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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.

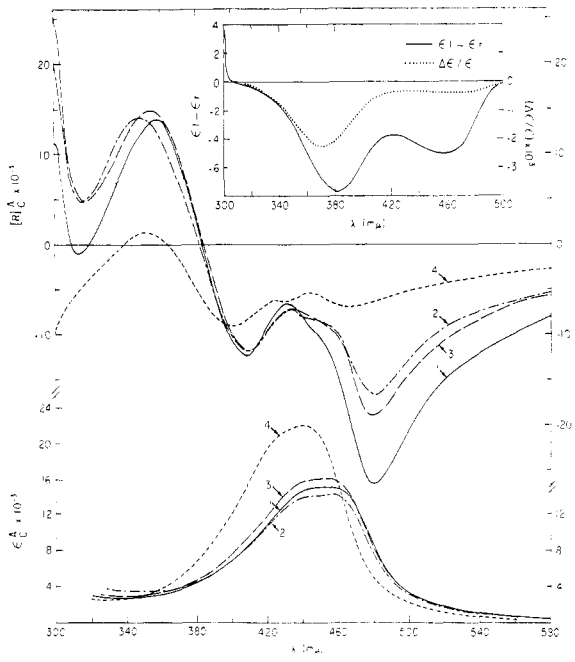

FIGURE 1: Optical rotatory dispersion, circular dichroism, and absorption of mixtures of AMD with DNA at P/A 15. (1) Native AMD–DNA complex, ——; (2) heated (AMD–DNA) complex, —·—·—; (3) AMD–heated DNA complex, — — —; and (4) AMD without DNA, - - - -.

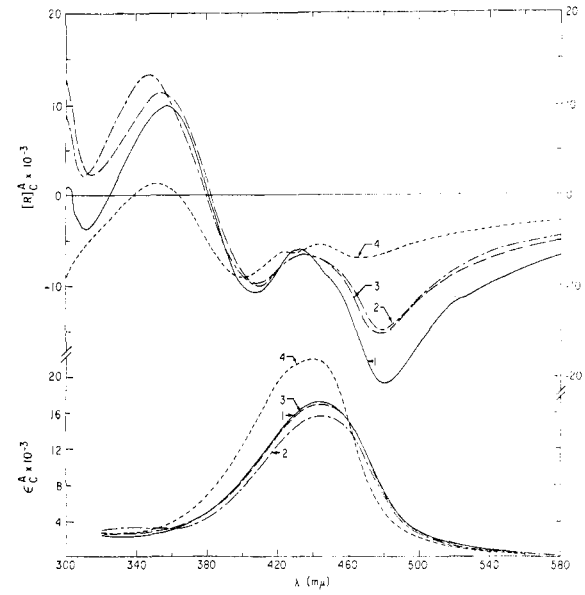

FIGURE 2: Optical rotatory dispersion and absorption curves of mixtures of AMD with DNA at P/A 6. (1) Native AMD–DNA complex, ——; (2) heated (AMD–DNA complex), —·—·—; (3) AMD-heated DNA complex, — — —; and (4) AMD without DNA, - - - -.

Cotton effects with the characteristics of the complex between AMD and native DNA. Some measurements were extended into the ultraviolet region, where the anomalous optical rotatory dispersion of the bound DNA component is appreciable, in order to find out if the DNA Cotton effect is also affected by complex formation.

Cavalieri and Nemchin (1964) and Gellert *et al.* (1965) have shown that more than one type of binding site exists in AMD–DNA complex.[2] Since the optical activity associated with these different sites would not be expected to be identical, it was hoped that a more detailed examination of the AMD–DNA complex at different P/A ratios would help define binding sites.

The specificity of AMD is remarkable in that although the chromophore (actinocin) is structurally similar to that of various dyes, it shows no evidence of binding to DNA and is biologically inactive (Mueller, 1962). The molecular shape of AMD is so critical that hydrolysis of the lactone rings results in a complete loss of biological activity (Kirk, 1960). To determine whether or not both lactone rings are required for binding with DNA tests were made by hydrolyzing only one of the lactones present in AMD and examining the optical properties of this compound in the presence of DNA. It is also possible that the AMD with one or both lactone rings opened does not bind to DNA because of the negative charge on the carboxyl group formed in hydrolysis. To test this hypothesis the methyl esters of both the monolactone–monocarboxylic acid (AMD monoacid) and the dicarboxylic acid (AMD diacid) were prepared and their interaction with DNA was investigated.

Experimental Section

Materials. Actinomycin D was given by Merck & Co. The AMD monoacid and AMD diacid derivatives were prepared as described by Ziffer *et al.* (1968). The methyl esters were prepared by methylation with diazomethane and used without further purification.

A highly polymerized calf thymus DNA was purchased from Worthington Biochemical Corp., Freehold, N. J. A concentrated stock solution (3.5 mg/ml) was dialyzed against 1×10^{-4} N NaCl at 4° for 24 hr. The stock solution was diluted in a 1×10^{-2} N NaCl containing 1×10^{-3} M sodium phosphate buffer at pH 7.0–6.9 prior to use. The final concentration of DNA was 1.8×10^{-4} M in terms of DNA phosphate residues (ϵ 6400). The native AMD–DNA complex was prepared by adding AMD solution dropwise to DNA solution with stirring. This complex was heated in a tightly capped test tube in a boiling-water bath for 15 min and then quickly cooled in an iced bath at 0° for experiments on the heated AMD–DNA complex. The AMD–heated DNA complex was prepared by mixing AMD with heat-denatured DNA (treated as above) at room temperature. Hyperchromicities of the heat-denatured DNA in 1×10^{-2} and 1×10^{-4} N NaCl were 29 and 36% at 25°.

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

Calculation of Data. All the observed optical rotation

[2] P/A ≈ 15 for strong binding and P/A ≈ 5 for weak binding.

TABLE I: The Summary of the Optical Rotatory Dispersion Data of the AMD–DNA Complex.[a]

	P/A	Native (AMD–DNA)	Heated (AMD–DNA)	AMD–Heated DNA
Trough (position and net molar rotation)	15	480 mμ (−26,500)	480 mμ (−16,700)	480 mμ (−18,900)
		409 mμ (−12,300)	409 mμ (−11,800)	409 mμ (−11,800)
	15[b]			479 mμ (−11,000)
				408 mμ (−11,600)
	6	480 mμ (−20,700)	480 mμ (−14,900)	479 mμ (−15,300)
		408 mμ (−10,700)	409 mμ (−10,000)	408 mμ (−9,700)
Peak (position and net molar rotation)	15	431 mμ (−6,500)	434 mμ (−7,200)	434 mμ (−7,200)
		358 mμ (13,900)	348 mμ (14,100)	355 mμ (14,900)
	15[b]			448 mμ (−7,900)
				354 mμ (8,500)
	6	432 mμ (−6,000)	435 mμ (−6,600)	435 mμ (−6,600)
		357 mμ (10,000)	347 mμ (13,400)	354 mμ (11,400)
Apparent inflection point and amplitude	15	456 mμ (−20,000)	457 mμ (−9,500)	457 mμ (−11,700)
		384 mμ (−26,200)	379 mμ (−25,900)	382 mμ (−26,700)
	15[b]			464 mμ (−3,900)
				381 mμ (−20,100)
	6	456 mμ (−14,700)	458 mμ (−8,300)	457 mμ (−8,700)
		383 mμ (−20,700)	378 mμ (−23,400)	381 mμ (−21,100)

[a] At each P/A the first row is for the longer wavelength Cotton effect. Amplitude is defined as $[R]_C^A$(trough) − $[R]_C^A$(peak). Inflection point is defined as $\frac{1}{2}(\lambda_{trough} + \lambda_{peak})$. The uncertainty involved in the determination of positions is ±1 mμ. [b] The complex in 1×10^{-4} N NaCl without buffer.

was expressed in terms of net molar rotation

$$[R]_C^A = \frac{10}{M_A}\frac{\alpha_C - \alpha_{DNA}}{l} \quad (1)$$

where α_C and α_{DNA} are the observed rotation in degrees of AMD–DNA complex and control DNA minus the solvent blank, respectively. The path length of a cell (l) is in decimeters. M_A is the molar concentration of AMD in solution; thus, the molar rotation of an AMD–DNA complex solution is only an *apparent* rotation when an equilibrium exists between bound and free AMD. The concentration of the control DNA was kept the same as that of the DNA in AMD–DNA complex in all cases.

Similarly, the molar extinction coefficient (ϵ_C^A) for the AMD–DNA complex solution is given by

$$\epsilon_C^A = \frac{1}{M_A}\frac{OD_C - OD_{DNA}}{d} \quad (2)$$

where OD_C and OD_{DNA} are the optical densities of the AMD–DNA complex solution and of the control DNA, respectively, and d is the path length in centimeters. Finally the molar circular dichoism, $\epsilon_l - \epsilon_r = \Delta\epsilon$, is given by

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

where ΔOD is the difference in optical densities of left and right circularly polarized light.

Results

Optical Rotatory Dispersion, Circular Dichroism, and Absorption of AMD–DNA Complex above 300 mμ. The optical rotatory dispersion and absorption spectra of three kinds of AMD–DNA complexes were studied at two different ratios (P/A) of phosphate residue to AMD. The results are shown in Figure 1 (P/A 15) and in Figure 2 (P/A 6), and the data are summarized in Table I.

Under present experimental conditions, the addition of DNA to the AMD solution affects the major absorption band of free AMD in all systems (AMD–native DNA, heated AMD–DNA, and AMD–heated DNA) by decreasing the maximum intensity at 441 mμ and shifting the entire band bathochromically (bottom of Figures 1 and 2), while the optical rotatory dispersion curve of AMD is strikingly changed in both magnitude and sign (top of Figures 1 and 2).

In the optical rotatory dispersion curve of every AMD–DNA complex, the 460-mμ absorption band is associated with a strong negative Cotton effect which is absent in the curves of free AMD (water and organic solvents). It should be noted that the sign of this Cotton effect is opposite to that of free AMD (*cf.* Figures 1–3, Ziffer *et al.*, 1968) and the magnitude of the effect has increased significantly. In addition, the negative Cotton effect at 376 mμ of free AMD shifts a few millimicrons toward the red and becomes much stronger. The circular dichroism measurements (insert in Figure 1) confirm the optical rotatory dispersion results. The g value or $\Delta\epsilon/\epsilon$ (Kuhn, 1958) for a native AMD–DNA complex has been calculated and is independent of wavelength

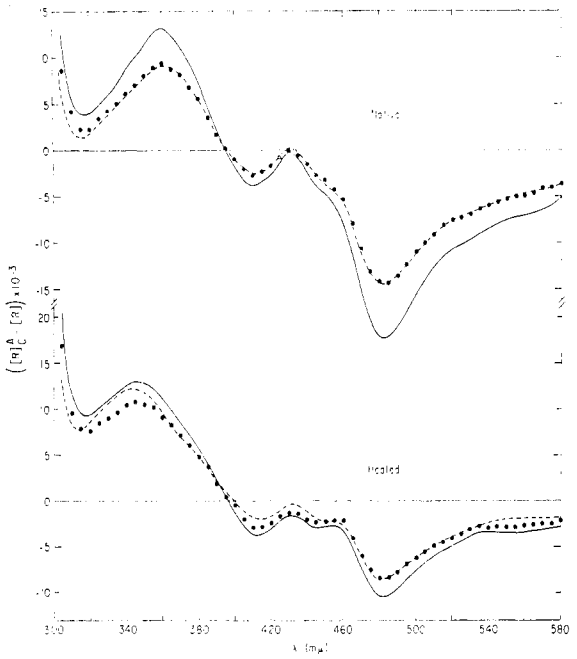

FIGURE 3: Difference optical rotatory dispersion curves of AMD–DNA complexes of P/A 15 and 6. Symbols are: ——, P/A = 15; - - - -, P/A = 6. Filled circles were calculated using eq 7.

only above 410 mμ. At shorter wavelengths it is markedly wavelength dependent.

The magnitude of the trough at 480 mμ is sensitive to the kind of AMD–DNA complex; the largest trough is obtained with native AMD–DNA. The magnitude of this trough also varies with the P/A ratio. The second Cotton effect at 380 mμ, however, is insensitive to the state of the DNA in the AMD–DNA complex (Table I). No significant difference is found in the optical rotatory dispersion curves of heat-denatured complexes prepared by heating DNA either in presence or absence of AMD. These Cotton effects are quite large and exhibit all the characteristics observed for the native AMD–DNA complex. AMD is thermolabile and when heated at 100° in water its absorption spectrum decreases uniformly between 500 and 350 mμ and a new band with a weak maximum at *ca.* 330 mμ appears. These effects are also observed in the absorption spectrum of the heated AMD–DNA complex (Figures 1 and 2).

Mixing AMD monoacid or its methyl ester with either native or heat-denatured DNA did not change either the absorption spectra (wavelength shift or the appearance of new bands) or optical rotatory dispersion curves (600–300 mμ). The absorption spectra and optical rotatory dispersion curves of AMD diacid and its methyl ester in the presence of either native or heat-denatured DNA again showed no evidence for interaction. These results indicate that both lactone rings are required for interaction of AMD with DNA.

Difference Optical Rotatory Dispersion and Difference Absorption Spectrum of AMD–DNA Complex. The optical rotatory dispersion curves of the AMD–DNA complex at P/A ratios of 15 and 6, which are representative of strong and weak bindings, are qualitatively very similar (Figures 1 and 2). Since there is an equilibrium between bound and unbound AMD in the mixture of AMD and DNA (Cavalieri and Nemchin, 1964; Gellert *et al.*, 1965), it is necessary to separate the contribution of the various components to the observed molar rotation. The approach employed in this work is somewhat similar to that of Maestre and Tinoco (1967) and the pertinent expressions are briefly derived at the end of this section.

The term "difference optical rotatory dispersion" is defined here as the difference between the optical rotatory dispersion of the AMD–DNA complex solution (thus, actually AMD–DNA complex and unbound AMD in equilibrium) and that of the AMD solution (*cf.* the left-hand side of eq 5 and 6). If the molar fraction of the *bound* AMD (f^B) is known, this difference optical rotatory dispersion curve at a particular P/A can explicitly and quantitatively describe the optical rotatory behavior of the AMD *bound* to DNA at the P/A in the visible and near the ultraviolet (above *ca.* 310 mμ) region where the small and monotonic background rotation of the bound DNA can be assumed to be the same as that of the unbound (control) DNA. Without knowing f^B, it is still possible to determine whether the optical activity of *bound* AMD remains constant at different P/A values, since in these circumstances the difference optical rotatory dispersion curves at any two P/A ratios are related by a proportionality constant K (*cf.* eq 7). A similar analysis (eq 8) was also made for the difference absorption spectra.

The difference optical rotatory dispersion curves of native and heated AMD–DNA complexes at two P/A values (15 and 6) are shown in Figure 3. Since the difference optical rotatory dispersion curve at P/A 6 is smaller in magnitude than that at P/A 15 both for native and heated system, quantitative comparisons were made using eq 7. The curve at P/A 6 calculated from the experimental one at P/A 15 with $K = 0.713$ (which was found by trial and error) is shown for the native system in the upper half of Figure 3 (filled circles). For the AMD–native DNA complex the agreement between the calculated and experimental values is very good between 580 mμ and 320 mμ.[3] For the heat-denatured system the experimental curve of P/A 6 was compared with the one calculated from the P/A 15 curve with $K = 0.82$ (the best averaged value) and the result is shown in the lower half of Figure 3 (filled circles). The fit is somewhat less satisfactory.

[3] If the two difference optical rotatory dispersion curves at 6 and 15 are identical, with $K = 1$, then the equilibrium (or binding) constant remains independent of the P/A value and the optical activity of the *bound* AMD does not change at all in the spectral range concerned. If the P/A 15 curve is superimposable on the P/A 6 curve with $K < 1$ (as is the case here), the equilibrium between AMD–DNA complex and free AMD is less favorable at the lower P/A (as noted by Cavalieri and Nemchin, 1964; Gellert *et al.*, 1965); nevertheless, each and every bound AMD molecule should possess the identical optical rotatory power regardless of the mixing ratio of P to A. If the P/A 15 curve deviates from the P/A 6 curve at some sections of the spectral range with $K < 1$ (as observed for the heated system), the optical activity of the bound AMD no longer remains constant but varies with the P/A as does the equilibrium constant.

The difference optical rotatory dispersion curves in Figure 3 show that the 380-mμ Cotton effect at both P/A ratios is essentially unaffected by heating, whereas the 460-mμ negative Cotton effect is sensitive to the state of the denaturation of the AMD–DNA complex. The data suggest that the optical activity shown by the *bound* AMD in the heat-denatured AMD–DNA complex varies between P/A 6 and 15 ($[R]_B(6) \neq [R]_B(15)$) and, at either value of P/A, is different from the optical activity of the *bound* AMD in the native AMD–DNA complex.

Figure 4 shows the difference absorption spectra (540–340 mμ) for both native and heated AMD–DNA complex solutions. For the former an isosbestic point was observed at 460.5 mμ and the whole shape of the difference absorption spectrum with a hump around 445 mμ in each case is very similar to those previously reported (Kersten and Kersten, 1962; Goldberg *et al.*, 1962). Equation 8 was used to compare the difference absorption spectrum of the native AMD–DNA complex at P/A 6 with the one at P/A 15. With a value of 0.678 for the proportionality constant (K') the observed difference spectrum at P/A 6 could be reproduced quite well (filled circles in the upper half of Figure 4) in the intense absorption band. In the heat-denatured system, however, as in the difference optical rotatory dispersion, the fit was less satisfactory ($K' = 0.706$). Finally, it should be noted that the proportionality constant K or K' is higher for the heat-denatured complex than for the native one, although the absolute magnitude of the difference optical rotatory dispersion or difference absorption is larger in the latter.

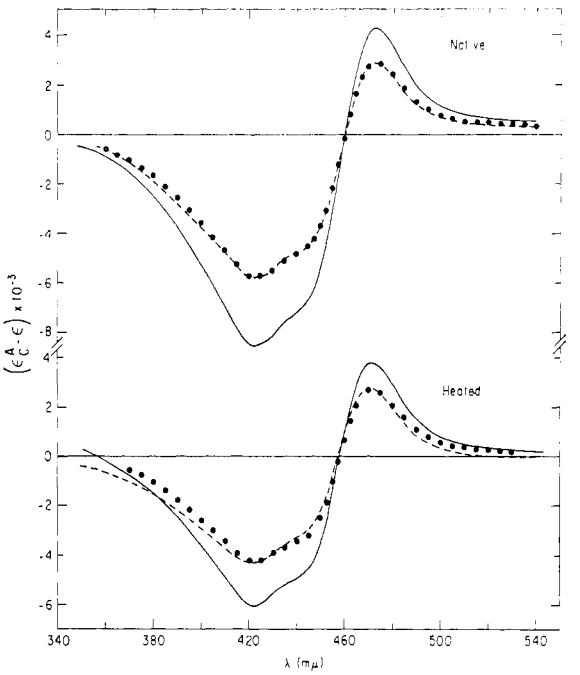

FIGURE 4: Difference absorption spectra of AMD–DNA complexes at P/A 15 and 6. Symbols are: ——, P/A = 15; - - - -, P/A = 6. Filled circles were calculated using eq 8.

Derivation

If in the AMD–DNA complex solution at P/A 15 there exists an equilibrium between unbound AMD and the AMD bound to a single type (but not multiple type) of site of the DNA (namely, AMD (unbound) $\rightleftarrows$ AMD (bound)), then the net molar rotation, $[R]_C^A(15)$, which is calculated with eq 1 from experimental data, is related to the molar rotation of the unbound AMD, $[R]_U(15)$, and the molar rotation of the bound AMD, $[R]_B(15)$, at any wavelength λ

$$M_A[R]_C^A(15) = M_B[R]_B(15) + M_U[R]_U(15) \quad (3)$$

where M_B and M_U are the molar concentrations of bound and unbound AMD at equilibrium. In terms of the molar fractions of bound (1) f_{15}^B (1) and unbound (1) f_{15}^U (1) AMD present at equilibrium in the AMD–DNA complex solution

$$[R]_C^A(15) = f_{15}^B[R]_B(15) + f_{15}^U[R](15) \quad (4)$$

Since $f_{15}^B + f_{15}^U = 1$, eq 4 can be rewritten as

$$[R]_C^A(15) - [R]_U(15) = f_{15}^B\{[R]_B(15) - [R]_U(15)\} \quad (5)$$

Also for the AMD–DNA complex solution at P/A 6

$$[R]_C^A(6) - [R]_U(6) = f_6^B\{[R]_B(6) - [R]_U(6)\} \quad (6)$$

Since the molar rotation of the unbound AMD in the complex solution may be assumed to be that of AMD in the same solvent, $[R]$ (*cf.* Ziffer *et al.*, 1968), regardless of the P/A value (*i.e.*, $[R] \equiv [R]_U(15) = [R]_U(6)$), the left-hand side of eq 5 or 6 is the quantity defined as difference optical rotatory dispersion. If the molar rotation of the bound AMD at P/A 15 is identical with that of P/A 6, *i.e.*, $[R]_B(15) = [R]_B(6)$, then from eq 5 and 6 the following can be obtained.

$$[R]_C^A(6) - [R] = K\{[R]_C^A(15) - [R]\} \quad (7)$$

That is, the difference optical rotatory dispersion curves at two different P/A values are linearly related by a constant K ($=f_6^B/f_{15}^B$).

The equivalent expression for the difference absorption spectra is

$$\epsilon_C^A(6) - \epsilon = K'\{\epsilon_C^A(15) - \epsilon\}. \quad (8)$$

Optical Rotatory Dispersion and Absorption of the AMD–DNA Complex between 310 and 220 mμ. As shown earlier, the difference optical rotatory dispersion and absorption spectra of the AMD–DNA complex at P/A 6 above *ca.* 310 mμ (where the contribution of the bound AMD is by far more significant than that of the bound DNA) could satisfactorily be calculated from the observed values at P/A 15 (Figures 3 and 4). The calculation was therefore extended to the ultraviolet region (310–220 mμ). Since much of the observed rotation and absorption of the AMD–DNA complex in this region can be attributed to the bound DNA, discrepancies between observed and calculated values of the molar extinction as well as molar rotation for the smaller

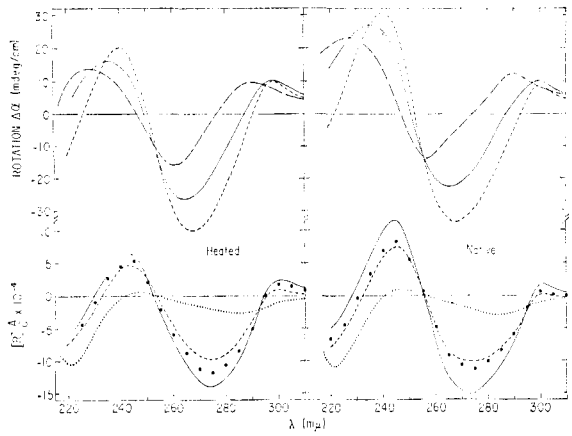

FIGURE 5: Optical rotatory dispersion of AMD–DNA complexes at P/A 15 and 6 in the ultraviolet region. Symbols are: ——, P/A = 15; - - - -, P/A = 6. For comparison — — —, control DNA; · · · ·, AMD. Filled circles were calculated using eq 7.

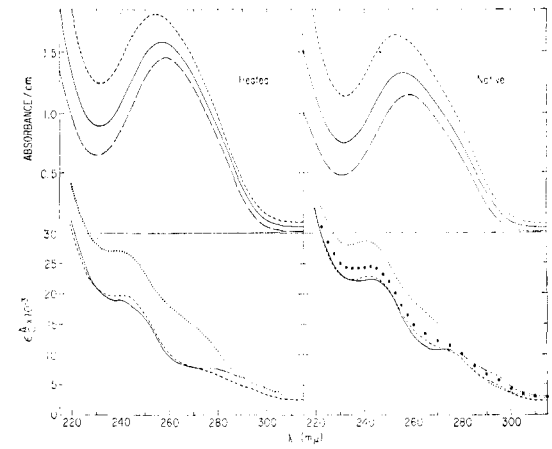

FIGURE 6: Absorption of AMD–DNA complexes at P/A 15 and 6 in the ultraviolet region. Symbols are: ——, P/A = 15; - - - -, P/A = 6. For comparison — — —, control DNA; · · · ·, AMD. Filled circles were calculated using eq 8.

P/A ratio may be construed as reflecting changes in the conformation of the bound, native DNA with decrease in P/A. This comparison was made using the proportionality constants obtained in the preceding section.

The results are given in Figure 5 (optical rotatory dispersion curves) and in Figure 6 (absorption spectra). In upper halves of Figure 5 experimentally obtained optical rotations in millidegrees per centimeter are plotted both for native complex solutions and the control DNA (right-hand side) and for the heat-denatured complexes and the control DNA (left-hand side). The net molar rotation, $[R]_C^A$, of the native or heated AMD–DNA complex solution was then calculated by subtracting the observed rotation of the control DNA from that of the complex solution. These results are shown in lower halves of Figure 5, together with molar rotations of AMD, both unheated and heated, in the buffered aqueous medium.

As noted previously, once AMD is bound to either native or denatured DNA, its molar rotation in the visible greatly exceeds that of AMD in absence of DNA (Figures 1 and 2). The binding of AMD to DNA also changes the optical rotatory dispersion curves in the ultraviolet region. The peaks at *ca.* 300 and 245 mμ and trough at *ca.* 275 mμ are intensified.

Using eq 7 and $K = 0.713$ as previously obtained for wavelengths above 310 mμ, the optical rotatory dispersion curve of the native complex solution at P/A 6 was calculated from the experimental curve at the higher P/A below 310 mμ and the result was plotted in Figure 5 (filled circles, right bottom). The discrepancies between the calculated and experimental curves in the ultraviolet region are greater, although the experimental error is about the same. This disagreement possibly reflects changes in conformation of the bound, native DNA, as P/A decreases from 15 to 6, since the contribution of the peptide chromophore of the bound AMD to the total optical rotation above 230 mμ is expected to be monotonic at two values of P/A. For comparison a similar calculation was also made for the heat-denatured AMD–DNA system (left bottom).

The absorption spectra of the AMD–DNA complex solution and the control DNA are shown in Figure 6. The spectra show no detail except for a broad maximum between 260 and 250 mμ. However, once corrections for the absorbance of the DNA are made according to eq 2, there appear three distinctive shoulders at *ca.* 295, 275, and 240–245 mμ in all four absorption spectra of the AMD–DNA complex (native and heated at P/A 15 and 6, as plotted in lower halves of Figure 6). These three shoulders are shifted a few millimicrons toward the red. The 295-mμ shoulder is hyperchromic and the other two are hypochromic relative to the free AMD. The absorption spectrum of AMD in water does not show any discrete band at 295 mμ, although the circular dichroism curve in water and absorption spectra in organic solvents do indicate it. It is therefore evident that the absorption bands of AMD are all affected upon its binding to DNA, *i.e.*, not only those in the visible but also those in the ultraviolet region.

Using eq 8 and the value of 0.678 for K', an attempt was made to detect any possible change in the conformation of the bound, native DNA with decrease in P/A. The points calculated for the spectrum of the native complex at P/A 6 compared with the one at 15 are shown with filled circles (right bottom). From this result, as similarly obtained from the optical rotatory dispersion data, the absorption of the bound, native DNA varies with lowering P/A and is different from the absorption of the native control DNA.

Optical Rotatory Dispersion and Absorption of the AMD–Heated DNA Complex in 10^{-4} N NaCl. Since some conflicting statements have appeared in the literature as to whether or not AMD binds to denatured DNA (Cavalieri and Nemchin, 1964; Gellert *et al.*, 1965), an AMD–DNA complex was examined under the condition in which a DNA solution of moderate concentration undergoes spontaneous partial denaturation at room temperature (Doty *et al.*, 1959). The stock DNA solution was diluted in 1×10^{-4} N NaCl without phosphate buffer at 0° (in this reduced ionic strength, the optical density at 259 mμ steadily increases at room

temperature). The DNA solution was subjected to a heating–rapid cooling cycle at pH 6.5 (hypochromicity 36% at 25°) and then AMD was added to this doubly denatured DNA solution at room temperature. The result is shown in Figure 7, together with data for free AMD. This mixture of AMD with doubly denatured DNA at P/A 15 still shows qualitatively the optical features characteristic of complex formation both in optical rotatory dispersion and absorption, as observed for other AMD–DNA complexes (Figures 1 and 2). For instance, both the 480-mμ trough and the 380-mμ negative Cotton effect are clearly seen.

Extraction of AMD from AMD–DNA Complexes. It may be thought that short segments of the double-stranded helical structure of DNA can either remain protected by AMD present in AMD–DNA complex when heated or be reconstituted by AMD added to the heat-denatured DNA. In that case the AMD bound to those helical regions would be responsible for the appearance of pronounced Cotton effects observed even in denatured complexes (Figures 1–3 and 7). To test this hypothesis an experiment based on the extraction of AMD from AMD–DNA complex was carried out.

AMD can conveniently be extracted from either kind of denatured complexes (heated AMD–DNA and AMD–heated DNA) in aqueous solution by shaking with chloroform (Spectrograde). Complete extraction was checked by the disappearance of the absorption band of AMD from the aqueous phase in the visible region. This aqueous solution of the DNA was subsequently subjected to a further heating–quenching cycle. The absorption spectra of the extracted DNA and of the control DNA between 300 and 230 mμ at 25° after reheating were, within experimental error, identical with those before reheating, *i.e.*, no additional hyperchromism was shown.

It may be concluded from this experiment that the *permanent* recovery of base pairing in register by the addition of AMD to any once-denatured DNA solutions is unlikely, although some ordered local structure may be retained temporarily by AMD bound to DNA. This would also be true for DNA heated in presence of AMD, since the Cotton effects observed in these two kinds of complexes are essentially identical.

Discussion

Interaction of AMD, AMD Monoacid, and AMD Diacid with DNA. In contrast to the ord of free AMD, two large negative Cotton effects appear when AMD is mixed with DNA (Figures 1 and 2). The trough (*ca.* 480 mμ) and a peak (*ca.* 360 mμ) found earlier (Permogorov and Lazurkin, 1965) belong to two different Cotton effects. The association of the spectral shift from 440 to 460 mμ of the major absorption band of AMD with the appearance of a strong negative circular dichroism band or Cotton effect at 460 mμ is convincing evidence that the actinocin chromophore indeed participates in bonding to a DNA site, presumably deoxyguanosine residues (Reich and Goldberg, 1964). The transition along the longest axis of a conjugated chromophore generally corresponds with the longest wavelength absorption band. The increase in the optical activity of the longest wavelength absorption band as well as the importance of the 3-amino and 2-quinonoid oxygen in binding (Reich and Goldberg, 1964) indicates that 460-mμ transition lies along the direction of the quinoidal oxygen and the imino nitrogen and in the plane of the chromophore.

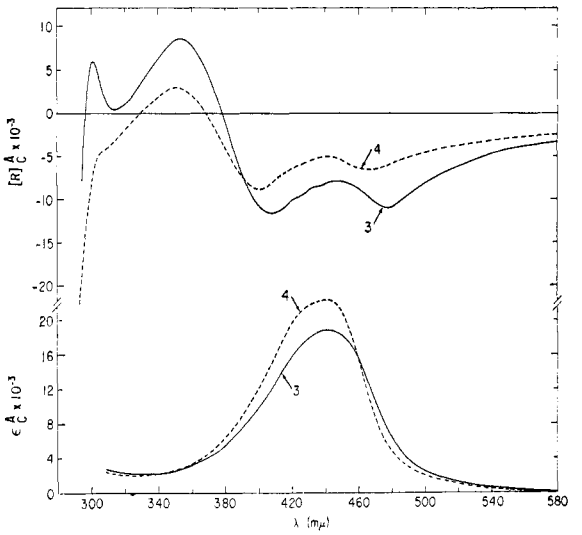

FIGURE 7: Optical rotatory dispersion and absorption of the mixture of AMD and doubly denatured DNA in water containing 1×10^{-4} N NaCl at pH 6.3. Symbols and numbers are: —— (3), AMD-doubly denatured complex and - - - - (4), AMD.

The 376–379-mμ negative Cotton effect shifts only slightly toward the red when AMD is bound to DNA (Table I), but the magnitude becomes comparable with that of free AMD in ethanol or benzene considering that not all AMD molecules are bound to DNA. These observations indicate that the 380-mμ transition is involved only indirectly in binding, and that its transition moment may lie perpendicular to the 460-mμ transition.

According to hypothesis b the origin of the optical activity of AMD (Discussion of Ziffer *et al.*, 1968), the orientation of the two lactone rings must be modified, once AMD binds to DNA, in such a way that the new steric arrangement of the rings introduces more distortion on the actinocin chromophore. This new steric orientation of the rings may be due to binding of two peptide NH groups in each ring through hydrogen bonds to the phosphate oxygen atoms of DNA (Hamilton *et al.*, 1963). The present study has shown that both lactone rings are required for AMD to bind to DNA. As actinocin and some of its derivatives neither bind to DNA nor exhibit biological activity (Mueller, 1962), the present findings strongly indicate that some specific interaction must exist between the two cyclodepsipeptides of AMD and DNA (possibly phosphodeoxyribose units). Although involvement of the actinocin chromophore in binding to DNA has been emphasized by such model experiments as the binding of AMD with constituents of nucleic acids (Kersten, 1961; Reich, 1964) or acridine dyes with DNA (Liersch and Hartmann, 1964, 1965; Permogorov and Lazurkin, 1965), it is now clear that the study of the

interaction of AMD with maromolecules containing phosphate groups (but void of purines) is equally important to understand the binding of AMD to DNA.

Although the present experiments were not designed to test the model proposed by Hamilton *et al.* (1963) for the AMD–DNA complex, some of these results do not appear easily explicable by their model. Their model does not appear to account for the necessity of having both depsipeptide rings intact for interaction with DNA. Furthermore, as the interaction between denatured DNA and AMD is reduced only by approximately a factor of two, a model that depends on the major or minor groove of the DNA helix appears untenable. However, the X-ray finding by Hamilton *et al.* that the plane of the AMD chromophore is inclined at about 70° to the helical axis of DNA would account for the long-wavelength band of AMD (460 mμ) becoming strongly optically active in the complex but being very weak in AMD *per se*. If roughly the same orientation were maintained in binding to denatured DNA as was to native DNA, local asymmetry or dissymmetry could be expected to produce a significant Cotton effect for the long-wavelength transition.

By thermal and acid denaturation of AMD–DNA complexes, Gellert *et al.* (1965) concluded that *disordered* DNA has no measurable affinity for AMD. A similar observation was reported by Reich (1964). On the other hand, Cavalieri and Nemchin (1964) among others found that heat-denatured DNA interacts with AMD at room temperature. Mueller (1962) also demonstrated that a number of actinomycins and their derivatives interact with totally or partially denatured calf thymus DNA. Recently Liersch and Hartmann (1965) reported that single-stranded fd phage DNA can interact with AMD. The heat-denatured AMD–DNA complexes prepared by two different methods still exhibit optical rotatory dispersion curves resembling those of the native complexes at room temperature, as observed in the present study (Figures 1–3 and 7). These two apparently incompatible results could be reconciled by assuming that heated DNA or DNA plus AMD in relatively high salt concentration (1×10^{-2} N NaCl plus 1×10^{-3} M phosphate buffer) can form some kind of *partly ordered structure* (see Results) upon quick quenching, whereas at elevated temperatures no such ordered structure prevails and/or the dissociation of bound AMD occurs, as suggested by Gellert *et al.* (1965).

A close comparison of the shapes and magnitudes of the Cotton effects of both heated AMD–DNA and AMD–heated DNA complexes with those of the native complexes reveals that the 460-mμ negative Cotton effect is much weaker, indicating a decrease or change in the interaction. The 380-mμ negative Cotton effect, in contrast, is remarkably unaffected by heat denaturation (Figures 1–3; Table I). Since the latter Cotton effect is most likely influenced by the steric rearrangement of the two intact depsipeptide rings, the structure of the heat-denatured DNA chain must have some *folded* or ordered segments in order for these rings to be able to interact with DNA. It is otherwise difficult to understand why decreased ionic strength decreases the strength or amount of interaction of AMD with denatured DNA (Results).

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