Pyrrolo[1,4]benzodiazepine Antibiotics. Proposed Structures and Characteristics of the in Vitro Deoxyribonucleic Acid Adducts of Anthramycin, Tomaymycin, Sibiromycin, and Neothramycins A and B†

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ABSTRACT: The pyrrolo[1,4]benzodiazepine antibiotics anthramycin, tomaymycin, sibiromycin, and neothramycins A and B are potent antitumor agents that bind to DNA in a unique manner, resulting in some unusual biological consequences. This paper describes results on which the points of covalent linkage between the drugs (carbinolamine carbon atom) and DNA (N-2 of guanine) are deduced, as well as Corey–Pauling–Koltun (CPK) models for the various drug–DNA adducts. Predictions based upon these CPK models have been tested, and the results are reported in this paper. These tested experimental predictions include (1) instability of the drug–DNA adducts to denaturation of DNA, (2) saturation binding limits, (3) effect of drug binding on the structure of DNA, (4) lack of unwinding and in vitro strand breakage of closed-circular supercoiled simian virus 40 (SV-40) DNA, (5) sensitivity of the secondary structure of DNA to drug binding, (6) hydrodynamic properties of the drug–DNA adducts, (7) hydrogen bonding of the 9-phenolic proton in anthramycin to DNA, (8) structure–activity relationships, and (9) biological consequences of DNA damage, including cumulative damage and slow excision repair, double-strand breaks in DNA in repair-proficient cells, and the selective inhibition of H-strand DNA synthesis in mitochondria. The results are completely in accord with our postulated space-filling models.

The pyrrolo[1,4]benzodiazepine antibiotics anthramycin, tomaymycin, sibiromycin, and neothramycins A and B are potent antitumor agents produced by various actinomycetes (Hurley, 1977). Examination of the structures of these agents (Figure 1) reveals that while all, except the neothramycins,[1] have in common the reactive carbinolamine (N-10, C-11) group, they differ in their aromatic substitution patterns, the degree of saturation in the pyrrolo ring, and the type of side chain at C-2. With the exception of sibiromycin, all other members of this group have an asymmetric carbon at C-11a, which provides the molecules with a 45° right-handed twist from the aromatic ring to the pyrrolo ring (Mostad et al., 1978). The carbinolamine group is highly reactive and with the exception of sibiromycin can be reversibly converted via the imine to the two C-11 isomers. In the case of sibiromycin, dehydration leads to the (1–11a) conjugated imine, which is less susceptible to nucleophilic attack due to its lower reactivity.

Addition of the pyrrolo[1,4]benzodiazepine antibiotics to cultures of prokaryotic or eukaryotic organisms leads to potent inhibition of nucleic acid synthesis (Horwitz & Grollman, 1968; Kann & Kohn, 1972; Maruyama et al., 1978; Gause & Dudnik, 1971, 1972; Kohn, 1975; Nishioka et al., 1972), which is almost certainly due to modification of the DNA template by these agents (Kohn et al., 1974; Hurley et al., 1977; Gause and Dudnik, 1971; Maruyama et al., 1979). Gause and co-workers have demonstrated that anthramycin and sibiromycin produce selective inhibition of H-strand synthesis in animal mitochondrial DNA (Gause & Dolgilevich, 1975; Gause et al., 1976). The reaction of anthramycin and related drugs with DNA appears to be unique to this group of agents (Hurley, 1977; Kohn et al., 1974; Hurley & Petrusek, 1979). For this reason, we have been actively investigating both the in vitro reaction of the anthramycin with DNA as well as the biological consequences of DNA damage in human cell lines, yeast, and bacterial cells. The results of the latter studies show that anthramycin binds to cellular DNA and induces unscheduled DNA synthesis in excision repair proficient cells (Hurley et al., 1979a). The drug undergoes excision removal from cellular DNA in excision-proficient cells, but at a slow rate in comparison to other DNA-reactive compounds such as AAAF[2] or BMBA. In the yeast *Saccharomyces cerevisiae*, anthramycin is only weakly mutagenic but is highly recombinogenic (Hannan et al., 1978). Mutants of *S. cerevisiae* defective in either excision repair or repair of double-strand breaks are unusually sensitive to anthramycin, tomaymycin, and sibiromycin (Hannan & Hurley, 1978).

The results described in this paper provide a strong basis for our proposed structures for the DNA adducts of anthramycin, tomaymycin, sibiromycin, and the neothramycins. Construction of CPK space-filling models for these various drug adducts leads to a number of predictions, some of which we have tested by using various biochemical techniques and others the results of which have already appeared in the literature. In all cases, the predictions are mimicked precisely in the practice. Some of these results have been published

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[1] The neothramycins possess masked carbinolamines, since when they are dissolved in aqueous solution nucleophilic addition of water produces the N-10, C-11 carbinolamine.

[2] Abbreviations used: AAAF, 2-(*N*-acetoxyacetylamino)fluorene; BMBA, 7-(bromomethyl)benz[*a*]anthracene; CPK, Corey–Pauling–Koltun molecular models; TLC, thin-layer chromatography; SSC, 0.15 M NaCl and 0.015 M sodium citrate, pH 7.4; BND-cellulose, benzoylated, naphthoylated DEAE-cellulose; MEM, minimal essential media; FCS, fetal calf serum; 0.3 M NET, 0.3 M NaCl, 10^{-4} M EDTA, and 0.01 M Tris-HCl, pH 7.5; 1.0 M NET, 1.0 M NaCl, 10^{-4} M EDTA, and 0.01 M Tris-HCl, pH 7.2; TEA, 150 mM Tris, 2 mM EDTA, 20 mM sodium acetate, and 18 mM NaCl, pH 8.1; SV-40 FI DNA, simian virus 40 form I DNA; SV-40 FII DNA, simian virus 40 form II DNA; SV-40 FIII DNA, simian virus 40 form III DNA; Hepes, *N*-2-(hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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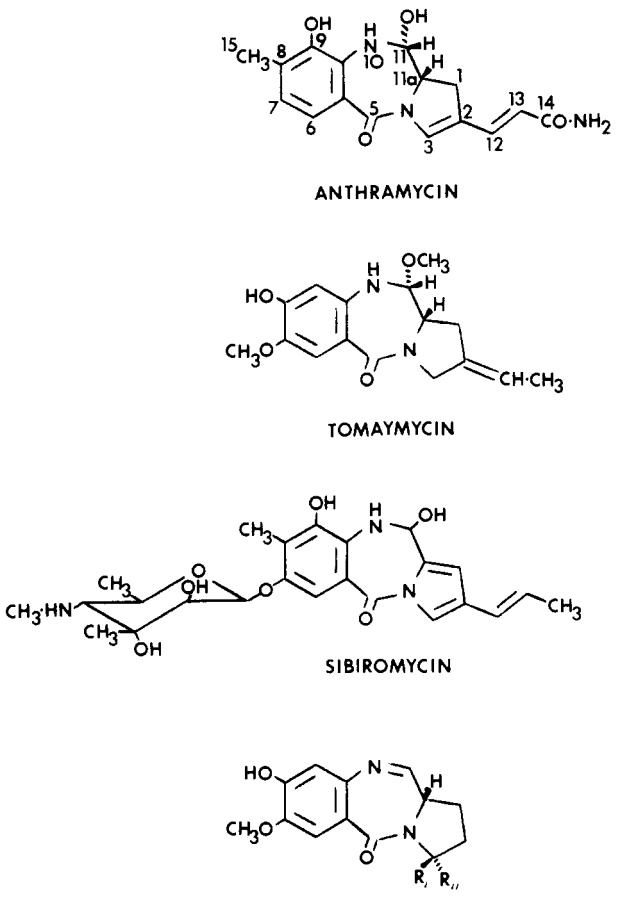

FIGURE 1: Structures of the pyrrolo[1,4]benzodiazepine antitumor antibiotics.

either in preliminary form (Hurley & Petrusek, 1979; Hurley et al., 1980) or as an abstract (Hurley et al., 1978).

Experimental Procedures

(*A*) *Chemicals and Supplies.* (*1*) *Drugs, Enzymes, Nucleic Acids, and Chromatographic Materials.* Anthramycin and sibiromycin were produced biosynthetically in this laboratory by previously published procedures (Hurley et al., 1975, 1979c). Tomaymycin and neothramycins A and B were generously provided by Dr. Aoki (Fujisawa Pharmaceutical Co., Ltd.) and Dr. J. Douros (Drug Development Branch, National Cancer Instutute), respectively.

S_1 nuclease and DNase I were purchased from Sigma Chemical Co. Cs_2SO_4 and CsCl were purchased from Kawecki Berylico Industry and Sigma Chemical Co., respectively. *Eco*RI and SV-40 DNA were purchased from BRL, and the latter was greater than 90% SV-40 FI DNA. Calf thymus DNA and *Clostridium perfringens* DNA were purchased from Sigma Chemical Co. T-4 DNA was purchased from Miles Laboratories. Poly(dG)·poly(dC) and poly(dG-dC)·poly(dC-dG) were purchased from P-L Biochemicals.

SV-40 FIII DNA was prepared from SV-40 FI DNA by incubation with *Eco*RI (10 units/100 μg of DNA) in a final solution of 500 μg/mL DNA in 100 mM Tris and 10 mM $MgCl_2$, pH 8.1. Digestions were allowed to proceed for 30 min at 37 °C and then stopped by the addition of an excess amount of EDTA.

(*2*) *Radiolabeled Material.* [15-^{3}H]Anthramycin and [15-^{3}H]sibiromycin were prepared biosynthetically from L-[*methyl*-^{3}H]methionine (NEN, 80 Ci/mmol) as described previously (Hurley et al., 1975, 1979c, respectively). Specific activities (μCi/μmol) of anthramycin and sibiromycin were based upon molar extinction coefficients of 36 800 at 333 nm and 21 800 at 310 nm in aqueous solution at pH 7.2, respectively.

[8-^{3}H]Guanine-labeled DNA was prepared by biosynthetic labeling. A human fibroblast cell line (TC-98) was established from an explant of a primary human skin fibroblast karyotypically normal and was maintained in MEM with 10% FCS and 20 mM Hepes buffer; 0.5 mCi of deoxy[8-^{3}H]guanine (1.9 Ci/mmol; Amersham Searle) was added to a 150-cm^2 Falcon flask. After a 12-h incubation, the DNA was extracted and purified by a CsCl-gradient method. The final DNA had a specific activity of 2.5×10^6 dpm/1.0 A_{260} unit. The specificity of labeling of the deoxy[8-^{3}H]guanosine in DNA was determined by enzymatic degradation of the DNA to the constituent deoxynucleosides. These were separated on a LH-20 column with H_2O as an eluate (Martin & Garner, 1977). The radioactivity was found exclusively in the deoxyguanosine functions.

^{14}C-Labeled SV-40 DNA was prepared by addition of 1 μCi/mL [*methyl*-^{14}C]thymidine (52 mCi/mmol; Amersham) to SV-40-infected cells of green monkey kidney cells (CV-1's) in 60-mm plates. Thymidine was added at 20-h postinfection and allowed to incubate for 22 h prior to harvesting by using a Hirt supernatant procedure (Hirt, 1967) and subsequently purified by velocity CsCl centrifugation.

(*B*) *General Methods.* Antibiotic to base ratios were determined by using a combined radioactivity (drug) and absorption (nucleic acid) method (Hurley et al., 1977). Nucleic acid concentrations were calculated by assuming E_{260nm} = 6412 (calf thymus DNA), E_{260nm} = 6225 (*C. perfringens*), E_{260nm} = 7300 (T-4 DNA), E_{254nm} = 8400 [poly(dG-dC)·poly(dG·dC)], and E_{253nm} = 7400 M^{-1} cm^{-1} [poly(dG)·poly(dC)]. In certain cases where drug concentrations were high enough, these ratios were also calculated by the absorbance method of Kohn & Spears (1970), and these values never varied from the combined radioactivity-absorption method by more than ±5%.

Absorption spectra were recorded on a Beckman Model 26 spectrophotometer. Radioactivity was determined by using a Packard 2425 liquid scintillation counter. Radiochemical purity of drugs was determined on a Packard Model 7201 radiochromatogram scanner (Hurley et al., 1975, 1979c).

(*1*) *Alkaline Cs_2SO_4 Gradient of [15-^{3}H]Anthramycin–Poly(dG)·Poly(dC).* The [15-^{3}H]anthramycin–poly(dG)·poly(dC) adduct was prepared by addition of 0.05 mL (0.155 μmol) of [15-^{3}H]anthramycin (1.0 μCi/μmol) to a 6-mL solution of poly(dG)·poly(dC) at 0.034 μmol/mL in 0.1 M SSC. The solution was dialyzed for 48 h against SSC, after binding for 24 h at room temperature. An aliquot was removed, and the OD at 253 nm and ^{3}H radioactivity were measured. The final solution used for alkaline denaturation contained 0.15 μmol of poly(dG)·poly(dC) and 0.015 μmol of [15-^{3}H]-anthramycin, giving an antibiotic to base ratio of 1:10. Alkaline denaturation was carried out by slow addition of 1 N NaOH to a final pH of 12.5. Denaturation was monitored by UV absorption changes. The denatured antibiotic–polymer solution (4.4 mL) was mixed with solid Cs_2SO_4 (2.95 g) to give a final density of 1.5. The solution was then centrifuged at 30 000 rpm for 44 h at 20 °C in the SW 50.1 rotor. Ten-drop fractions were collected. The OD of each fraction was measured, and ^{3}H radioactivity (cpm) was determined by addition of 150-μL aliquots of the fractions to 1 mL of 1 N

HCl in 10 mL of Aquasol. Refractive indices of selected fractions were read on a Zeiss refractometer.

(*2*) *Assay for Distillable Tritium from Anthramycin and Nitrogen Mustard Treated [8-^{3}H]Guanine-Labeled DNA.* This procedure was based largely upon the published method described by Tomasz (1970a). The incubation mixture consisted of 3×10^{-3} μmol of [8-^{3}H]guanine-labeled DNA in 200 μL of 5 mM Tris buffer (pH 7.4) plus 200 μL of a 1 μg/mL solution of either anthramycin or nitrogen mustard. The volume was made up to a total of 1 mL with 5 mM Tris buffer (pH 7.4) and allowed to react overnight at room temperature. Any released tritium was collected by vacuum distillation at 35–40 °C. Aquasol (10 mL) was added to the distillate (1 mL) and the radioactivity determined by liquid scintillation counting.

(*3*) *Electrophoresis and Analysis of Anthramycin Binding to SV-40 FI and FIII DNA.* Electrophoresis of SV-40 DNA was performed in 1% agarose gels (w/v) made up in TEA buffer. Aliquots of 10 μL each of glycerin and 0.1% bromophenol blue were added to each sample immediately prior to loading the gels. Electrophoresis was carried out at 1 mA/gel until the marker dye migrated approximately 7 cm. The buffer used for electrophoresis was the same as that used to make up the gels. After electrophoresis, the gels were removed and washed extensively in TEA buffer containing 1% ethidium bromide. The DNA bands were then located with a shortwave UV (254 nm) cutout, hydrolyzed in 1 mL of 0.5 N HCl, and counted in Aquasol (NEN). As a correction for variance in sample size, ^{14}C-labeled SV-40 DNA was used in these experiments. The samples were counted for dual label (^{3}H/^{14}C) and corrected for spillover of ^{14}C into the ^{3}H channel.

Results and Discussion

(*A*) *Formulation of CPK Models for the Pyrrolo[1,4]-benzodiazepine Antibiotic–DNA Adducts.* Since the anthramycin–DNA adduct is unstable to enzymatic denaturation of DNA (Hurley et al., 1979b), the usual approaches involving isolation of the nucleoside adducts to determination of the structures of the pyrrolo[1,]benzodiazepine nucleoside adducts could not be taken.

Our approach reported in this paper was therefore to obtain indirect evidence for the sites of covalent linkage between anthramycin and DNA.

(*1*) *Determination of the Anthramycin Reactive Base in DNA.* Previously reported results on anthramycin (Kohn et al., 1974) and the neothramycins (Maruyama et al., 1979) have shown that these antibiotics have a specificity for G–C base pairs. Alkaline denaturation of DNA, to which anthramycin, sibiromycin, or tomaymycin is bound, does not lead to loss of the drugs from the single-stranded DNA (Kohn & Spears, 1970; Hurley et al., 1977). Therefore, in order to determine whether anthramycin is bound to guanine or cytosine, we have denatured the anthramycin-modified homopolymer poly(dG)·poly(dC) under alkaline conditions and separated the two strands on a cesium sulfate gradient (see Figure 2). The results show that the radioactively labeled anthramycin is bound exclusively to the poly(dG) strand.

(*2*) *Alkylation Site on Poly(dG).* Since we had shown that anthramycin binds exclusively to the poly(dG) strand of the homopolymer poly(dG)·poly(dC), it now remained to pinpoint the location on guanine to which anthramycin was attached.

An alkylation assay designed by Tomasz (Tomasz, 1970b) was used to determine if alkylation at C-8 or N-7 of guanine occurred upon reaction of anthramycin with DNA. The labilization of the C-8 proton upon 7-alkylation has been demonstrated previously (Tomasz, 1970a). The results of our

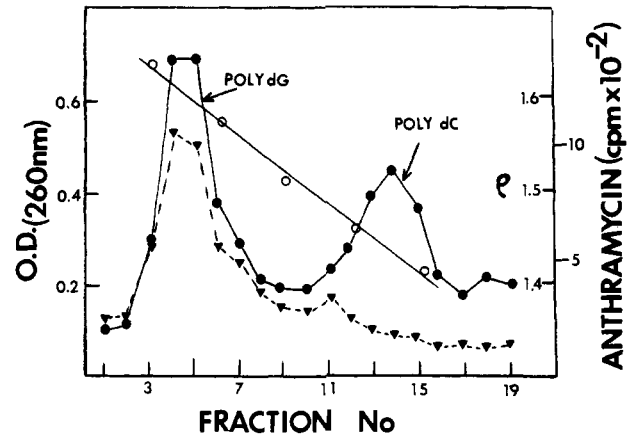

FIGURE 2: Alkaline cesium sulfate gradient on denatured [15-^{3}H]-anthramycin–poly(dG)·poly(dC). Conditions for preparation of [15-^{3}H]anthramycin–poly(dG)·poly(dC), alkaline denaturation, and gradient analysis are as described under Experimental Procedures. Literature density values (Szybalski & Szybalski, 1970a) for poly(dG)·poly(dC), poly(dG), and poly(dC) are 1.46–1.49, 1.54, and 1.40–1.42, respectively. The slightly higher density of the anthramycin–poly(dG) (1.59–1.61) than that of poly(dG) alone may be due to the anthramycin modification of the polymer. The small increase in cpm in fraction 11 (density = 1.48) coincides with the position of undenatured anthramycin–poly(dG)·poly(dC) (data not shown). OD_{260} (●); cpm (▼); ρ (○).

Table I: Release of Distillable Tritium from [8-^{3}H]Guanine-Labeled DNA upon Alkylation by Anthramycin or Nitrogen Mustard

sample	% release of tritium[a]
[8-^{3}H]guanine-labeled DNA[b] (untreated)	0.7
[8-^{3}H]guanine-labeled DNA[b] + anthramycin[c]	0.2
[8-^{3}H]guanine-labeled DNA[b] + *N*-mustard[c]	11.4

[a] Based on an average of two experiments. [b] 5×10^4 dpm of a 7.6 μCi/μmol (nucleotide) solution. [c] 200 μL of a 1 μg/mL solution.

experiments show that alkylation at either C-8 or N-7 can be eliminated from consideration, since insignificant amounts of distillable tritium above that found in control experiments were found upon in vitro reaction of an amount of anthramycin known to produce saturation binding (1:12.5 antibiotic to base ratio) with [8-^{3}H]guanine-labeled DNA (Table I). A control experiment with nitrogen mustard, which is known to alkylate N-7 of guanine (Tomasz, 1970a), showed the expected release of distillable tritium (Table I).

Alkylation of N-3 of guanine can also be eliminated since anthramycin-modified DNA does not undergo depurination (Hurley et al., 1978; Lown & Joshua, 1979) by neutral heating, a process known to depurinate N-7 and N-3 alkylated guanine (Singer, 1975).

Of the remaining sites on guanine N-2 or O-6 is potentially accessible in the minor and major groove of DNA, respectively. Binding experiments with T-4 phage, which is 100% glucosylated in the major groove and blocks O-6 as a potential binding site, have allowed us to distinguish between these possibilities. The results (Table II) show that T-4 phage was able to bind anthramycin to a final antibiotic to base ratio of 1:15.5 whereas *Clostridium perfringens* DNA, which has a similar G–C content, produced a binding ratio of 1:13.5.

We therefore can narrow the choice to just one site on guanine, i.e., N-2. Supporting evidence for this position for alkylation by guanine comes from the unreactivity of poly(dI)·poly(dC), which differs from poly(dG)·poly(dC) in that

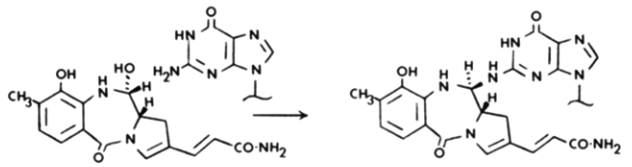

FIGURE 3: Proposed structure and mechanism for formation of the anthramycin–deoxyguanosine adduct in DNA.

it lacks a 2-amino group of guanine (Kohn et al., 1974), the fact that actinomycin D, which also binds in the narrow groove, prevents anthramycin binding (Kohn et al., 1974), and CPK model building studies which show adduct formation through O-6 of guanine is impossible.

(3) DNA Reactive Group on the Pyrrolo[1,4]benzodiazepine Antibiotics. The only *common* potentially reactive group on anthramycin, tomaymycin, sibiromycin, and the neothramycins A and B[1] is the N-10, C-11 carbinolamine. Further confirmatory evidence that the carbinolamine is the DNA reactive moiety is that oxidation to the amide (Horwitz et al., 1971; Kariyone et al., 1971), reduction to the methylenamine (Leimgruber et al., 1965), or dehydration to the imine in the case of sibiromycin (Mesentsev et al., 1974) all lead to loss of biological activity. In the latter case, the imine is conjugated into the pyrrolo ring and is therefore unreactive to nucleophilic attack. The facile conversion of anthramycin 11-methyl ether to the two isomers of anthramycin when dissolved in water is further evidence for the high reactivity of this group to nucleophilic attack. We have suggested earlier (Hurley et al., 1977) that anthramycin undergoes a nucleophilic attack via a S_N1ca mechanism from some reactive group (i.e., 2-amino group of guanine) on DNA (Figure 3). Lown has claimed the imine is the more likely DNA reactive species, based mainly upon the fact that the N-10 acetyl derivative is unreactive toward DNA (Lown & Joshua, 1979). However, this is not necessarily a valid argument since the N-10 acetyl group sterically prevents access of the carbinolamine to the exocyclic amino group of guanine.

Table II: Saturation Binding of [15-^{3}H]Anthramycin to Calf Thymus DNA, T-4 DNA, and *Clostridium perfringens* DNA[a]

origin of DNA	% G–C composition[b]	binding ratio[c] (mol of drug/mol of DNA) (nucleotide)
calf thymus	43	0.083
C. perfringens	31	0.074
T-4[d]	34	0.066

[a] Saturated [15-^{3}H]anthramycin-DNA adducts were prepared by incubation of 1 μmol of DNA with 4 μmol of [15-^{3}H]anthramycin (sp act. = 5.22×10^{-2} μCi/μmol) in 0.1 M SSC (1 mL) for 2 h at room temperature and overnight at 4 °C. Unbound anthramycin was extracted from anthramycin–DNA complex as described before (Hurley et al., 1977). [b] See Szybalski & Szybalski (1970b). [c] Binding ratios were determined independently by an absorption method (Kohn & Spears, 1970) and a mixed absorption–radioactivity determination (Hurley et al., 1977), and these values never differed by more than 4.8%. [d] 100% glucosylation (70% α- and 30% β-glucosyl groups on the hydroxymethyl derivatives of cytosine) in the major groove of DNA (Kornberg, 1962).

(4) CPK Space-Filling Models for the DNA Adducts of Anthramycin, Tomaymycin, Sibiromycin, and Neothramycins A and B. The results described so far allowed us to predict with some assurance the structure of the anthramycin–deoxyguanosine adduct (see Figure 3). X-ray diffraction studies on crystal of anthramycin methyl ether show that the molecule is twisted 40–50° from one end to the other along the long axis, and this might fit into one of the grooves of DNA (Mostad et al., 1978). Model building with CPK molecular models of DNA and anthramycin in the published crystals conformation demonstrated that adduct formation through N-2 of guanine and C-11 of anthramycin produces a remarkably snug fit (Hurley & Petrusek, 1979). In this model, the anthramycin molecule is attached through C-11 to N-2 of guanine by an aminal linkage and lies along the narrow

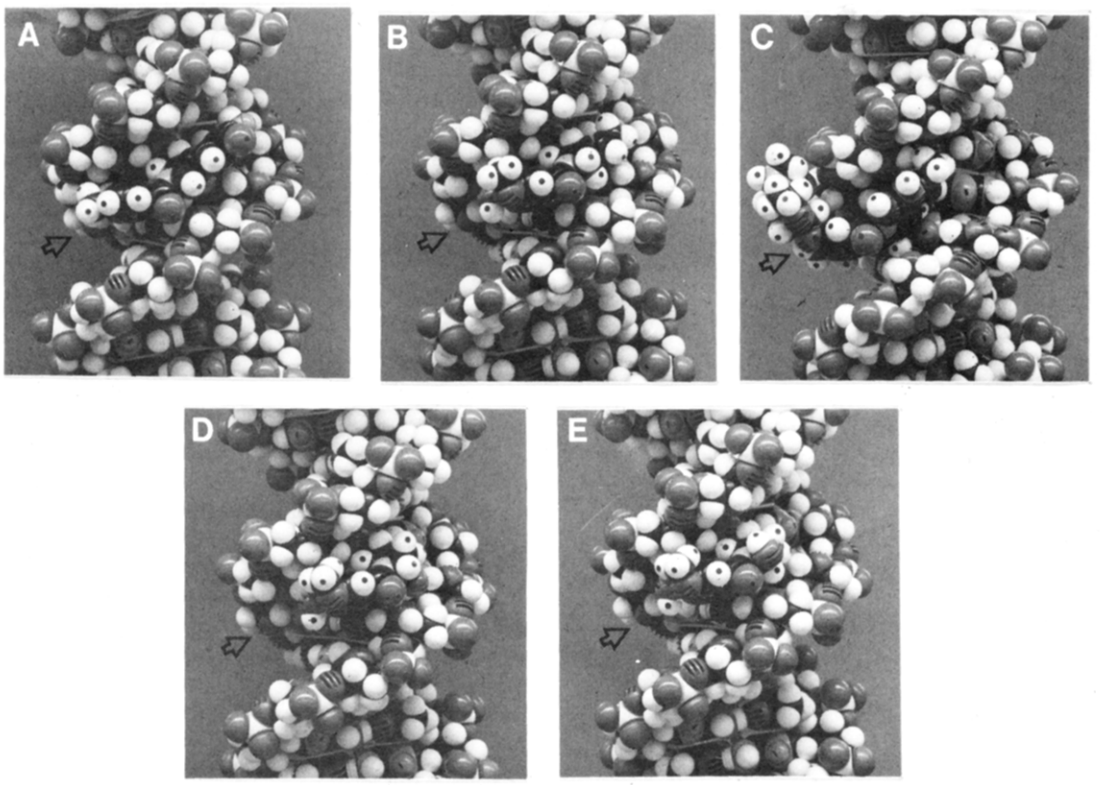

FIGURE 4: CPK space-filling model of the (A) anthramycin–, (B) tomaymycin–, (C) sibiromycin–, (D) neothramycin A–, and (E) neothramycin B–DNA adducts in which anthramycin is attached through C-11 to the N-2 group of guanine.

groove of DNA (Figure 4A). The model has been extended to include the tomaymycin, sibiromycin, and neothramycin A and B adducts (Figure 4B–E) (Hurley et al., 1980). In each of these CPK models, the drug fits snugly within the narrow groove without distortion of the helix. The bulky amino sugar of sibiromycin is the only part of any of the antibiotics which extends outside the groove of DNA (Figure 4C).

The CPK models predict hydrogen-bonding stabilization through both protons at N-10 and C-9 anthramycin and sibiromycin. With the exception of sibiromycin, the N-10 proton of each of the antibiotics should be hydrogen bonded to the 2-keto group of thymine or cytosine or N-1 of guanine or adenine in the adjacent base pair on the same strand to which the drug is covalently bound. The replacement of the common sp_3 carbon atom at C-11a in anthramycin, tomaymycin, and the neothramycins by an sp_2 carbon atom in sibiromycin should affect the conformation of the seven-membered ring such that the N-10 proton hydrogen bonds to the 2-keto group in cytosine in the same base pair to which the drug is covalently bound. The phenolic proton at C-9 of anthramycin should be hydrogen bonded to the 2-keto group in cytosine in the same base pair to which the drug is covalently bound whereas the equivalent proton in sibiromycin should be hydrogen bonded to the 2-keto group of thymine or cytosine or N-1 of guanine or adenine in the adjacent base pair on the same strand to which the drug is covalently bound. In the case of tomaymycin and the neothramycins, hydrogen-bond stabilization occurs through the N-10 proton in an analogous manner to that of anthramycin. In sibiromycin, the amino sugar may further stabilize the adduct by interaction with the deoxyribose phosphate backbone of DNA.

(*B*) *Evidence Which Supports the Proposed Structures of the Pyrrolo[1,4]benzodiazepine Antibiotic–DNA Adducts.* For substantiation of our proposed structures of the various drug–DNA adducts, predictions made from the CPK models were tested experimentally. In all cases, the experimental data support our proposed CPK models for the pyrrolo[1,4]-benzodiazepine–DNA adducts.

(*1*) *Stability of the Drug–DNA Adducts.* According to our models, the drugs are held to DNA by an aminal linkage plus secondary hydrogen-bonding stabilizing forces as previously described. As the aminal linkage is only stable when the exocyclic amine cannot be protonated (i.e., alkaline conditions), then in the absence of the secondary stabilizing forces (hydrogen bonding), the drugs should be released from their DNA adducts. We have previously shown that the anthramycin–, tomaymycin–, and sibiromycin–DNA adducts are unstable under acidic conditions in which denaturation of DNA occurs (Hurley et al., 1977). However, the drug–DNA adducts are reasonably stable at neutral and alkaline conditions, even at alkaline pHs at which denaturation occurs (Hurley et al., 1977). Significantly, the stability under acidic conditions increases in the order tomaymycin, anthramycin, and sibiromycin, which follows the order of increases in the ΔT_m values of each of the DNA adducts, a pattern which can be predicted on the basis of the number of secondary hydrogen-bonding interactions in our CPK models of the various drug–DNA adducts.

Although the anthramycin– and sibiromycin–DNA adducts are very stable at neutral pH at temperatures below the melting point of their DNA complexes, at temperatures in excess of their melting points rapid loss of drug occurs from the complexes (Hurley et al., 1979b; L. H. Hurley and R. L. Petrusek, unpublished experiments). The tomaymycin–DNA adduct is less stable to neutral conditions, presumably due to its lower inherent hydrogen-bonding stabilization, but again this adduct is unstable to temperatures above the melting point of the drug–DNA complex (L. H. Hurley and R. L. Petrusek, unpublished experiments). Finally, we have demonstrated that enzymatic denaturation of DNA with DNase and venom phosphodiesterase at 37 °C leads to release of intact anthramycin (Hurley et al., 1979b).

Irrespective of whether acidic, thermal, or enzymatic conditions lead to denaturation of DNA and loss of radiolabeled product from the ^{3}H-labeled drug–DNA adducts, we have demonstrated by chromatography that the released material is unchanged antibiotic.

Whereas acid denaturation of DNA leads to complete release of anthramycin, tomaymycin, or sibiromycin from DNA, both heat and enzymatic degradation appear to lead to incomplete release, based upon butanol extraction procedures. The apparent incomplete loss in the case of thermal or enzymatic denaturation (37 °C for 8 h) we feel is most likely due to the extreme chemical instability of free anthramycin. We cannot, however, completely rule out at this time a minor adduct between these drugs and guanine which is acid labile but stable to heat and enzymatic degradation in addition to the major adduct described in this paper.

(*2*) *Saturation Binding of the Drug–DNA Adducts.* According to our CPK models, the binding of anthramycin and releated drugs to DNA covers three base pairs. With the assumption of a random distribution of guanine, occurring at a frequency of 21.5% in calf thymus DNA, then a statistical analysis of binding sites shows that a maximum of 69.3% of the bases will be covered by anthramycin; i.e., the maximum binding ratio will be about 1:9.6. In practice, at maximum binding the sibriomycin, anthramycin, and tomaymycin drug to base ratios are 1:8.8, 1:12.9, and 1:18.2, respectively (Hurley et al., 1977). The sibiromycin/base ratio is almost exactly what we would predict, while the somewhat lower binding of anthramycin and tomaymycin may be due to the more critical conformational selectivity of these drugs toward DNA, compared to sibiromycin.[3]

(*3*) *Effect of Drug Binding on the Structure of DNA.* One of the most attractive features of our CPK models of the various drug–DNA adducts is that the covalent binding does not require any distortion of DNA, unlike DNA adducts with such compounds as AAAF, BMBA, and intercalating agents. Furthermore, with the exception of the amino sugar of sibiromycin, no protrusion of any of the drug molecules outside the helix is predicted. In order to probe the modified DNA for distortion, S_1 nuclease digestions and benzoylated, naphthoylated DEAE-cellulose (BND-cellulose) chromatography were carried out. The S_1 nuclease results (Hurley & Petrusek, 1979; Hurley et al., 1980) on the anthramycin–, tomaymycin–, sibiromycin–, and neothramycin–DNA adducts show that this enzyme was unable to reveal any regions of distortion in the drug-modified DNA, and, in fact, release of nucleotides was inhibited with increasing amounts of drug modification. The BND-cellulose chromatography results with anthramycin (Hurley & Petrusek, 1979) and sibiromycin (L. H. Hurley and R. L. Petrusek, unpublished experiments) demonstrated lack of any single strandedness of DNA induced by these drugs. Although the amino sugar of sibiromycin protrudes outside the DNA helix according to our CPK models, this did

[3] According to our CPK models, the 11a sp_3 carbon atoms of anthramycin, tomaymycin, and neothramycins dictate a closer and snugger fit along the narrow groove of DNA than the equivalent sp_2 carbon in sibiromycin, and this may explain their somewhat lower reactivity toward DNA.

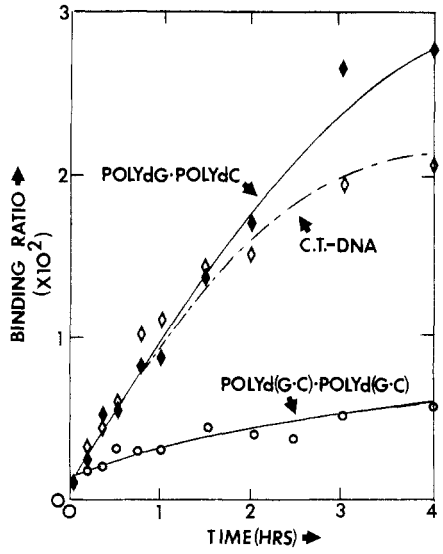

FIGURE 5: Time-course binding of anthramycin to calf thymus DNA, poly(dG)·poly(dC), and poly(dG-dC)·poly(dG-dC). [15-^{3}H]-Anthramycin (sp act. = 2.63 μCi/μmol) in the amount of 7.80×10^{-2} μM was added to 4.5 mL of a 5×10^{-5} M solution of DNA of synthetic polydeoxynucleotide in SSC buffer (pH 7.2) at 23 °C. Aliquots (0.2 mL) of the reaction mixture were removed at various intervals, diluted into 0.8 mL of water, and rapidly extracted twice with 2 × 1 mL of water-saturated 1-butanol to remove unreacted antibiotic, and a 0.5-mL aliquot was removed from the aqueous layer for radioactivity determination.

not cause the sibiromycin–DNA adduct to adhere to the resin.

(*4*) *Unwinding and Strand Breakage of SV-40 DNA in Vitro.* Our CPK models predict no unwinding or strand breakage in vitro of the drug-modified DNA. Covalently closed superhelical DNA such as SV-40 FI DNA is a sensitive probe for detection of single- and double-strand scission as well as a means to monitor unwinding of DNA. A single-strand break permits the DNA strands to unwind, resulting in the relaxed FII DNA. Introduction of another break adjacent to the first, but on the opposite strand, gives the linear FIII DNA.

Binding of anthramycin to SV-40 DNA up to maximum drug to base ratios of 1:125 did not produce any increase in the proportion of SV-40 FII DNA or SV-40 FIII DNA over the untreated control (Hurley et al., 1978). The absence of intermediate bands between SV-40 FI and FII DNA on the gel eliminates the possibility that anthramycin causes helix unwinding of DNA. Similar results have been obtained with sibiromycin (Kozmyan et al., 1978).

(*5*) *Sensitivity of the Secondary Structure of DNA to Drug Binding.* The CPK space-filling models of the various pyrrolo[1,4]1benzodiazepine–DNA adducts show that the drugs fit very snugly into the narrow groove of DNA. The right-handed twist of anthramycin, tomaymycin, and the neothramycin molecules follows the contour of the narrow groove so closely that it almost appears that the drugs were tailor-made to accommodate specifically to DNA in the B form as a molecular target. It is therefore anticipated that the drugs will show a high specificity for DNA in the correct configurational form and might even have sequence preferences if these different sequences produced local regions of slightly changed secondary structure in DNA. Kohn and co-workers had shown earlier that anthramycin shows a strict specificity for DNA or deoxyguanosine in a double-stranded template (Kohn et al., 1974). We have extended these results to compare the reactivity of poly(dG)·poly(dC) and poly(dG-dC)·poly(dG-dC) with calf thymus DNA in form B. The results (Figure 5) show that anthramycin has a much higher reactivity toward poly(dG)·poly(dC) and linear DNA than toward poly(dG-dC)·poly(dG-dC). Significantly, in the case of poly(dG-dC)·poly(dG-dC), after extended periods of time (24 h), the final extent of binding was almost exactly one-half of that of the homocopolymer or calf thymus DNA [i.e., antibiotic/base ratio 1:25 for poly(dG-dC)·poly(dG-dC) vs. 1:12 and 1:12.5 for the homocopolymer and calf thymus DNA, respectively]. Anthramycin, tomaymycin, and the neothramycins have two phases of reaction with DNA, an initial fast reaction which takes place over a 20–50-min period followed by a slower second phase which takes up to 24 h to reach saturation binding under the conditions described in the legend to Figure 5. We feel this may very well represent binding to guanines in at least two different sequences. In the case of the fast reaction, this may be reaction of anthramycin with a G-G sequence, such as occurs in poly(dG)·poly(dC), which proceeds much more rapidly than with the G-C sequence found in poly(dG-dC)·poly(dG-dC). The slow reaction then consists of reaction of anthramycin with the sequence G-X (X may be C, A, or T). Superimposition of the fast (G-G sequence) and slow (G-x sequence) reactions then leads to the decrease in the exponential reaction found in native DNA. These results suggest that different base sequences occurring in DNA lead to slightly different regional configurational forms, which can be distinguished by drugs such as anthramycin which fit very snugly along a contour of DNA.

X-ray diffraction patterns on fibers of poly(dG)·poly(dC) (Arnott & Selsing, 1974) and poly(dG-dC)·poly(dG-dC) (Arnott et al., 1974) show that both species have molecular structures rather different from classical DNA forms and also from one another. The poly(dG)·poly(dC) has a preference for the A conformation (which has the C3-endofuranose ring) while the poly(dG-dC)·poly(dG-dC) produces a diffraction pattern called D-DNA, with eight residues per turn. More recent results on poly(dG-dC)·poly(dG-dC) by X-ray diffraction of orientated fibers (Arnott et al., 1980) and on the fragment d(CpGpCpGpCpG) by X-ray diffraction of a crystal (Wang et al., 1979) have shown that these forms exist in left-handed DNA helices (Z-DNA). In view of these markedly different molecular structure for poly(dG)·poly(dC) and poly(dG-dC)·poly(dG-dC), it is not surprising that anthramycin shows significantly different reaction kinetics with these two forms. Studies with other drugs have other characteristics; for example, while mitomycin C binds equally well to poly(dG)·poly(dC) and poly(dG-dC)·poly(dG-dC) (Lipman et al., 1978), actinomycin D binds appreciably better to DNAs which contain both purines and pyrimidines in both strands (Wells, 1969; Wells & Larson, 1970). The fact that there are obviously sequence specificities due either to direct base–drug interactions or to the effect of base sequence on the configuration of DNA may be important in understanding the mechanism of action of these compounds.

During the course of our experiments with closed-circular supercoiled SV-40 FI DNA to examine intercalation as a possible mechanism for drug binding, we discovered the SV-40 FI DNA had a much lower reactivity toward anthramycin than SV-40 FIII DNA (see Figure 6). Several explanations for the lower reactivity of anthramycin to supercoiled DNA vs. linear DNA can be proposed. However, we would like to suggest that the torsional stress in supercoiled DNA which can induce helix coil transitions (Vinograd et al., 1968; Burke & Bauer, 1980), which move up and down the macromolecule between G–C-rich areas, may reduce anthramycin binding. The slowness of the reaction of anthramycin with DNA relative to the helix coil transition or, alternatively, the melting and unzipping of long lengths of DNA may result in loss of

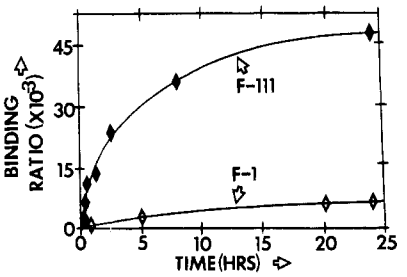

FIGURE 6: Time-course binding of [15-^{3}H]anthramycin to SV-40 FI and FIII DNA. [15-^{3}H]Anthramycin (sp act. = 86.6 μCi/μmol) in the amount of 4.45×10^{-3} μM in 10 μL or 0.6×10^{-3} μM in 59 μL of methanol was added to 1.2 μg in 287 μL (SV-40 FI DNA) or 1.2 μg in 1.2 mL (SV-40 FIII DNA) in 0.1 M SSC and 2 mM EDTA buffer, pH 8, at 23 °C. At various times, aliquots of the reaction mixtures were removed and extracted twice with twice the volume of H_2O-saturated *n*-BuOH. Half of the aqueous phase was then electrophoresed and assayed for anthramycin binding as described under Experimental Procedures.

already bound anthramycin molecules and would explain our results. We cannot, however, exclude other possibilities such as changes in the rotation angle of the DNA helix (Upholt et al., 1971), distortion due to bending under torsional stress (Bauer & Vinograd, 1970), or even regions of Z-DNA (Davies & Zimmerman, 1980) in supercoiled DNA.

(*6*) *Physical Properties of the Anthramycin–DNA Adduct.* The physical properties of drug-modified DNA should be predictable from the CPK models of the pyrrolo[1,4]benzodiazepine–DNA adducts. According to our CPK models, saturation binding of the pyrrolo[1,4]benzodiazepine antibiotics to DNA results in the formation of an "artificial third strand" within the narrow groove of DNA made up of end-to-end antibiotic molecules. The most obvious effect of this type of binding would be stiffening, without causing lengthening, of the helix. Therefore, increases in the viscosity of high molecular weight DNA at a relatively high degree of binding would be anticipated. On the other hand, low molecular weight DNA, which already exists in short rodlike structures, will not be expected to undergo viscosity increases upon anthramycin binding. These anticipated results are exactly mimicked in practice (Glaubiger et al., 1974). These same authors did not report any significant changes in the sedimentation of either low or high molecular weight DNA upon binding of anthramycin, which they state argues against any lengthening effect of drug binding. This is also in accord with our proposed CPK model.

Direct measurements from our CPK model show that the angle of the chromophore of anthramycin is inclined at 45–55° relative to the helix axis. Electric dichroism measurements reported by Glaubiger et al. (1974) indicate the chromophore is inclined less than 54.7° and at an angle of approximately 36° relative to the helix axis, which is in reasonable agreement with our model.

(*7*) *Hydrogen Bonding of the 9-Phenolic Proton of Anthramycin.* Examination of the CPK models of the anthramycin–DNA adduct reveals that the phenolic group at C-9 is hydrogen bonded to the 2-keto group of cytosine in the same base pair to which the drug is covalently attached. It would therefore be anticipated that this phenolic hydroxy proton would not be available for titration when the drug is bound to DNA. In practice, pH titration curves for the anthramycin–DNA adduct (Kohn et al., 1974) demonstrate that the phenolic proton is absent. Circular dichroism (CD) measurements on anthramycin and its adduct with native and denatured DNA reveal some pertinent results, related to the absence or presence of the phenolic hydroxy proton (Glaubiger et al., 1974). By comparison of the CD spectra of anthramycin at pH 10.3, which would be n the deprotonated form (pK_a = 8.7), with those of the DNA adduct, these authors conclude that anthramycin is in the high-pH or deprotonated form when complexed to DNA. Equally important is that examination of the published CD spectra of anthramycin bound to denatured DNA shows that in this case the anthramycin appears to have partially reverted to the protonated form. This again is precisely what would be predicted from our CPK models, since in the denatured form the drug is bound only to one strand and the secondary hydrogen-bonding interaction between the phenolic hydroxy proton and the 2-keto group on cytosine on the opposite strand would be absent.

(*8*) *Structure–Activity Relationships for Derivatives of the Pyrrolo[1,4]benzodiazepine Antibiotics.* An important consequence of our CPK model is the prediction of structure–activity relationships for derivatives of the pyrrolo[1,4]-benzodiazepine antibiotics. Fortunately, a number of derivatives of anthramycin, tomaymycin, sibiromycin, and the neothramycins are available, which have been evaluated for their ability to react with DNA. Most importantly, these derivatives in some cases bear only minor changes in structure (e.g., isomeric forms) but show drastic differences in their ability to react with DNA. The experimental results reported in this section fit precisely and without exception into our proposed CPK models, supplying compelling evidence for our proposed structures for these DNA adducts.

(*i*) *Substituents on the Aromatic Ring.* According to our proposed models, substituents at C-9 point directly in toward the narrow groove of DNA whereas substituents at C-6, C-7, or C-8 point away from the groove and therefore should not affect drug binding. In practice, the bulky amino sugar of sibiromycin at C-7 does not prevent binding to DNA and may in fact stabilize the adduct by hydrogen bonding to the deoxyribose phosphate backbone of DNA. According to our CPK model, the phenolic group at C-9 of anthramycin and sibiromycin is sterically allowed and should in fact stabilize the adduct due to hydrogen bonding. This may be one of the reasons why the sibiromycin– and anthramycin–DNA adducts are more stable than the tomaymycin– or neothramycin–DNA adduct, which lacks a phenolic group at C-9. The 9-methoxy derivatives of anthramycin and sibiromycin do not react with DNA and are biologically unreactive (Horwitz et al., 1971; Stefanovic, 1968; Kozmyan et al., 1977), which is predictable from a CPK model since the methoxy group at C-9 is too large to be accommodated within the narrow groove of DNA with concomitant bond formation.

(*ii*) *Substituents on the Carbinolamine Group.* The CPK models show that the carbinolamine group of all the antibiotics is in direct contact with the inside of the narrow groove. Therefore, as expected, the *N*-acetyl derivative of anthramycin does not react with DNA (Lown & Joshua, 1979). As previously described in this paper, variations on the carbinolamine structure, such as an amide, methylenamine, or imine in the case of sibiromycin and 1,11a-didehydroanhydroanthramycin (Malhotra et al., 1981), are unreactive toward DNA because this is the alkylation group on the pyrrolo[1,4]benzodiazepine antibiotics (Hurley, 1977; Lown & Joshua, 1979). 11-*O*-Alkyl substituents such as in the 11-methoxy or 11-ethoxy derivatives of anthramycin will react with DNA; however, these groups are lost either during the reaction with DNA or prior to this reaction by hydrolysis in aqueous media.

(*iii*) *Stereochemistry at C-11a of Anthramycin, Tomaymycin, and Neothramycins A and B.* The stereochemistry at C-11a of these antibiotics determines whether the drugs have

a right- or left-handed twist along the length of the molecules (Mostad et al., 1978). Since the correct configuration is required to follow the right-handed twist of DNA in the B form, then only those molecules having the 11a-*R* configuration should, according to our model, react with DNA. As anticipated, the only naturally occurring forms of the pyrrolo[1,4]benzodiazepine antibiotics to be isolated are the 11a-*R* isomers.

(*iv*) *Substituents on the Pyrrolo Ring.* The pyrrolo ring has various degrees of unsaturation in tomaymycin and neothramycins (none), anthramycin (one), and sibiromycin (two). Where sp_3 carbon atoms exist at C-2 or C-3, then the steroechemistry of the substituents becomes an important factor in determining whether the drugs will react with DNA. Substituents at C-2 and C-3 which point toward the inside of the groove can only be accommodated if they are small. On the other hand, substituents at C-2 and C-3 can be much larger, providing they project away from the narrow groove. Derivatives in which there are substituents other than hydrogens at C-1 should not react with DNA; however, these are not available, and, therefore, speculations remain untested for these cases. However, in the case of C-3, an ideal series of compounds have been prepared and tested for reactivity toward DNA. These are the isomeric neothramycins A and B and their 3-methoxy and -butoxy derivatives which have been tested by Maruyama et al. (1979), using fluorescence enhancement meausurements, for their ability to bind the calf thymus DNA. Individual members of the three isomeric pairs show surprisingly different DNA binding abilities, such that while neothramycin A binds *more* strongly to DNA than do neothramycin B and its 3-methoxy and 3-butoxy derivatives, the 3-methoxy and 3-butoxy derivatives of neothramycin A *do not* bind to DNA. Reassuringly, this is *precisely* what would be predicted from the CPK models of the neothramycin A and B adducts, since only in the case of neothramycin A is hydrogen bonding between the 3-hydroxy proton and O-1′ of the deoxyribose phosphate backbone of DNA possible. This explains the stronger binding of neothramycin A relative to neothramycin B and its derivatives. Furthermore, steric hindrance due to methylation or butylation of neothramycin A at position 3 prevents *only* these derivatives of this isomer of neothramycin from binding to DNA.

The presence of an sp_2 carbon atom at C-2 of anthramycin, tomaymycin, and sibiromycin projects substituents at this position directly along the narrow groove of DNA. Therefore, the ethylidene, propylidene, and acrylamide side chains of tomaymycin, sibiromycin, and anthramycin do not prevent drug binding to DNA, and it is possible that the amide protons may help stabilize the anthramycin–DNA adduct by hydrogen bonding to base pairs one or two removed from the point of covalent attachment.

(*9*) *Biological Consequences of DNA Damage by the Pyrrolo[1,4]benzodiazepine Antibiotics.* The in vitro studies on anthramycin and related drugs reveal certain unique features of the adducts which might be predicted to cause unusual biological consequences. For example, because the drugs are well hidden within the narrow groove, and do not cause detectable distortion of DNA, then recognition of the drug lesion and also identification of the damaged strand may be difficult for excision-repair complexes. A second peculiarity of the adducts is their instability to denaturation at physiological pH, and the fact that upon denaturation the active drug is released. This may result in cumulative damage as the drug "walks the DNA" through cycles of excision removal and rebinding to DNA. A third potentially important feature of the reaction between anthramycin and related drugs with DNA is the selective ability to bind only to a double-stranded template. Therefore, reactions which take place on an already single-stranded template, such as the synthesis of the L strand of mitochondrial DNA (Robberson et al., 1972; Berk & Clayton, 1974), will not be inhibited by these drugs whereas those in which a double-stranded template is required will be inhibited by anthramycin.

These properties can be used to rationalize a number of previously unexplained biological effects of anthramycin. The relatively slow excision repair (Hurley et al., 1979) and high potency can be attributed to a slow endonucleolytic recognition and/or the ability of the drug to undergo cycles of binding and excision removal to and from DNA. The production of excision repair dependent double-strand breaks in DNA in human cells exposed to anthramycin (L. H. Hurley, unpublished experiments) may be rationalized on the proposed inability of repair complexes to identify only one of the two strands of the duplex as carrying the anthramycin lesion. Consequently, simultaneous repair occurring on opposite strands toward a single anthramycin lesion may result in near-overlapping repair patches and double-strand breaks in DNA. Lastly, Gause and co-workers (1975, 1976) have shown that both anthramycin and sibiromycin selectively inhibit H-strand synthesis in mitochondrial DNA from animal cells. The explanation for this is believed to be that the synthesis of the L strand, which proceeds on a single-strand template, is relatively resistant to drug action while the H-strand synthesis, which proceeds on a double-stranded template to which drug is bound, is specifically inhibited (Gause et al., 1976).

Conclusions

The most important conclusions from this paper are the following: (1) Circumstantial evidence suggests that the points of covalent attachment between the pyrrolo[1,4]benzodiazepine antibiotics and DNA are C-11 on the drug molecules and N-2 of guanine. (2) CPK models of these drug–DNA adducts lead to predictions concerning the properties of the drug-modified DNA and biological consequences. Experimental results either described in this paper or already published in the literature support, without exception, these CPK models and thus provided strong evidence that our proposed structures are correct. (3) The CPK models of the various drug–DNA adducts described in this paper have important implications in both design of new synthetic pyrrolo[1,4]benzodiazepine antitumor agents and chemical carcinogenesis. Present and future research is directed along both of these lines.

Added in Proof

We have recently confirmed the points of covalent adduct formation reported in this paper. In these studies we utilized ^1H NMR on anthramycin–dinucleotide adducts and ^{13}C NMR on carbon-13-enriched anthramycin–DNA adducts (Ostrander et al., 1981).

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