridines with DNA. In this model, the positively-charged nitrogen of the acridine ring associates closely with the negatively-charged phosphate group of DNA, and the flat acridine ring is inserted between adjacent bases on the same polynucleotide chain. In both models, the structure of acridine derivatives can be expected to influence their binding to DNA. Therefore, it is important to study the effect of the acridine structure on the interaction between acridines and DNA by using various acridine derivatives and various techniques.

In the present study, the DNA–acridine complexes were investigated by the fluorescence depolarization method,[10–13] using five derivatives of 3,6-diaminoacridine. The aims of this study are: (1) to obtain some information on the characteristics of the complex related to the rotation or internal motion of the bound acridine and (2) to reveal the relation between these properties and the structure of 3,6-amino groups.

Experimental

Materials. A highly-polymerized calf thymus DNA was purchased from the Worthington Biochemical Corporation. The concentration of DNA was determined spectrophotometrically at 259 nm, with an extinction coefficient per molar DNA phosphate ($\varepsilon_P=6600$). The hyperchromicity was 35% at room temperature and at an ionic strength of 0.01; this means that the DNA used was native. The thermal denaturation of DNA was performed by heating the DNA solutions for 20 min in boiling water and by then rapidly cooling them in ice water.

Acridine orange (AO) and proflavine (PF) were obtained from Chroma and British Drug Houses respectively. 3,6-Bis(methylamino)acridine (Ac[NHMe]$_2$), 3,6-bis(ethylamino)acridine (Ac[NHEt]$_2$), and 3,6-bis(diethylamino)-acridine (Ac[NEt$_2$]$_2$) were prepared according to the method described by Albert.[14] These acridines were purified by repeated recrystallizations and by chromatography.

Measurements. The solutions of the complexes were made up in a 0.005 M phosphate buffer (ionic strength of 0.01) at pH 6.8. This low ionic strength was used to minimize the amount of unbound acridine molecules. The acridine concentrations were $(1—2.5)\times10^{-6}$ M.

The absorption spectra were measured with a Shimadzu MPS-50L spectrophotometer.

The fluorescence spectra and polarization were measured with a Hitachi MPF-2A spectrophotofluorometer, with a pair of Polacoat dichroic filters as the polarizer and the analyzer. With the incident beam vertically polarized, the degree of polarization (P) is given by:[15,16]

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$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}} \quad (1)$$

where I_{VV} and I_{VH} are the measured fluorescence intensities with the analyzer vertically and horizontally oriented; the correction factor, $G = I_{HV}/I_{HH}$, is obtained with the incident beam horizontally polarized.

The fluorescence lifetimes were measured by means of a JASCO FL-10 phase fluorometer equipped with a 500-W xenon lamp and a CT-20P prism monochromator; the cut-off color and interference filters were used to select appropriate emission bands. The arrangement was similar to the one described in detail in an earlier paper.[17] The light emerging from the monochromator was modulated at a frequency of 13.56 MHz. The phase shifts of the fluorescent samples were measured by comparison with the phase shift of light scattered from a colloidal solution of silica.[18]

The measurements of the flow dichroism were carried out at 25 °C by the use of a Shimadzu QV-50 spectrophotometer equipped with an attachment. The datails of the theory and the experiment procedures have previously been reported by Wada and Kozawa.[19]

All the measurements except those of the flow dichroism were made at temperatures from 10 to 70 °C.

Results and Discussion

A typical fluorescence polarization spectrum is shown for the DNA–Ac[NHMe]$_2$ complex in Fig. 1. No change in the P value was observed at the wavelengths from 400 to 490 nm; this indicates that only one electronic transition exists in this wavelength region.[7,20] The other complexes gave similar polarization spectra. Therefore, the complexes were excited in this wavelength region. Further, no significant change in the P value was observed at N/A values higher than *ca.* 100. Our equilibrium dialysis and absorption data[21] showed that almost all the acridine molecules are bound to DNA at a high N/A value and even at elevated temperature up to 65 °C. Accordingly, the value of P at a sufficiently high N/A value can be expected to reflect the rotational characteristics of the unit to which

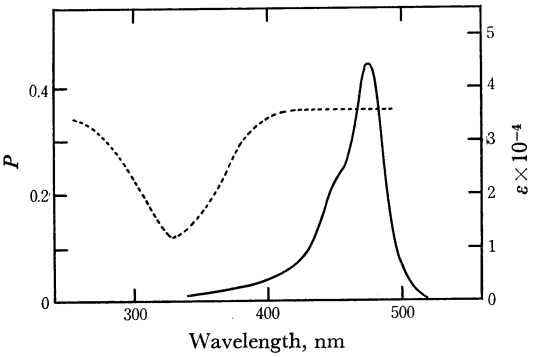

Fig. 1. Fluorescence polarization (·····) and absorption (——) spectra of a native DNA–Ac[NHMe]$_2$ complex at 25 °C. Ac[NHMe]$_2$: 2.2×10^{-6} M, N/A: 240

Table 1. Polarization of the DNA-acridine complexes at 25 °C

Actridine	Native DNA		Denatured DNA	
	N/A	P	N/A	P
PF	270	0.34_2	300	0.27_4
AO	260	0.34_5	240	0.27_6
Ac[NHMe]$_2$	240	0.35_7	270	0.28_1
Ac[NHEt]$_2$	260	0.35_4		
Ac[NEt$_2$]$_2$	240	0.32_3		

an acridine molecule is attached. The polarization of the complex at 25 °C is tabulated in Table 1. At every temperature investigated, the value of P in the case of denatured DNA was lower than that in the case of native DNA, and the value of P for Ac[NEt$_2$]$_2$ was smaller than those for other acridines.

For plane-polarized exciting light, the Perrin equation[10] relates the value of P to the molecular characteristics on the assumption that the molecule undergoing the Brownian rotation is spherical:

$$1/P - 1/3 = (1/P_0 - 1/3)(1 + RT\tau/\eta V) \quad (2)$$

where P_0 is the limiting value of P when $T/\eta = 0$; R, the gas constant; T, the absolute temperature; τ, the lifetime of fluorescence; η, the viscosity of the solvent, and V, the molar volume of the rotating molecule. The rotational relaxation time of this molecule (ρ_0) equals $3V\eta/RT$. If the molecule is ellipsoidal in shape the expression for P is given by:[11–13]

$$1/P - 1/3 = (1/P_0 - 1/3)(1 + 3\tau/\rho_h) \quad (3)$$

where $\rho_h = 3\eta V_e/RT$ is the harmonic mean of the three principal rotational relaxation times and where V_e is the effective hydrodynamic volume of the molecule.

If V and τ are constant, both equations predict a linear relation between $1/P$ and T/η, and the rotational relaxation time can be calculated by the determination of the slope (β) of such a plot:

$$\rho_h = 3(1/P_0 - 1/3)/(\beta \times T/\eta) \quad (4)$$

If V changes with the temperature, or if two or more rotational units of distinctly different volumes are present, deviations from linearity are to be expected.[11]

Figures 2 and 3 show some results of the plots of $1/P$ against T/η; η was assumed to be that of water and was interpolated from the values given in the literature.[22] For all the native DNA-acridine complexes studied in this work, there exists a linear relation between $1/P$ and T/η over the temperature range from 10 to *ca.* 60 °C. However, there is an abrupt increase in the value of $1/P$ above *ca.* 60 °C; this implies that the bound acridine now reflects the new rotational unit associated with the denaturation of DNA. The $1/P$ *vs.* T/η curves of the complexes are similar in shape to those reported by Anufrieva *et al.*[23] for DNA–AO complexes and by Ellerton *et al.*[24] for DNA–PF complexes. However, all the P values of Anufrieva

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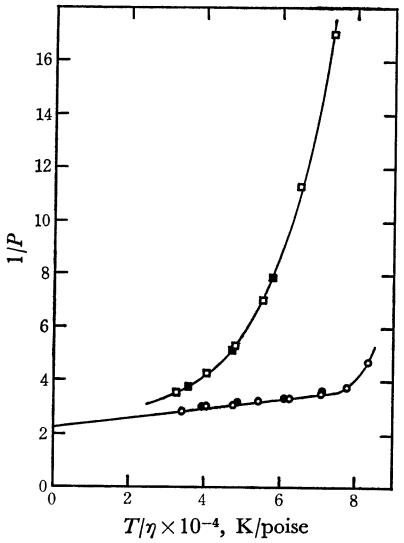

Eig. 2. Plots of $1/P$ *vs.* T/η for DNA–Ac[NHMc]$_2$ complexes.
Ac[NHMe]$_2$: 2.2×10^{-6} M
○ ●: native DNA (N/A=240)
□ ■: denatured DNA (N/A=270)
Symbols of ○ and □ denote data obtained on heating the solution, while those of ● and ■ denote data obtained on cooling the solution; the same notation is used in Fig. 3.

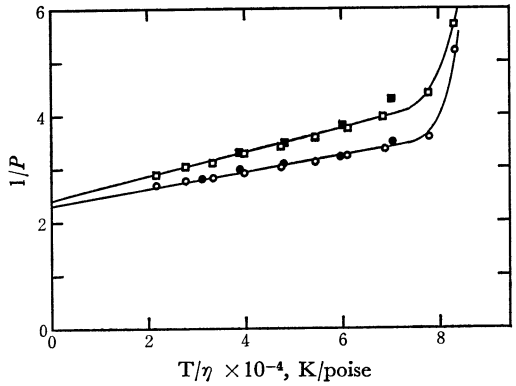

Fig. 3. Plots of $1/P$ *vs.* T/η for native DNA–Ac[NHEt]$_2$ and native DNA–Ac[NEt$_2$]$_2$ complexes.
○ ●: Ac[NHEt]$_2$ (2.0×10^{-6} M, N/A=260)
□ ■: Ac[NEt$_2$]$_2$ (2.2×10^{-6} M, N/A=240)

et al. are much lower than ours and those of Ellerton *et al.*

On the other hand, the value of P in the case of denatured DNA remarkably decreased with an increase in the temperature. As may be seen in Fig. 2, the plots of $1/P$ *vs.* T/η showed a marked departure from linearity. This phenomenon is probably due to the structural change in denatured DNA resulting from the collapse of the double-helical regions at elevated temperatures.[25]

The results obtained on cooling the solutions of the complexes from the maximum temperature (69 °C in all systems) are shown in Figs. 2 and 3. The temperature effects were in general reversible between 10 and 69 °C; a further elevation of the maximum temperature led to an irreversibility of the temperature

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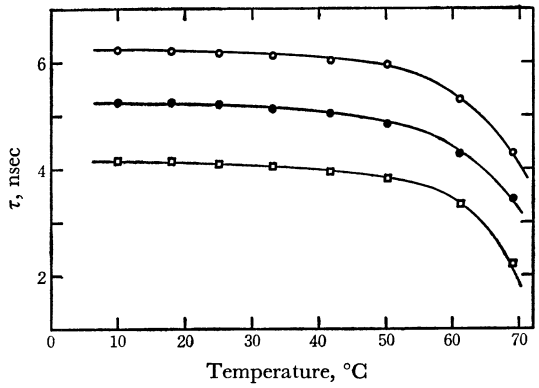

Fig. 4. The variation of fluorescence lifetimes (τ's) with temperature.
○: native DNA–PF (N/A=270)
●: native DNA–AO (N/A=260)
□: native DNA–Ac[NEt$_2$]$_2$ (N/A=240)

effects. This implies that, under the present experiment conditions, the tertiary structure of native DNA is not significantly lost as the polarization of the bound acridine is altered.

Next, the fluorescence lifetimes of the complexes were measured under each of the experimental conditions in order to ascertain the proper values for use in calculating the rotational relaxation times. Figure 4 shows the dependence of the fluorescence lifetimes on the temperature for the DNA–PF, DNA–AO, and DNA–Ac-[NEt$_2$]$_2$ complexes. The value of τ for each complex declines very slowly with the temperature up to *ca.* 60 °C, but it sharply decreases above *ca.* 60 °C; this temperature coincides with the temperature at which the value of P rapidly decreases. Similar results were obtained in the cases of Ac[NHMe]$_2$ and Ac[NHEt]$_2$. Since almost all acridine molecules are bound to DNA under the present experiment conditions, the decrease of τ above *ca.* 60 °C may be ascribed to a certain change in the binding site associated with the denaturation of DNA. Further studies of the variation in τ with the temperature are in progress.

Table 2. Depolarization results at 25 °C

Acridine	N/A	τ (nsec)	P_0	ρ_h (nsec)
PF	470	6.1	0.43_5	$52._6$
	270	6.1	0.43_9	$52._0$
AO	930	5.2	0.44_4	$47._9$
	260	5.2	0.44_0	$48._4$
Ac[NHMe]$_2$	420	4.0	0.44_2	$47._9$
	240	4.0	0.43_9	$48._3$
Ac[NHEt]$_2$	260	4.0	0.43_5	$45._4$
Ac[NEt$_2$]$_2$	240	4.0_5	0.42_0	$32._1$

Because of the small variation in the value of τ below *ca.* 60 °C, the mean rotational relaxation times were evaluated by Eq. (4), using an average value of τ for each system and the slope of the linear portion of the $1/P$ *vs.* T/η plots. The calculated values are summarized in Table 2. The depolarization results in Table 2 show that: (1) the values of P_0 and ρ_h are independent of N/A, (2) the value of ρ_h is of almost the same order of magnitude except for Ac[NEt$_2$]$_2$,

(3) the value of ρ_h for $Ac[NEt_2]_2$ is much smaller than those for the other acridines, and (4) the value of P_0 for $Ac[NEt_2]_2$ is also lower than those for the others.

The ρ_h value of the bound acridine (30—50 nsec) is much larger than the value for the free acridine in water (a few tenths of 1 nsec), but it is much too small to correspond to the rotation of the whole DNA molecule.[26] Since almost all acridine molecules are bound and are very fara part under the present experimental conditions (high N/A value and low ionic strength), any depolarization due to the free acridine or energy transfer between bound acridine molecules may be ruled out. Furthermore, it has previously been shown[27] that the rate constant of an intercalation reaction between native DNA and PF is about 10^4—10^2 sec^{-1}; therefore, the dissociation of the bound acridine could not occur during the lifetime of the excited state (4—6 nsec).

From the measurements of the nanosecond anisotropy, Wahl *et al.*[28] determined the rotational relaxation time of DNA–ethidium bromide (EB) in water to be 28 nsec; this value is also much too small for the rotation of the whole DNA molecule. Wahl *et al.* proposed that the depolarization is due either to a restricted motion of the EB molecule in its binding site on DNA, without any motion of the DNA, or to a local deformation motion of the DNA, and they showed that the latter is the only likely hypothesis that fits the restraints of the intercalation model.[4,29]

On the other hand, studies of the fluorescence quenching[30] and equilibrium dialysis[31] of the DNA-acridine complexes showed that the binding constants of PF, AO, $Ac[NHMe]_2$, and $Ac[NHEt]_2$ are of the same order of magnitude, while that of $Ac[NEt_2]_2$ is much smaller than those of the others. This suggsets that the bulky diethylamino groups produce some steric hindrance of the binding.

Table 3. Flow dichroism at 25 °C[a]

Acridine	λ_{max}[b] (nm)	$B(\alpha)$[c] at λ_{max}
PF	462	−0.84
AO	502	−0.82
$Ac[NHMe]_2$	475	−0.84
$Ac[NHEt]_2$	476	−0.82
$Ac[NEt_2]_2$	508	−0.70

a) The molar concentration of DNA phosphate (M_P) is 1.38×10^{-3} M_P. $N/A=15$ to 50.
b) The maximum wavelength of absorption spectrum of the complex in the visible region.
c) The reduced dichroism at a perfect orientation. The value of $B(\alpha)$ was obtained according to the method described by Wada.[32]

In view of these findings, the depolarization results of $Ac[NEt_2]_2$ in Table 2 can be interpreted to show that the $Ac[NEt_2]_2$ molecule, when intercalated between base pairs, leads to a larger local change in the DNA helix and, hence, a larger local deformation motion than the other acridines. If this is the case, some difference in the orientation of the bound acridines can be expected to occur between $Ac[NEt_2]_2$ and the others. In practice, preliminary results on flow dichroism reveal that the magnitude of the dichroism for the DNA–$Ac[NEt_2]_2$ complex is much smaller than those for the other complexes (see Table 3). Further studies by flow techniques are now in progress to clarify the orientation of the bound acridines; the results will be presented in a subsequent paper.

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31) Preliminary results at ionic strength of 0.01 and at 25 °C revealed that the binding constant of $Ac[NEt_2]_2$ is the order of 10^5 M^{-1}, while those of other acridines the order of 10^6 M^{-1}.
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