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Optical Properties of Actinomycin D. I. Influence of the Lactone Rings on Its Optical Activity*

Herman Ziffer, Kiwamu Yamaoka, and Anthony B. Mauger†

ABSTRACT: The optical activity of actinomycin D (AMD) as well as the ring-opened monolactone monocarboxylic acid and the dicarboxylic acid has been determined in several solvents between 580 and 190 mμ. The optical activity of the long-wavelength major absorption band (450–440 mμ) of AMD is weak. However, a strong optically active band is present at ∼380 mμ. This band has been shown to require the presence of both lactone rings. Possible explanations for the optical activity of this band are discussed.

Several investigators have commented on the structural similarity of the chromophore actinocin of actinomycin D (AMD)[1] to the acridine dye, proflavine (Reich and Goldberg, 1964; Liersch and Hartmann, 1964, 1965; Cavalieri and Nemchin, 1964). In this context AMD may be considered, to a first approximation, as an optically inactive conjugated aromatic dye chemically bound at two sites to nonhelical but rigid cyclodepsipeptides. It would, therefore, be of some importance to determine if the absorption bands associated with the chromophore, which is insulated from the nearest asymmetric carbon atom by an amide group, are optically active and if any optical activity present is significantly affected by *internal* changes in the rigidity of depsipeptides as a result of chemical scission of either one or both lactone linkages. In this study the optical activity of the monocarboxylic (AMD monoacid) and dicarboxylic (AMD diacid) acid derivatives was measured and compared with that of AMD. Since a solvent can frequently change the position, magnitude, and sign of Cotton effects in an optical rotatory dispersion curve, such *external* factors as polarity and geometry of a solvent may conceivably alter the optical rotatory dispersion curve of AMD in changing the conformation of the molecule. Furthermore, since these perturbations may affect the interaction between AMD and DNA (Yamaoka and Ziffer, 1968), the optical rotatory dispersion, circular dichroism, and absorption spectrum of AMD were examined in three solvents (water, ethanol, and

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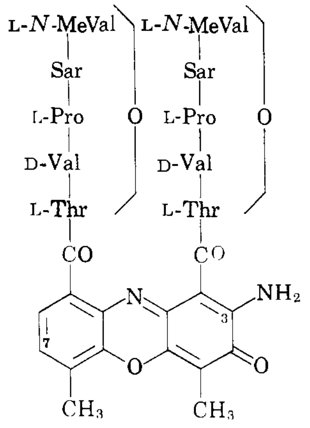
† Supported by the National Institutes of Health for Grant No. AI07662.

[1] Abbreviation used: AMD, actinomycin D.

benzene) of different polarity, dielectric constant, and molecular size.



Experimental Section

Materials. Actinomycin D was given by Merck & Co.

Preparation of Mono- and Dicarboxylic Acid Derivatives of Actinomycin D. AMD (108 mg) was dissolved in 15 ml of 0.1 N NaOH in methanol and kept at 37° for 70 min at which time water (50 ml) was added and the solution was extracted twice with chloroform (50 ml each). The aqueous solution was acidified with HCl, saturated with NaCl, and extracted three times with ethyl acetate (50 ml each). The ethyl acetate extracts were combined, washed with a saturated aqueous NaCl solution (25 ml), dried over Na_2SO_4, and concentrated *in vacuo*. The red residue was dissolved in a small volume of methanol and purified by high-voltage paper electrophoresis on Whatman No. 3MM paper at pH 6.5 at 1000–1200 V. The fastest moving yellow zone (AMD diacid) was cut out and eluted with methanol. Its absorption spectrum in ethanol and water was very similar (but not identical) to that of AMD and hence, the concentration of the AMD diacid was obtained by assuming the molar extinction coefficient at 445 mμ (ϵ 22,000) in ethanol to be the same as that of AMD. The yellow zone that moved one-half as fast as the AMD diacid was also cut out, eluted with methanol, and its concentration was determined in the same way as the dicarboxylic acid. Each zone was hydrolyzed with hydrochloric acid and the amino acid composition was determined on a Beckman amino acid analyzer at pH 3.1. Each zone contained the expected amino acids essentially in the ratios required, indicating that the partial hydrolysis did not result in the removal of *N*-methylvaline and sarcosine in addition to opening the lactone ring. Neither compound could be obtained in crystalline form. In the case of the AMD monoacid (monolactone monoacid), no attempt was made to determine if the compound was a mixture of two possible monolactones or if one of the lactones was preferentially hydrolyzed. A small quantity of each hydrolysis product was dissolved in ether, methylated with diazomethane, and used without further purification.

Instruments. Optical rotatory dispersion, circular dichroism, and absorption curves were measured at 25–27° with a Cary 60 spectropolarimeter, a Durrum-Jasco spectropolarimeter, and a Cary 14 spectrophotometer, respectively.

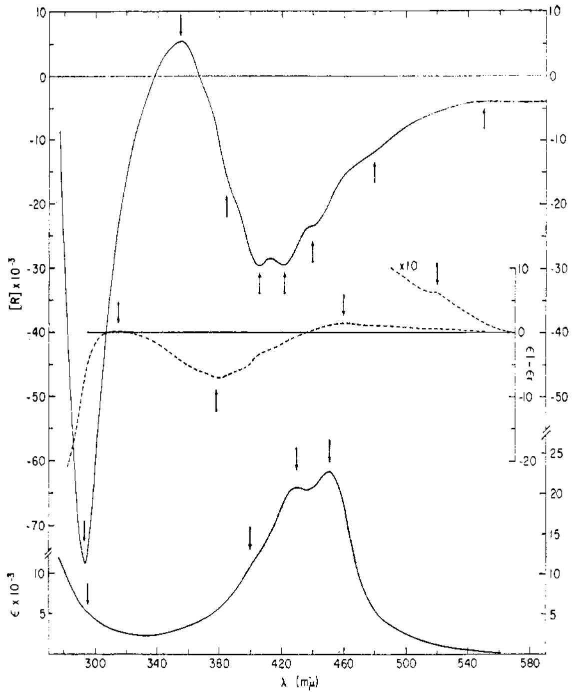


FIGURE 1: Optical rotatory dispersion, circular dichroism, and absorption of AMD in benzene. Symbols are: ——, optical rotatory dispersion (top) and absorption (bottom); – – – –, circular dichroism (middle). Arrows indicate peaks, troughs, maxima, and shoulders. The concentration of AMD was covered between 2.8×10^{-4} and 5.6×10^{-5} M. No deviation from Beer's law was observed.

Calculation of Observed Data. All the observed optical rotation was expressed in terms of molar rotation, [*R*], for AMD and its derivatives.

$$[R] = \frac{10}{M_A}\frac{\alpha_A}{l} \qquad (1)$$

where M_A is the concentration of AMD (or derivative) in moles per liter, l is the path length of the cell in decimeters, and α_A the observed rotation in degrees. The molar circular dichroism, $\epsilon_l - \epsilon_r = \Delta\epsilon$, is given by

$$\epsilon_l - \epsilon_r = \frac{1}{M_A}\frac{\Delta OD}{d} \qquad (2)$$

where ΔOD is the difference in optical densities of left and right circularly polarized light, and d is the path length in centimeters.

Results

Optical Rotatory Dispersion, Circular Dichroism, and Absorption of AMD. The optical rotatory dispersion, circular dichroism, and absorption spectrum of AMD were measured in three solvents (benzene, ethanol, and water) of different polarity and are shown in Figures 1–3. The pertinent data are given in Table I. AMD clearly

TABLE I: Results of Optical Rotatory Dispersion, Circular Dichroism, and Absorption of Actinomycin D in Three Solvents.[a]

Benzene	Ethanol	Water
	Optical Rotatory Dispersion[b]	
∼550 mμ (−3,800) p		
480 mμ (−12,000) s, 440 mμ (−23,500) s, 422.5 mμ (−29,500) t	444 mμ (−21,500) s, 425 mμ (−27,300) t	465 mμ (−7,000) t, ∼444 mμ (−5,500) p
406 mμ (−29,600) t, 385 mμ (−15,900) s, 367 mμ c, 355 mμ (5,300) p, 338 mμ c	410 mμ (−28,900) t, 390 mμ (−19,500) s, 376 mμ c, 353 mμ (5,200) p, 388 mμ c	402 mμ (−9,000) t, 364 mμ c, 351 mμ (1,300) p
293.5 mμ (−75,700) t	293 mμ (−64,000) t, 276 mμ c	
	251 mμ (105,000) p, 239.5 mμ c	284 mμ (−29,000) t, 256 mμ c, 248 mμ (9,400) p, 241 mμ c
	228 mμ (−80,200) t, 227.5 mμ c	221 mμ (−109,000) t, 211.5 mμ c, 194 mμ (409,000) p
	Circular Dichroism[b]	
∼520 mμ (0.6) s		
∼460 mμ (1.3) m	455 mμ (1.3) m	Ripples between 460 and 400 mμ
379 mμ (−7.5) m	378 mμ (−8.0) m	376 mμ (−3.0) m
	277 mμ (−24.9) m	263 mμ (−7.0) m
	239 mμ (14.7) m, ∼210 mμ (−62 ± 18) m	233 mμ (4.0) m, 207mμ (−59) m
	Absorption[b]	
451 mμ (22,700) m, 430 mμ (20,700) m, ∼400 mμ (10,900) s	444 mμ (21,900) m, 427 (mμ 20,700) m, ∼395 mμ (11,300) s	441 mμ (21,900) m, ∼420 mμ (9,500) s
∼295 mμ (5,300) s	∼294 mμ (4,800) s	
	∼275 mμ (12,200) s	∼270 mμ (15,500) s
	240 mμ (32,000) m	240 mμ (29,000) m
		199 mμ (68,500) m

[a] p, peak; t, trough; c, crossover; s, shoulder; m, maximum. [b] The magnitudes are given in parentheses. Absorption is expressed in terms of molar extinction coefficients.

shows strong optical activity in all three solvents and in both visible and ultraviolet regions. In all cases a strong Cotton effect appears at 376–379 mμ as can be seen from the circular dichroism curves (middle portion of Figures 1–3). The position and magnitude of this Cotton effect are quite unexpected, since the intense absorption bands are located at considerably longer wavelengths (see Table I and Figures 1–3).

In comparison with the negative Cotton effect at 376–379 mμ, the optical activity associated with the strong electronic transition above 390 mμ is exceedingly weak. As the circular dichroism curve in benzene or ethanol (Figure 1 or 2) shows, the major absorption maximum at 451–444 mμ is optically active and the sign of the Cotton effect is positive. Although much weaker, the two successive subbands are also optically active, but the exact position and sign of the Cotton effects could not be determined from the present circular dichroism or optical rotatory dispersion curves. These Cotton effects are indicated with arrows (top portion of each figure).

Between 600 and 500 mμ, the optical rotatory dispersion of AMD is weak but anomalous and the corresponding circular dichroism shows a positive shoulder around 520 mμ, as is seen most clearly in benzene (Figure 1). The existence of one or more weak absorption bands above 500 mμ is also reflected by the fact that the limb of the absorption spectrum of AMD in benzene or ethanol is broader than in water.

In the ultraviolet region, there are at least four absorption bands: shoulders at 295 (not observed in water) and 270–275 mμ, and maxima at 240 and 199 mμ (only in water). These bands are all optically active, as confirmed by both circular dichroism and optical rotatory dispersion measurements, and their signs are positive at 295 mμ, negative at 270–275 mμ, positive at 235–240 mμ, and probably positive at 199 mμ. In addition, the circular dichroism curves in ethanol and in water show negative Cotton effects around 210 mμ, although there is no distinctive shoulder in the corresponding absorption spectra (Table I and Figure 3).

The amplitude of the negative Cotton effect calculated from the observed value of the circular dichroism maximum at 378 mμ in ethanol is −32,000, whereas that observed in the optical rotatory dispersion curve is −34,000 (see Table II in Discussion). This agreement is quite

TABLE II: The 380-mμ Negative Cotton Effect of Actinomycin D in Three Solvents.

	Benzene	Ethanol	Water
Trough (position and magnitude)	406 mμ (−29,600)	410 mμ (−28,900)	402 mμ (−9,100)
Peak (position and magnitude)	355 mμ (5,300)	353 mμ (5,200)	351 mμ (1,300)
Inflection point and amplitude calculated from optical rotatory dispersion[a]	381 mμ (−34,900)	381 mμ (−34,100)	378 mμ (−10,400)
$\frac{3}{n^2+2}$ = 380 mμ[b]	0.689	0.771	0.788
$CD_{\max}$ (position and magnitude)[c]	379 mμ (−7.5)	378 mμ (−8.0)	376 mμ (−3.0)
Amplitude calculated from $CD_{\max}$[d]	30,000 ± 2,000	32,000 ± 2,000	12,000 ± 2,000

[a] Calculated as follows: inflection point = $\frac{1}{2}(\lambda_{\text{trough}} + \lambda_{\text{peak}})$; amplitude = $[R]_{\text{first extremum}} - [R]_{\text{second extemum}}$. [b] Calculated from data in International Critical Tables VII. Solvent correction may be made by multiplying this factor to amplitude. [c] Experimental errors are ±2 and ±0.5 at 380 mμ. [d] Amplitude of optical rotatory dispersion = 4028 × $CD_{\max}$.

good, considering that these measurements were made with different instruments. Finally, it should be noted that the optical rotatory dispersion, circular dichroism, and absorption curves of AMD are not affected in changing the solvent from ethanol to benzene. The corresponding curves taken in water, although similar in shape and sign, do differ significantly in amplitude (Table II).

Optical Rotatory Dispersion, Circular Dichroism, and Absorption of AMD Derivatives. Opening the lactone rings of AMD by hydrolysis has a profound effect on the optical rotatory dispersion curves, although there is little change in the corresponding absorption spectra (Figure 4). The ord curves of both AMD monoacid and AMD diacid derivatives no longer show any measurable Cotton effect associated with the 380-mμ transition. Both derivatives show multiple Cotton effects between 200 and 300 mμ; however, the amplitudes of the Cotton effects are so reduced in comparison with those of AMD that the optical rotation of the dicarboxylic acid derivative is barely measurable. The trough at 220 mμ, which is probably associated with a peptide transition, remains little changed in going from the dilactone (AMD) to the monolactone and finally to the dicarboxylic acid (Figures 3 and 4). The methylation of the terminal carboxyl group(s) of the derivatives did not affect the optical rotatory dispersion or absorption curves. The position, sign, and magnitude of the Cotton effect in ethanol were practically indistinguishable from those in water for both AMD monoacid and AMD diacid and their methyl esters.

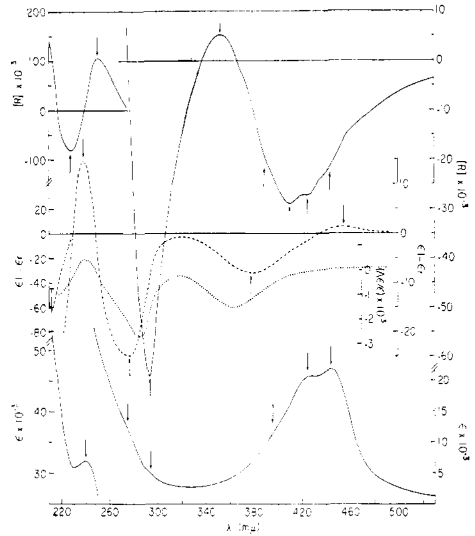


FIGURE 2: Optical rotatory dispersion, circular dichroism, and absorption of AMD in ethanol. Symbols are the same as in Figure 1 except: . . . ., dissymmetric factor (Δε/ε). Note the difference in scales on ordinates. The left-hand scales apply only to those data in the ultraviolet indicated by discontinuity. The concentration of AMD was varied between 1 × 10⁻⁵ and 1 × 10⁻⁶ M. No dilution effect on spectra was detected.

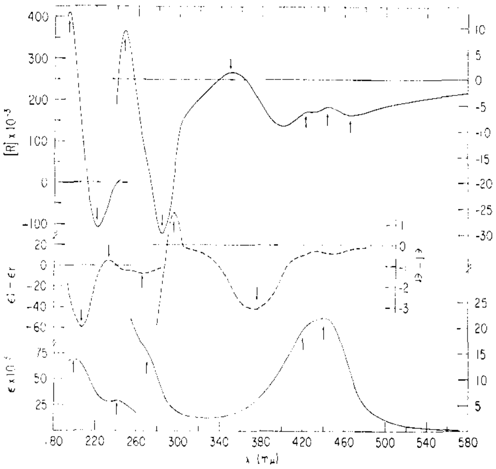


FIGURE 3: Optical rotatory dispersion, circular dichroism, and absorption of AMD in water at pH 7. Symbols are: ——, optical rotatory dispersion (top) and absorption (bottom); ----, circular dichroism (middle). The concentration of AMD was *ca.* 3 × 10⁻⁵ M in 10⁻² N NaCl plus 10⁻³ M sodium phosphate buffer. The maximum extinction (441 mμ) varied from 22,200 at 2 × 10⁻⁶ M to 20,500 at 3 × 10⁻⁴ M. This dependence was negligible when the concentration was 4 × 10⁻⁵ M or less.

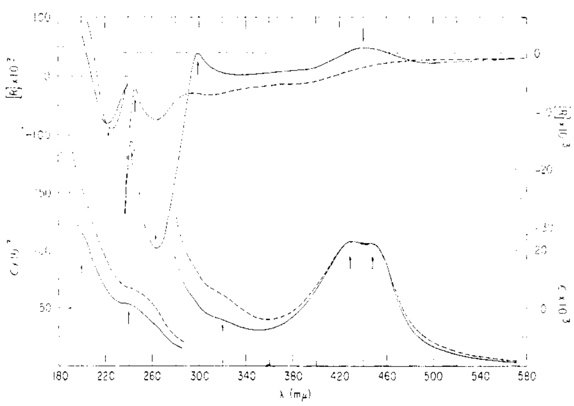


FIGURE 4: Optical rotatory dispersion and absorption of AMD monoacid and AMD diacid in water at pH 7. Symbols are: ——, optical rotatory dispersion (top) and absorption (bottom) of AMD monoacid; – – – –, optical rotatory dispersion (top) and absorption (bottom) of AMD diacid. The concentration of AMD derivatives was *ca.* 4 × 10^{-5} M in 10^{-2} N NaCl plus 10^{-3} M sodium phosphate buffer. No deviation from Beers law was observed between 4 × 10^{-4} and 4 × 10^{-5} M.

Discussion

Optical Rotatory Dispersion, Circular Dichroism, and Absorption of AMD and Its Derivatives. Comparison of the ord curves of AMD with its two derivatives, AMD monoacid and AMD diacid, clearly points out the importance of both cyclodepsipeptides attached to the actinocin chromophore for the long-wavelength Cotton effects at 445 and 380 mμ. As noted earlier, the absorption bands of actinocin need not be optically active simply because it is bound to an optically active amino acid residue through an amide linkage. In fact, the principal absorption band (500–400 mμ) of AMD monoacid is only very weakly optically active and that of AMD diacid is practically inactive. Moreover, the optical rotatory dispersion curves of both derivatives are only plain between 400 and 300 mμ, with no detectable Cotton effect (Figure 4). In contrast with its derivatives, however, AMD itself shows two Cotton effects: a relatively weak positive effect associated with the principal band and, unexpectedly, a strong negative effect at 380 mμ (Figures 1–3).

Since the attachment of the threonine residues to actinocin as well as the absolute configuration of the α-carbon atoms are the same in all three compounds, the aforementioned Cotton effects must somehow involve the intact depsipeptide rings. Furthermore, the 380-mμ Cotton effect requires both lactone rings, as AMD monoacid no longer shows it. Although many interpretations for this observation are possible, two plausible explanations may be offered. (a) As a result of steric interaction between the two lactone rings, there are only a few energetically favorable conformations possible for the molecule and all other conformations are much more energetic (*i.e.*, unfavorable). In such a situation the molecule as a whole could behave essentially as a dissymmetric chromophore and fairly large optical rotations are possible, even though actinocin *per se* is undistorted and remains symmetric (contains a plane of symmetry). The chemical scission of one of the lactone rings greatly increases the number of possible conformations and, since some would be associated with positive Cotton effects and others with negative effects, the over-all effect is greatly reduced. The magnitude of the Cotton effect based on this model clearly depends on the relative energy levels of the possible conformations. (b) As a result of steric interaction between the two lactone rings, the planarity of the actinocin chromophore is distorted sufficiently for it to become a dissymmetric chromophore. In this case, the opening of one of the two identical lactone rings at once destroys their definite steric arrangement (which may be symmetric or nonsymmetric) and releases the strain imposed upon actinocin. This chromophore would then become planar and hence no longer appreciably optically active. Other explanations are also possible and, in order to distinguish between them, more detailed model compounds will have to be made, and experiments designed to distinguish many possibilities.

Optically Active Transitions of AMD. In spite of the earlier report by Permogorov and Lazurkin (1965), the present study disclosed that all observed absorption bands of AMD are optically active (as indicated with arrows in Figures 1–3). The magnitude (rotational strength) of the observed Cotton effect of each optically active transition, however, is not always proportional to the intensity of the corresponding absorption band (or the dipole strength), as shown by the dissymmetric factor or *g* value (Figure 2). The weak positive circular dichroism shoulder around 520 mμ, most easily seen in benzene (Figure 1), could belong to an n–π^* transition of the actinocin chromophore, although reduced activity hindered observation of this effect in both derivatives.

The solvent dependence of the Cotton effect associated with the 376–379-mμ transition (Table II) may be summarized as (i) the position moves no more than 3 mμ toward shorter wavelengths when water is the solvent compared to benzene or ethanol, (ii) the amplitude is much lower in water than in the organic solvents, and (iii) the sign of the ∼380-mμ Cotton effect is negative, while the 440-450-mμ circular dichromism band is positive. It is not likely that the ∼380-mμ transition is an n–π^* transition of a carbonyl group since the solvent dependence of such a band is large (Jaffé and Orchin, 1962) and no such dependence is observed.

For a complicated molecule like AMD, it is not possible to make unequivocal assignments of each optically active band. However, the negative circular dichroism band centered at 205–210 mμ (trough at about 225 mμ in optical rotatory dispersion) is most likely associated with the cyclic or straight-chain pentapeptide part of the molecule. The maximum molar extinction coefficient per amide of AMD, as noted at 200–198 mμ (Figure 3) is about 7000. This value is in good agreement with previously reported values for random coil polypeptides and some amides (Imahori and Tanaka, 1959; Tinoco *et al.*, 1962). With the above information of the optical properties of AMD, one can now undertake a detailed study of the optical properties of the AMD–DNA complex (Yamaoka and Ziffer, 1968).

Acknowledgment

The authors are grateful to Professor C. Hammer for permitting the use of a Durrum-Jasco spectropolarimeter at Georgetown University. They are also indebted to Drs. E. Charney and M. Gellert of this Institute for reading the manuscript and giving valuable comments.

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The Optical Properties of Actinomycin D. II. Optical Activity of the Deoxyribonucleic Acid Complex*

Kiwamu Yamaoka and Herman Ziffer

ABSTRACT: Studies of the optical activity of the complex of actinomycin D (AMD) with deoxyribonucleic acid (DNA), native and denatured, have shown that they exhibit anomalous rotatory dispersion. The long-wavelength absorption band (~440 mμ) of AMD which is only weakly optically active becomes strongly optically active on complexing with DNA. There are no qualitative differences between the complex of AMD with native or denatured DNA, although some quantitative differences exist. Although there may be more than one binding site for AMD on a DNA molecule, the optical activity associated with these binding sites must be very similar. Quantitative studies of the optical rotatory dispersion curves at different phosphate residue to AMD (P/A) ratios revealed that the shape and magnitude of the curve at a P/A ratio of 6 could be calculated from the data obtained at a P/A ratio of 15. Both lactone rings of AMD are required for complexing with DNA.

The antibiotic actinomycin D (AMD)[1] has been shown to function, at low concentrations, by selectively inhibiting DNA-dependent RNA synthesis (for review, see Reich and Goldberg, 1964). Mueller (1962) has been able to correlate the biological activity of AMD and its derivatives with changes in the absorption spectra observed on mixing them with partially or totally denatured DNA. The changes in the absorption spectrum have been ascribed to formation of a complex between AMD and DNA (Goldberg *et al.*, 1962), and possible structures for this complex have been proposed (Hamilton *et al.*, 1963). Although the nature of the bonding between AMD and DNA is still not very well understood, studies of the effects of salts and of ions (Kersten and Kersten, 1962), optical titrations of AMD with DNA (Gellert *et al.*, 1965), and other physicochemical properties of the complex (Kahan *et al.*, 1963; Liersch and Hartmann, 1964, 1965; Cavalieri and Nemchin, 1964) have placed constraints on the structure that any proposed model is required to satisfy.

Although studies of the Cotton effect induced in acridine dye–DNA complexes have contributed to a better understanding of the binding mechanism of these planar dyes to the sites of DNA (Yamaoka and Resnik, 1966, 1967, and references cited therein; Blake and Peacocke, 1966; Gardner and Mason, 1967), only one such study of the AMD–DNA complex has been reported (Permogorov and Lazurkin, 1965). In this study the optical activity of AMD was not detected and only a partial optical rotatory dispersion curve of the AMD–DNA complex was measured. In the present work optical rotatory dispersion measurements were carried out for three kinds of AMD–DNA complexes (AMD–native DNA, heated AMD–DNA, and AMD–heated DNA) to determine if the disordered DNA also shows

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[1] Abbreviation used: AMD, actinomycin D.