MECHANISM OF ACTION OF ACLACINOMYCIN A
II. THE INTERACTION WITH DNA AND WITH TUBULIN

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Aclacinomycin A was observed to effect the thermal denaturation of DNA and to increase *Tm*. The visible absorption spectrum of the antibiotic showed bathochromic and hypochromic shifts upon reaction with native and heat-denatured DNA. [^{14}C]Aclacinomycin A was demonstrated by equilibrium dialysis to bind to DNA. Native calf thymus DNA appeared to possess one binding site per *ca.* 6 nucleotides for the antibiotic with an apparent association constant of *ca.* 1.2×10^{6} M^{-1}. Heat-denatured DNA showed much less affinity for the antibiotic: one binding site per *ca.* 6 nucleotides with an apparent binding constant of *ca.* 3.5×10^{4} M^{-1}. The difference of association constants between double- and single-stranded DNAs suggested that the antibiotic may be intercalated between base pairs of the DNA double helix. [^{14}C]Aclacinomycin A exhibited higher affinity for poly(dAdT) than for poly(dIdC). The antibiotic showed a significant difference spectrum with porcine tubulin, indicating an interaction with tubulin. The binding to tubulin was also demonstrated by equilibrium dialysis of [^{14}C]aclacinomycin A and tubulin.

The effects of aclacinomycin A, a tumor-inhibitory anthracycline antibiotic, on macromolecular syntheses have been studied, using mouse tumor cells and *Escherichia coli*, and the results have been reported in a previous paper[1]. The antibiotic produces a preferential inhibition of RNA synthesis over DNA synthesis both *in vitro* and *in vivo*. The difference in degree of inhibition between RNA and DNA is greater than with adriamycin or daunorubicin. The blockage by aclacinomycin A of RNA polymerase action is reversed by increasing amounts of the template DNA but not significantly by increased enzyme, suggesting that the inhibition may be caused by the interaction with template DNA.

The binding of aclacinomycin A to DNA has been investigated, using thermal denaturation, visible absorption spectrum, and the binding of [^{14}C]aclacinomycin A to DNA; and the interaction with tubulin has been followed by absorption spectrum and the binding of [^{14}C]aclacinomycin A. The results are presented in this publication.

Materials and Methods

Calf thymus DNA and salmon sperm DNA were products of P-L Biochemicals, Milwaukee, Wis. Poly (dAdT) was purchased from Miles Lab., Elkhalt, Ind., and poly (dIdC) from Boehringer Mannheim, Germany. Tubulin was prepared from porcine brain by the method of SCHELANSKI *et al.*[2]

Radiolabelled and unlabelled aclacinomycin A were kindly supplied by Dr. T. OKI, Central Research Lab., Sanraku-Ocean Co., Ltd., Fujisawa, Kanagawa-ken. [^{14}C]Aclacinomycin A was biosynthesized by introducing [^{14}C]methionine and [^{14}C]propionate into the culture of *Streptomyces galilaeus* MA144-M1, and purified by solvent extraction, silica-gel column and thin-layer chromatography[3]. The specific activity was 1.61 mCi/mmole, and radiochemical purity was *ca.* 92%.

Determination of *Tm* of DNA:

Ultraviolet absorbance at 260 nm of calf thymus DNA 35 μg/ml with or without aclacinomycin A 8.5 μg/ml in 1 ml of 15 mM NaCl and 1.5 mM sodium citrate, pH 6.4, in a quartz cuvette of 1-cm light

path was recorded in a Gilford spectrophotometer, as the temperature was raised at a rate of 1°C per 5 minutes up to 97°C.

Difference spectrum of aclacinomycin A in the presence of native salmon sperm DNA or porcine tubulin:

Difference in visible absorption spectrum between aclacinomycin A and the antibiotic-DNA complex was observed in 0.1 M phosphate buffer, pH 7.0 (salmon sperm DNA-P 1 mM and aclacinomycin A 0.1 mM) in a quartz cuvette of 1-cm light path, using a Shimazu spectromonitor UV-202. Difference spectrum between the antibiotic and the tubulin-antibiotic complex was measured in MES buffer [2-(N-morpholino)ethanesulfonic acid 100 mM, KCl 50 mM, $MgCl_2$ 0.5 mM and GTP 1 mM, pH 6.8] (tubulin 14.2 μM and aclacinomycin A 350 μM).

Equilibrium dialysis:

The interaction of [^{14}C]aclacinomycin A with DNA was measured by equilibrium dialysis.[4] Native or heat-denatured calf thymus DNA 100 μM in 0.1 ml of a buffer (150 mM NaCl and 15 mM sodium citrate, pH 6.4) was placed in a chamber; and various concentrations (8~170 μM) of [^{14}C]-aclacinomycin A in 0.1 ml of the same buffer in the other chamber. The chambers were separated by Visking cellulose tubing membrane. The apparatus was kept at 20°C for 24 hours with gentle shaking. The period was long enough for equilibrium. The radioactivity in both chambers was determined in a liquid scintillation counter. The difference was taken to represent the amount of bound aclacinomycin A. For porcine tubulin, MES buffer, pH 6.8, was employed, and dialysis was performed at 4°C for 24 hours (tubulin 10 μM), because of lability of tubulin.

Results

Thermal Transition for DNA-aclacinomycin A

The interaction of aclacinomycin A with DNA was observed by thermal denaturation. As illustrated in Fig. 1, the thermal transition curve of calf thymus DNA was markedly shifted in the presence of aclacinomycin A. The difference of *Tm* in the absence and presence of the antibiotic was approximately 13°C. The result indicated that aclacinomycin A binds to and stabilizes the double strand structure of DNA to denaturing effect of heat.

Fig. 1. Thermal transition for DNA-aclacinomycin A at a total antibiotic to DNA-P ratio of 0.1.
D: calf thymus DNA alone, *Tm*=74°C
D-A: DNA-aclacinomycin A complex, *Tm*=87°C
$\frac{A_{T°}}{A_{50°}} = \frac{\text{Absorbance at T°C}}{\text{Absorbance at 50°C}}$

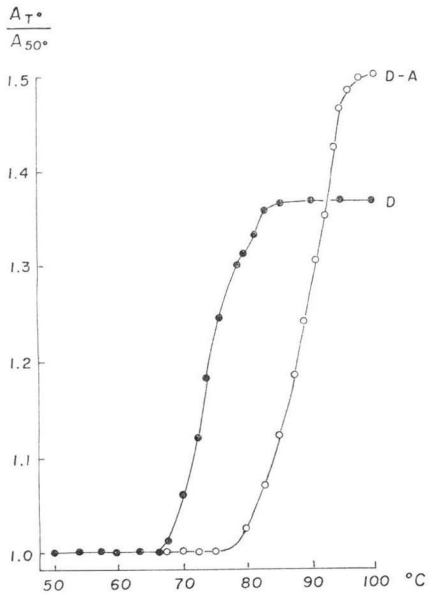


Fig. 2. Difference spectrum of aclacinomycin A in the presence of native salmon sperm DNA.

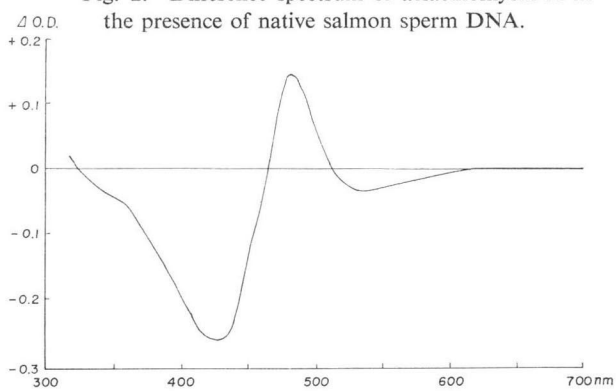


Fig. 3. [^{14}C]Aclacinomycin A concentration dependence for binding to native or heat-denatured calf thymus DNA.

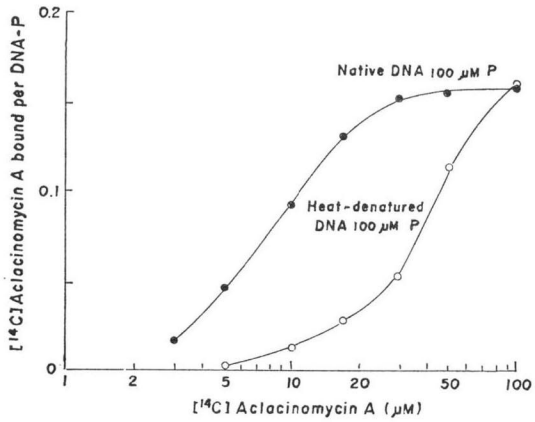
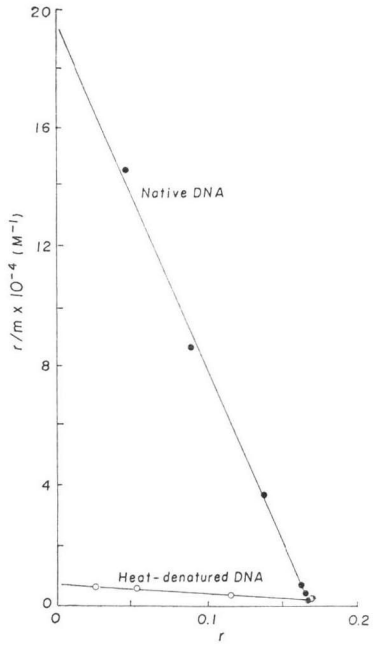


Fig. 4. SCATCHARD plots for equilibrium binding of [^{14}C]aclacinomycin A to calf thymus DNA.



Difference Spectrum of Aclacinomycin A in the Presence of DNA

The visible absorption spectrum of aclacinomycin A showed bathochromic shift and hypochromic change upon reaction with native and heat-denatured DNA of salmon sperm and calf thymus. The difference spectrum of the antibiotic in the presence and absence of native salmon sperm DNA is presented in Fig. 2. The results, again, showed the interaction of aclacinomycin A with DNA.

Binding of [^{14}C]Aclacinomycin A to DNA

[^{14}C]Aclacinomycin A was demonstrated to bind with calf thymus DNA by equilibrium dialysis. For the purpose of determining the association constant and number of binding sites on DNA, equilibrium dialysis experiments were carried out over a range of concentrations of [^{14}C]aclacinomycin A (Fig. 3). The antibiotic was observed to exhibit a stronger affinity for native DNA than for heat-denatured DNA. Fig. 4 shows SCATCHARD plots[5] of data for equilibrium binding of the antibiotic to DNA. There appeared to be a linear relationship between r and r/m, where r is moles of bound [^{14}C]aclacinomycin A per mole of nucleotide, and m is molar concentration of the free [^{14}C]antibiotic. Native calf thymus DNA seemed to possess one binding site for aclacinomycin A per *ca.* 6 nucleotides with an apparent association constant of approximately 1.2×10^6 M^{-1}. The binding constant was estimated from the slope of SCATCHARD plot. Heat-denatured DNA appeared to have one binding site for the antibiotic per *ca.* 6 nucleotides with an apparent association constant of about 3.5×10^4 M^{-1}. The difference of binding constants between double- and single-stranded DNAs suggested that the antibiotic may be intercalated between base pairs of the double helix of DNA, as in the case of daunorubicin, adriamycin, and other anthracyclines[6,7]. The apparent association constants and number of binding sites were of the same order of magnitude as those of daunorubicin and adriamycin[6,7].

Base Specificity in the Binding of [^{14}C]Aclacinomycin A with DNA

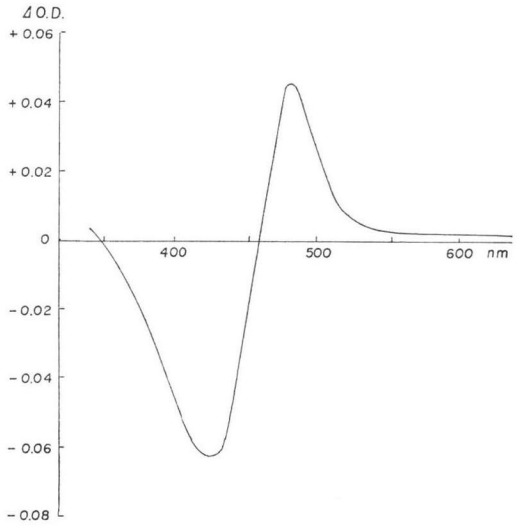
The binding of [^{14}C]aclacinomycin A to synthetic polynucleotides was determined by the equilibrium dialysis method, using 10~100 μM [^{14}C]antibiotic and 100 μM polynucleotide-P. As presented

Table 1. Binding of [^{14}C]aclacinomycin A to polynucleotides.

[^{14}C]Aclacinomycin A	Polynucleotide		
	poly (dAdT)	poly (dIdC)	Native calf thymus DNA
100 μM	0.33*	0.04	0.17
30	0.31	0.03	0.16
10	0.27	0.01	0.09

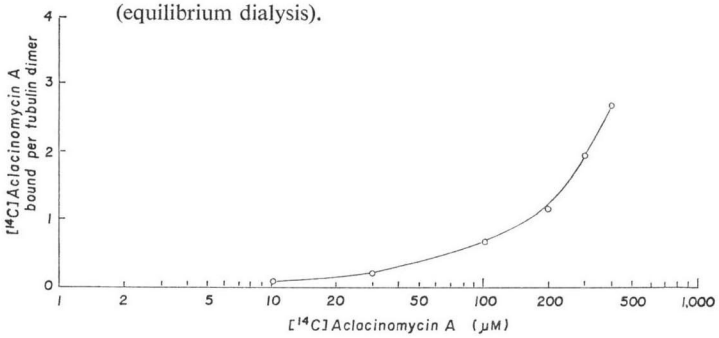
* The number represents molecules of [^{14}C]aclacinomycin A per polynucleotide-P. The data were obtained by equilibrium dialysis at 20°C for 24 hours. The buffer used consisted of 150 mM NaCl and 15 mM sodium citrate, pH 6.4, and the concentration of polynucleotides was 100 μM.

Fig. 5. Difference spectrum of aclacinomycin A in the presence of porcine tubulin.



in Table 1, more [^{14}C]aclacinomycin A was observed to bind to poly(dAdT) than native calf thymus DNA. The binding to poly(dIdC) was much less significant. The results indicated that the antibiotic possesses much higher affinity for poly(dAdT) than for poly (dIdC); and it seemed to be in accord with the base specificity in DNA-dependent RNA polymerase reaction[1].

Fig. 6. Binding of [^{14}C]aclacinomycin A to porcine tubulin (equilibrium dialysis).



Difference Spectrum of Aclacinomycin A in the Presence of Tubulin

Addition of porcine tubulin to aclacinomycin A perturbed the absorption spectrum of the antibiotic. The difference spectrum in the presence of porcine tubulin is illustrated in Fig. 5. The results suggested that aclacinomycin A bound to tubulin.

Binding of [^{14}C]Aclacinomycin A to Tubulin

[^{14}C]Aclacinomycin A was found to bind to porcine tubulin by equilibrium dialysis (Fig. 6). The stoichiometry of the antibiotic-tubulin interaction could not be determined precisely, because tubulin was not stable enough during 24-hour dialysis.

Discussion

The current results, obtained by equilibrium dialysis and by thermal denaturation, suggest that aclacinomycin A may intercalate between base pairs of the double helix of DNA. It is consistent with the results with other anthracyclines (*cf.* a review by DI MARCO *et al.*[6]).

Concerning base requirement for the interaction of anthracyline antibiotics with DNA, there are many reports with disparate results (see the above review[6]). Aclacinomycin A has been found to

possess greater affinity for poly (dAdT) than for poly (dIdC). It is in accord with the previous observation[1] that the antibiotic impairs the template activity of poly (dAdT) for RNA polymerase reaction, but not significantly that of poly (dIdC). The results seem to be consistent with those of nogalamycin, reported by BHUYAN *et al.*[8] and by WARD *et al.*[9] The base specificity may be attributed to the mode of antibiotic-DNA interaction, which remains to be determined.

The activity of aclacinomycin A is more similar to that of actinomycin than to those of adriamycin and daunorubicin, because aclacinomycin A shows a preferential inhibition of RNA over DNA polymerase reaction by interacting with template DNA[1]. However, actinomycin does not interact with poly (dAdT), and the base specificity of actinomycin (binding to deoxyguanosine moiety of DNA, *cf.* a monograph by GALE *et al.*[10]) is different from that of aclacinomycin A.

The binding of aclacinomycin A to tubulin seems to be in accord with the finding by NA and TIMASHEFF with daunorubicin[11]. However, the significance of the interaction with tubulin for the mechanism of action remains to be determined.

Acknowedgements

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