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Biochemistry

Hydrogen bonding, overlap geometry, and sequence specificity in anthracycline antitumor antibiotic·DNA complexes in solution

[daunomycin/11-deoxydaunomycin/poly(dA-dT)/intercalation/upfield complexation shifts]

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ABSTRACT We have deduced structural aspects of the intercalation complex of the anthracycline antitumor antibiotic daunomycin and its analogs with the synthetic DNA poly(dA-dT) by ^{1}H and ^{31}P NMR in high-salt solution. We demonstrate that the base pairs are intact at the antibiotic binding site and that the anthracycline phenolic hydroxyls form intramolecular hydrogen bonds with the quinone carbonyls and are shielded from solvent in the intercalation complex. The complexation shifts of the exchangeable phenolic and nonexchangeable aromatic protons demonstrate that rings B and C of the anthracycline chromophore overlap with adjacent base pairs, while anthracycline ring D passes right through the intercalation site in the complex. We observe two resolved ^{31}P resonances attributable to the dA-dT and dT-dA phosphodiester linkages in the phosphorus spectra of the neighbor-exclusion daunomycin·poly(dA-dT) complex. This suggests that the anthracycline antitumor antibiotic exhibits a sequence specificity in its intercalation complex with alternating purine-pyrimidine synthetic DNAs in solution. These conclusions on hydrogen bonding and overlap geometry at the intercalation site and sequence specificity for the daunomycin·poly(dA-dT) complex in solution are in agreement with the structure of the daunomycin·dC-dG-dT-dA-dC-dG hexanucleotide duplex crystalline complex at atomic resolution published recently [Quigley, G. J., Wang, A. H.-J., Ughetto, G., van der Marel, G., van Boom, J. H. & Rich, A. (1980) *Proc. Natl. Acad. Sci. USA* 77, 7204–7208].

The antitumor properties of the antibiotics daunomycin (Fig. 1, structure A) and adriamycin have been associated with intercalation of the planar portion of the anthracycline ring into the DNA of rapidly proliferating neoplastic cells and subsequent blocking of RNA synthesis (1–4).

Several groups have attempted to evaluate structural aspects of the daunomycin·DNA complex on the basis of an analysis of fiber diffraction x-ray patterns (5) and spectroscopic (6, 7) investigations. The proposed models differ as to the overlap geometry at the intercalation site and as to whether the antibiotic sugar residue is located in the minor or major groove (2–7).

A seminal x-ray investigation of a daunomycin·dC-dG-dT-dA-dC-dG complex containing two antibiotics per hexamer duplex has been completed in A. Rich's laboratory (8). The structure of the complex has been solved to atomic resolution and provides the details of the antibiotic–DNA interaction (8).

Our laboratory has reported a high-resolution proton NMR analysis of the nonexchangeable antibiotic and nucleic acid resonances in the daunomycin·poly(dA-dT) as a function of the nucleotide-to-drug ratio (Nuc/D) in 1 M NaCl solution (9). These studies demonstrated that the proton markers on anthracycline ring D (Fig. 1) underwent small upfield shifts on formation of the daunomycin·poly(dA-dT) complex, requiring that ring D not overlap with adjacent base pairs at the intercalation site. There were no nonexchangeable protons on anthracycline rings B and C, and hence these planar ring systems were not monitored in the NMR spectrum of the complex in ^2H_2O solution. Similar conclusions have been deduced from NMR studies of daunomycin complexes with self-complementary tetranucleotide (10) and hexanucleotide (11) duplexes.

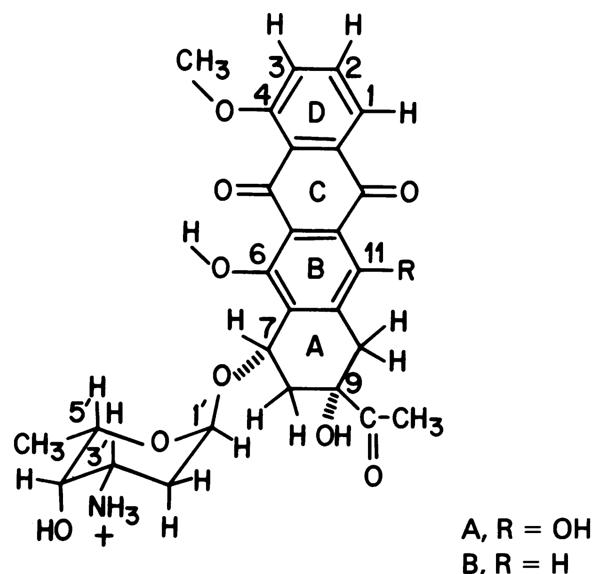

FIG. 1. Chemical formula of daunomycin (A) and 11-deoxydaunomycin (B).

RESULTS AND DISCUSSION

Daunomycin·polydA-dT) complex

This paper summarizes further NMR investigations on the daunomycin·poly(dA-dT) complex and attempts to monitor the hydrogen-bonded nucleic acid and antibiotic protons in H_2O solution and the phosphodiester linkages by ^{31}P NMR spectroscopy. These results put additional constraints on possible overlap geometries at the intercalation site in solution and provide some insight into the sequence specificity of complex formation.

Throughout the text and figures, chemical shifts are given in ppm relative to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) for protons and trimethylphosphate for phosphorus resonances.

The high-resolution NMR investigations were undertaken in high-salt (1 and 2 M NaCl) solution because salt facilitates intercalative binding over stacking along the exterior of the duplex (3, 12).

Watson–Crick Base Pairing. The base-paired duplex state in nucleic acids can be readily characterized by monitoring the

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Abbreviation: Nuc/D, nucleotide-to-drug ratio.

exchangeable imino protons in H_2O solution (13, 14). Earlier studies have demonstrated that the thymidine H-3 imino proton of nonterminal base pairs in nucleic acid duplexes are in slow exchange with solvent H_2O and resonate between 12 and 15 ppm (13, 14). The exchangeable proton NMR spectra (10–15 ppm) of poly(dA-dT) and the Nuc/D = 12 daunomycin·poly(dA-dT) complex in 2 M NaCl/10 mM sodium phosphate/H_2O at 57°C are presented in Fig. 2, spectra A and B, respectively. The stronger resonance in the spectra corresponds to the thymidine H-3 proton, which shifts 0.15 ppm to higher field on formation of the Nuc/D = 12 complex, and these results demonstrate that the base pairing is intact in the daunomycin·poly(dA-dT) complex.

Anthracycline Phenolic Exchangeable Protons. We observe two exchangeable resonances (designated by asterisks in spectrum B of Fig. 2) between 11 and 12.5 ppm in the Nuc/D = 12 daunomycin·poly(dA-dT) proton NMR spectrum in 2 M NaCl solution at 57°C. Their area increases relative to the thymidine H-3 resonance with increasing daunomycin concentration and they are hence assigned to the anthracycline phenolic protons at positions 6 and 11 on the basis of their low field position.

These phenolic hydroxyl protons cannot be observed in the NMR spectrum of daunomycin in H_2O due to rapid exchange with solvent. Their observation in the daunomycin·poly(dA-dT) complex suggests that contributions from intramolecular hydroxyl–carbonyl hydrogen bonds and shielding from solvent after intercalation of the anthracycline ring between base pairs stabilizes these hydroxyl protons against exchange with water.

The anthracycline ring B phenolic hydroxyl proton chemical shifts of 12.23 ppm and 11.46 ppm in the Nuc/D = 12 daunomycin·poly(dA-dT) complex in 2 M NaCl, H_2O solution (Fig. 2, spectrum B) may be compared with the published chemical shifts of 13.85 ppm and 13.15 ppm for *N*-acetyldaunomycin in nonpolar chloroform solution (15). Both exchangeable protons shift upfield by ≈1.6 ppm on formation of the daunomycin·poly(dA-dT) complex with the large magnitude of the antibiotic upfield complexation shift characteristic of intercalation complexes (16). Such a comparison is somewhat limited by the unknown solvent correction that would have to be applied to correlate the antibiotic chemical shifts in chloroform solution with those of the antibiotic·synthetic DNA complex in aqueous solution.

The daunomycin (Fig. 1, structure A) phenolic hydroxyls at position 6 and 11 on ring B are intramolecularly hydrogen-bonded to their adjacent carbonyl groups on ring C and hence serve as proton markers for rings B and C. These data require that anthracycline rings B and C overlap with adjacent base pairs at the intercalation site and that the phenolic hydroxyls experience upfield ring current contributions from nearest- and next-nearest-neighbor base pairs (17, 18). By contrast, previous studies from our laboratory demonstrated that the anthracycline ring D of daunomycin does not overlap with adjacent base pairs of poly(dA-dT), on the basis of the small (0.1– 0.3 ppm) upfield complexation shifts of the nonexchangeable protons of this ring system on complex formation (9).

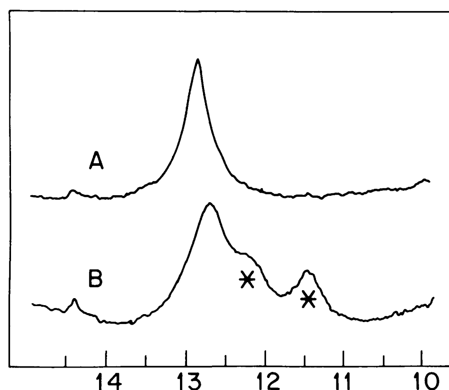

FIG. 2. The 360-MHz correlation proton NMR spectra of poly(dA-dT) (spectrum A) and the Nuc/D = 12 daunomycin·poly(dA-dT) complex (spectrum B) in 2 M NaCl/20 mM sodium phosphate/5 mM EDTA/ 4:1 (vol/vol) H_2O:^2H_2O solution at 57°C. The strong resonance corresponds to the thymidine H-3 proton of the nucleic acid, while the weaker resonances (designated by asterisks) correspond to hydroxyl protons at positions 6 and 11 on ring B of the anthracycline ring of daunomycin in the complex.

Phosphodiester Linkages. The synthetic DNA poly(dA-dT) contains dA-dT and dT-dA phosphodiester linkages, which do not exhibit resolved resonances at the polynucleotide level in solution (16). By contrast, partially resolved resonances have been reported for 150-base-pair $(dA\text{-}dT)_n$ in solution (19) and for poly(dA-dT) in 1 M $(CH_3)_4NCl$ solution (20).

We have observed partially resolved resonances in the proton noise-decoupled 145.7-MHz ^{31}P NMR spectra of the daunomycin·poly(dA-dT) complex in 1 M NaCl solution (Fig. 3). One of the resonances in the complex exhibits the same chemical shift (≈4.1 ppm) as in the synthetic DNA alone, whereas the other resonance shifts downfield by 0.3 ppm in the Nuc/D = 11.8 complex (Fig. 3, spectrum A) and 0.45 ppm in the Nuc/D = 5.9 complex (Fig. 3, spectrum B).

The results suggest that daunomycin intercalates at either dT-dA or dA-dT sites in the poly(dA-dT) duplex, resulting in a downfield shift of the ^{31}P resonance of the corresponding phosphodiester groups at the intercalation site.

The observed ≈0.45 ppm downfield ^{31}P shift for the phosphodiester grouping at the intercalation site for the 1 drug per 3 base pair daunomycin·poly(dA-dT) complex parallels similar observations in the ^{31}P NMR spectra of the proflavine·poly(dA-

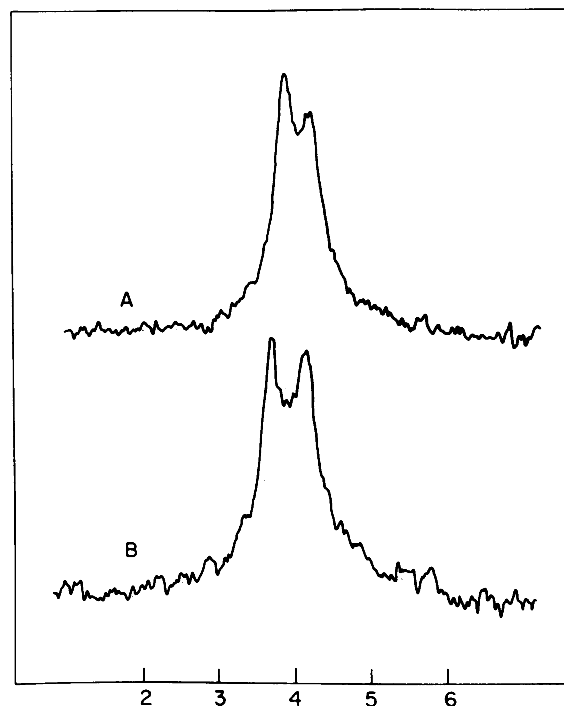

FIG. 3. Proton noise-decoupled 145.7-MHz ^{31}P NMR spectra of the daunomycin·poly(dA-dT) complex in 1 M NaCl/10 mM sodium cacodylate/10 mM EDTA/^2H_2O. Spectrum A corresponds to the Nuc/D = 11.8 complex at pH 6.0 and 67°C, and spectrum B corresponds to the Nuc/D = 5.9 complex at pH 6.05 and 67°C. The chemical shifts are upfield from standard trimethylphosphate.

dT) complex (16) and more recent observations in the nitroaniline dication reporter molecule·poly(dA-dT) complex (21) in solution.

11-Deoxydaunomycin·poly(dA-dT) complex

A daunomycin analog in which the exchangeable hydroxyl group at position 11 on anthracycline ring B has been replaced by a nonexchangeable proton has been isolated from fermentation broths of *Micromonospora pencetica sp. nov.* (22). 11-Deoxydaunomycin (Fig. 1, structure *B*) was found to be biologically active, and the x-ray analysis of 11-deoxydaunomycin aglycone triacetate demonstrated that rings B, C, and D are planar, while the A ring is in the half-chair conformation (22); these results are similar to earlier observations on daunomycin (23, 24) and carminomycin (25, 26). We have investigated the thermal dissociation of the 11-deoxydaunomycin·poly(dA-dT) complex in high-salt solution in order to compare the relative complexation shifts of the nonexchangeable H-11 marker on ring B with the nonexchangeable H-1, H-2, H-3, and OCH_3-4 markers on ring D. These studies should provide additional constraints on the overlap geometry between the anthracycline ring and adjacent base pairs at the intercalation site.

Hydrogen Bonding. The 360-MHz proton NMR spectrum of the Nuc/D = 12 11-deoxydaunomycin·poly(dA-dT) complex in 2 M NaCl/20 mM phosphate solution at 57°C is presented in Fig. 4. The thymidine H-3 proton in the complex resonates 0.11 ppm upfield relative to its position in the synthetic DNA and indicates intact base pairing at the binding site. The phenolic hydroxyl proton at position 6 on ring B of 11-deoxydaunomycin is observed at 11.82 ppm in the Nuc/D = 12 poly(dA-dT) complex (Fig. 4). This does not correlate with either the 12.23 ppm or the 11.46 ppm chemical shift of the 6- and 11-phenolic hydroxyl groups of the Nuc/D = 12 daunomycin·poly(dA-dT) complex (Fig. 2, spectrum B) but is consistent with intramolecular hydrogen bonding and solvent shielding of this exchangeable resonance in an intercalated complex. We believe that the antibiotic 6-hydroxyl resonance at 11.82 ppm reflects its intrinsic chemical shift in the synthetic DNA complex rather than a different overlap in the 11-deoxydaunomycin·poly(dA-dT) complex and that this antibiotic analog can be utilized to obtain additional information on the anthracycline antibiotic·DNA complexes in solution.

Nonexchangeable Proton Spectra. The aromatic region (4.5–8.5 ppm) nonexchangeable proton spectra of the Nuc/D = 12 11-deoxydaunomycin·poly(dA-dT) complex in 2 M NaCl solution in the intact complex (77.5°C), near the transition midpoint (83.5°C), and in the dissociated complex (87.5°C) are presented in Fig. 5. Because the nucleic acid is in excess, the stronger resonances correspond to the base and sugar protons, whereas the weaker resonances (designated by asterisks) correspond to the antibiotic protons.

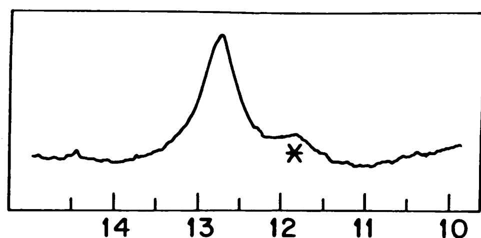

FIG. 4. The 360-MHz correlation proton NMR spectrum of the Nuc/D = 12 11-deoxydaunomycin·poly(dA-dT) complex in 2 M NaCl/20 mM phosphate/5 mM EDTA/4:1 H_2O:^2H_2O solution at 57°C. The strong resonance corresponds to the thymidine H-3 proton of the nucleic acid, whereas the weaker resonance (designated by an asterisk) corresponds to the hydroxyl at position 6 on ring B of the anthracycline ring of 11-deoxydaunomycin in the complex.

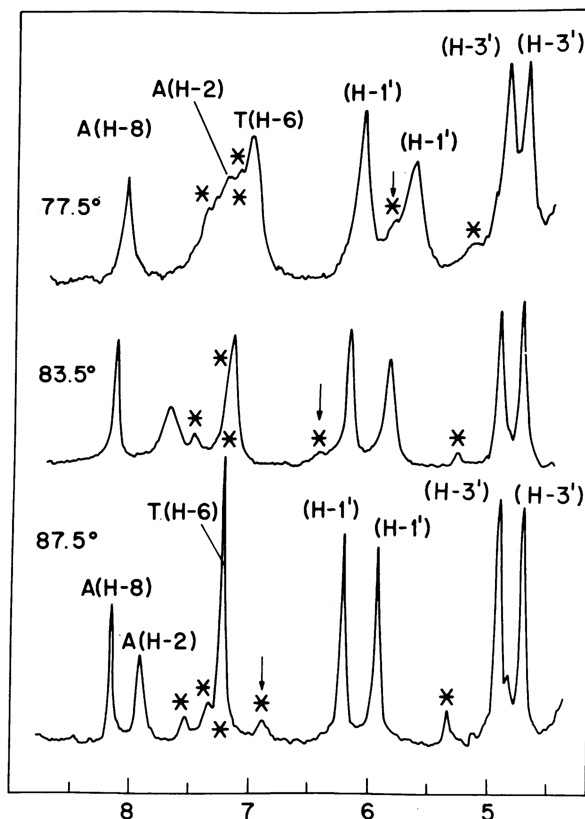

FIG. 5. Temperature dependence of the Fourier transform 360-MHz proton NMR spectra (4.5–8.5 ppm) of the Nuc/D = 12 11-deoxydaunomycin·poly(dA-dT) complex in 2 M NaCl/20 mM phosphate/5 mM EDTA/^2H_2O solution. The resonances of the 11-deoxydaunomycin protons are designated by asterisks and the resonance of the H-11 proton by an arrow.

There are three nonexchangeable aromatic protons on ring D of 11-deoxydaunomycin with the H-1 and H-3 doublets resolved from the H-2 multiplet in the spectrum of the synthetic DNA complex at high temperature. The remaining aromatic proton at position 11 gives a singlet in the 11-deoxydaunomycin complex and can be differentiated from the sugar anomeric H-1′ proton, which exhibits similar behavior in the 11-deoxydaunomycin and daunomycin synthetic DNA complexes.

The temperature dependence of the nucleic acid and antibiotic resonances in the Nuc/D = 12 11-deoxydaunomycin·poly(dA-dT) complex are plotted in Fig. 6, with all the resonances shifting as average peaks during the temperature-dependent dissociation of the complex.

Antibiotic Protons. The magnitude of the upfield shifts of the 11-deoxydaunomycin resonances on complex formation with poly(dA-dT) are summarized in Table 1. It is readily apparent that H-11 undergoes a very large upfield shift (≈1.42 ppm) compared to the much smaller upfield shifts (0.15–0.35 ppm) observed at H-1, H-2, H-3, and OCH_3-4 (Table 1 and Fig. 6). These results unambiguously demonstrate that H-11 on ring B must be located in the shielding region of the base pairs (17, 18) and most likely is stacked directly over a purine ring to account for this large and upfield shift. By contrast, the markers on ring D (positions 1, 2, 3, and 4) undergo much smaller upfield shifts, implying that ring D does not overlap with adjacent base pairs and that its protons project onto the periphery of the ring current contours (17, 18).

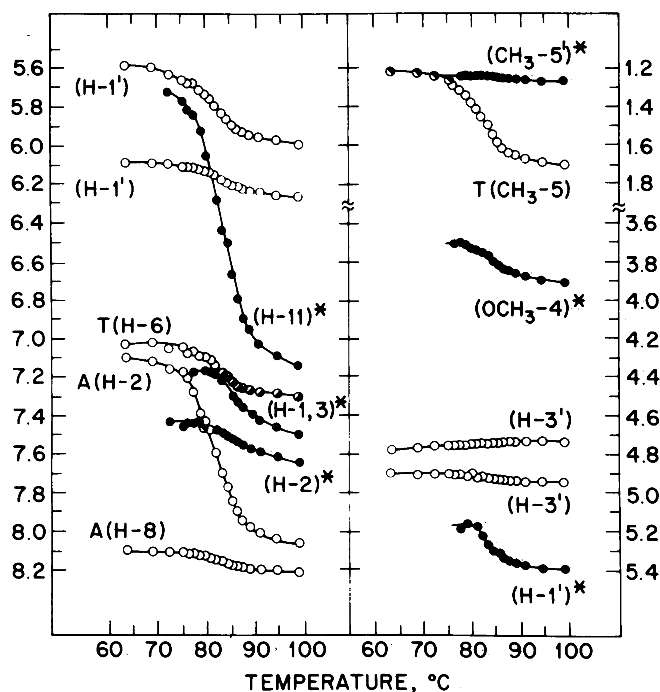

FIG. 6. Temperature dependence of the nucleic acid (○) and the antibiotic (●) resonances in the Nuc/D = 12 11-deoxydaunomycin·poly(dA-dT) complex in 2 M NaCl/20 mM phosphate/5 mM EDTA/^2H_2O solution.

The 5.4 ppm resonance of the anomeric H-1′ proton of 11-deoxydaunomycin shifts to 5.15 ppm on formation of the poly(dA-dT) complex (Fig. 6). This suggests that this anomeric proton and by extension ring A are influenced by the base pairs at the intercalation site. By contrast, the sugar CH_3-5′ group of the antibiotic at 1.25 ppm is unperturbed on complex formation, an observation that suggests there is no interaction between this nonpolar group and the base pair edges.

Nucleic Acid Protons. The temperature dependence of the nucleic acid chemical shifts during the dissociation of the Nuc/D = 12 11-deoxydaunomycin·poly(dA-dT) complex in 2 M NaCl (Fig. 6) exhibits behavior similar to that for the daunomycin complex. The chemical shift changes reflect conversion from a duplex state in the intact complex to the single-strand state in the dissociated complex.

Table 1. Experimental upfield complexation shifts on formation of the 11-deoxydaunomycin·poly(dA-dT) complex (t_m = 83.6°C) in solution*

	Resonance, ppm		Upfield
Assignment	Complex at 72.5°C	Complex at 99.0°C	complexation shift,† ppm
H-11 ring B	≈5.72	7.14	≈1.42
H-1/3 ring D‡	≈7.15	7.30	≈0.15
H-1/3 ring D‡	≈7.15	7.50	≈0.35
H-2 ring D	≈7.43	7.64	≈0.21
OCH_3-4 ring D	≈3.70	3.81	≈0.11
H-1′ sugar	≈5.15	5.40	0.25
CH_3-5′ sugar	1.24	1.27	0.03

* Data for the Nuc/D = 12 11-deoxydaunomycin·poly(dA-dT) complex in 2 M NaCl/20 mM phosphate/5 mM EDTA/^2H_2O solution.

† The experimental complexation shift of the antibiotic protons is the difference between the values for the intact complex at 72.5°C and the dissociated complex at 99.0°C.

‡ We are unable to differentiate between the H-1 and H-3 protons located on anthracycline ring D of the 11-deoxydaunomycin.

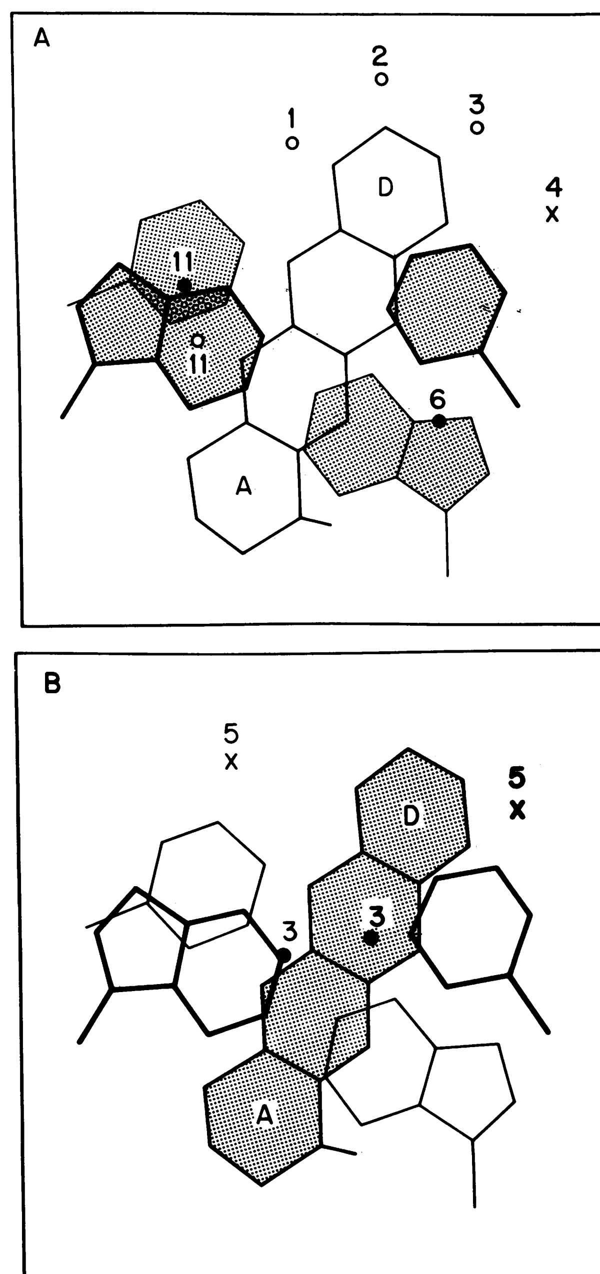

FIG. 7. Overlap geometry between the anthracycline ring system and adjacent base pairs observed at atomic resolution (8) for intercalation of the antibiotic at dC-dG sites in the two-antibiotics per duplex daunomycin·dC-dG-dT-dA-dC-dG crystalline complex. (*A*) The nonexchangeable protons at positions 1, 2, 3, and 11, methoxyl carbon at position 4, and exchangeable protons at positions 6 and 11 on the anthracycline ring are represented by ○, ×, and ●, respectively. This view down the helix axis locates the projection of these antibiotic proton markers onto the planes of the shaded base rings at the intercalation site. (*B*) The nonexchangeable CH_3-5 carbons and exchangeable H-3 thymidine protons are represented by × and ●, respectively. This view down the helix axis locates the projection of these base proton markers onto the plane of the shaded anthracycline ring system at the intercalation site.

As noted previously (9), the thymidine CH_3-5 group with its resonance at 1.3 ppm in the poly(dA-dT) duplex shifts 0.1 ppm upfield to 1.2 ppm on formation of the neighbor-exclusion anthracycline antibiotic·poly(dA-dT) complex. This demonstrates that the ring currents of the anthracycline ring more than compensate those of a base pair displaced as a result of intercalation. This result contrasts with no net shift at the thymidine CH_3-5

resonance in neighbor-exclusion proflavine (27) and ethidium (28) intercalation complexes. This suggests that the orientation of the intercalating anthracycline chromophore differs from the orientations of the intercalating acridine and phenanthridine chromophores, in which the long axis of the aromatic ligand was collinear with the direction of the Watson–Crick hydrogen bonds (28, 29).

Crystallographic and solution data on daunomycin·DNA complexes

This section analyzes the NMR data on the daunomycin·poly(dA-dT) complex in light of the available x-ray structure of the daunomycin·hexanucleotide complex (8), which was kindly provided by A. Rich prior to publication.

Overlap Geometry at Intercalation Site. The antibiotic nonexchangeable protons at positions 1, 2, 3, and 11, the methoxyl carbon at position 4, and the exchangeable phenolic protons at positions 6 and 11 are indicated in Fig. 7*A*, which shows the crystallographic overlap geometry for daunomycin intercalation at dC-dG sites in the hexanucleotide duplex (8).

The NMR data on the daunomycin and 11-deoxydaunomycin complex with poly(dA-dT) in solution are in agreement with the overlap geometry observed in the daunomycin·hexanucleotide crystal (8). Thus, the NMR parameters predict that anthracycline rings B and C overlap with adjacent base pairs as monitored by the exchangeable hydroxyl protons at position 6 and 11 in the daunomycin complex (Fig. 1) and the nonexchangeable proton at position 11 in the 11-deoxydaunomycin complex (Fig. 6 and Table 1), while they predict that anthracycline ring D projects right through the intercalation site as monitored at nonexchangeable protons 1, 2, 3, and methoxyl 4 in the daunomycin complex (9) and the 11-deoxydaunomycin complex (Fig. 6 and Table 1).

The thymidine CH_3-5 carbon and H-3 exchangeable protons are indicated in Fig. 7*B*, which shows the crystallographic overlap geometry for daunomycin intercalation at dC-dG sites in the hexanucleotide duplex (8). The nucleic acid complexation shifts are the net contributions from the bound anthracycline ring less the base pair displaced as a result of intercalation. The thymidine H-3 imino proton of poly(dA-dT) shifts upfield on addition of daunomycin, consistent with intact base pairs in the complex and location of these Watson–Crick protons over the anthracycline aromatic ring system at the intercalation site. The upfield shift of the thymidine CH_3 on complex formation with daunomycin (9) and 11-deoxydaunomycin (Fig. 6) requires that one or both of the pyrimidine 5 positions project onto the ring current shielding region of the anthracycline ring. This can occur only if the anthracycline chromophore is approximately perpendicular to the Watson–Crick hydrogen bonds, a feature observed in the overlap geometry in the crystalline state (Fig. 7*B*).

Minor Groove Binding. The x-ray data on the daunomycin·hexanucleotide complex demonstrate binding through the minor groove, because the sugar ring is located in this groove (8). It appears that this crystallographic observation can be extended to solution, because we have previously demonstrated that stabilization of the synthetic DNA helix by bound daunomycin is unperturbed by the introduction of bulky halogen substituents at the pyrimidine 5 position, which faces the major groove (9).

Sequence Specificity. Rich and coworkers found that daunomycin intercalates at dC-dG sites in the dC-dG-dT-dA-dC-dG hexanucleotide duplex (8). The pyrimidine(3′-5′)purine specificity in the crystalline state suggests that the observation of resolved ^{31}P NMR spectra in the daunomycin·poly(dA-dT) complex (Fig. 3) may reflect intercalation at either the dT-dA or dA-dT site, resulting in a downfield ^{31}P shift at that phosphodiester linkage.

A correlation between the observed downfield ^{31}P shift assigned to the phosphodiester at the intercalation site in the daunomycin·poly(dA-dT) complex in solution with the phosphate backbone at the binding site in the daunomycin·hexanucleotide complex must await further details of the x-ray structure.

The 11-deoxydaunomycin was a gift of Dr. F. Arcamone of Farmitalia, Italy. The proton correlation 360-MHz spectra on exchangeable protons in H_2O solution were recorded at the Mid-Atlantic Regional NMR facility at the University of Pennsylvania Medical School (sponsored by National Institutes of Health Grant RR542). Prof. Alexander Rich kindly provided a preprint of his daunomycin·hexanucleotide crystallographic research prior to publication.

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