with stirring for 60 h. The course of the reaction was monitored by ^1H NMR. An additional 36 mmol of Br_2 was added, and heating was continued at 110 °C for an additional 12 h. The reaction was cooled, and excess Br_2 was removed by sweeping with N_2. A 5% aqueous Na_2CO_3 solution (w/v) was added carefully with stirring, and 8 N NaOH was added until the pH was basic. The alkaline solution was washed 3 times with equal volumes of $(C_2H_5)_2O$ and the pH of the aqueous phase was carefully lowered to <2 with 6 N HCl. The solution was extracted 4 times with equal volumes of $(C_2H_5)_2O$, and the combined organic extracts were dried with $MgSO_4$ and concentrated in vacuo to give 4.65 g of the product $Br_2CH^{13}CO_2H$ (21 mmol, 59% yield). The acid reduced with $LiAlH_4$ (1.20 g, 32 mmol) in 100 mL of dry $(C_2H_5)_2O$ under N_2 using the procedure of Sroog and Woodburn[21] to give the product $Br_2CH^{13}CH_2OH$ in 47% yield. ^1H and ^{13}C NMR spectroscopy indicated that the product was contaminated with ~10% $BrCH_2{}^{13}CH_2OH$; only the carbinol carbon was labeled in each case (^{13}C signal at 70 ppm for $Br_2CH^{13}CH_2OH$, 63 ppm for $BrCH_2{}^{13}CH_2OH$). The J value for the ^{13}C induced splitting of the Br_2CHCH_2OH proton doublets in the ^1H NMR spectrum was 140 and 1.4 Hz for the Br_2CH-CH_2OH triplet members.

5,6-Dihydro-1-methyl-5-oxo-6-β-D-ribofuranosylimidazo[1,2-c]pyrimidine Chloride (3,N^4-Etheno-N^4-methylcytidinium Chloride) (13). The procedure was adopted from Barrio et al.[16] N^3,4-Ethenocytidine·HCl (**2**) (25 mg, 82 μmol) was stirred in 1 mL of distilled $(CH_3)_2NCHO$ with $(C_2H_5)_3N$ (160 μmol) and CH_3I (320 μmol) overnight at 23 °C under Ar. HPLC analysis (Ultrasphere 5 μm octadecasilyl, 10 × 250 mm, 5% CH_3OH in 25 mM NH_4HCO_2, pH 5.5) (Beckman, San Ramon, CA) indicated that the reaction was ~90% complete as judged by A_{267} measurements (formation of a major new polar peak). A portion of the total preparation was purified using the same HPLC column and eluant in the absence of CH_3OH: ^1H and ^{13}C NMR assignments are presented in the legends for Figures 4 and 5.[16] UV (25 mM aqeuous NH_4HCO_2, pH 5.5): apparent broad λ_{max} at 292 nm, second derivative analysis showed peaks at 248, 257, 274, 285, 295, and 307 nm.

Preparation of ^{13}C-Labeled Etheno Derivatives. The general procedure involved mixing $Br_2CH^{13}CH_2OH$ (200 mM), N-ethylmorpholine acetate (200 mM, pH 9.2), and either cytidine (200 mM), adenosine (35 mM), guanosine (50 mM), or O^6-ethylguanosine (50 mM) and shaking at 37 °C under Ar in a Teflon-sealed amber glass vial for 5-7 days. When guanosine or O^6-ethylguanosine was used, 50% (v/v) $(CH_3)_2SO$ was added to improve solubility. The solution was washed four times with an equal volume of $(C_2H_5)_2O$ and concentrated by lyophilization prior to separation of components by HPLC (vide infra).

HPLC. Most HPLC was done using a Beckman octadecasilyl semi-prep column (5 μm, 10 × 250 mm) and mixtures of CH_3OH in 25 mM NH_4HCO_2 (pH 5.5) as modified from the literature.[12] Some of the analytical work on the formation of products of the reaction of N^6-methyladenosine was done using a Zorbax octadecasilyl column (3 μm, 6.2 × 80 mm, Mac-Modd, Chadds Ford, PA). All of the compounds under consideration could be separated using either isocratic or gradient conditions, e.g., see indicated references for examples of separations.[12,24]

Acknowledgment. This work was supported in part by United States Public Health Service Grants CA 44353 and ES 00267. We thank Dr. W. G. Humphreys and Professor T. M. Harris for advice and helpful discussions.

Communications to the Editor

Cooperative Binding of Distamycin-A to DNA in the 2:1 Mode

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Received August 9, 1991

The preference of distamycin-A for A-T rich binding sites has been recognized for many years.[1] NMR was first used to show unambiguously that the closely related drug netropsin bound in the minor groove of B-form DNA.[3a] Both NMR and crystallographic studies of complexes of distamycin-A,[2] netropsin,[3] Hoechst 33258,[4] and SN-6999[5] have been carried out. In all of these studies to date, the drugs are bound deep in the minor groove of an A-T segment. Where the drug is bound, the structure of the minor groove closely matches the shape and width of the drug molecule. In several of the DNAs studied crystallographically, the groove is found to be equally narrow without the drug present.[6] NMR studies have shown that the sequences CGCAAATTGGC[7] and CGCAAATTTGCG[8] bind a single drug at low drug ratios (analogous to complexes characterized crystallographically). At higher amounts of added drug, new complexes were formed with two distamycin molecules bound side by side in the same region of the minor groove. This indicates a significant degree of adaptability in the minor groove width, since an increase in groove width of about 3.5 Å is required to accommodate the second drug. It was found that the second drug bound somewhat less tightly than the first for AAATT, but was somewhat tighter than the first for AAATTT.

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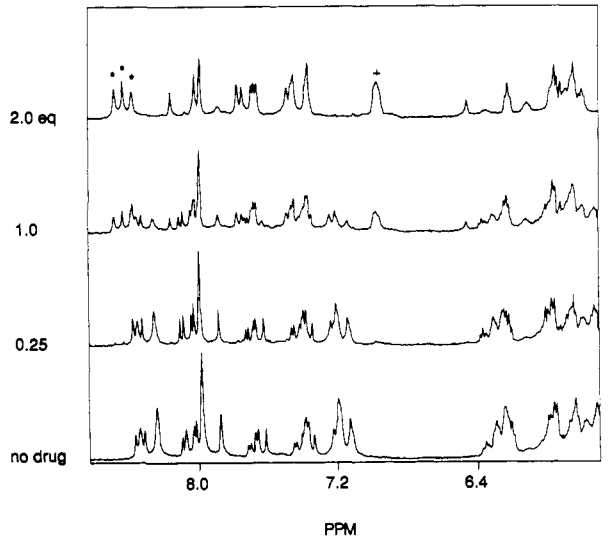

Figure 1. The downfield regions of the ^{1}H NMR spectra for CGCATATATGCG shown with increasing amounts of added distamycin-A. Ratios of drug to DNA duplex are indicated; DNA, ~2 mM duplex concentration in 10 mM phosphate buffer, pH 7.0, 25 °C. The asterisks (*) indicate the downfield-shifted adenosine H8 resonances which are from the 2:1 form of the complex.

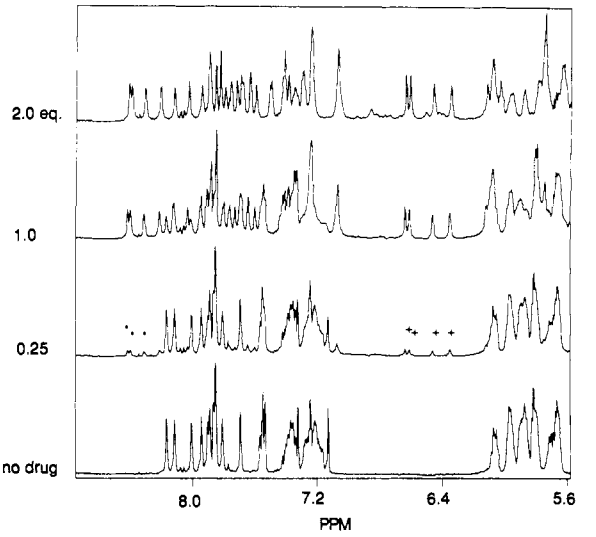

Figure 2. The downfield regions of the ^{1}H NMR spectra of CGCIIICCGGC + GCCIICCCGCG shown with increasing amounts of added distamycin-A. Ratios of drug to DNA duplexes are indicated; DNA, ~2 mM duplex concentration in 10 mM phosphate buffer, pH 7.0, 15 °C. The asterisks (*) indicate the downfield-shifted inosine H8 resonances in the 2:1 complex, and the plus signs indicate the drug pyrrole resonances of the 2:1 complex. A small amount of free drug is seen in the 2:1 ratio spectrum.

We have now examined distamycin binding to d(CGCATATATGCG)$_2$, for which a structure has been determined by X-ray diffraction.[6c] In the AT region the groove is somewhat wider than is seen in oligomers with central sequences AATT and AAAAAA.[6] Standard 2D methods were used to assign all of the resonances for the duplex.[3] Upon addition of distamycin, there was broadening and appearance of new resonances, shown in Figure 1, as has been seen in other distamycin titrations. The resonances of the complex were reassigned after the addition of 2 mol of distamycin/mol of duplex oligomer. The NOESY spectrum clearly indicates a symmetric 2:1 complex, with two drugs bound side by side, based upon drug–DNA and drug–drug cross peaks (supplementary material). It is important to note that peaks from the 2:1 complex appear well below stoichiometric addition of drug, indicating a positive cooperativity in binding. The degree of cooperativity is somewhat higher than for AAATTT.[8] This suggests that the wider groove in free ATATAT leads to weaker binding in the 1:1 mode, but still allows facile binding in the 2:1 mode. Relative to the minor groove width seen in the crystal structure, it is still necessary to widen the groove to accommodate two drugs binding side by side.

We have also examined the binding of distamycin to d(CGCIIICCGGC) + d(GCCIICCCGCG), where I represents inosine (2-desaminoguanosine). An I-C base pair has minor groove functional groups which are completely equivalent to an A-T pair, although the major groove functional groups and stacking interactions are more equivalent to G-C pairs. Since the functional groups available for minor groove recognition are equivalent in A-T and I-C base pairs, any difference in distamycin binding should arise from structural rather than functional differences. In particular if the groove width is similar to that typical of G-C-rich regions (6–7 Å), rather than the narrow value seen in A-T-rich regions (3–4 Å), changes in drug affinity may be expected. The electrostatic potential of I-C pairs is probably much closer to that of G-C than A-T, which could contribute to differences in binding affinity.

The NMR spectrum of IIICC was also assigned with standard 2D methods. The titration of this sequence with distamycin is shown in Figure 2. There is no broadening upon addition of drug, just growth of new resonances from complex with concomitant disappearance of the free DNA, up to a stoichiometry of 2:1. The spectrum was reassigned at this point, clearly indicating a side-by-side 2:1 complex (supplementary material), completely analogous to the complex with AAATT. The major difference is that for AAATT several forms of 1:1 complex are observed at low levels of added drug, the 2:1 complex not appearing until nearly 1 drug has been added per DNA. However, for IIICC there are never any peaks seen for the 1:1 complex, only for the 2:1, indicating a very high degree of cooperativity for the drugs binding to this sequence. This suggests that the free DNA has an intrinsically wide minor groove, in which a single drug would make contact only with one side, lowering its binding affinity. It also indicates that there is a substantial energetic cost to reducing the groove width in order to reestablish contact on both sides of the drug. In the 2:1 complex, however, the groove is filled, and the two drugs make close contacts with both sides of the groove. From the lack of exchange broadening at intermediate ratios of added drug, it is clear that the affinity of this 2:1 complex is similar to that of the AAATT analogue, arguing that electrostatic difference is not the primary factor responsible for the differences.

These studies indicate that the drug affinity in the 1:1 binding mode is affected by the width of the minor groove. The groove can expand to accommodate a second drug in the 2:1 complex, but it appears to be energetically unfavorable to narrow a wide groove even by a small amount, as would be required for ATATAT. Our observations suggest that the sequence IIICC has a wider groove than AAATT, leading to different drug binding despite the fact that the functional groups in the minor groove are identical in these two sequences. The difference in the shape of the groove is not unexpected, since the stacking of I-C pairs should be more like G-C than A-T, leading to a groove more typical of a G-C-rich segment. This work also suggests that substitution of I-C for A-T pairs may provide a mechanism for characterization of sequence-dependent backbone interactions, such as those suggested in some repressor–operator complexes.

Acknowledgment. We thank Dr. Jeffrey Pelton for help with early work on inosine-containing sequences. This work was supported by NIH Grant GM-43129 (D.E.W.) and an NSF graduate fellowship (P.F.) and through instrumentation grants from the U.S. Department of Energy, DE FG05-86ER75281, and the National Science Foundation, DMB 86-09305 and BBS 87-20134.

Supplementary Material Available: Two figures showing the assignments and intermolecular contacts for the 2:1 complexes of distamycin-A with (ATATAT)$_2$ and IIICC + IICCC (2 pages). Ordering information is given on any current masthead page.