

# THE INTERACTION OF ACTINOMYCIN WITH DNA: REQUIREMENT FOR THE 2-AMINO GROUP OF PURINES

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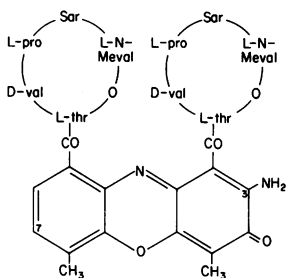
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The formation of complexes between actinomycin (AM) (Fig. 1) and DNA is known to depend on specific structures in both the antibiotic and the polydeoxynucleotide.<sup>1</sup> These include the base guanine in the helical conformation of DNA, and the amino group, the quinoidal oxygen, and the intact peptide lactones of the antibiotic. A model for the structure of AM-DNA complexes which accounts for the participation of these functional groups has been proposed.<sup>2</sup> According to this model, AM is located in the minor groove of the DNA helix, where three hydrogen bonds may be formed between the chromophore of the antibiotic and deoxyguanosine in DNA. The specificity of the interaction is attributed to a hydrogen bond between the quinoidal oxygen of AM and the 2-amino group of guanine.

The model has already been subjected to a variety of tests. For example, several lines of evidence show that the N-7 of guanine is probably not involved in complexing AM, since extensive substitution of this position by mustard gas<sup>3</sup> or other alkylating agents<sup>4</sup> does not diminish the actinomycin binding capacity of DNA; conversely, AM does not affect the rate of alkylation of DNA by mustard gas.<sup>3</sup> Another experimental test of the model has been conducted with the synthetic DNA polymer dIdC.<sup>3</sup> The structure of this polymer corresponds exactly to that of dGdC except for the absence of the 2-amino group of guanine. As judged by the results of spectral and enzymatic assays, the removal of this amino group is correlated with a loss of the ability to interact with AM.<sup>3</sup> These and other findings are therefore fully in accord with predictions which can be derived from the model.

In this paper we describe the results of another experimental test of the model.



Actinomycin C<sub>1</sub> (D)

FIG. 1.—Structure of actinomycin D. L-thr, L-threonine; D-val, D-valine; L-pro, L-proline; sar, sarcosine; and L-N-Meval, L-N-methylvaline.

The principle of the experiments is outlined in Figure 2. As noted above, the removal of the 2-amino group of guanine from the AM-sensitive G-C base pair yields the AM-resistant I-C pair. The A-T base pair is known not to interact with AM;<sup>1</sup> however, the structure of this base pair permits the insertion of an amino group at the position in the helix normally occupied by the amino group of guanine. Such an insertion can be accomplished by substituting 2,6-diaminopurine or 2-aminopurine for adenine, since we find that the corresponding deoxynucleotides serve as substrates for DNA polymerase. This finding has permitted us to produce and isolate a deoxynucleotide copolymer (dDAP-T) with an alternating sequence of diaminopurine and thymine residues. If the specificity of AM-binding is determined simply by a purine amino group

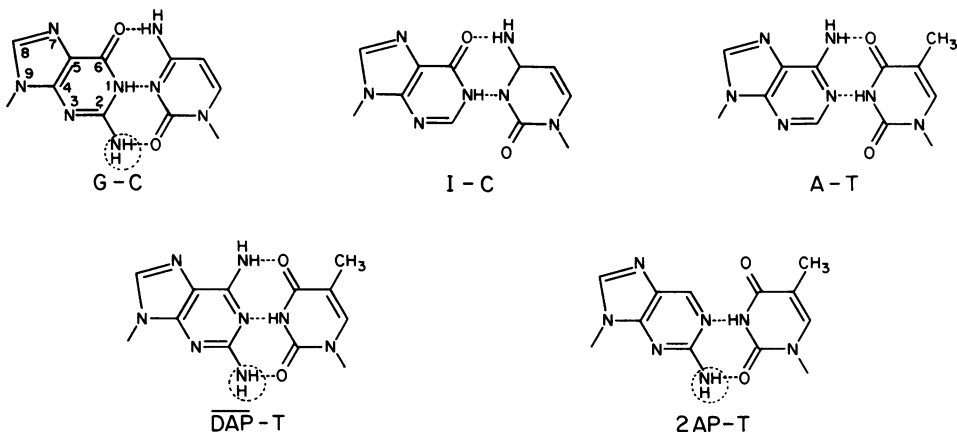


Fig. 2.—Structure of hydrogen-bonded purine-pyrimidine base pairs; G-C, guanine-cytosine; I-C, hypoxanthine-cytosine; A-T, adenine-thymine; DAP-T, 2,6-diaminopurine-thymine; and 2AP-T, 2-aminopurine-thymine. The 2-amino group of the purine components is encircled.

suitably located in the minor groove, this polymer should interact with AM. As predicted by the model, the formation of complexes between d-DAP-T and AM can be demonstrated by means of a variety of techniques.

Previous work<sup>5</sup> has shown that the Mithramycin group of antibiotics, like AM, require the 2-amino group of guanine for DNA binding; dDAP-T also interacts with these.

*Materials and Methods.*—Radioactive and nonradioactive nucleotides were obtained from Schwarz BioResearch and P-L Laboratories, respectively. 2,6-Diaminopurine deoxynucleoside was converted to the corresponding 5'-triphosphate by standard chemical procedures.<sup>6, 7</sup> Actinomycin D, Mithramycin, and Nogalamycin were generously provided by Merck, Sharp and Dohme, Inc., Chas Pfizer and Co., and the Upjohn Co., respectively.

DNA polymerase from *Escherichia coli* was prepared according to Richardson *et al.*<sup>8</sup> Fraction VII (sp. act. 3750 units/mg of protein) was used for polymer synthesis. dAT copolymer was isolated from a *de novo* synthesis according to Schachman *et al.*<sup>9</sup> For the synthesis of dDAP-T, dAT primer was replicated 11-fold with DAP-deoxynucleoside triphosphate (dDAP-TP) and thymidine triphosphate as substrates. The purine content of the final product was therefore equal to 90 per cent DAP and 10 per cent A. A polymer containing a ratio of A/DAP = 10 was also prepared; in this case dAT primer was replicated 10-fold, with a corresponding ratio of triphosphate precursors; control experiments had shown that the input ratio of triphosphates was faithfully reflected in the composition of the polymer; in the present instance this was verified by the use of  $\alpha$ -P<sup>32</sup>-dDAP-TP and dTTP-H<sup>3</sup> as radioactive tracers. Full details of the synthesis of these polymers will be published elsewhere.<sup>10</sup> RNA polymerase of *E. coli* was prepared according to Chamberlin and Berg,<sup>11</sup> and assayed as previously described.<sup>5</sup> Ultracentrifugation in density gradients of CsCl was performed as previously described.<sup>12</sup>

*Results.*—*Effect of actinomycin on buoyant density of dDAP-T:* When dDAP-T is synthesized, the appearance of a new polymer can be observed by analytical ultracentrifugation of the reaction mixture in gradients of CsCl. As seen in Figure

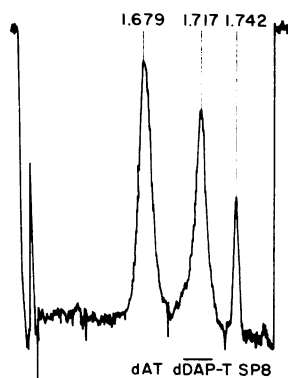


Fig. 3.—Analytical equilibrium centrifugation of  $d\overline{\text{DAP-T}}$  in  $\text{CsCl}$  density gradients. The reference markers are bacteriophage SP-8 DNA and synthetic dAT.

3, a symmetrical band can be distinguished with a density corresponding to  $\rho = 1.717$ . This band is clearly separated from dAT ( $\rho = 1.679$ ).

Since binding of AM is associated with significant decreases in the buoyant density of DNA,<sup>13, 14</sup> a sample of  $d\overline{\text{DAP-T}}$  containing AM was centrifuged under conditions identical with those used for the experiment illustrated in Figure 3. No band corresponding to the  $d\overline{\text{DAP-T}}$  polymer could be seen in the cell containing AM, and this unexpected finding was confirmed on repetition of the run. Since the polymer was radioactive, its presence in the analytic cell in acid-precipitable form at the end of centrifugation was verified. This established that no significant degradation had occurred.

In order to characterize more fully the effect of AM, preparative centrifugation in  $\text{CsCl}$  gradients was performed. The results of such an experiment are shown in Figure 4. In the absence of AM, the radioactivity of the polymer, which was due to incorporated  $d\overline{\text{DAP-MP}}^{32}$ , was distributed in a single symmetrical band with a density corresponding to  $\rho = 1.715$ . The addition of AM caused a remarkable alteration in the sedimentation of the polymer; one-half the radioactivity was located at the very top of the gradient, where AM normally forms a micelle; the other half was found in small yellow droplets which adhered to the centrifuge tube at the level of the meniscus.

As a result of these observations, analytical density-gradient centrifugation was performed on a polymer with a purine ratio  $\text{A/DAP} = 10$ . As seen in Figure 5, the buoyant density of this polymer is indistinguishable from that of dAT. The addition of AM to the centrifuge cell leads to the appearance of a new, symmetrical band at a density  $\rho = 1.661$  which differs significantly from that of the control

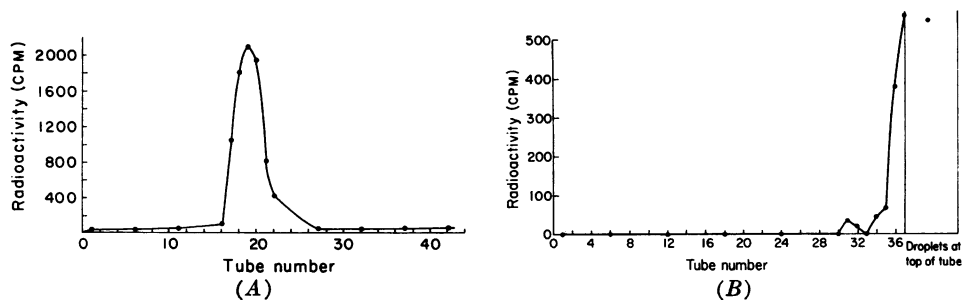


Fig. 4.—Preparative equilibrium centrifugation in  $\text{CsCl}$  density gradients. Each tube contained 5 ml.  $\text{CsCl}$  ( $\rho = 1.710$ ), and 0.14 OD units ( $260 \text{ m}\mu$ ) of radioactive  $d\overline{\text{DAP-T}}$ , a total of 20  $\text{m}\mu\text{moles}$  of polymer nucleotide.

(A) Control. (B) As in A, but containing a total of 20  $\text{m}\mu\text{moles}$  of actinomycin D. The specific radioactivity of the polymer in A was twice that in B. Following 120 hr of centrifugation (Spinco SW-39-rotor, 33,000 rpm,  $25^\circ$ ) the tubes were punctured, 2-drop fractions were collected, and the acid-insoluble radioactivity of the indicated samples was determined refractometrically. The density of fraction 19 (A) corresponded to  $\rho = 1.715$ .

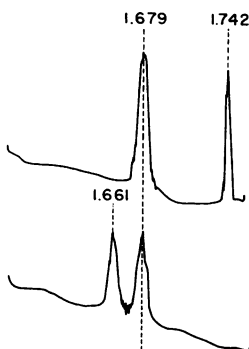


FIG. 5.—Analytical equilibrium centrifugation of dAT (A/DAP = 10) in CsCl density gradient.

The analytic cell corresponding to the upper densitometer tracing contained dAT (A/DAP = 10) 0.01 OD unit (260  $m\mu$ ); pure dAT and bacteriophage SP-8 DNA were present as reference standards.

The buoyant density of dAT (A/DAP = 10)  $\rho = 1.679$  is not detectably different from that of pure dAT.

The cell corresponding to the lower tracing contained dAT (A/DAP = 10) and pure dAT as above, with added actinomycin (4  $m\mu$ moles/ml).

A new band is seen at  $\rho = 1.661$ . This band is considered to represent dAT (A/DAP = 10) with bound actinomycin.

Actinomycin is known not to effect the buoyant density of dAT.

specimen. This is additional evidence for the interaction of AM with DAP incorporated in a polydeoxynucleotide.

*Effect of AM on thermal denaturation of  $d\overline{DAP}$ -T:* Previous work<sup>15, 16</sup> has shown that bound AM significantly stabilizes DNA to denaturation by heat; elevations of the transition temperature ( $T_m$ ) due to AM of up to 12–15° have been observed with naturally occurring native DNA's. The data in Figure 6 show a substantial increase in  $T_m$  of dAT containing 10 per cent DAP on addition of AM; the corresponding increase for  $d\overline{DAP}$ -T (Fig. 7) is almost 40°—a change far greater than that previously recorded for any DNA.

*Spectral changes in actinomycin solutions produced by  $d\overline{DAP}$ -T:* The change in the spectrum of AM solutions which occurs on addition of guanine-containing DNA's is a characteristic parameter of the AM-DNA interaction.<sup>17</sup> The difference spectrum shown in Figure 8 demonstrates that  $d\overline{DAP}$ -T, like DNA, produces alterations in the spectrum of AM.

*Effect of antibiotics on template function of  $d\overline{DAP}$ -T with RNA polymerase:* The results of experiments which will be described in detail elsewhere show that  $d\overline{DAP}$ -T possesses an alternating sequence of DAP and T residues, and functions as a template for RNA synthesis catalyzed by *E. coli* RNA polymerase. Although a much less effective template than dAT,  $d\overline{DAP}$ -T directs the formation of RNA containing only A + U in perfectly alternating sequence. The synthesis of RNA is linear

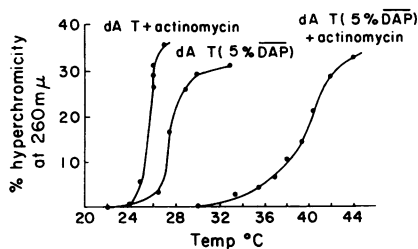


FIG. 6.—Effect of actinomycin on thermal denaturation of dAT (A/DAP = 10). dAT (A/DAP = 10) 36  $m\mu$ moles/ml was dissolved in 0.001 *M* Na<sup>+</sup>. AM, where present, was added to a final concentration of 6.1  $m\mu$ moles/ml; this concentration of AM did not alter the  $T_m$  of pure dAT.

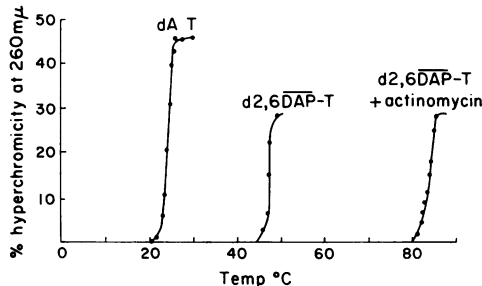


FIG. 7.—Effect of actinomycin on thermal denaturation of  $d\overline{DAP}$ -T.  $d\overline{DAP}$ -T, 54  $m\mu$ moles/ml, was dissolved in 0.001 *M* Na<sup>+</sup>. Where present, AM was added to a final concentration of 10.5  $m\mu$ moles/ml.

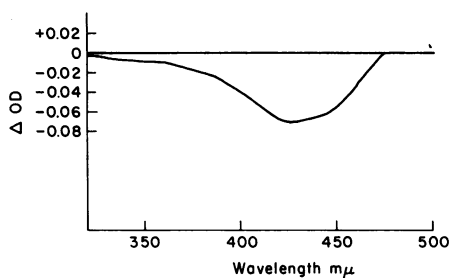


FIG. 8.—Difference spectrum of AM in the presence of dDAP-T.

A solution of AM (0.85 OD/ml at 440  $m\mu$ ) containing dDAP-T (24  $m\mu$ moles/ml) was read against a control solution of AM in Cary model 14 spectrophotometer.

for several hours; the concentration of polymer selected for the experiment reported here was on the linear portion of the velocity/template relationship.

The effect of AM on RNA synthesis directed by dDAP-T and by dAT (A/DAP = 10) is shown in Figure 9. The template function of both polymers is progressively inhibited by increasing concentrations of AM. The ratio AM/DNA-nucleotide which provides 50 per cent inhibition of synthesis is 1:100 for dDAP-T, and 1:100 for dAT (A/DAP = 10); whereas this ratio for native pneumococcal DNA is 1:800. Two points concerning the effect of AM on dDAP-T may be noted. First, a small fraction of RNA synthesis appears absolutely resistant to AM. Secondly, the inhibition curve shows a single, very steep slope as the concentration of AM is increased. With naturally occurring native DNA's the effect of AM is usually biphasic—an initial steep decline is seen at low AM concentration, followed by a more gradual inhibition.<sup>17, 18</sup> It is suggested that this difference may be due to the greater heterogeneity of the structure, sequences, and configuration of calf thymus DNA as compared with the perfectly regular structure of dDAP-T.

Since the specificity of the Mithramycin-DNA interaction closely resembles that previously established for AM,<sup>5</sup> the effect of Mithramycin on the template functions of dDAP-T was examined. As seen in Figure 10, Mithramycin strongly inhibits RNA synthesis directed by dDAP-T. As in the case of AM, a single, steep slope of inhibition is observed, and a totally antibiotic-resistant component remains.

Nogalamycin binds to A-T base pairs in native DNA, particularly when these are arranged in alternating sequences characteristic of dAT.<sup>19</sup> It was of interest

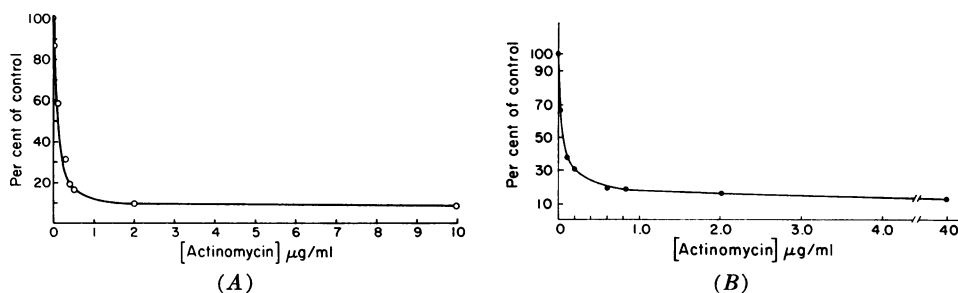


FIG. 9.—Effect of AM on RNA synthesis directed by (A) dDAP-T and (B) dAT (A/DAP = 10).

Enzyme incubations were performed at 37° for 30', in a final volume of 0.125 ml, containing 9  $m\mu$ moles/ml of the respective polymer templates.

100% incorporation corresponds in (A) to 11.6  $m\mu$ moles/ml of H<sup>3</sup>-UMP and in (B) to 39.2  $m\mu$ moles/ml H<sup>3</sup>-AMP.

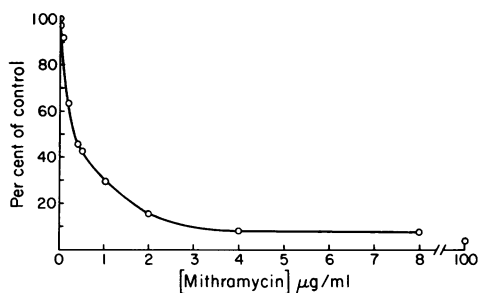


FIG. 10.—Effect of Mithramycin on RNA synthesis directed by dDAP-T. Conditions as for Fig. 9. 100% incorporation corresponds to 10.7  $\mu\text{moles/ml}$  of  $\text{H}^3\text{-UMP}$ .

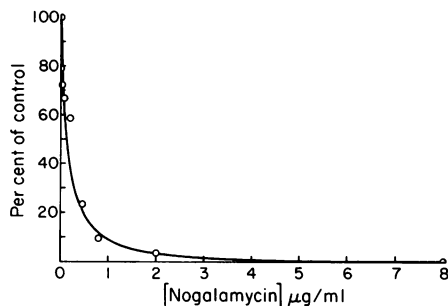


FIG. 11.—Effect of Nogalamycin on RNA synthesis directed by dDAP-T. Conditions as for Fig. 9. 100% incorporation corresponds to 11.9  $\mu\text{moles/ml}$  of  $\text{H}^3\text{-UMP}$ .

to determine whether the DAP-T base pair, which resembles the G-C pair in its stability, in having a purine amino group in the narrow groove, and in the presumed formation of three H-bonds, would interact with Nogalamycin. The data in Figure 11 show that Nogalamycin strongly inhibits the template function of dDAP-T. In contrast to the results with AM and Mithramycin, no antibiotic-resistant synthesis is apparent. Since Nogalamycin complexes strongly with dAT, whereas AM and Mithramycin do not, the AM-resistant and Mithramycin-resistant syntheses are considered to represent the template activity of the residual dAT which was used to prime dDAP-T formation and which accounts for approximately 10 per cent of the total polymer template.

*Discussion and Summary.*—In contrast to the A-T base pair, with its two H-bonds, the DAP-T (Fig. 2), like the G-C pair, might be expected to form three. The higher  $T_m$  of dDAP-T, as compared with dAT, is consistent with this assumption.<sup>20</sup> It is of interest that the formation of the additional H-bond in this case, as in the G-C base pair, is correlated also with a substantial increase in buoyant density of the polymer.

Due to the location of its two amino groups, DAP is structurally similar in some respects to both adenine and guanine. However, its chemical and biochemical properties make DAP an analogue of adenine, not of guanine. Thus, in its pK values,<sup>21</sup> base pairing pattern,<sup>10</sup> and substrate behavior for enzymes<sup>10</sup> DAP closely resembles only adenine, not guanine. Moreover, the 2-amino group of DAP differs significantly from that of guanine in its susceptibility to chemical deamination. These facts make it reasonable to conclude that the DAP-T base pair is analogous to the the A-T base pair chemically, as well as in the distribution of its functional groups; the outstanding property which DAP-T and G-C pairs have in common is a purine 2-amino group and its location in the minor groove of helical DNA.

When AM forms complexes with native DNA, the spectrum of the antibiotic changes, and the buoyant density, thermal stability, and template function of the DNA are markedly affected. The interaction of AM with dDAP-T faithfully reproduces (and in some respects exaggerates) each of these qualities of AM-DNA complexes. dAT does not interact with AM, and dDAP-T differs from dAT in its possession of the additional purine 2-amino groups. Thus, the introduction of a

purine 2-amino group into the minor groove of helical DNA is sufficient, and perhaps the sole requirement, for converting a base pair from AM resistance (and Mithramycin resistance) to AM sensitivity (and Mithramycin sensitivity).

*Abbreviations.*—The following abbreviations are used: A, G, T, C, I, DAP, 2-AP—adenine, guanine, thymine, cytosine, hypoxanthine, 2,6-diaminopurine, 2-aminopurine, respectively; DNA, RNA—deoxyribonucleic and ribonucleic acid, respectively; d $\overline{\text{DAP}}$ -MP, d $\overline{\text{DAP}}$ -TP—5' monophosphate and triphosphate, respectively, of 2,6-diaminopurine deoxynucleoside; AMP, UMP—adenylic acid and uridylic acid; dDAP-T—deoxynucleotide polymer, in which 90% of the residues are arranged in alternating sequence of DAP + T, the remaining 10% consisting of alternating A + T residues; dAT—alternating deoxynucleotide copolymer of A + T; dAT (A/DAP = 10)—dAT copolymer in which 10% of the purine residues are occupied by DAP, 90% by A.

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<sup>20</sup> A similar observation has been described by Howard *et al.* (*J. Biol. Chem.*, **241**, 4293 (1966)), who reported that the  $T_m$  of homopolyribonucleotide complexes of DAP and U was substantially higher than that of the corresponding A + U complexes.

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