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Optical Activity of Nucleic Acid-Thionine Complexes[1)]

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The absorption spectra, optical rotatory dispersion, and circular dichroism (CD) of the DNA- and RNA-thionine complexes were measured at the nucleic acid phosphate to dye ratios (P/D) 0.01—50 and at the temperature range 20—85°C. The Cotton effects of P/D >3 complexes were observed in the wavelength region 520—680 nm, being resolved at least into four partial Cotton effects. From their transient profiles with changing P/D values and the observed temperature effect, they were interpreted to be induced from the dye monomers bound near the asymmetric environment of nucleic acids. The Cotton effects of P/D <2 complexes in the wavelength region 450—580 nm are new and have not been reported for other DNA-dye systems. The trough of these Cotton effects completely disappeared at 50°C and the decreasing profile of its relative rotation with increasing temperature was roughly parallel to that of the dimer fraction. The newly developed Cotton effects are inferred to result from an interaction between the dye dimers aggregated in a helical fashion along the nucleic acid helices. The negative sign CD maximum observed in the wavelength region 300—330 nm is probably due to DNA-dye interactions, but its origin is not clear.

It is well known that some basic dyes show photodynamic and mutagenic actions for microorganisms.[2,3)] Binding of such dyes to biological macromolecules, such as nucleic acids and polysaccarides, induces the bathochromic and hypochromic effects in the visible electronic absorption region of the dyes.[4-7)] Concerning the nucleic acid-aminoacridine dye complexes, the binding mechanism and conformations have been studied extensively by many investigators using the optical rotatory dispersion (ORD) and circular dichroism (CD) methods.[8-11)] Acridine orange (AO) and/or proflavine is an optically inactive molecule. When either dye binds to a biological helical polymer, anomalous optical rotation (Cotton effect) is observed in the spectral region of the dye. Blake and Peacocke[9)] observed this "extrinsic Cotton effect" by means of ORD measurement, and Gardner and Mason[11)] by CD measurement under various experimental conditions, *viz.*, by changing the nucleic acid phosphate to dye ratio (P/D), pH, and ionic strength. In order to explain the induced anomalous optical rotations for nucleic acid-dye complexes, particularly for the DNA-AO complexes, the following models have been proposed: for their conformations, helical arrangement of the dye along the macromolecule or helical tangential extension of the dye hanging on the anionic residue of the macromolecule, and for their

1) Presented in part at the 23rd Annual Meeting of the Chemical Society of Japan, Tokyo, 1970.

2) N. Yamamoto, *Virus*, **6**, 510, 522, 531 (1956).

3) F. H. Crick, L. Barnett, S. Brenner, and R. J. Watts Robin, *Nature*, **192**, 1227 (1961).

4) F. W. Morthland, P. P. H. de Bruyn, and N. H. Smith, *Exp. Cell Res.*, **7**, 201 (1954).

5) M. Semmel and M. Daune, *Biochim. Biophys. Acta*, **145**, 561 (1967).

6) H. Ito and Y. J. I'Haya, *Intern. J. Quantum Chem.*, **2**, 5 (1968).

7) Teh Fu Yen, M. Davar, and A. Rembaum, *Biochim. Biophys. Acta*, **184**, 646 (1969).

8) A. R. Peacocke and J. N. H. Skerett, *Trans. Faraday Soc.*, **52**, 261 (1956).

9) A. Blake and A. R. Peacocke, *Biopolymers*, **4**, 1091 (1966).

10) K. Yamaoka and R. A. Resnik, *J. Phys. Chem.*, **70**, 4051 (1966).

11) B. J. Gardner and S. F. Mason, *Biopolymers*, **5**, 79 (1967).

interaction mechanism, dipole-dipole interaction between the stacked and/or intercalated dyes or asymmetric environment of the binding sites.

Much has been said for and against these models. In the DNA-AO complexes, there appear two or three negative CD maxima in the visible absorption region of the dye, in addition to the positive CD maximum in the longer wavelength region. However, the primary source of the Cotton effects, *i.e.*, whether they are induced from monomerically bound AO or from dimerically bound AO, has not been established.

Thionine forms molecular complexes with nucleic acids. In this paper we report results of the measurement of the induced Cotton effects and absorption spectra resulting from the interaction of thionine with native DNA and RNA. We show how Cotton effects change their profiles with P/D and temperature. Comparing the results with those obtained for the DNA-AO complexes, we will discuss the origin of the induced Cotton effects, in particular the contribution of the thionine dimer. Certain inferences will be made as to the binding conformations of the nucleic acid-thionine complexes.

Experimental

Commercial calf-thymus DNA (Sigma Type 1) was used without further purification. Unfractioned RNA was prepared from rat-liver according to Kirby's method.[12] Stock solutions of nucleic acids (30 mg/25 m*l*) were stored in the cold and diluted just prior to use. Thionine (3,7-diaminophenothiazonium chloride; Lauth's violet) (Tokyo Kasei) was purified by recrystallization twice from aqueous solution. In this purified dye, no detectable impurity was found through Sephadex LH-20 column chromatography. Stock solutions of the dye were stored in the dark in order to avoid photosensitized reactions.

Stock aqueous solutions of the dye and nucleic acids were dissolved in 0.01 M phosphate buffer (pH 6.84). The concentration of nucleic acids, expressed in terms of phosphate residues, was determined as usual by the measurement of the absorbance at 260 nm and using the known molar extinction coefficients $\varepsilon_P^{260}=6{,}650$ and $\varepsilon_P^{260}=8{,}190$ for DNA and RNA, respectively. Within the concentration range of $(1—3)\times10^{-5}$ M, $\varepsilon^{598}=54{,}200$ was obtained for thionine in the phosphate buffer. This value is nearly in accord with that given by Rabinowitch and Epstein.[13]

Total concentration of the dye in the complexes was selected to be from 1.0×10^{-5} M to 3.3×10^{-5} M, since the observed extrinsic Cotton effects of the complexes strongly depended upon the concentration of the dye. Using a Jasco ORD/UV-5 recording spectrophotometer, absorption, ORD, and CD spectra were measured at various temperatures (20—85°C). Temperature studies were carried out with a specially designed variable temperature cell. Sample temperatures were measured with a copper-constantan thermocouple while spectra were being taken.

Results

Morthland *et al.*[4] reported that the absorption spectrum of thionine shifts to the red, losing its intensity, upon formation of the complex with nucleic acids. Although our experimental conditions differ somewhat from theirs, analogous bathochromism and hypochromism are observed except for extremely low P/D complexes (Fig. 1). When P/D exceeds 10, a new absorption maximum appears at about 610 nm, while the absorption maximum at 598 nm is diminished in intensity and remains as a shoulder in this region. In contrast to such high P/D complexes, the extremely low P/D ones show slight broadening of the shorter wavelength limb together with slight depression of the absorption maximum at 598 nm. The absorption spectrum of the dye alone, when the concentration

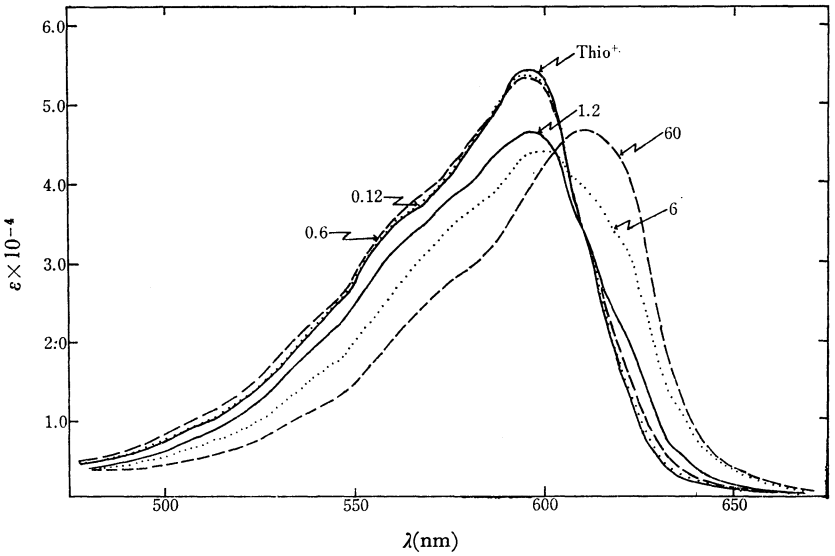

Fig. 1. Absorption spectra of Calf-thymus DNA-thionine complexes in 10^{-2} M phoshate buffer (pH=6.84). Total concentration of thionine, 2×10^{-5} M. Each figure shows P/D value of the complexes.

12) K. S. Kirby, *Biochem. J.*, **64**, 405 (1956).

13) E. Rabinowitch and L. F. Epstein, *J. Amer. Chem. Soc.*, **63**, 69 (1941).

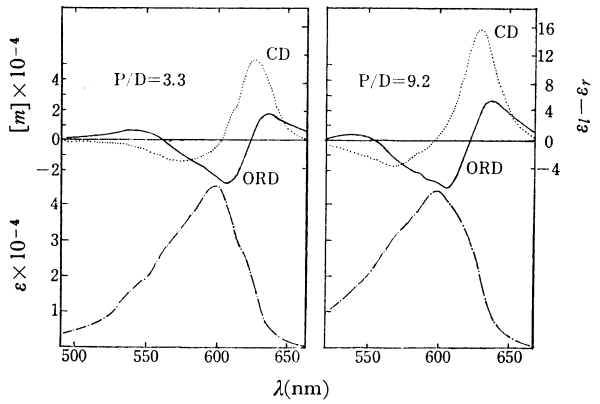

Fig. 2. ORD, CD (upper), and absorption (lower) spectra of DNA-thionine (left) and RNA-thionine (right) complexes at high P/D values. Total concentration of thionine, 1×10^{-5} M.

of the dye is sufficiently high (about 10^{-3} M), shows another maximum at 560 nm. This has been assigned to a dimer band of thionine.[13] The 610 nm (monomer) band of the high P/D and the 560 nm (dimer) band of the low P/D DNA-thionine complexes seem to correspond to the α-(or complex II) and β-(or complex I) bands of the DNA-AO complexes, respectively. At further increased P/D values, two isosbestic points are obtained at 608 nm and 602 nm. The former can be observed even when the concentration of DNA is not very high. The latter, however, can be observed only in the complexes of P/D values greater than 20. Metachromatic properties of the RNA-thionine complex are almost the same as those of the DNA-thionine complex described above.

Figures 2 and 3 show the representative ORD and CD spectra of the DNA- and RNA-thionine complexes. These data together with those for the absorption spectra are summarized in Table 1. At relatively high P/D values, the general shapes of these ORD and CD spectra resemble those of the DNA-AO complex.[9-11] It is apparent that there are one positive (*ca.* 626 nm) and one negative (*ca.* 576 nm) Cotton effect in the visible absorption spectral region of these complexes, as seen in Fig. 2 and Table 1. When P/D decreases to less than 2, new Cotton effects are observed in the region of 450—580 nm (Fig. 3). These types of Cotton effects have not been observed in other DNA-dye complexes. It should be noted that even in the DNA-thionine system the following conditions are required for observation: low P/D value (less than 2), high total concentration of the dye (at least, more than 2×10^{-5} M), and moderate temperature (below 50°C).

The decrease in P/D gives rise to the decrease in molar rotation of the peak at 636 nm and the trough at 606 nm and the increase in that of the trough at 525 nm. After passing intermediate P/D values where all these peaks and troughs are detected, only the trough

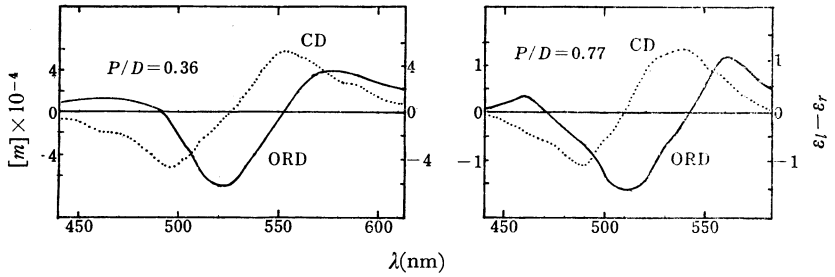

Fig. 3. ORD and CD spectra of DNA-thionine (left) and RNA-thionine (right) complexes at low P/D values. Total concentration of thionine, 3.3×10^{-5} M.

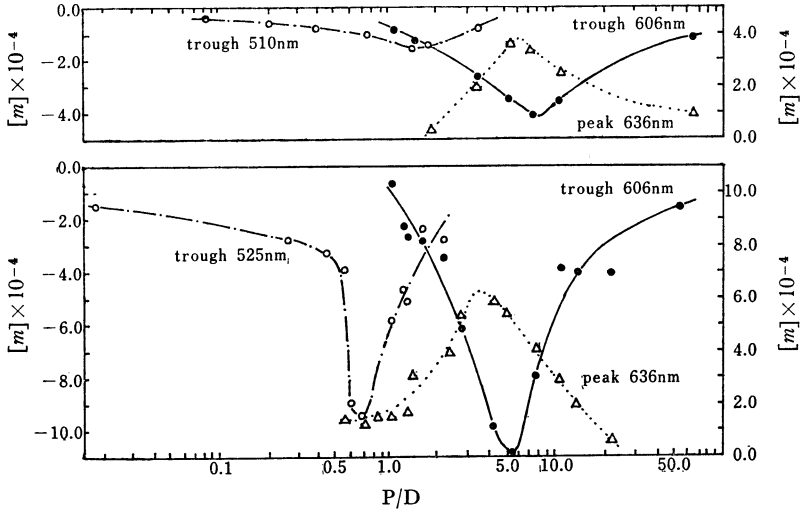

Fig. 4. Plot of molar rotation against P/D value for RNA-thionine (upper, thionine conc., 2×10^{-5} M) and DNA-thionine (lower, thionine conc., 3×10^{-5} M) complexes. Left hand scale applies to each trough and right hand scale to each peak.

Table 1. Absorption, ORD, and CD spectral data for DNA- and RNA-thionine complexes (nm)

Thionine-DNA

Total conc. of thionine (M)	1.00×10^{-5}	2.95×10^{-5}	2.95×10^{-5}	2.95×10^{-5}	3.33×10^{-5}
P/D	3.3	0.27	1.36	5.43	0.36
Absorption					
peaks	602	596	596	602	596
shoulders	~570, ~615	~565	~540, ~565	~565, ~615	~565
ORD[a]					
peaks	635 (1.69)		635 (3.39)	637 (5.42)	
					~580 (2.36)
troughs	607 (−2.83)		604 (−2.71)	609 (−11.19)	
		527 (−2.71)	525 (−5.08)		522 (−6.31)
shoulder				590 (−6.11)	
inflextion points	623	560	622		490, 556
CD[b]					
(+) maxima	626 (11.88)				555 (4.80)
(−) maxima	576 (−3.20)				494 (−4.50)

Thionine-RNA

Total conc. of thionine	1.00×10^{-5}	2.30×10^{-5}	2.30×10^{-5}	2.30×10^{-5}	3.33×10^{-5}
P/D	9.2	0.37	1.85	11.13	0.77
Absorption					
peaks	599	596	596	601	592
shoulders	~560, ~620	~565	~510, ~565	~575, ~620	~565
ORD[a]					
peaks	636 (2.50)		640 (0.43)	640 (2.60)	
		555 (0.65)	550 (0.65)		562 (1.05)
troughs	607 (−3.13)			606 (3.47)	
		510 (−0.65)	506 (−1.30)		512 (−1.65)
shoulder					
inflextion points	622	535	622, 565, 520	624	541
CD[b]					
(+) maxima	627 (16.26)				540 (1.20)
(−) maxima	570 (−3.96)				490 (−1.05)

a) Values in braces show molar rotations [m] in units of degrees mol^{-1} decimeter^{-1} $ml\times10^{-4}$.
b) Values in braces show circular dichroic absorptions $\varepsilon_l-\varepsilon_r$ in units of l cm^{-1} mol^{-1}.

at 525 nm can be observed clearly even at extremely low P/D values. These variations in the ORD profile are illustrated in Fig. 4, together with those of the RNA-thionine complexes.

Figure 5 shows the effect of temperature on the ORD curves. The relative amplitudes (or relative molar rotations) at various temperatures are plotted in Fig. 6 where the value of the amplitude (or molar rotation) at 20°C is taken to be unity. The relative amplitude of the P/D=2.6 complex begins to decrease at about 40°C, reaches a value 0.5 at 60°C, but is still observable even at 85°C, beyond which the measurements become impossible to be carried out (Fig. 6a). This trend is quite similar to that of the DNA-AO complex.[14] For the P/D=0.36 complex, the effect of temperature is more drastic. At about 35°C the re-

14) M. Zama and S. Ichimura, *Biopolymers*, **9**, 53 (1970).

lative molar rotation of this complex reaches a value 0.5 and vanishes completely at temperatures higher than 50°C (Fig. 6b).

Thionine shows absorption also in the ultraviolet absorption region of nucleic acids. A negative CD band, which seems to correspond to the absorption shoulder of the dye at 310 nm, appears in the DNA-thionine complex. Figure 7 shows the Cotton effects of the complex and DNA alone developing in this spectral region, together with the absorption spectra of the complex and thionine alone. These CD spectra

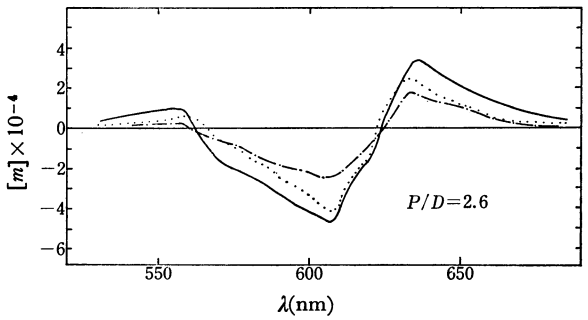

Fig. 5a. Effect of temperature on the ORD curves of the DNA-thionine P/D=2.6 complex. ——: 25°C, ·····: 50°C, —·—·: 70°C.

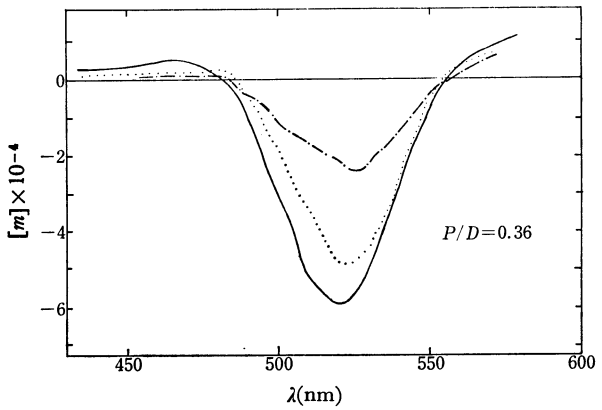

Fig. 5b. Effect of temperature on the ORD curves of the DNA-thionine P/D=0.36 complex. ——: 25°C, ·····: 32°C, —·—·: 40°C.

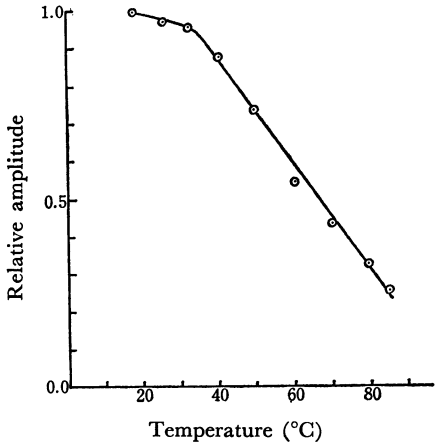

Fig. 6a. Temperature dependence of the relative amplitude of the DNA-thionine P/D=2.6 complex. Values are normalized against that at 20°C.

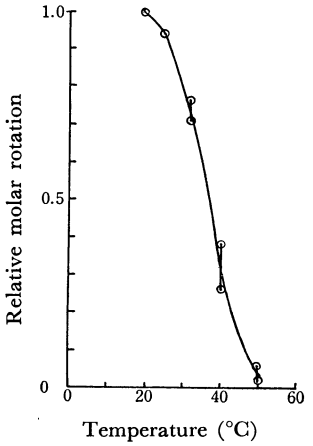

Fig. 6b. Temperature dependence of the relative molar rotation at 250 nm of the DNA-thionine P/D=0.36 complex. Vertical lines show experimental errors.

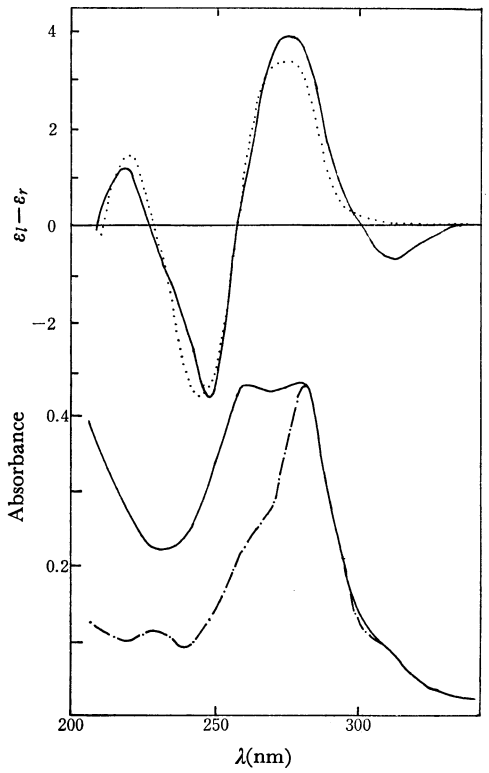

Fig. 7. CD spectra of DNA (upper, ——) and of the DNA-thionine P/D=3.2 complex (upper, ·····) and absorption spectra of thionine (lower, —·—) and of the DNA-thionine complex (lower, ——).

are corrected in magnitude in order that the negative CD maximum of the complex around at 250 nm coincides with the corresponding maximum of DNA. Similar effects are observed in the RNA-thionine complex. No quantitative measurements with respect to these Cotton effects developing in the ultraviolet absorption region of the complexes have been carried out.

Discussion

Interactions between nucleic acids and dyes have been a subject of both experimental and theoretical

studies concerning the effect on the electronic spectra, the change in the thermodynamic parameters, hydrodynamic characters and other physical and chemical properties of complexes.[15] A problem of current interest in chemistry of macromolecule-dye complexes is the study of the induced optical activity of these systems. Many workers have studied the Cotton effects of complexes such as DNA-aminoacridine dyes,[8-11] DNA-actinomycine,[16] and polysaccaride-various dyes complexes by means of ORD and CD measurements.[17]

Yamaoka and Resnik[10] and Gardner and Mason[11] independently suggested the existence of the four partial Cotton effects observable in the visible absorption spectral region of the DNA-AO complex. Their concepts, however, are not in accord with each other with respect to the signs of Cotton effects. Yamaoka and Resnik resolved the resultant CD curves, derived from the experimental ORD spectrum by the Kroning-Kramers transform, into four components (partial CD bands) *viz.*, a positive CD band located at the longest wavelength and three successive negative CD bands. Gardner and Mason, on the contrary, directly measured the CD spectrum and concluded that there are one positive and two negative Cotton effects, of which the longer wavelength positive CD band possesses two positive components. Yamaoka and Resnik ascribed the interaction mechanism to the asymmetric environment due to the asymmetric carbon atoms of the macromolecule, in accordance with model I proposed and rejected by Stryer and Blout.[18] According to their interpretation these four CD bands correspond to four monomer transitions. The positive CD maximum is assigned to the ^1L_a forbidden transition of the free AO molecule and the other three negatives to the 0–0, 0–1, and 0–2 vibrational transitions of the ^1L_b allowed band.

The ORD and CD spectra of the DNA-thionine P/D=3.3 complex (Fig. 2) resemble very closely those of the DNA-AO complex (for example the P/D=5 complex given in Fig. 4 of Ref. 9). The binding conformation of the DNA-thionine complex is supposed to be quite similar to that of the DNA-AO complex, being supported by the flow dichroism experiment of Nagata *et al.*[19] on the complexes of DNA-phenothiazine dyes, such as methylene blue and toluidine blue, and of DNA-acridine dyes. From these similarities, the observed Cotton effects of the DNA-thionine P/D>3 complexes are inferred to be resolved into at least four components, all of them being due to monomer dye-asymmetric site and/or monomer dye-monomer dye interactions (vide post).

The Cotton effects newly developed at low P/D values (Fig. 3) seem to be caused by an interaction between the dimerically bound or more aggregated dyes in view of the following results experimental findings. (1) The Cotton effects are found near the dimer band. (2) Occurrence of this phenomenon strongly depends upon the total concentration of the dye. (3) At low P/D values, the visible absorption spectrum of the complex shows a slight blue shift (Fig. 1). (4) The Cotton effects of low P/D complexes undergo more rapid and more drastic temperature effect than those of high P/D complexes.

A question arises with respect to items (1) and (2). Is the existence of two CD maxima, a positive one at 555 nm and a negative one at 494 nm, consistent with the assumption that the Cotton effects of the low P/D complexes are due to the 560 nm dimer band of the dye? This can be answered by the concept of exciton splitting. The dimer transition may split into two transitions through interaction between the dimerically bound dyes. In the present case, an absorption spectrum corresponding to the negative CD maximum is uncertain, but it should be observed under some optimum experimental conditions, for example at lower temperature.

At extremely low P/D values, it appears that the amount of the dye dimers greatly increases since the dye monomers will closely bind to almost all possible binding sites of nucleic acids. This is confirmed by the fact that the longer wavelength Cotton effects, assigned to the monomerically bound dye molecules, disappear at extremely low P/D values (Fig. 4). From item (3), the bound dye dimer may form a face-to-face arrangement, the so-called stacking model, along the helix of nucleic acid.

The Cotton effects developing in the spectral region of 450—580 nm at low P/D values are ascribable to the dimer or higher aggregates of thionine. This is supported more strongly by the transient profiles of these Cotton effects accompanied with increasing temperature (item (4)). The decrease in the relative amplitude with increasing temperature, for the Cotton effects of the DNA-thionine P/D=2.6 complexes (Fig. 6a), appears to be due to the detachment of the bound dye from DNA and/or to a relatively mild structural change not so drastic as to induce the denaturation of DNA. Since the melting temperature of calf-thymus DNA is 85.2°C (in 0.15 M NaCl+0.05 M Na citrate solution),[20] we may infer that the decomposition of the DNA helices in the complex occurs at temperatures higher than 85°C, although the accurate melting temperature of the complex is not known. The fact that the relative amplitude is still non-zero even at this limiting temperature suggests that the Cotton effects of the P/D=2.6 complexes may be induced not from an interaction between the dye molecules aggregated in a helical fashion but to the dye bound near the asymmetric carbon atoms of nucleic acids. The bound dye is supposed to be primarily of the monomeric form, since there may exist quite a small amount of dye dimers at such high temperatures. This conclusion is also supported by Yamaoka and Resnik's experiment on the sodium poly-α, L-glutamate-AO complexes which shows optical activity

15) G. Löber, *Z. Chem.*, **9**, 252 (1969).
16) Y. Courtois, W. Guschlbauer, and P. Fromagect, *Eur. J. Biochem.*, **6**, 106 (1968).
17) A. L. Stone, *Biopolymers*, **3**, 617 (1965).
18) L. Stryer and E. R. Blout, *J. Amer. Chem. Soc.*, **83**, 1411 (1961).
19) C. Nagata, M. Kodama, Y. Tagashira, and A. Imamura, *Biopolymers*, **4**, 409 (1966).
20) S. Yabuki and A. Wada, *Seibutsu Butsuri*, **6**, 31 (1966).

even at very high P/D values (P/D $\sim 10^4$).

In the case of the P/D=0.36 complex, the trough of the newly developed Cotton effects at 520—525 nm completely disappears at about 50°C (Fig. 6b). This critical temperature becomes lower with the decrease of the total concentration of dye. Using the data for the dimerization constants of thionine in aqueous solution,[13] the dimer fraction for the 3.3×10^{-5} M solution is calculated and plotted in Fig. 8 against temperature. The dimer fraction of the dye undergoes rapid reduction with increasing temperature. The trend resembles quite well the decrease in relative molar rotations. Since the dimerization constants used are of pure thionine in aqueous solution, there remains the question whether it is correct to use these values to estimate the dimer fraction of the dye in the DNA-thionine complex. We may suppose that the amount of the dimeric dyes bound to DNA in the complex is roughly proportional to the amount of the dye dimers in aqueous solution of the dye alone.

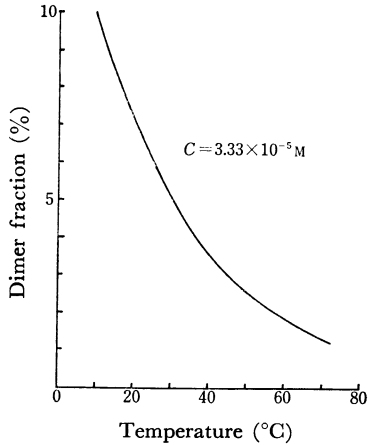

Fig. 8. Plot of dimer fraction of thionine in aqueous solution against temperature.

It appears reasonable therefore, to conclude that these newly developed Cotton effects for the low P/D complexes are ascribable to the dye dimer and that the disappearance of optical activity at about 50°C is due to the absence of dye dimer bound to DNA. Since DNA may still possess their helical structures at 50°C, there is possibly a model in which the dye dimers are bound in a repetitive fashion along the DNA helix to interact with each other. This model is supported by the general shapes of the ORD and CD curves of the low P/D complexes. We tentatively conclude, therefore, that the primary source of the optical activity newly found at 450—580 nm may be an electrostatic interaction between the dye dimers bound to DNA probably in a repetitive fashion to form a dye dimer super-helix. Vicinal disymmetry might play a sufficient role to induce optical activity, just as the case of the 520—650 nm Cotton effects.

A negative CD maximum appearing at 300—330 nm for the P/D=3.2 complex may be assigned to the absorption shoulder of thionine (Fig. 7). This has not been observed in the DNA-AO system. It was reported that DNA has a negative CD maximum in the same spectral region,[21] but we did not observe this. However, if it could be observed, it should be as small as one-tenth of the CD maximum of the complex. Therefore, the latter is undoubtedly due to an interaction between DNA and thionine. Our data are insufficient to estimate its origin, however.

It should be added that all of the foregoing discussion on the DNA-thionine complexes equally applies to the RNA-thionine complexes.

Conclusion

The results on the optical activity of the DNA- and RNA-thionine complexes are summarized as follows:

(a) The Cotton effects of the high P/D complexes are observed in the wavelength region 520—680 nm (Optical Activity I), both of them being resolved at least into four components.

(b) The Cotton effects of the low P/D complexes are observed in the wavelength region 450—580 nm (Optical Activity II). These are new types of Cotton effects which have not been observed in other DNA (or RNA)-dye systems.

(c) Optical Activity I which is predominant at the P/D values 4—5 diminishes rapidly with decreasing P/D values and Optical Activity II develops at about P/D=2.

(d) The effect of temperature on Optical Activity I differs from that on Optical Activity II. The former is observed even at 85°C, the latter completely disappears at 50°C.

(e) Unlike the DNA-AO complex, a negative CD maximum appears in the wavelength region 300—330 nm.

Interpretation of the results is summarized as follows:

(1) Optical Activity I is induced from the dye monomers bound near the asymmetric carbon atoms of nucleic acids.

(2) Optical Activity II results from an interaction between the dye dimers aggregated probably in a helical fashion along the nucleic acid helices.

(3) A negative CD maximum developing at 300—330 nm is probably due to DNA-dye interactions, but its origin is uncertain.

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21) Jen Tsi Yang and T. Samejima, *Biochem. Biophys. Res. Commun.*, **33**, 793 (1968).