Biochemistry

 Volume 21, Number 3 February 2, 1982

# Molecular Mechanisms of Binding and Single-Strand Scission of Deoxyribonucleic Acid by the Antitumor Antibiotics Saframycins A and C†

J. William Lown,* Alummoottil V. Joshua, and Jeremy S. Lee

ABSTRACT: The quinone antitumor antibiotics saframycins A and C bind to duplex DNA and G,C containing complementary oligodeoxyribonucleotides at pHs below 5.5 via selective protonation of the antibiotics at the N-12 positions in an equilibrium process. This binding shows minor groove specificity to T4 DNA for both antibiotics. Saframycin A exhibits an additional mode of covalent binding to DNA, which is reversible by heating, via an aminal linkage with the $NH_2$ group of guanine. Covalent binding of saframycin A is enhanced by prior reduction of the quinone moiety via a different mechanism with concomitant elimination of the nitrile group and plausibly involves an intermediate quinone methide. Both saframycin A and saframycin C when reduced in situ with NADPH or sodium borohydride cause single-strand breaks, but not double-strand breaks, in PM2 covalently closer circular DNA. This latter reaction proceeds via intermediate semiquinones, the identity and conformation of which are established by EPR. The DNA scission process which requires oxygen is mediated by the generation of $O_2^{-}\cdot$, $H_2O_2$ and $OH\cdot$, the latter of which species was identified by spin trapping. The strand scission of DNA is pH dependent and, unlike the binding of the antibiotics, is strongly inhibited by $Mg^{2+}$ and $Zn^{2+}$ ions.

Saframycins A and C (Figure 1) are members of a family of antibiotics isolated from the streptothricin-producing strain of *Streptomyces lavendulae* (Arai et al., 1977, 1980). The saframycins are yellow crystalline solids structurally related to the heterocyclic quinone antibiotics which include mitomycin C (Szybalski & Iyer, 1967), streptonigrin (Rao et al., 1963), and naphthridinomycin (Kluepfel et al., 1975). Unlike the latter examples, the saframycins contain two heterocyclic quinone moieties. These microbial metabolites exhibit wide-spectrum antimicrobial activity, but more particularly saframycin A is attracting interest because it exhibits extreme cytotoxicity toward cultured cells and shows antitumor activity against several experimental tumors including leukemias L1210 and P388 and Ehrlich carcinoma both in ascites and in solid forms (Arai et al., 1977; Ishiguro et al., 1978). Saframycin A has been shown to block RNA synthesis in cultured L1210 cells (Ishiguro et al., 1978). There is evidence then that DNA is one of the principal cell targets. The cellular action of saframycin A, in common with that of other quinone-containing antibiotics, has been shown to involve interaction with a DNA template rather than interference with replicative enzymes (Ishiguro et al., 1978).

One of the modes of binding of the saframycins to DNA templates is specifically promoted by acids, and the sites of protonation on the antibiotics have been identified (Lown et al., 1981). However, the precise nature of the interaction or reaction between different saframycins and DNA has not be elucidated, and it has been suggested (Ishiguro et al., 1978) that in order to clarify the reactions a more sensitive assay employing covalently closed circular DNA should be carried out to detect any single-strand DNA breaks, double-strand breaks, or interstrand cross-linking.

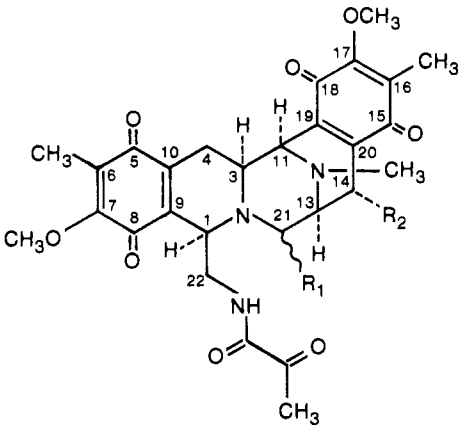
We report a further investigation of the binding of saframycins A and C to DNA demonstrating the specificity for binding in the minor groove. We also report the detection of single-strand scission of supercoiled covalently closed circular PM2-DNA by reductively activated saframycins A and C and the study of its chemical mechanism. This involves the detection, characterization, and the assignment of conformation of the antibiotic semiquinone intermediates and identification of the superoxide anion, hydrogen peroxide and the hydroxyl radical which is the ultimate species giving rise to DNA lesions.

## Materials and Methods

*Chemicals and Enzymes.* Saframycins A and C were kindly supplied by Dr. Tadashi Arai, Division of Chemotherapy, Chiba Cancer Center Research Institute, Chiba, Japan. Saframycins A and C were further purified by preparative thin-layer chromatography on silca gel using ethyl acetate as

† From the Department of Chemistry (J.W.L. and A.V.J.) and the Department of Biochemistry (J.S.L.), University of Alberta, Edmonton, Alberta T6G 2G2, Canada. *Received July 1, 1981.* Part 27 of the series Studies Related to Antitumor Antibiotics. This investigation was supported by grants (to J.W.L.) from the National Cancer Institute of Canada, the Natural Sciences and Engineering Council of Canada, and the Department of Chemistry of the University of Alberta. J.S.L. acknowledges financial support from the Medical Research Council of Canada.

0006-2960/82/0421-0419$01.25/0 



| | | |
|---|---|---|
| Saframycin A | $R_1 = CN$, | $R_2 = H$ |
| B | $R_1 = H$, | $R_2 = H$ |
| C | $R_1 = H$, | $R_2 = OCH_3$ |
| S | $R_1 = OH$, | $R_2 = H$ |

FIGURE 1: Structures for saframycins A, B, C, and S.

eluant and by recrystallization from a mixture of ethyl acetate and petroleum ether (1:1). Ethidium bromide and superoxide dismutase (EC 1.15.1.1) were obtained from Sigma Chemical Co., St. Louis, MO, and catalase (EC 1.11.1.6) (beef liver) was from Aldrich Chemical Co., Milwaukee, WI. PM2 covalently closed circular (CCC) DNA (92% CCC) was prepared as described previously (Morgan & Pulleyblank, 1974). Calf thymus DNA was a product of Worthington while T4 and λ DNAs were purchased from Miles Biochemicals. The repeating sequence DNA polymers were synthesized and purified as described previously (Morgan et al., 1979).

*Antibiotic–DNA Binding Studies.* Thermal denaturation profiles ($T_m$ measurements) were measured on a Gilford Model 2400 spectrophotometer equipped with an insulated cell compartment attached to a Haake circulating waterbath. When the temperature was raised 0.5–1.0 °C/min, the $T_m$'s were reproducible to ±1 °C. DNA samples (0.3 mL) in the appropriate buffer were purged with helium and overlaid with paraffin oil. The buffers used were 40 mM sodium acetate–acetic acid at pH 5.0 or pH 5.5 and 40 mM sodium phosphate at pH 6.0.

*Ethidium Fluorescence Determination of Cleavage of PM2-CCC-DNA.* The fluorometric method of measuring strand breakage of PM2 covalently closed circular DNA (CCC-DNA) and its inhibition by enzymes and free radical scavengers has been described (Morgan & Pulleyblank, 1974; Cone et al., 1976; Lown et al., 1976, 1977). When the pH is raised to around 11.8, duplex DNA can still be detected very sensitively (as little as 0.1 μg) by the enhanced fluorescence of ethidium bromide which specifically intercalates duplex DNA. At these high pHs, any short intramolecular base pairing of denatured DNA is destabilized, and it assumes a true single-stranded conformation. At this high pH, CCC-DNA gives a quantitative reading of fluorescence (at lower pHs a denatured form of CCC-DNA is obtained), and this has been exploited as a sensitive assay for measuring breaks introduced into CCC-DNA by either nucleases or chemical means (Morgan & Pulleybank, 1974).

Ethidium bromide binds intercalatively to negatively supercoiled DNA and in consequence suffers an enhancement of fluorescence which is recorded. The conversion of PM2-CCC-DNA to nicked or open-circular (OC) DNA results in release of topological constraints, allowing more ethidium to intercalate, and consequently a characteristic 30% increase in fluorescence is observed in the pH 11.8 ethidium assay solution (see below). After heat denaturation (96 °C, 4 min) and cooling to 22 °C since the strands are now separable, a loss of fluorescence is observed in contrast to that of the control CCC-DNA which, we have seen, returns to duplex register. Therefore at any given time the loss of fluorescence after the heating and cooling cycle, compared to that of the control is proportional to the percentage of DNA suffering single-strand scission (Morgan & Pulleybank, 1974; Cone et al., 1976).

All measurements were performed on a Tuner Associate Model 430 Spectrofluorometer equipped with a cooling fan to reduce fluctuations in the xenon lamp source. Wavelength calibration was performed as described in the manual for the instrument. One-centimeter-square cuvettes were used. The excitation wavelength was 525 nm, and the emission wavelength was 600 nm. The 30× and 100× scales of medium sensitivity were generally used, and water was circulated between the cell compartment and a thermally regulated bath at 22 °C.

The reaction mixtures were buffered to the appropriate pH with potassium phosphate. The reactions were carried out in a total volume of 100 μL at 37 °C in deionized water. The reaction solution contained approximately 1.50 $A_{260}$ units of PM2-CCC-DNA (92% CCC and 8% OC), 50 mM buffer, and the appropriate concentrations of saframycin. At intervals, 10-μL aliquots were withdrawn and added to 2 mL of assay solution which contained 20 mM potassium phosphate, pH 11.8, 0.2 mM ethylenediaminetetraacetic acid (EDTA), and 0.5 μg/mL ethidium bromide (Morgan & Paetkau, 1972). The fluoresence was measured by using a blank without added sample. The solution was then heat denatured at 96 °C on a Temp-Blok for 4 min and cooled rapidly in an ice bath and then in a thermostated water bath at 22 °C for 5 min, and the fluorescence was read again. In a control experiment it was shown that none of the components interfered with the ethidium fluorescence.

*Agarose Gel Electrophoresis Determination of Single-Strand Scission of PM2-CCC-DNA by Reduced Saframycins A and C.* At intervals, 10-μL aliquots of the reaction mixture described above were withdrawn and added to 2 mL of the ethidium assay solution at pH 11.8, and the extent of DNA scission was monitored as described previously. Treatment of the PM2-CCC-DNA in this way was continued until the fluorescence assays showed that >90% of the initial CCC form of the PM2 DNA had been eliminated. The original reaction mixture was then analyzed by electrophoresis on 0.6% agarose gel run at a constant voltage of 25 V for 22 h. After electrophoresis, the gels were stained with ethidium and the different DNA bands visualized by their fluorescence under UV irradiation. The components identified in the different lanes are shown in Figure 5.

*Ethidium Fluorescence Assay for Detecting Covalently Linked Complementary (CLC) Sequences in DNA.* The fluorometric method of detecting CLC sequences in λ DNA has been described (Morgan & Paetkau, 1972; Lown et al., 1976). Cross-linking of DNA creates a nucleation site which allows renaturation of λ DNA after heat denaturation (96 °C/3 min) and rapid cooling and thus provides intercalation sites for ethidium. That this assay procedure detects the formation of CLC-DNA as a result of a chemical cross-linking event has been confirmed by experiments with the enzyme endonuclease $S_1$ (Morgan & Paetkau, 1972; Lown et al., 1976). This enzyme specifically cleaves single-stranded DNA, is essentially inactive on duplex DNA and therefore distin-

Table I: Characteristics of Binding of Saframycins to DNA

| DNA | control (°C) | saframycin A ($D/P$ = 0.12) (°C) | pH | $\Delta T_m$ (°C) | saframycin C ($D/P$ = 1.2) |
|---|---|---|---|---|---|
| calf thymus (42% G+C) | 74.0 | 80.0 | 5.0 | +6.0 | +2.1 |
| | 74.0 | 78.0 | 5.5 | +4.0 | 0.0 |
| | 74.0 | 74.5 | 6.0 | +0.5 | 0.0 |
| | 74.0 | 74.0 | 6.5 | 0.0 | 0.0 |
| $d(T\text{-}T\text{-}G)_n \cdot d(A\text{-}A\text{-}C)_n$ | 72.5 | 80.0 | 5.0 | +7.5 | |
| $d(T\text{-}A\text{-}C)_n \cdot d(G\text{-}T\text{-}A)_n$ | 70.5 | 76.0 | 5.0 | +5.5 | |
| $d(A\text{-}T\text{-}C)_n \cdot d(G\text{-}A\text{-}T)_n$ | 73.5 | 78.5 | 5.0 | +5.0 | |
| $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ | 78.5 | 83.0 | 5.0 | +4.5 | |
| $d(A\text{-}T)_n$ | 54.5 | 54.5 | 5.0 | 0.0 | |
| $dA_n \text{-} dT_n$ | 58.5 | 58.5 | 5.0 | 0.0 | |

guishes DNA which is renaturable by virtue of a chemical cross-link and DNA which separates into single strands on heating.

In the cross-linking assay a 20-$\mu$L aliquot was taken at intervals from the reaction mixture (50 mM potassium phosphate, pH 7.2; 1.0 $A_{260}$ unit of $\lambda$ DNA; $4 \times 10^{-4}$ M saframycin; total volume 200 $\mu$L) at 37 °C and added to the standard assay mixture (which was 20 mM potassium phosphate, pH 11.8, 0.2 mM EDTA, and 0.5 $\mu$g/mL ethidium). The fluorescence after the heating and cooling cycle compared with that of control times 100 gives the percentage of CLC-DNA in a sample. For a standard set of conditions (i.e., type and concentration of DNA, pH, ionic strength, and the temperature), the accuracy of the CLC assay is determined by the precision of the fluorescence readings. Overall accuracy of the CLC assay is estimated at ±2%.

*Electron Paramagnetic Resonance.* The EPR spectra were obtained on a Bruker ER-400 spectrometer fitted with a Varian V3601 12-in. magnet with VFR 2503 Hall effect controller operating at a nominal frequncy of 9.64 GHz. The microwave power incident on the cavity was attenuated to 8 dB below maximum. Hyperfine couplings were measured by comparison with peroxylaminedisulfonate (spacing 13.0 G) and $g$ values were obtained by direct Fieldial measurement.

(*a*) *Generation and Detection of Saframycin Semiquinones.* Stock solutions of the antibiotics with concentration of 1 mM were prepared in $Me_2SO$. The experiments were performed at room temperature in a total volume of 500 $\mu$L containing $3 \times 10^{-3}$ M saframycin and $1.2 \times 10^{-2}$ M aqueous $NaBH_4$, with access to atmospheric oxygen, and the EPR spectra of the semiquinones of the antibiotics were recorded. Control solutions prepared as above but containing no antibiotic showed that no radical species were generated.

Computer simulation (Lown et al., 1978) of the EPR spectrum of saframycin C semiquinone was carried out by using Lorentzian line shapes and with a line width of 0.2 G (Figure 6).

(*b*) *Spin Trapping and EPR Detection of Radical Species from Saframycin Semiquinones.* After the EPR spectra of the semiquinones were recorded and after the $NaBH_4$ was completely discharged, the solution was made $1 \times 10^{-3}$ M in aqueous phenyl-*N*-*tert*-butylnitrone (PBN) and 60 mM potassium phosphate buffer, pH 7.0. After incubation for 0.5–2 h in the presence of oxygen, the EPR spectrum of the PBN-OH radical was recorded. The presence of any remaining $NaBH_4$ to react with PBN would have been revealed by the generation of benzyl *tert*-butyl nitroxide, with an EPR spectrum of a triplet of triplets; hyperfine splitting values of $a^N$ = 16.2 G and $a^H$ = 10.5 G (Lown et al., 1978).

*Electrochemistry.* Polarographic studies on the saframycin antibiotics were carried out with a Princeton Applied Research Model 9300-9301 polarographic cell employed in a three-electrode configuration as described previously (Lown et al., 1979; Rao et al., 1977, 1978). The temperature was 37.5 °C throughout, and all potentials were measured with respect to the aqueous SCE but are reported with respect to the hydrogen ion electrode at pH 7. The drop time in the polarographic runs was 2 s; all solutions were deaerated with purified $N_2$ for at least 10 min prior to study.

Table II: Binding of Saframycins A and C to T4 DNA in Acetate Buffer at pH 5.0

| antibiotic | DNA | $D/P$ | $\Delta T_m$ (°C) |
|---|---|---|---|
| saframycin A | T4 | 0.04 | +2.0 |
| | | 0.12 | +5.0 |
| | | 0.40 | +6.5 |
| saframycin C | T4 | 0.04 | +1.0 |
| | | 1.2 | +2.2 |
| | | 2.5 | +3.5 |

## Results

*Binding of Saframycins A and C to DNA Templates.* Table I shows the effect of saframycins A and C on the $T_m$ of calf thymus and T4 DNAs at various pHs. For saframycin A binding to calf thymus DNA at pH 5.0, there is a maximum increase in $T_m$ of approximately 9 °C at a $D/P$ value of 0.8 (Table I). Although it is recognized to be difficult to estimate binding constants from $T_m$ data (McChee, 1976; Lee & Waring, 1978), the large $D/P$ value required to induce this change in $T_m$ suggests only weak or readily reversible binding under these conditions. The work of Ishiguro et al. (1981) and the present work have demonstrated that saframycin A can become covalently bound to DNA under certain conditions. It was next determined if the above data represented an equilibrium process. This was accomplished by preincubating calf thymus DNA with saframycin A ($D/P$ = 0.12) at pH 5 at 50 °C for either 45 or 90 min. It was found that this treatment had no additional effect on the $T_m$ of the DNA, suggesting that this was an equilibrium process. This does not rule out the contribution from covalent binding since we have found this to be reversible by heating by fluorescence measurements (see below). The equilibrium may already have been established by mixing the DNA with the drug and taking the complex up to the melting temperature.

The effect of pH on the binding of saframycin A to DNA is quite marked, and at pH values higher than 6.0, no change in $T_m$ can be detected. Comparison with saframycin C shows that even at pH 5 the change in $T_m$ is much smaller than with saframycin A and at higher pHs no binding can be detected.

The effect of saframycin A on the $T_m$ of T4 DNA at pH 5 is qualitatively similar to that of calf thymus DNA, although the changes are rather smaller (Table II). Since T4 DNA has glycosylation of the 5-(hydroxymethyl)cytidine residues which occlude the major groove (Erickson & Szybalski, 1964),

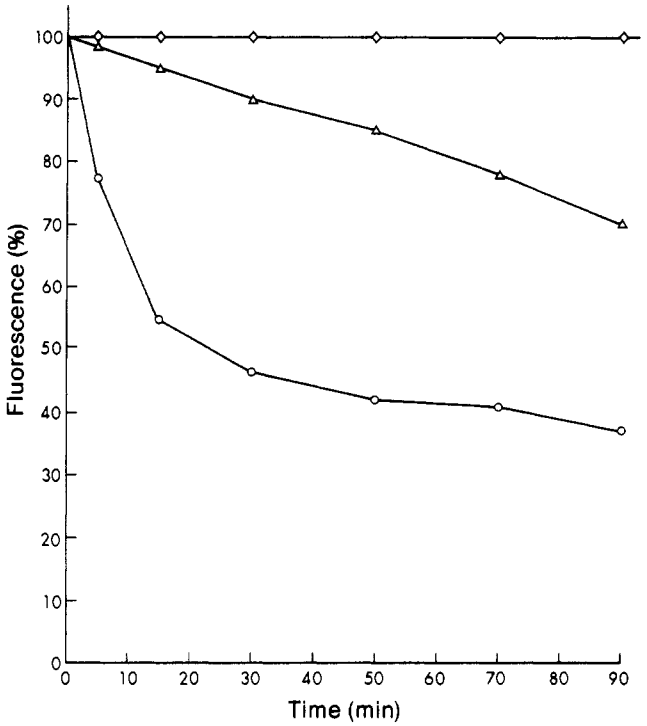



FIGURE 2: Binding of saframycin A to λ DNA at pH 4.7. The reaction mixture contained 22.0 μM antibiotic and 1.0 OD unit of λ DNA in 50 mM potassium phosphate buffer at pH 4.7 in 10% acetonitrile in aqueous solution. Additional components were (△) none, (○) 2.6 × $10^{-3}$ M sodium borohydride, and (◇) control or 22.0 μM saframycin A, or 22.5 μM saframycin C and 2.6 × $10^{-3}$ M sodium borohydride.

it would appear that saframycin A binds to DNA in the minor groove. A small but significant effect has also been found for saframycin C binding to T4 DNA (Table II) which therefore also shows minor groove specificity.

Table I also shows the effect of saframycin A on the $T_m$ of various repeating sequence DNAs. All DNAs containing G-C base pairs bind the drug while $d(A\text{-}T)_n$ and $dA_n \cdot dT_n$ do not. Similarly, it was found that saframycin C does not affect the $T_m$ of $d(A\text{-}T)_n$. Thus it would also appear that the saframycins require a G-C base pair for binding to DNA, and since this binding occurs in the minor groove, the 2-amino group of guanine is implicated in this process. In view of the preference for binding to G-C base pairs, the greater $T_m$ changes for saframycin binding to calf thymus DNA (42% G+C) compared with T4 DNA (34% G+C) are not unexpected.

The binding of saframycin A to DNA was independently determined by the prevention of intercalative binding of ethidium to DNA with the concomitant suppression of the characteristic fluorescence. This technique has the advantage over $T_m$ measurements of allowing the kinetics of the covalent binding process to be measured. This binding was found to be faster at lower pH values. Thus saframycin A at a concentration of 44.5 μM at 37 °C suppresses the ethidium fluorescence of calf thymus DNA by 30% and 12% at pH 4.7 and 5.0, respectively, in 2 (Figure 2). The reversibility of this type of binding is indicated by the recovery of the ethidium fluorescence of the PM2-CCC-DNA after the heat-denaturation–renaturation cycle. In contrast saframycin C shows no evidence for such covalent binding to DNA by this technique, although as described above, like saframycin A, it exhibits the equilibrium binding to DNA under lower pH conditions.

*Binding of Reduced Saframycins A and C to DNA Templates.* The inhibitory action of saframycin A against the template activity of DNA on *Escherichia coli* RNA polymerase in vitro is potentiated by the presence of reducing agents (Ishiguro et al., 1978). Accordingly we have also investigated the effects of reducing agents on the DNA binding of the saframycins. The efficiency of covalent binding of saframycin A (as evidenced by the suppression of ethidium fluorescence) is much greater under reducing conditions (Figure 2). For example, in the presence of 2.6 × $10^{-3}$ M sodium borohydride at 37 °C and pH 4.7, 22 μM saframycin A suppressed the fluorescence of the DNA–ethidium complex by 63% in 90 min compared with 30% in the absence of the reducing agents. Saframycin C had no detectable effect on the ethidium fluorescence under either nonreducing or reducing conditions at any pH in the range 4.7–8.0.

The covalent binding of reduced saframycin A to DNA shown by the suppression of ethidium fluorescence was not affected by the presence of the divalent metal ions $Mg^{2+}$ and $Zn^{2+}$ (data not shown) in contrast to their effects on the single-strand cleavage of DNA (see below). There was no evidence for interstrand cross-linking of λ DNA by either saframycin A or C in either oxidized or reduced form.

*Single-Strand Scission of DNA by Reduced Saframycins A and C and Its Chemical Mechanism.* For investigation of the nature of the chemical reaction of reduced saframycins on DNA, suitable reducing agents had to be selected. Although thiols such as dithiothreitol or 2-mercaptoethanol were the logical first choice by analogy with the in vivo situation, they were unsatisfactory in the in vitro experiments in that they led to significant background nicking of DNAs both of PM2-CCC-DNA in our experience and of φX174 RFI circular DNA or *Bacillus subtilis* DNA in the experience of the Japanese workers (K. Ishiguro, personal communication) and others (Bode, 1967). For this reason NADPH or sodium borohydride reduction was used since in controls no background nicking of PM2-CCC-DNA could be detected.

While neither component alone affected supercoiled circular DNA, saframycin A at a concentration of 4.5 × $10^{-5}$ M, and reduced in situ with 2.6 × $10^{-3}$ M sodium borohydride, caused 85% single-strand scission of PM2-CCC-DNA at pH 7 and 37 °C in 60 min (Figure 3). Treatment of the DNA with saframycin A prereduced with sodium borohydride produces exactly the same extent of DNA cleavage (Figure 3). Under comparable conditions saframycin C at 4.5 × $10^{-5}$ M produces 95% DNA single-strand scission in 60 min. As in the cases of the similar damage to DNA by daunorubicin or adriamycin (Lown et al., 1977), streptonigrin (Cone et al., 1976), and mitomycins B and C (Lown et al., 1976), the reductively activated saframycins react in the presence of oxygen to produce species which are capable of cleaving the strands of DNA. Although saframycins A and C produce comparable extents of strand scission in the presence of sodium borohydride at pH 7.0, saframycin C was more efficient in the presence of NADPH (Figure 4). Thus 45 μM saframycin C with 1.0 × $10^{-3}$ M NADPH afforded 92% nicking of PM2-CCC-DNA in 90 min at pH 7.0 and 37 °C whereas saframycin A under comparable conditions caused only 58% nicking. The difference in the rates of DNA scission may be related to the reduction potentials of the quinone moieties in the two antibiotics (Table III). Saframycin A has a slightly more positive reduction potential (on the hydrogen scale) for its quinone moiety.

Gel electrophoresis was applied to analyze the different forms of DNA, in order to confirm the single-strand scission of DNA by activated saframycins by an independent experiment and to test for double-strand breaks. PM2-CCC-DNA was treated with reduced saframycin A or C until the

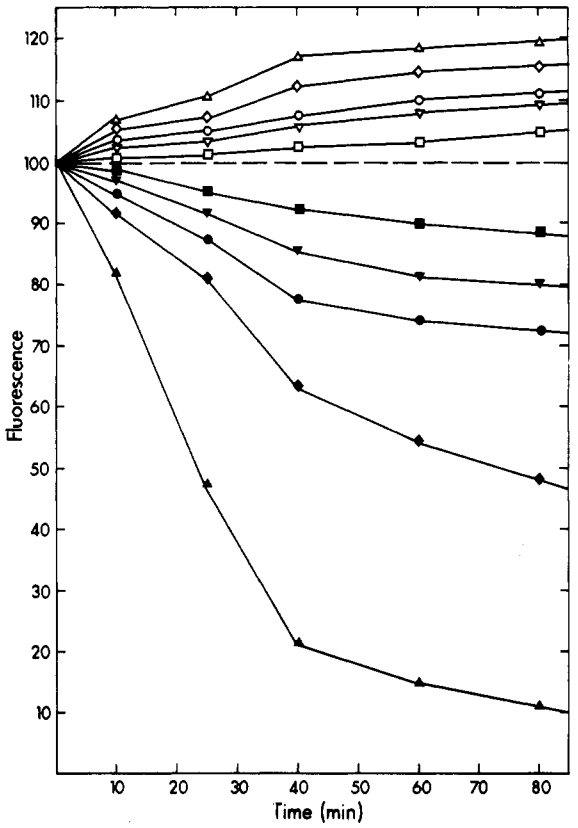



FIGURE 3: Strand scission of PM2-CCC-DNA by reduced saframycin A and its selective enzymatic inhibition. Reactions were performed at 37 °C in 40 mM potassium phosphate buffer, pH 7.0, and contained 1.0 OD unit/mL PM2-CCC-DNA (92% CCC), 45 μM of saframycin A in 10% acetonitrile, and 2.6 mM of sodium borohydride. The before heat-denaturation fluorescence readings are shown as open symbols, and the closed symbols are fluorescence readings after heat denaturation of the DNA at 96 °C for 4 min followed by rapid cooling to 22 °C. Additional components were (△) none or prereduced saframycin A, (○) $5 \times 10^{-5}$ g/mL catalase, (▽) $5 \times 10^{-5}$ g/mL superoxide dismutase, (□) 0.6 M isopropyl alcohol, (◇) $1.5 \times 10^{-2}$ M sodium benzoate, and (---) control.

Table III: Redox Characteristics of Saframycins A and B and Related Antibiotics and Reductants

| antibiotic | $E'_0$ (pH 7) (V) quinones | side chain |
|---|---|---|
| saframycin A | +0.4885[a] | −0.3685 |
| saframycin C | +0.4795[a] | −0.3485 |
| streptonigrin | +0.34[a] | |
| mitomycin C | +0.2935[b] | |
| mitomycin B | +0.4615[b] | |
| daunorubicin | +0.0415[c] | |
| adriamycin | +0.0415[c] | |
| glutathione | −0.23[d] | |
| $NAD^+$/NADH | −0.32[d] | |

[a] Present work. [b] Rao et al. (1977). [c] Rao et al. (1978). [d] Mahler & Cordes (1966).

fluorescence assay showed that >90% of the initial CCC had been removed. Analysis by electrophoresis on agarose gels revealed substantial formation of nicked or open-circular DNA but no detectable amounts of linear DNA that would result from double-strand breaks (Figure 5). The chemical mechanism of the single-strand breakage of DNA was investigated by adding selective inhibitors to the PM2-CCC-DNA solution containing the reduced saframycins.

The conclusion that the reduced saframycins react with dissolved oxygen to generate reactive species including hydrogen peroxide, superoxide anion, and hydroxyl radicals is

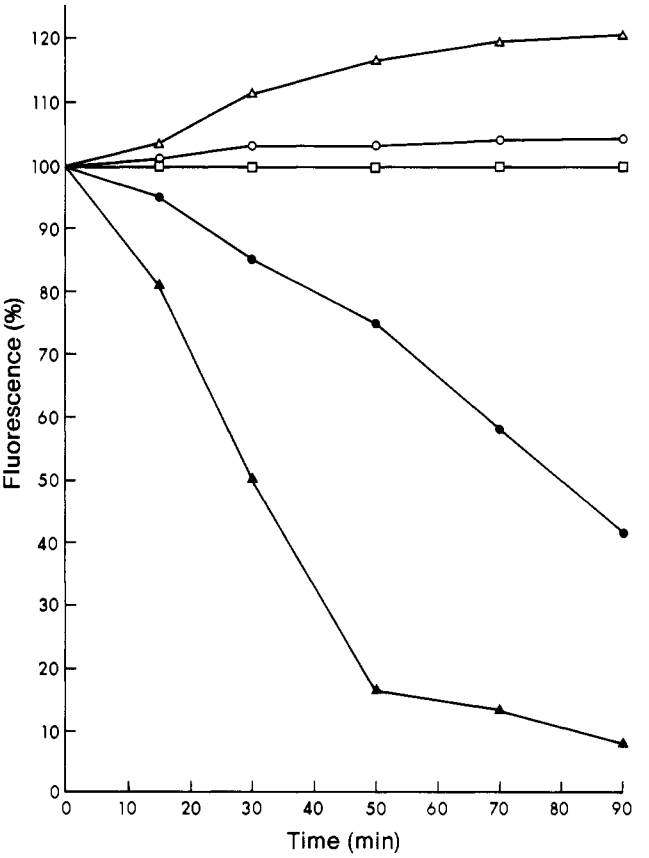



FIGURE 4: Single-strand scission of PM2-CCC-DNA by saframycins A and C in the presence of NADPH. Reactions were performed at 37 °C in 50 mM potassium phosphate buffer, pH 7.0, and contained 1.27 OD units of PM2-CCC-DNA (92% CCC), 45 μM of the antibiotic, and 1 mM of NADPH in 10% acetonitrile. The before heat-denaturation fluorescence readings are shown as open symbols, and the closed symbols are fluorescence readings after heat denaturation of the DNA at 96 °C for 4 min followed by rapid cooling to 22 °C: (○) saframycin A; (△) saframycin C; (□) control.

confirmed by the selective inhibition in the rate of DNA scission produced by catalase, superoxide dismutase, and general free radical scavengers such as isopropyl alcohol or sodium benzoate (Figure 3), respectively (Cone et al., 1976). In addition the fact that the heat-denatured enzymes catalase and superoxide dismutase under otherwise identical conditions do not inhibit the single-strand scission demonstrates that the previous inhibition was not due to nonspecific protein effects (Cone et al., 1976). The intermediacy of the hydroxyl radicals in the scission process is confirmed by spin-trapping experiments (Janzen, 1971; Harbour et al., 1974) in conjunction with EPR spectroscopy. Addition of the spin trap phenyl-*N*-*tert*-butylnitrone (PBN) in 50% aqueous dimethyl sulfoxide resulted in the generation of the EPR spectrum of PBN-OH spin adduct with its characteristic pattern of a triplet of doublets of $a^N = 16.0$ G, $a_\beta^H = 3.4$ G, and $g = 2.0061$ (Lai & Piette, 1977; Lown & Sim, 1977).

Similarly the intermediacy of the saframycin semiquinones was confirmed by their EPR spectra and computer simulation in the case of saframycin C (Figure 6). The computer simulation which indicates a $a_H^{Me} = 1.65$ G, $a_H^{4,5} = 1.55$ G, and $a_H^{8,9} = 1.40$ G signifies coupling to the quinone ring methyl group, but not appreciably to the methoxy, and to only four benzylic protons. This suggests an oscillation of the unpaired electron in the semiquinone species between the proximate quinone rings. The magnitude of the hyperfine couplings to the benzylic hydrogens also suggests a molecular geometry whereby benzylic protons H-4, H-5, H-8, and H-9 are in planes

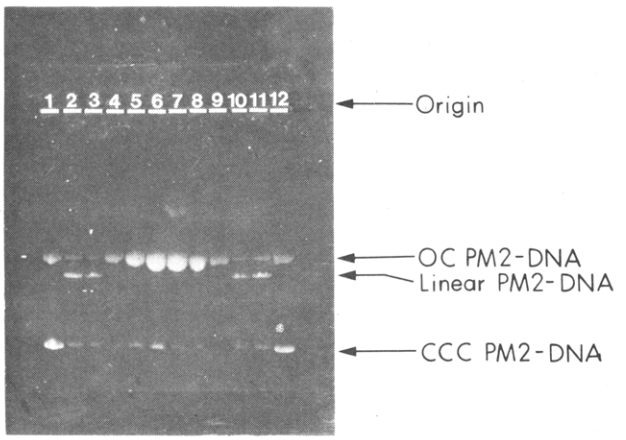



FIGURE 5: Agarose gel electrophoresis of PM2-CCC-DNA treated with in situ reduced saframycin A or C. PM2-CCC-DNA (92% CCC) at 1.0 OD unit in 50 mM potassium phosphate buffer, pH 7.0, was treated with 45 $\mu$M saframycin A or C and 2.6 mM sodium borohydride until the fluorescence assay showed that >90% of the initial CCC form had been eliminated. The reaction mixture was subjected to electrophoresis at 25 V for 22 h, and the bands on the washed gel were visualized by staining with ethidium bromide solution and UV irradiation. (Lanes 1 and 2) Initial PM2-CCC-DNA before the treatment with the antibiotic; (lanes 2, 3, 10, and 11) standard DNAs showing OC (open circular), linear, and CCC forms; (lanes 4–6) increasing amounts of PM2-treated with reduced saframycin C showing only OC- and CCC-DNAs; (lanes 7–9) increasing amounts of PM2-DNA treated with saframycin A showing only OC- and CCC-DNAs.

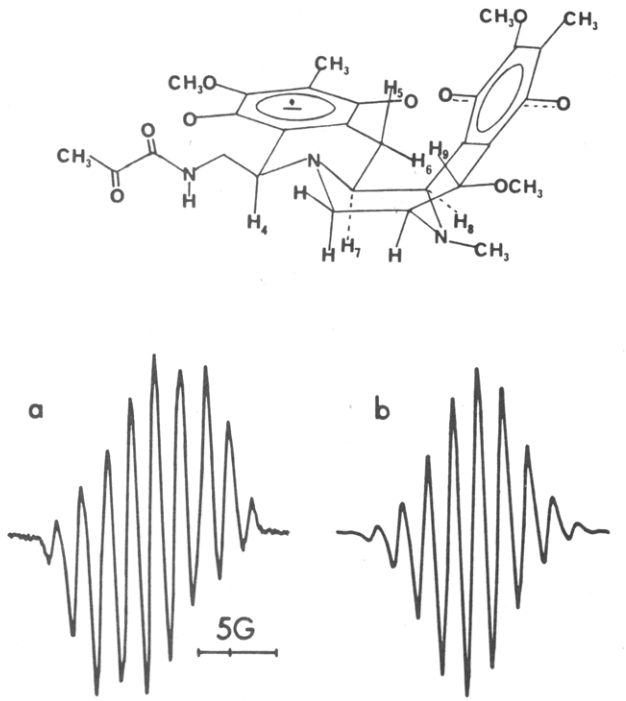



FIGURE 6: EPR spectrum (microwave power 12 dB, modulation amplitude 0.63 G, and scan time 1000 s) obtained from $3 \times 10^{-3}$ M saframycin C in $(CH_3)_2SO$–$H_2O$ (1:1) with reduction by $1.2 \times 10^{-2}$ M sodium borohydride in 50 mM potassium phosphate buffer, pH 7.0, in the presence of atmospheric oxygen. (a) Observed EPR spectrum with a $g$ value of 2.0046. (b) Computer simulation with Lorentzien line shapes with line widths of 0.35 G and hyperfine splitting values of $a_H^{Me} = 1.65$ G, $a_H^{4,5} = 1.55$ G, and $a_H^{8,9} = 1.40$ G.

in which they may couple to the unpaired electron in the aromatic rings but H-6, whose coupling is zero, must lie in an orthogonal plane as shown in Figure 6.

*Effect of pH on the Single-Strand Scission of PM2-CCC-DNA by Reduced Saframycins.* In view of the marked effects of pH changes on the extent of both the noncovalent and the

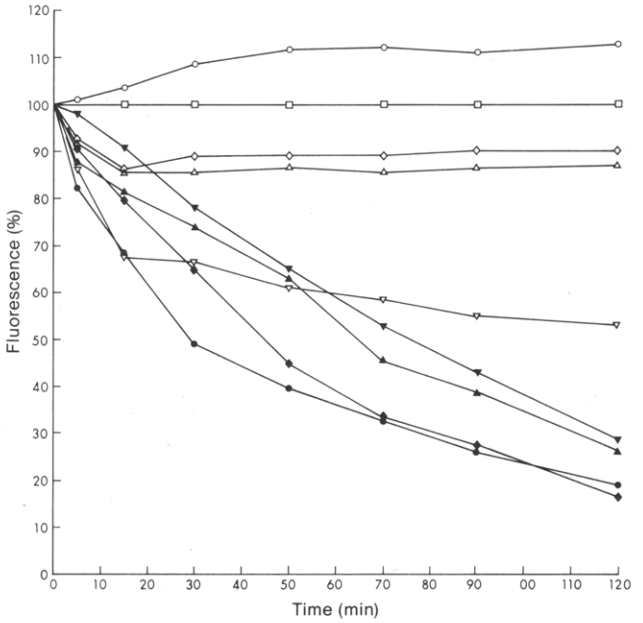



FIGURE 7: Effects of changes in the reaction medium pH on the extent of single-strand scission of DNA by in situ reduced saframycin A. Reactions were performed at 37 °C in 50 mM potassium phosphate buffers and contained 0.8 OD unit of PM2-CCC-DNA (92% CCC), 22.0 $\mu$M saframycin A, and 2.6 mM sodium borohydride in 10% acetonitrile in aqueous solution. The before heat-denaturation fluorescence readings are shown as open symbols and the closed symbols are readings after heat denaturation of the DNA at 96 °C for 4 min followed by rapid cooling to 22 °C. The pH of the medium was (○) 7.0, (△) 6.0, (◇) 5.4, and (▽) 4.7. (□) Controls without antibiotic present.

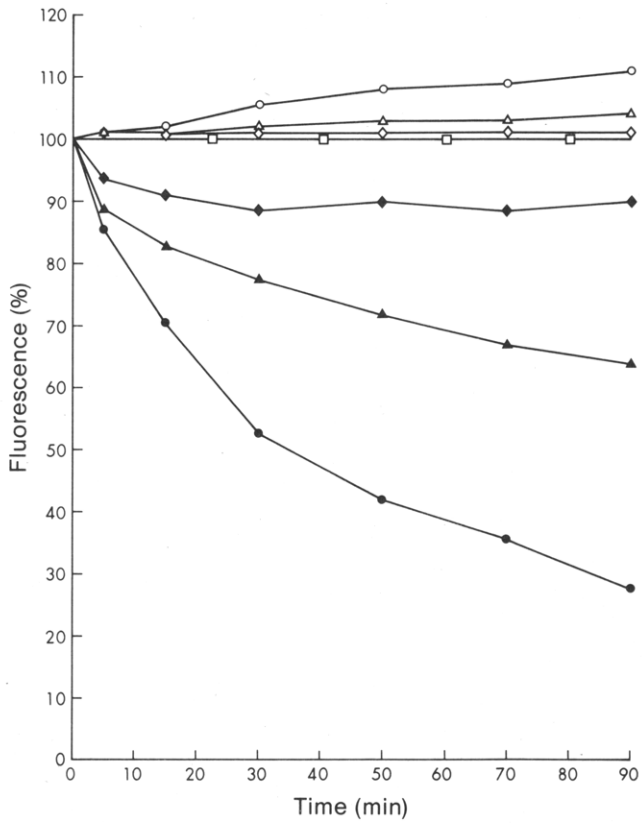



FIGURE 8: Effects of added divalent metal ions on the extent of single-strand scission of PM2-CCC-DNA by in situ reduced saframycin A. Reactions were performed at 37 °C in 50 mM potassium phosphate buffer, pH 7.0, and contained 0.8 OD unit of PM2-CCC-DNA (92% CCC), 22.0 $\mu$M saframycin A, and 2.6 mM sodium borohydride. Additional components were (○) none, (△) 1 mM $MgCl_2$, and (◇) 1 mM $ZnCl_2$.

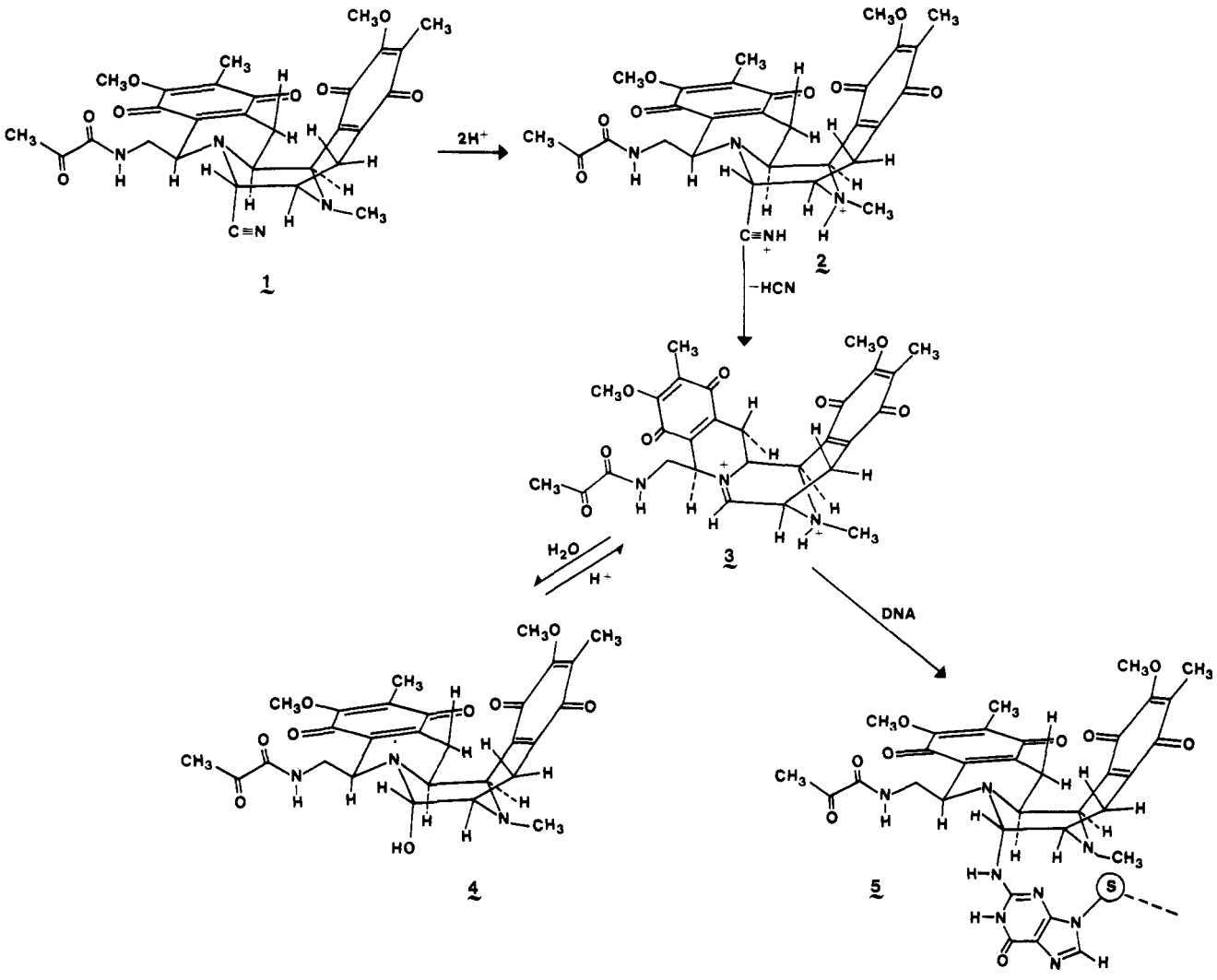


FIGURE 9: Reaction scheme showing acid-promoted loss of nitrile and subsequent covalent binding of intermediate immonium ion 3 to DNA.

covalent binding of saframycin A and of the noncovalent binding of saframycin C, the effects of pH on the single-strand scission process by the reduced antibiotics were investigated. The single-strand cleavage of PM2-CCC-DNA by both reduced saframycins at pH is faster initially whereas at pHs 6.0, 5.6, and 4.7 the cleavage is relatively slower initially and then shows an increase. Thus saframycin A at a concentration of 22 $\mu$M in the presence of $2.6 \times 10^{-3}$ M sodium borohydride at 37 °C nicks PM2-DNA by 26%, 35%, and 22% at pH 6.0, 5.6, and 4.7, respectively, in 30 min compared to 51% at pH 7.0 (Figure 7). The corresponding values after 2 h are 81%, 74%, 84%, and 72% at pH 7.0, 6.0, 5.6, and 4.7, respectively. Similarly saframycin C under comparable conditions nicks PM2-CCC-DNA by 37%, 10%, 7%, and 6%, respectively, in 30 min at pH 7.0, 6.0, 5.6, and 4.7 whereas the values are 63%, 63%, 54%, and 30% after 2 h. In both cases the extent of cleavage was lower at pH 4.7 than at higher pH values. The values at pH 4.7 also demonstrate the reversibility of binding of the antibiotic to DNA under reducing conditions since the fluorescence values after heat denaturation and cooling are higher than those before heating (Figure 7).

*Effect of Divalent Metal Ions on the Single-Strand Scission of DNA by Saframycin.* The cleavage of PM2-CCC-DNA by both saframycin A and C is inhibited by the divalent metals $Zn^{2+}$ and $Mg^{2+}$. At a concentration of 1 mM, $Zn^{2+}$ inhibits 84% of the cleavage of PM2-CCC-DNA by 22 $\mu$M saframycin A at pH 7.0 and 37 °C in the presence of 2.6 mM sodium borohydride in 90 min. Similarly 1 mM $Mg^{2+}$ under comparable conditions inhibits the cleavage by 50% (Figure 8). In the case of saframycin C at 22 $\mu$M, 1 mM $Zn^{2+}$ and 1 mM $Mg^{2+}$ under comparable conditions inhibit the DNA scission by 67% and 10%, respectively. Thus $Zn^{2+}$ is evidently more effective in inhibiting DNA scission than $Mg^{2+}$, especially in the case of saframycin C. No enhancement, inhibition, or change in the extent of cleavage was observed in the presence of added $Fe^{2+}$ ion.

## Discussion

Both saframycins A and C under low pH conditions are protonated at the N-12 position (Lown et al., 1981). The resulting conjugate acids bind by electrostatic attraction to DNA as indicated by both elevation of $T_m$ of DNA and the immediate suppression of ethidium fluorescence. Saframycin A binds more strongly than saframycin C. An additional and much slower mode of binding operates for saframycin A corresponding to covalent attachment. We have previously demonstrated in the case of mitomycin C that similar slow covalent binding of the antibiotic to DNA shows a quantitative relationship between the amount of drug bound and the extent of displacement of ethidium (Lown et al., 1976).

Under acid catalysis and by assistance from the lone pair on the nitrogen (N-2), the cyano group at position 21 in species 2 (Figure 9) is eliminated, and the cyclic immonium ion 3 is formed, which can act as an electrophile toward nucleophilic centers in DNA, e.g., 5. Our $^1H$ NMR studies established N-12 as the site of protonation and that N-2 is not protonated

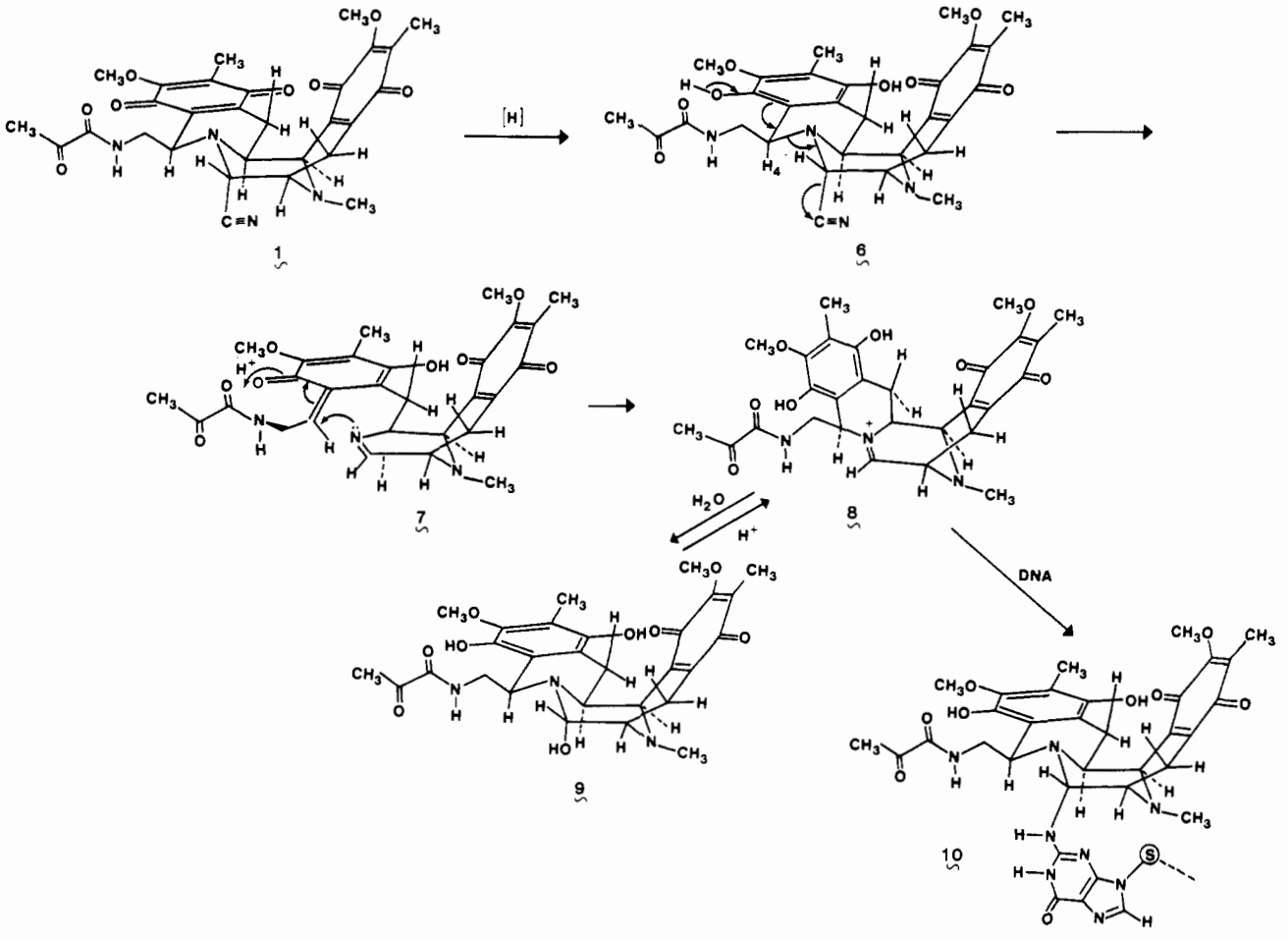



FIGURE 10: Reaction scheme showing reduction of quinone moiety with concomitant loss of leaving group, ring closure, and DNA binding.

so that the N-2 lone pair is available to assist the elimination. A similar mechanism involving a cyclic immonium intermediate has been proposed for the interconversion of saframycins A and S under mildly acidic conditions (Ishiguro et al., 1978). The fact that this type of behavior is not observed with saframycin C, which lacks a leaving group, is also consistent with this interpretation.

The acid-promoted binding of both antibiotics shows minor groove specificity and a preference for G+C containing sequences. This suggests but does not prove the formation of an aminal linkage between the guanine 2-$NH_2$ and the immonium bond of **3** in the case of saframycin A.

The observed slow reversibility of the binding of saframycin A toward heat and lower pH conditions is characteristic of covalent binding and is in accord with the above mechanism. This mechanism is analogous to that of pyrrolo[1,4]benzodiazepine antitumor antibiotics (e.g., anthramycin; Hurley, 1977; Lown & Joshua, 1979) and maytansenoids (Lown et al., 1977) where acid-catalyzed elimination of a neutral molecule, e.g., water or methanol, from the carbinolamine or carbinolamide moiety gives rise to an azomethine or azomethine lactone which can act as a powerful electrophile toward biological nucleophiles.

The binding of saframycin A to DNA increases substantially after reduction, which electrochemical evidence confirms takes place at the quinone moiety. The formation of the hydroquinone (**6**, Figure 10) facilitates the departure of the cyano group with concomitant cleavage of the adjacent ring via a transient quinone methide, **7**. The latter which will rapidly ring close to the hydroquinone-cyclic immonium ion **8** thereby generates an electrophilic center to permit binding to, e.g., a guanine group in the minor groove of DNA, e.g., **10**. The lack of any leaving group in saframycin C accounts for its inability to bind covalently to DNA under either reducing or nonreducing conditions. The possibility of interstrand cross-linking of DNA by saframycins had been suggested by the Japanese workers (Ishiguro et al., 1978). No covalent interstrand cross-linking of λ DNA by reduced saframycin A was observed. Although the intermediate **7** possesses two electrophilic centers and is potentially capable of producing cross-links, the stereochemical constraints and entropic factors may well dictate rapid ring closure to **8**, thus preventing cross-linking from being observed. Neither of the mechanisms of binding to DNA, noncovalent nor covalent, is affected by divalent metal ions.

The saframycins have been shown to inhibit nucleic acid synthesis in vivo in a process involving interaction with template DNA and to require reductive activation for their cytotoxic action. If reduction takes place while the antibiotic is in close proximity to the cell receptor, the present results point to the following mechanism for the generation of radicals and subsequent single-strand (but not double-strand) cleavage of DNA:

$$SA + x[H] \rightarrow SAH_x \quad (1)$$

$$SAH_x + O_2 \rightarrow SAH_{x-1}\cdot + HO_2\cdot \quad (2)$$

$$HO_2\cdot \rightleftarrows O_2^{-}\cdot + H^+ \quad (3)$$

$$2O_2^{-}\cdot \xrightarrow{SOD} H_2O_2 + O_2 \quad (4)$$

$$2H_2O_2 \xrightarrow{catalase} 2H_2O + O_2 \quad (5)$$

$$H_2O_2 + CMXFe(II) \rightarrow CMXFe(III) + OH\cdot + OH^- \quad (6)$$

$$CMXFe(III) + O_2^{-}\cdot \rightarrow CMXFe(II) + O_2 \quad (7)$$

$$H_2O_2 + O_2^{-}\cdot \rightarrow OH\cdot + OH^- + O_2 \quad (8)$$

$$OH\cdot \rightarrow \text{DNA strand breakage} \quad (9)$$

where SA = saframycin, $SAH_x$ = quinone ring reduced saframycin, $SAH_{x-1}\cdot$ = saframycin semiquinone, SOD = superoxide dismutase, and CMXFe(II), iron complexed with protein or ATP. It may be seen from Table III that when redox potentials are recorded on the hydrogen scale at pH 7, both antibiotics show two reduction waves, corresponding to facile reduction of the two quinone moieties and at redox potentials comparable with that of streptonigrin that bears a quinone group with a very similar substitution pattern. The saframycins show an additional wave near −0.3 to −0.4 V tentatively attributed to reduction of the α-dione side chain. It may be seen from Table III that thermodynamically at least, reduction of the quinone moieties by glutathione or a comparable thiol or NADPH is feasible in vivo. The proposed mechanism is similar to that suggested for the DNA strand scission by streptonigrin (Cone et al., 1976), the anthracyclines (Lown et al., 1977), mitomycin C (Lown et al., 1976), and bleomycin (Lown & Sim, 1977), with, however, some significant differences. Unlike other quinone antibiotics, the saframycins contain two such rings. It is not clear at present if one or both of these are involved in the electron transfer processes which give rise to $O_2^{-}\cdot$ or if the reduction in vivo involves one or two electrons in either step. Additionally, as has been pointed out previously, the Haber–Weiss reaction (step 8), frequently quoted as a source of OH· radicals, has been shown to be quite slow ($K < 0.3\ M^{-1}\ s^{-1}$) (van Hemmen & Meuling, 1977; McClune & Fee, 1976; Halliwell, 1976). While this rate permits its contribution in vitro, it is unlikely to compete for $O_2^{-}\cdot$, in reaction 4 for example, in vivo. In such cases it has been suggested rather that in vivo the OH· radical is produced by reaction of $O_2^{-}\cdot$ with Fe(III) complexed with either protein, ATP (designated CMXFe(III) in reaction steps 6 and 7) (Czapski & Ilan, 1977), or, in the case of the glycopeptides bleomycin and tallysomycin, the antibiotic itself (Sausville et al., 1976; Lown & Sim, 1977). In the present case there was no spectroscopic evidence for binding of Fe(II) or Fe(III) to the saframycins. It seems likely therefore that OH· generation in vitro results from reaction of $H_2O_2$ with traces of adventitious Fe(II) in the aqueous system, as is found to be the case for mitomycins C and B (Lown et al., 1976), and other quinone antibiotics (Lown & Chen, 1981; Lown et al., 1978). Metal chelating agents such as EDTA suppress but do not entirely eliminate hydroxyl radical production. This is in accord with Czapski & Bielski's observation (1963) that Fe(II) is a potent catalyst of the Haber–Weiss or Fenton reaction even in the presence of a 50-fold excess of EDTA. The effects of changing the pH of the medium on the rates and extents of single-strand scission by saframycin A may be rationalized in terms of the different modes of binding and of the mechanism of the scission process involving the generation of reactive oxygen species. Lower pHs will promote both the binding of the N-12 protonated saframycin and the covalent binding via the immonium ion **3**. Once bound in the minor groove, the antibiotic may well be less accessible to the reducing agent that is responsible for producing the reactive oxygen species accounting for the slow rate of scission at the lower pHs. In contrast reduction of the quinone moiety of saframycin C may occur freely at pH 7.0. The resulting reduced antibiotic can react with dissolved oxygen and thereby generate $O_2^{-}\cdot$ and OH· which diffuse to the DNA, causing strand scission of the deoxyribose moieties (Dizdaroglu et al., 1975). Thus pH 7.0 or 7.2 may well represent the optimum pH for the antibiotic for inducing oxidative lesions in susceptible sites in the minor groove of DNA.

The observed effects of divalent metal ions $Zn^{2+}$ and $Mg^{2+}$ are noteworthy. Since they have no effect on the binding process but a marked inhibitory effect on the single-strand scission process, this suggests a specific interaction between these metal ions and the reduced antibiotics, conceivably involving chelation of the hydroquinone moiety and the methyl α-dione side chain. This specific effect had the distinct experimental advantage of permitting clean separation of the processes of covalent binding of the reduced antibiotic and of the single-strand scission process. A more extensive electrochemical study of these effects of particular metal ions will be reported subsequently. These results should assist in the interpretation of the mechanism of anticancer activity of these potentialy useful antibiotics.

Acknowledgments

We thank Glen Bigam for technical assistance in obtaining the EPR spectra, Kam Kong for the EPR computer simulations, Susan Ranson for the electrochemical determinations, and Dennise Dombroski for technical assistance.

References

Arai, T., Takahashi, K., & Kubo, A. (1977) *J. Antibiot. 30*, 1015–1018.

Arai, T., Takahashi, K., Ishiguro, K., & Yazawa, K. (1980) *J. Antibiot. 33*, 951–960.

Bode, V. C. (1967) *J. Mol. Biol. 26*, 125–129.

Cone, R., Hasan, S. K., Lown, J. W., & Morgan, A. R. (1976) *Can. J. Chem. 54*, 219–223.

Czapski, G., & Bielski, H. J. (1963) *J. Phys. Chem. 67*, 2180–2187.

Czapski, G., & Ilan, Y. A. (1977) International Conference on Singlet Oxygen and Related Species in Chemistry and Biology, Pinawa, Manitoba, Canada, Aug 21–26, Abstract C-11.

Dizdaroglu, M., von Sonntag, C., & Schulte-Frohlinde, D. (1975) *J. Am. Chem. Soc. 97*, 2277–2278.

Erickson, R. L., & Szybalski, W. (1964) *Virology 22*, 111–124.

Halliwell, B. (1976) *FEBS Lett. 72*, 8–10.

Harbour, J. R., Chow, V., & Bolton, J. R. (1974) *Can. J. Chem. 52*, 3549–3553.

Hurley, L. H. (1977) *J. Antibiot. 30*, 349–370.

Ishiguro, K., Sakiyama, S., Takahashi, K., & Arai, T. (1978) *Biochemistry 17*, 2545–2550.

Ishiguro, K., Takahashi, K., Yazawa, K., Sakiyama, S., & Arai, T. (1981) *J. Biol. Chem. 256*, 2162–2167.

Janzen, E. G. (1971) *Acc. Chem. Res. 4*, 31–40.

Kluepfel, D., Baker, H. A., Piattoni, G., Sehgal, S. N., Sidorowicz, A., Singh, K., & Vezina, S. (1975) *J. Antibiot. 28*, 497–502.

Lai, C. S., & Piette, L. H. (1977) *Biochem. Biophys. Res. Commun. 78*, 51–59.

Lee, J. S., & Waring, M. J. (1978) *Biochem. J. 173*, 129–144.

Lown, J. W., & Sim, S. K. (1977) *Biochem. Biophys. Res. Commun. 77*, 1150–1157.

Lown, J. W., & Joshua, A. V. (1979) *Biochem. Pharmacol. 28*, 2017–2026.

Lown, J. W., & Chen, H. H. (1981) *Can. J. Chem. 59*, 390–395.

Lown, J. W., Begleiter, A., Johnson, D., & Morgan, A. R. (1976) *Can. J. Biochem. 54*, 110–119.

Lown, J. W., Majumdar, K. C., Meyers, A. I., & Hecht, A. (1977) *Bioorg. Chem. 6*, 453-463.
Lown, J. W., Sim, S. K., & Chen, H. H. (1978) *Can. J. Biochem. 56*, 1042-1047.
Lown, J. W., Chen, H. H., Plambeck, J. A., & Acton, E. M. (1979) *Biochem. Pharmacol. 28*, 2563-2568.
Lown, J. W., Joshua, A. V., & Chen, H. H. (1981) *Can. J. Chem. 59*, 2945-2952.
Mahler, H. R., & Cordes, E. H. (1966) *Biological Chemistry*, p 207, Harper and Row, New York.
McChee, J. D. (1976) *Biopolymers 15*, 1345-1375.
McClune, G. J., & Fee, J. A. (1976) *FEBS Lett. 67*, 294-298.
Morgan, A. R., & Paetkau, V. (1972) *Can. J. Biochem. 50*, 210-216.
Morgan, A. R., & Pulleyblank, D. E. (1974) *Biochem. Biophys. Res. Commun. 61*, 396-403.
Morgan, A. R., Lee, J. S., Pulleyblank, D. E., Murray, N. L., & Evans, D. H. (1979) *Nucleic Acids Res. 7*, 547-569.
Rao, K. V., Biemann, K., & Woodward, R. B. (1963) *J. Am. Chem. Soc. 85*, 2532-2533.
Rao, G. M., Begleiter, A., Lown, J. W., & Plambeck, J. A. (1977) *J. Electrochem. Soc. 124*, 199-202.
Rao, G. M., Lown, J. W., & Plambeck, J. A. (1978) *J. Electrochem. Soc. 125*, 534-539.
Sausville, E. A., Peisach, J., & Horwitz, S. B. (1976) *Biochem. Biophys. Res. Commun. 73*, 814-822.
Szybalski, W., & Iyer, V. N. (1967) in *The Mitomycins and Porfiromycins* in *Antibiotics 1. Mechanism of Action* (Gottlieb, D., & Shaw, P. D., Eds.) pp 211-245, Springer-Verlag, New York.
van Hemmen, J. J., & Meuling, W. J. S. (1977) *Arch. Biochem. Biophys. 182*, 743-748.

# Premelting and Melting Transitions in the d(CGCGAATTCGCG) Self-Complementary Duplex in Solution†

Dinshaw J. Patel,* Sharon A. Kozlowski, Luis A. Marky, Chris Broka, Janet A. Rice, Keiichi Itakura, and Kenneth J. Breslauer

ABSTRACT: We have characterized structural and dynamic aspects of the d($C_1G_2C_3G_4A_5A_6T_6T_5C_4G_3C_2G_1$) 12-mer duplex based on an analysis of the proton and phosphorus nuclear magnetic resonance parameters in the premelting and melting transition region. The self-complementary dodecanucleotide sequence forms a 12 base-paired duplex with 2-fold symmetry in solution. The six Watson–Crick imino protons are well resolved between 12.5 and 14.0 ppm and have been assigned to individual positions in the sequence. Fraying of the ends of the duplex is most pronounced at terminal base pair 1. This fraying extends into the interior of the duplex up to and including base pair 3 with increasing temperature in the premelting transition region. A temperature-dependent transition is detected in the dA·dT-containing tetranucleotide core of the duplex in the premelting temperature range, which is not observed at the flanking dG·dC-containing tetranucleotide regions. The 11 phosphodiesters in the 12-mer duplex exhibit $^{31}P$ chemical shifts spread over a 0.45-ppm chemical shift range, suggestive of a distribution of O–P torsion angles and/or O–P–O bond angles at individual phosphodiester linkages along the sequence. Well-resolved resonances of nonexchangeable base protons are observed in the 12-mer duplex spectrum. Partial assignment of these resonances to specific base pairs in the 12-mer was accomplished with spin decoupling, nuclear Overhauser effect, and chemical modification studies. These base protons shift as average resonances during the helix–coil transition of the 12-mer duplex in 0.1 M phosphate solution with a transition midpoint of 72 ± 2 °C at the 10 nonterminal base pairs. Upfield shifts are observed at the base protons of the dodecanucleotide sequence on duplex formation, which reflect the stacking interactions in the double-helical state. The thermally induced transitions of the right-handed 12-mer duplex in 0.01 and 0.1 M NaCl were investigated by differential scanning calorimetry. In 0.01 M NaCl an enthalpy change of 90 kcal (mol of double strand)$^{-1}$ was measured with a melting temperature of 65.5 °C. In 0.1 M NaCl an enthalpy change of 102 kcal (mol of double strand)$^{-1}$ was measured with a melting temperature of 71.3 °C. Analysis of the shapes of the calorimetric heat capacity curves yields van't Hoff enthalpies of 94 kcal in 0.01 M NaCl and 74 kcal in 0.1 M NaCl. Thus, we conclude that in 0.01 M NaCl the transition approaches two-state behavior ($\Delta H_{cal} = \Delta H_{V.H.}$) while in 0.1 M NaCl the transition from duplex to strands involves intermediate states ($\Delta H_{V.H.} < \Delta H_{cal}$). The ratio of the van't Hoff and calorimetric enthalpies allows specification of the size of the cooperative unit. Thus, in 0.1 M NaCl 9 ± 1 base pairs of the 12-mer melt in a cooperative manner.

We have begun a program in which nuclear magnetic resonance (NMR) and differential scanning calorimetry (DSC) have been used to investigate the conformation, dynamics, and thermodynamics of order–disorder transitions in a series of DNA duplexes containing at least one turn of helix in which the structures systematically differ due to selected modifications.

Our initial studies focused on the self-complementary dodecanucleotide d(CGCGAATTCGCG) duplex (Chart I) since parallel crystallographic investigations have been undertaken (Wing et al., 1980; Drew et al., 1981; Dickerson & Drew, 1981) and should permit a direct comparison between data obtained in solution with that obtained at atomic resolution in the crystalline state.

The application of high-resolution nuclear magnetic reso-

† From Bell Laboratories, Inc., Murray Hill, New Jersey 07974 (D.J.P., S.A.K., and J.A.R.), the Department of Chemistry, Douglass College, Rutgers University, New Brunswick, New Jersey 08903 (L.A.M. and K.J.B.), and the City of Hope National Medical Center, Duarte, California 91010 (C.B. and K.I.). *Received May 22, 1981; revised manuscript received September 3, 1981.* K.J.B. and L.A.M. gratefully acknowledge support from the National Institutes of Health, Grant GM-23509, the Research Corporation, and the Charles and Johanna Busch Memorial Fund.

0006-2960/82/0421-0428$01.25/0 