Cleland, W. W. (1979) *Methods Enzymol. 63*, 103.
Cleland, W. W. (1982) *CRC Crit. Rev. Biochem. 13*, 385.
Cleland, W. W. (1987) *Bioorg. Chem. 15*, 283.
Hackney, D. D., Stempel, K. E., & Boyer, P. D. (1980) *Methods Enzymol. 64*, 60.
Hansen, D. E., & Knowles, J. R. (1985) *J. Am. Chem. Soc. 107*, 8304.
Hegarty, A. F., Bruice, T. C., & Benkovic, S. J. (1969) *J. Chem. Soc., Chem. Commun.*, 1173.
Hermes, J. D., Roeske, C. A., O'Leary, M. H., & Cleland, W. W. (1982) *Biochemistry 21*, 5106.
Hudsen, P. J., Keech, D. B., & Wallace, J. C. (1975) *Biochem. Biophys. Res. Commun. 65*, 213.
Kaziro, Y., Hass, L. F., Boyer, P. D., & Ochoa, S. (1962) *J. Biol. Chem. 237*, 1460.
Kluger, R., Davis, P. P., & Adawadkar, P. D. (1979) *J. Am. Chem. Soc. 101*, 5995.
Midelfort, C. F., & Rose, I. A. (1976) *J. Biol. Chem. 251*, 5881.
O'Leary, M. H. (1980) *Methods Enzymol. 64*, 83.
Polakis, S. E., Guchhait, R. B., & Lane, M. D. (1972) *J. Biol. Chem. 247*, 1335.
Polakis, S. E., Guchhait, R. B., Zwergel, E. E., Lane, M. D., & Cooper, T. G. (1974) *J. Biol. Chem. 249*, 6657.
Sauers, C. K., Jencks, W. P., & Groh, S. (1975) *J. Am. Chem. Soc. 97*, 5546.
Schowen, R. L. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) p 64, University Park Press, Baltimore, MD.
Tipton, P. A., & Cleland, W. W. (1988a) *Arch. Biochem. Biophys. 260*, 273.
Tipton, P. A., & Cleland, W. W. (1988b) *Biochemistry* (preceding paper in this issue).
Tipton, P. A., & Cleland, W. W. (1988c) *J. Am. Chem. Soc.* (in press).
Wehrli, W. E., Verheyden, D. L. M., & Moffat, J. G. (1965) *J. Am. Chem. Soc. 87*, 2265.
Wimmer, M. J., Rose, I. A., Powers, S. G., & Meister, A. (1979) *J. Biol. Chem. 254*, 1854.

Atypical Abasic Sites Generated by Neocarzinostatin at Sequence-Specific Cytidylate Residues in Oligodeoxynucleotides†

Lizzy S. Kappen, Chang-qing Chen,‡ and Irving H. Goldberg*
Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115
Received December 8, 1987; Revised Manuscript Received February 22, 1988

ABSTRACT: Neocarzinostatin chromophore produces alkali-labile, abasic sites at cytidylate residues in AGC sequences in oligonucleotides in their duplex form. Glutathione is the preferred thiol activator of the drug in the formation of these lesions. The phosphodiester linkages on each side of the abasic site are intact, but when treated with alkali, breaks are formed with phosphate moieties at each end. Similar properties are exhibited by the abasic lesions produced at the purine residue to which the C in AGC is base-paired on the complementary strand. The abasic sites at C residues differ from those produced by acid-induced depurination in the much greater lability of the phosphodiester linkages on both sides of the deoxyribose, in the inability of $NaBH_4$ to prevent alkali-induced cleavage, and in the relative resistance to apurinic/apyrimidinic endonucleases. The importance of DNA microstructure in determining attack site specificity in abasic site formation at C residues is shown not only by the requirement for the sequence AGC but also by the findings that substitution of G by I 5′ to the C decreases the attack at C, whereas placement of an I opposite the C markedly enhances the reaction. Quantitation of the abstraction of ^3H into the drug from C residues in AGC specifically labeled in the deoxyribose at C-5′ or C-1′,2′ suggests that, in contrast to the attack at C-5′ in the induction of direct strand breaks at T residues, abasic site formation at C residues may involve attack at C-1′. Each type of lesion may exist on the complementary strands of the same DNA molecule, forming a double-stranded lesion.

The DNA-damaging antibiotic neocarzinostatin (NCS)[1] consists of a labile nonprotein chromophore complexed with its apoprotein [reviewed in Goldberg (1986)]. The biologically active nonprotein chromophore has a structure comprised of three subunits: a 5-methyl-7-methoxynaphthoate and a 2,6-dideoxy-2-(methylamino)galactose moiety interlinked by a C_{12} subunit containing a bicyclo[7.3.0]dodecadiyne system bearing acyclic carbonate moiety and an epoxide. NCS chromophore binds to DNA by an intercalative mechanism and causes a variety of lesions of which spontaneous base release (T > A ≫ C > G) and single-strand breaks are the most predominant. The mechanism of base release is not clearly understood. The strand breaks occur in a thiol- and O_2-dependent reaction primarily at thymidylic and deoxyadenylic acid residues (T > A ≫ C > G) and have mainly (>80%) a nucleoside aldehyde at their 5′-ends and a PO_4 at their 3′-termini. A reaction mechanism proposed to account for these products

† This work was supported by U.S. Public Health Service Research Grant GM12573 from the National Institutes of Health and an award from the Bristol-Myers Co.
‡ Present address: Shanghai Institute of Biochemistry, Academia Sinica, 320 Yue-yang Road, Shanghai 200031, China.

[1] Abbreviations: NCS, neocarzinostatin; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography.

0006-2960/88/0427-4331$01.50/0

involves the abstraction of a hydrogen from the 5′-carbon to generate a carbon-centered radical, which upon addition of dioxygen forms a peroxyl radical species that undergoes reduction to give rise to nucleoside aldehyde and PO_4 (Kappen & Goldberg, 1985). When misonidazole substitutes for O_2 in an anaerobic reaction, base release (thymine) is enhanced with the generation of a gap bonded by PO_4 at both 3′- and 5′-termini (Kappen & Goldberg, 1984). The primary attack site in this reaction is also the 5′-carbon; the subsequent steps, however, involve the intermediacy of a high-energy form of formate, i.e., 3′-formyl phosphate ended DNA (Chin et al., 1987). A similar lesion accounts for <20% of the strand breaks occurring in the O_2-dependent reaction.

Another lesion induced in DNA by NCS is the alkali-dependent strand scission associated with cytosine release at certain cytosine residues, preferentially in the sequence AGC (Povirk & Goldberg, 1985). The apyrimidinic sites produced by the drug are much less sensitive than acid-produced apurinic sites to cleavage by several apurinic/apyrimidinic endonucleases. In studies using the *lacI* gene of *Escherichia coli* (Povirk & Goldberg, 1985) and the *cI* gene of λ phage (Povirk & Goldberg, 1986), a correlation was found between the occurrences of the alkali-labile lesion at cytidylate residues in AGC and the frequencies of GC to AT transitions. These results provided strong evidence for a role for these lesions in NCS-induced mutagenesis.

In the present study the goal is to characterize further the alkali-labile lesion induced by NCS at cytidylic acid residues and to elucidate the mechanism of its formation. We have used oligonucleotides as substrate models in the study of the DNA–drug interactions. The data reveal several features of the reaction: (1) cytosine release creates an equivalent number of apyrimidinic sites, which upon alkali treatment generate breaks having 3′- and 5′-phosphoryl termini; (2) base pairing of the target C residue with I on the opposite strand greatly enhances cytosine release and strand breakage, whereas an I residue 5′ to the C decreases the attack at the C residue; (3) the product generated by cytosine release is a full-length molecule containing abasic sites, which are distinctly different from acid-induced apurinic sites in the increased lability of their sugar–phosphoryl linkage at both the 3′- and 5′-sides and in their failure to be protected by $NaBH_4$ against alkali-induced cleavage; (4) nevertheless, the product of endonuclease IV digestion also has a 3′-hydroxyl group; (5) the reaction requires O_2, but under anaerobic conditions misonidazole can substitute for O_2. These data taken together with the results of ^3H abstraction from the DNA deoxyribose into the drug support a mechanism involving an attack at the C-1′ position. Another interesting finding in this study is that NCS also induces alkali-dependent breaks at the base (G or I) opposite the C residues in AGC.

Materials and Methods

The materials and their sources are as follows: protected deoxynucleoside–CPG (controlled pore glass) substrates (A, C, G, T), American Bionuclear; protected deoxynucleoside cyanoethyl phosphoramidites (A, C, G, I, T), Cruachem; all other reagents used in oligonucleotide synthesis, Aldrich; [5-^{3}H]dCTP (22.8 Ci/mmol), [5′,5-^{3}H]dCTP (55.5 Ci/mmol; 5′-^{3}H, 62%; 5-^{3}H, 38%), [γ-^{32}P]ATP, and other ^{32}P-labeled deoxynucleoside triphosphates (>3000 Ci/mmol), New England Nuclear; [1′,2′,5-^{3}H]dCTP (62 Ci/mmol: 1′-^{3}H, 27%; 2′-^{3}H], 41%; 5-^{3}H, 32%), Amersham. Since the ^{3}H at the 5′-carbon is randomly distributed (H_R, H_S), the specific activity for product calculations is half that given above. Polynucleotide kinase and DNA polymerase I large fragment were purchased from New England Biolabs. *E. coli* endonuclease IV was a generous gift from Bruce Demple and Tomas Lindahl. NCS was a gift from W. T. Bradner of Bristol Myers.

Synthesis of Oligonucleotides and Preparation of Radiolabeled Substrates. Oligodeoxyribonucleotides were synthesized manually by the phosphoramidite method (Sinha et al., 1984) on a controlled pore glass support. Reactions were carried out in a 1-μmol column through which solvents and reagents were forced to flow by argon pressure. Average coupling efficiency was about 97%. The products were purified by electrophoresis on a 20% polyacrylamide gel containing 8.3 M urea, and the oligomers were eluted from the gel as described (Fritz et al., 1978). Their sequences were confirmed by the standard DNA sequencing procedure after the 5′-ends were labeled with ^{32}P (Maxam & Gilbert, 1980). In order to introduce a label at the 3′-end, the oligomers were first annealed in the reaction buffer with a complementary strand longer by a few nucleotides so as to provide the appropriate template in the subsequent reaction with DNA polymerase I and radiolabeled deoxynucleoside triphosphates. The labeled oligomers were purified by gel electrophoresis.

Drug Reaction. NCS chromophore was extracted from the native drug as previously described (Kappen & Goldberg, 1985). The radiolabeled oligonucleotides were first annealed to their complementary strands of the same length or longer (0.1–0.65 A_{260} unit) in a 5× reaction buffer (<100 μL) by heating at 92 °C for 2 min and subsequent slow cooling to room temperature. The annealed mixture was then distributed for drug reactions. After the addition of glutathione (final 5 mM) and dilution with H_2O required to make up the final volume, the mixture was chilled in ice for at least an hour prior to the addition of NCS chromophore. A control reaction containing no drug received an equal volume of methanol (final maximum 20%). The reaction was allowed to proceed in the dark for 30 min in ice. A standard reaction contained 10–50 mM Tris-HCl or Hepes/NaOH, pH 8.0, and 0.1–0.5 mM EDTA. The buffer concentrations were varied to minimize salt interference in the subsequent assays. These variations did not significantly affect the extent of the reactions.

Alkali Treatment, Reduction, and Depurination. Alkali treatment was by heating in 0.1 M NaOH or 4 M NH_4OH at 90 °C for 30 min in a sealed tube. NaOH-treated samples were neutralized with HCl, and NH_4OH was removed by lyophilization.

Reduction with $NaBH_4$ was carried out as described (Kappen & Goldberg, 1983) with Hepes buffer, pH 7.0, instead of Tris buffer. After the addition of carrier tRNA, the oligomers were freed of salts by ethanol precipitation.

The oligomers were depurinated by heating in formic acid (33%) at 37 °C for 1 h. The samples were then extracted with ether 4 times and lyophilized. Lyophilization was repeated after the addition of 15 μL of distilled H_2O to the pellet.

Product Analyses. In order to analyze for strand breaks, aliquots of the reaction mixture were lyophilized in duplicates, one of which was subjected to alkali treatment and was relyophilized. The residue, dissolved in the loading buffer (80% formamide and 1 mM EDTA containing marker dyes), was electrophoresed on a denaturing 20% DNA sequencing gel (Maxam & Gilbert, 1980). Since the alkali-labile lesion at the C residues was found to be somewhat labile to Tris, in several analyses Tris–borate buffer was replaced by Hepes–NaOH, pH 7.9. In order to quantitate strand breaks, the autoradiographs were scanned with a Quick Scan densitometer (Helena Laboratories), and the peak areas corresponding to

Table I: Oligodeoxynucleotides Used as Substrates for NCS[a]

GAGCG	CGCTCICTCGCT
GGAGCGG	$(AGCG)_3$
AGCGAGCG	$(AGCC)_3$
GAGCGAGCG	$(GGCT)_3$
AGCGAICGAGCG	$G(AGC)_4G$

[a] The complementary strands in the duplex are not shown.

the band of interest were cut out and weighted in an analytical balance.

In order to isolate the material from a gel band, the crushed gel slice, suspended in 0.3 M sodium acetate–1 mM EDTA containing carrier tRNA, was stirred at room temperature for 8 h. After the removal of the gel slices by centrifugation, the product was recovered by ethanol precipitation.

Cytosine released in the reaction was separated by (a) thin-layer chromatography on cellulose plates or paper chromatography in the solvent system 1 M sodium acetate–ethanol (3:7) and by (b) HPLC (see ^3H Abstraction into NCS Chromophore). The radioactivity in the chromatography strips and in HPLC fractions was determined by liquid scintillation counting.

^3H Abstraction into NCS Chromophore. Standard DNA-damage reactions (200 μL) containing the appropriately ^3H-labeled substrates were carried out as described. An aliquot was removed for determination of cytosine. After the addition of carrier tRNA and sodium acetate to 0.3 M, the rest of the sample was ethanol precipitated at −20 °C. After centrifugation (15000*g*, 15 min), the supernatant containing the drug and released cytosine was lyophilized. The pellet was dissolved in 10% methanol for HPLC analysis using a μBondapak C_{18} column, as previously described (Kappen & Goldberg, 1985). The elution positions of cytosine and NCS chromophore were identified by UV absorption and/or fluorescence. The radioactivity in the fractions was determined by liquid scintillation counting.

Results

General Features of the Reaction of NCS with Oligonucleotides. Since it was known from previous work (Povirk & Goldberg, 1985) that alkali-dependent breaks at C residues occurred preferentially in the sequence AGC, several ^{32}P-end-labeled oligonucleotides containing one or more AGC triplets, as well as sequences with different flanking residues at the 5′- and the 3′-sides of the C residues, were screened in their double-stranded form for NCS-induced alkali-labile strand break at the C residues. Those that were studied in detail are listed in Table I. It was found that neither the 3-mer AGC nor the 4-mer GAGC annealed to a 12-mer was a substrate for the drug under the conditions used (data not shown). The pentamer GAGCG and several longer oligomers containing the AGC sequence served as good substrates. With the 5-mer and the 7-mer (Figure 1A) alkali-dependent strand scission due to NCS occurred at the C residues in AGC (lanes 6 and 10, arrows). In the absence of alkali treatment there is a band with mobility slightly faster than that of the starting material (lanes 5 and 9, arrows); this appears to be a full-length molecule containing abasic sites at C residues, as judged by its disappearance in the alkali-treated sample and the concomitant appearance of a band at the appropriate C with a 3′-phosphoryl terminus (hereafter referred to as C–PO_4 band). Acid depurination of these and other oligomers generated a similar band (later proved to be the full-length, depurinated form) moving slightly ahead of the starting material (data not shown). It should be noted that in these and subsequent gel analyses there is considerable smearing from the origin to the

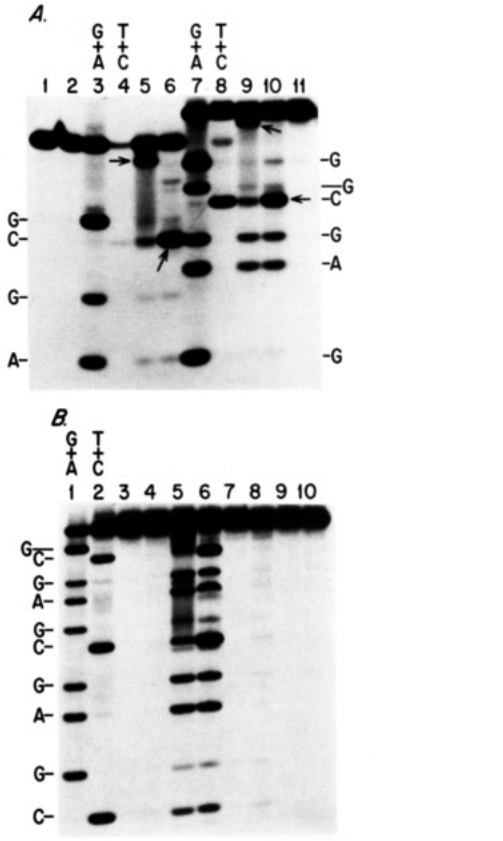

FIGURE 1: (A). Alkali-dependent strand scission by NCS at C residues in AGC. 5′-^{32}P-end-labeled GAGCG annealed to $(CGCT)_3$ or GGAGCGG annealed to CCGCTCC was the substrate for NCS (40 μM) in the standard reaction. Alkali treatment and analysis of the samples on a Hepes-containing gel were as described under Materials and Methods. Lanes 1–6, the 5-mer; lanes 7–11, the 7-mer. Lanes 1 and 2 are (−drug) controls without and with alkali, respectively. Lanes 5, NCS; lane 6, NCS + alkali; lane 9, NCS; lane 10, NCS + alkali; lane 11, (−) drug control + alkali. The arrows in lanes 5 and 9 indicate the putative abasic intermediate, and those in lanes 6 and 10 show the drug-dependent C–PO_4 bands following alkali treatment. Maxam–Gilbert markers are as indicated in lanes 3, 4, 7, and 8 and are shown in a similar manner (G+A, T+C) in the other figures. (B) Requirement for duplex DNA for NCS action. 5′-^{32}P-end-labeled $(AGCG)_3$ annealed to $(CGCT)_3$ (lanes 3–6) or $(AGCG)_3$ alone (lanes 7–10) was the substrate for NCS (64 μM) in the standard reaction. The reaction mixture was analyzed on a Tris-containing gel. Lanes 3 and 4 are (−) drug controls without and with alkali, respectively. Lane 5, NCS; lane 6, NCS + alkali; lane 7, NCS; lane 8, NCS + alkali. Lanes 9 and 10 are (−) drug controls without and with alkali, respectively.

involved C residue in the lane containing samples not treated with alkali, indicative of cleavage occurring during the course of the electrophoresis. This will be discussed later when the stability properties of the DNA damage products are described. Duplex structure in the oligomers is an absolute requirement for the drug reaction (compare lanes 6 and 8 in Figure 1B). There was no strand breakage, spontaneous or alkali dependent, in the single-stranded oligomer.

In contrast to direct strand scission, where several different thiols are equally efficient activators at their optimum concentrations, there is a strong preference for glutathione in the NCS-induced alkali-labile strand cleavage at a C (Povirk & Goldberg, 1985). The experiments in Figure 2 using 5′-^{32}P-end-labeled $(AGCC)_3$ as substrate confirm that glutathione (lane 6) is much more effective than dithiothreitol (lane 8) or 2-mercaptoethanol (lane 12); $NaBH_4$ (lane 10) is nearly as good a cofactor as glutathione. The alkali-labile strand breaks occur only at the C residues that are 3′ to G (indicated by arrows), and of the three sensitive sites, C in the central

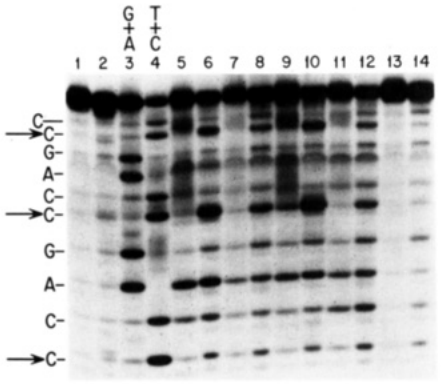

FIGURE 2: Effect of different activating agents on NCS-induced alkali-dependent strand scission at C residues. Standard reactions containing 5′-^{32}P-end-labeled $(AGCC)_3$, annealed to $(GGCT)_3$, and NCS (40 μM) were carried out in the presence of glutathione (5 mM), dithiothreitol (2 mM), $NaBH_4$ (20 mM), or 2-mercaptoethanol (10 mM), as described under Materials and Methods. Half of the samples were subjected to alkali treatment. Samples were analyzed on a Tris-containing gel. Arrows indicate the alkali-labile C sites. Lanes 1 and 2 are (−) drug controls containing $NaBH_4$ without and with alkali treatment, respectively. Lane 5, glutathione + NCS; lane 6, glutathione + NCS + alkali; lane 7, NCS + dithiothreitol; lane 8, NCS + dithiothreitol + alkali; lane 9, NCS + $NaBH_4$; lane 10, NCS + $NaBH_4$ + alkali; lane 11, NCS + 2-mercaptoethanol; lane 12, NCS + 2-mercaptoethanol + alkali; lane 13, (−) drug control with glutathione; lane 14, control + glutathione + alkali. Control reactions containing 2-mercaptoethanol or dithiothreitol but no drug produced patterns similar to those in lanes 13 and 14 (not shown).

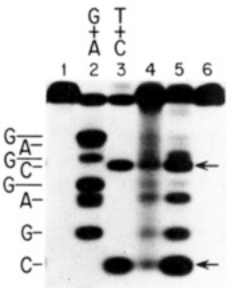

FIGURE 3: Analysis of the 5′-termini of the NCS-induced alkali-labile breaks at C residues. 3′-^{32}P-end-labeled GAGCGAGCG annealed to $(CGCT)_3$ was treated with NCS (40 μM) under standard conditions. Alkali treatment and analysis of the samples on a Hepes-containing gel were as described under Materials and Methods. Lanes 1 and 6 are (−) drug controls without and with alkali treatment, respectively. Lane 4, NCS; lane 5, NCS + alkali. Arrows indicate the alkali-labile C sites.

AGC triplet is the one most favored for attack.

The preceding experiments show that the breaks generated at a C upon alkali treatment most likely have 3′-phosphoryl termini. In order to examine the moiety at the 5′-ends of the breaks, a 9-mer with a ^{32}P label at its 3′-end was used as a substrate for the drug. As illustrated in Figure 3, the product of strand scission at the C (lane 5, arrows) has the same mobility as that of the standard marker for strand cleavage at the same C and known to have 5′-phosphoryl termini (Maxam & Gilbert, 1980). These results strongly suggest that NCS-induced alkali-dependent breaks have 5′-phosphoryl ends. Further, the absence of an intermediate, slower moving band suggests that the original lesion (before alkali) does not possess a sugar residue at the 5′-phosphate, due to cleavage 5′ to the sugar. As was found with the 5′-end-labeled substrate (Figure 1A), in the absence of alkali there is a drug-produced band moving slightly faster than the starting material.

As in the case of direct strand breaks (Kappen & Goldberg, 1978; Burger et al., 1978), O_2 is required for the alkali-labile lesion at C, and under anaerobic conditions misonidazole can substitute for O_2 (data not shown). The sequence specificity of alkali-labile strand breakage at C residues in oligonucleotides is in agreement with that reported for restriction fragments (Povirk & Goldberg, 1985). In order to obtain alkali-dependent strand scission at a C, it has to be flanked by a minimum of three nucleotides on its 5′-side and by one on its 3′-side. Furthermore, in oligomers with more than one susceptible C residue there is a variation in the extent of strand scission at the different sites (e.g., Figure 2). The direct breaks in oligomers occurred generally with the same base specificity (T > A ≫ C > G) as that reported for strand breakage in large DNA substrates (Goldberg, 1986).

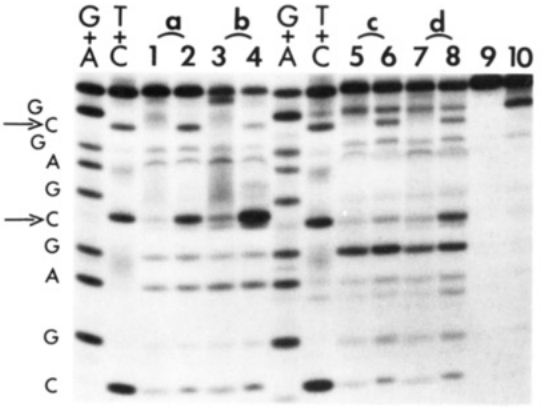

FIGURE 4: Effect of substituting I for G on the formation of alkali-labile lesions at C. Procedures for the reactions, alkali treatment, and analysis of the samples on a Tris-containing gel were as described under Materials and Methods. Lanes 1, 3, 5, and 7 are with NCS and without alkali treatment, and lanes 2, 4, 6, and 8 are with NCS and alkali treatment. Lanes 9 and 10 represent alkali-treated controls (containing no drug) of a and c, respectively. Arrows indicate alkali-dependent C–PO_4 bands. The standard markers (G+A, T+C) in the left and central lanes are of the ^{32}P-labeled strands and a + b and c + d, respectively. In lanes 5, 6, 7, and 8 the band immediately 5′ to the lower arrow is at the I residue, instead of the G residue. The following were substrates (in their double-stranded form) in standard reactions containing 40 μM NCS:

^{32}P-AGCGAGCGAGCG	^{32}P-AGCGAGCGAGCG
TCGCTCGCTCGC	TCGCTCICTCGC
a	b
^{32}P-AGCGAICGAGCG	^{32}P-AGCGAICGAGCG
TCGCTCGCTCGC	TCGCTCICTCGC
c	d

Effect of Substitution of G with I. In the preceding experiments the ^{32}P-end-labeled (+) strands containing the target C residues in AGC were annealed with their (−) strands to obtain the standard base pairing. Since G is the base, opposite and also at the 5′-side of the alkali-labile sites of the C residues in the several oligomers used in this study, it was of interest to investigate the effect of substitution of G with I (lacking the 2-amino group of guanine) on the alkali-labile lesion at C. With the 5′-^{32}P-end-labeled 12-mers $(AGCG)_3$ and AGCGAICGAGCG as the substrates, substitution of G with I opposite and/or on the 5′-side of the central target C results in four combinations:

GC	GC	IC	IC
CG	CI	CG	CI
a	b	c	d

The data presented in Figure 4 show that placement of I opposite the C residue (b) enhanced the alkali-dependent strand breakage at the C (bottom arrow) about 5-fold (lane 4) over that obtained with the standard base pairing (a) (lane 2). In (c), where I is 5′ to the C, the strand breakage at C was significantly reduced (lane 6), and in (d) it remained about the same (lane 8) as that in (a). These changes in cleavage

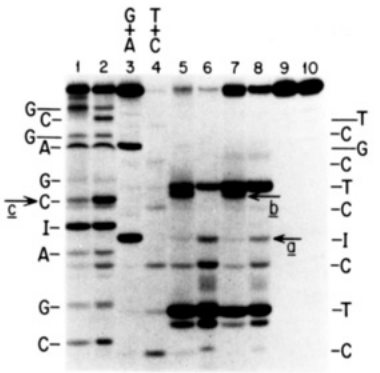

FIGURE 5: Comparison of strand breakage by NCS in the (+) and the (−) strand of an oligonucleotide duplex containing I. Standard reactions in lanes 1 and 2 had 5′-^{32}P-end-labeled (+) strand AGCGAICGAGCG annealed to the (−) strand CGCTCICTCGCT. In lanes 3–10, 5′-^{32}P-CGCTCICTCGCT annealed to AGCGAICGAGCG was the substrate. Alkali-treatment and analysis of the samples on a Tris-containing gel were as described under Materials and Methods. Lane 1, 40 μM NCS; lane 2, 40 μM NCS + alkali; lane 5, 40 μM NCS; lane 6, 40 μM NCS + alkali; lane 7, 8 μM NCS; lane 8, 8 μM NCS + alkali. Lanes 9 and 10 have (−) drug controls without and with alkali, respectively. Arrows are described in the text. The Maxam–Gilbert markers (lanes 3 and 4) are for the labeled (−) strand.

intensity are to be contrasted with their absence at the alkali-dependent C at the 3′-end (upper arrow), showing that the effects are not long range. It should also be noted that I, 5′ to C in (c) and (d) (lanes 5 and 7), is cut more than its counterpart G in (a) and (b) (lanes 1 and 3). The intensity of the band for strand scission at I is somewhat enhanced on hot alkali treatment. Furthermore, with the increase in cutting at I, there is a marked reduction in strand breakage at the A 5′ to it.

Lesions on the Complementary (−) Strand. In order to examine the lesions opposite the alkali-labile C sites, both (+) and (−) strands (5′ ^{32}P end labeled) were annealed to the appropriate unlabeled complementary strands and were used as substrates for the drug. In the first set of experiments ^{32}P-labeled $(AGCC)_3$ (+), containing three potential alkali-labile C sites, and its (−) strand ^{32}P-labeled $(GGCT)_3$ were the substrates (data not shown). In the (−) strand, in the absence of alkali treatment, strand scission occurred only at the G residues opposite the alkali-labile C sites and not at those opposite the alkali-insensitive C residues (see below).

In the experiment shown in Figure 5, the 12-mers ^{32}P-AGCGAIC*GAGCG and its (−) strand ^{32}P-CGCTCICTCGCT, which in the annealed form provides two I·C base pairs, were substrates for the drug. We were particularly interested in the lesion at the I residue opposite the central target C (C*) in the (+) strand. It can be seen that there is alkali-dependent formation of breaks at the I residue (lanes 6 and 8, arrow a) and an alkali-dependent disappearance of a band (lanes 5 and 7, arrow b) moving slightly ahead to the T residue. It is possible that this band represents a species formed after direct cleavage 3′ to the deoxyribose, resulting in a 5′-end-labeled fragment possessing deoxyribose at its 3′-end. It seems more likely, however, that because of the heavy cleavage at the T residue, the band with the slightly faster mobility is a fragment generated by cleavage at the T residue, but containing an abasic site at I. With alkali treatment this is converted into a break at I. In support of this formulation is the finding that the intensity of this band is greater than that of the alkali-labile I, suggesting that it contains the precursor of other alkali-dependent breaks in still smaller fragments than those made by cleavage at the I (e.g., see C that is 5′ to I). The pattern of strand breakage in the (+) strand (lanes 1 and 2, arrow c) is similar to that reported in Figure 4. The alkali-labile C is represented by a band stronger than that of the alkali-labile I on the opposite strand.

Table II: NCS-Dependent Cytosine Release in the Presence of Various Activating Agents[a]

activating agent	cytosine released (%) −alkali	cytosine released (%) +alkali
glutathione (5 mM)	21	24
2-mercaptoethanol (10 mM)	10	11
dithiothreitol (2 mM)	8	8
$NaBH_4$ (20 mM)	23	26

[a] [5-^{3}H]cytidine-labeled AGCGAGC*G (C* carries the ^{3}H label in its cytosine moiety) annealed to $(CGCT)_3$ was the substrate in the standard reaction containing 66 μM NCS and the indicated activators. Alkali treatment and quantitation of cytosine were as described under Materials and Methods. Cytosine release obtained (0.45%) in control reactions, containing the thiols or $NaBH_4$ but no drug, has been subtracted from all values; 100% cytosine represents 4434 cpm.

Table III: Correlation between NCS-Induced Cytosine Release and Alkali-Dependent Strand Scission at the C Residue in AGC[a]

	cytosine release (%)	strand breakage (%)
−alkali	15.9	3.5
+alkali	15.1	15.8

[a] A mixture of [5′,5-^{3}H]cytidine-labeled AGCGAGC*G (C* denotes the target having the ^{3}H label) and 3′-^{32}P-end-labeled AGCGAGCG annealed to $(CGCT)_3$ was the substrate for NCS (33 μM) in the standard reaction. The procedures for the determination of cytosine release and strand breakage are given under Materials and Methods. In the absence of the drug, 0.6 and 1.2% cytosine was released without and with alkali, respectively. These values have been subtracted from those obtained in the presence of the drug. 100% cytosine represents 9783 cpm (corrected for the ^{3}H in the 5′-position in the deoxyribose). In the absence of the drug, there was no measurable strand breakage with or without alkali treatment.

Relation between Cytosine Release and Alkali-Dependent Strand Scission. In the earlier studies correlating the alkali-dependent strand breakage at C to cytosine release, *E. coli* DNA, randomly labeled with [^{3}H]cytosine, was used as the substrate for the drug (Povirk & Goldberg, 1985). This has the obvious limitation of dealing with a random population of labeled cytosine residues, leaving an element of uncertainty as to whether the abasic and alkali-labile sites are one and the same. To clarify this issue we used as substrate an oligomer containing a single target C [AGCGAGC*G, (*) denotes the target]; by having a single C residue labeled with ^{3}H in its base and a ^{32}P end label, one can determine both cytosine release and strand cleavage at the same site.

Since thiols were found to vary in their efficiency to promote NCS-induced alkali-labile strand scission (Figure 2), we first determined cytosine release by NCS from [5-^{3}H]cytidine-labeled substrate in the presence of various thiols and $NaBH_4$. The data in Table II show a close parallel between the relative efficiency of the activating agents in cytosine release and that in the induction of alkali-dependent strand cleavage (compare Figure 2); further, cytosine release is not affected by alkali. Glutathione-dependent cytosine release and strand scission were then determined in reactions containing ^{3}H- and/or ^{32}P-labeled substrate. The results in Table III show that the extent of cytosine release and the degree of strand breakage are the same. It is likely that the small amount of strand breakage (3.5%) obtained in the absence of alkali treatment is primarily due to the breakdown of the labile intermediate during the workup. These results confirm that alkali-dependent strand scission at the C residues is a consequence of cytosine release.

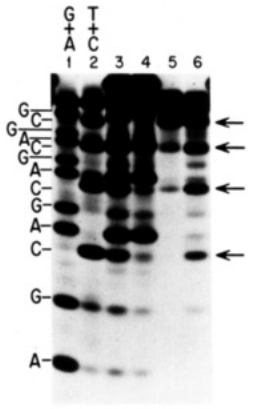

FIGURE 6: Generation of alkali-dependent breaks at the C residues in the isolated intermediate of drug treatment. 5′-^{32}P-end-labeled 14-mer G(AGC)$_4$G, annealed to C(GCT)$_4$C, was treated with NCS (40 μM) under standard conditions, and the reaction mixture was run on a Hepes-containing sequencing gel. The band of mobility slightly faster (putative intermediate) than that of the starting material was isolated as described under Materials and Methods. The isolated material was subjected to alkali treatment. The samples were analyzed on a Hepes-containing sequencing gel. Layered in lanes 3 and 4 were aliquots of the original drug reaction with and without alkali treatment, respectively. Lane 5, isolated precursor; lane 6, isolated precursor + alkali. The arrows indicate the alkali-dependent C–PO$_4$ bands. In control reactions containing no drug there were no bands (not shown).

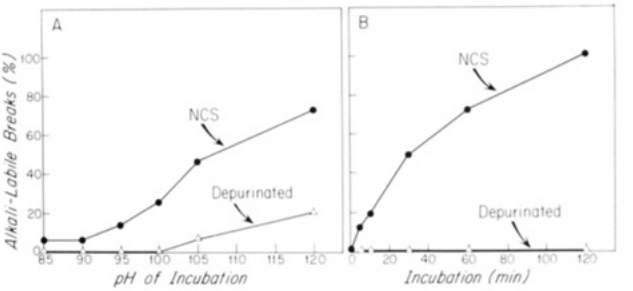

FIGURE 7: Comparison of hydrolysis profiles of NCS- and acid-depurinated abasic sites. 5′-^{32}P-end-labeled GAGCG annealed to (CGCT)$_3$ was the substrate for NCS (40 μM) in the standard reaction containing 10 mM Hepes buffer, pH 8.0, and 0.2 mM EDTA. Portions (3 μL) of the reaction mixture were mixed with sodium carbonate buffer (50 mM final) and incubated (37 °C at the indicated pH for 7 min in (A) and at pH 9.5 for the times shown in (B). At termination, the samples were neutralized with HCl. Another 3-μL reaction mixture was subjected to hot alkali (0.1 M NaOH) treatment. The depurinated substrate (^{32}P-GAGCG) to which all the components of the drug reaction except the drug had been added was similarly treated. The samples were analyzed on a Hepes-containing gel. Densitometric quantitation of strand breakage at C (C–PO$_4$) in the drug-treated and that at A (A–PO$_4$) in the depurinated oligomer was as described under Materials and Methods. Strand breakage obtained at the specified base on hot alkali treatment was taken as 100% which is 15.3% for C–PO$_4$ and 37.4% for A–PO$_4$. Strand breakage which occured at the respective base in the absence of high-pH treatment has been subtracted as a background in calculating alkali-dependent strand scission.

Evidence That Abasic Sites Are on Full-Length Molecules. As shown in Figure 1, gel analysis of several NCS-treated oligomers (less than 16 nucleotides long) revealed a band with a slightly faster mobility than that of the starting material; its disappearance on treatment with hot alkali concomitant with the appearance of the C–PO$_4$ band suggested it to be the precursor of C–PO$_4$. Although the results with the 5′- and 3′-^{32}P-end-labeled oligonucleotides, described above, pointed to the conclusion that the drug-induced lesion was an abasic site in full-length DNA, with short oligomers (e.g., 5–7-mer) where the target C is located one nucleotide short of its 3′-end, it is difficult to distinguish between a full-length molecule with an abasic site(s) and a fragment with a sugar residue attached to the phosphate only on the basis of mobilities in the gel.

This problem was resolved by use of as substrate a 5′-^{32}P-end-labeled 14-mer, G(AGC)$_4$G, in which there are four potential alkali-labile break sites. After drug treatment the sample was electrophoresed on a sequencing gel from which was isolated a band of mobility slightly faster than that of the starting material. The isolated material, which is presumed to be the full-length molecule with abasic sites, was treated with hot alkali and rerun on a sequencing gel (Figure 6). Also analyzed side by side were aliquots of the original reaction with (lane 3) and without (lane 4) alkali treatment. The film was overexposed to reveal the products from the isolated material. Alkali treatment of the original reaction generates four bands (arrows) corresponding to strand breakage at the four susceptible C residues (lane 3). Analysis of the isolated material (lane 5) without any further treatment shows a strong C–PO$_4$ band, corresponding to strand breakage at the C closest to the 3′-end (topmost arrow). Whether this was a contaminant present from the beginning because of its proximity to the starting material or was produced during the isolation procedure is not clear. Alkali treatment of the isolated material generated three additional C–PO$_4$ bands corresponding to strand scission at the other three target C residues (lane 6). No other bands were produced upon heating with alkali. Thus, the emergence of all the C–PO$_4$ bands, resulting from strand scission at the four potential C sites, from a common precursor strongly supports the existence of a full-length molecule as the product after cytosine release. The alternate possibility, that a break had already occurred on the 3′-side of the C residue following cytosine release, would require four different precursors widely differing in size and thus mobilities.

Stability Properties of the NCS-Induced Abasic Site. While hot alkali treatment of the drug-treated oligomer was required to obtain the full intensity of the C–PO$_4$ band, there was considerable smearing on the gel with the nonalkali-treated sample, indicative of product breakdown during electrophoresis. The smearing was somewhat reduced when Hepes replaced Tris as the buffer in the gel analysis. That Tris itself caused some breakdown of the precursor was confirmed in experiments (not shown) where the drug-treated oligomer, after incubation in Tris buffer at various pHs (7–9) at 37 °C, was electrophoresed on a Hepes-containing gel. Under the same conditions the acid-depurinated oligomer proved considerably more stable than the drug-treated substrate. We then compared the strand scission at a C (alkali-labile site) of the NCS-treated oligomer with that at a G or A of the depurinated substrate over a wide range of pH values. Shown in Figure 7A are the results obtained with a 5′-^{32}P-end-labeled substrate. There was a steady increase in the intensity of the drug-dependent C–PO$_4$ band as the pH was raised, in contrast to the virtual absence below pH 10.5 of fragments having 3′-phosphoryl termini due to strand breakage at the acid-generated abasic site at A (or G). In the latter case, there was instead, a band with a mobility slower than that expected for fragments having 3′-phosphoryl termini (not shown); this presumably represents a species with a sugar residue attached to the 3′-terminal phosphoryl group. Hot alkali did not produce this intermediate. These results and a time course (Figure 7B) of the strand scission at pH 9.5 demonstrate that the phosphoryl–sugar linkage on the 5′-side of the NCS-induced abasic site is much more labile than that at an acid-generated abasic site. Similar results were obtained when the stability of the 3′ sugar–phosphoryl linkage in NCS-induced abasic sites was compared with that of the acid-generated abasic site by

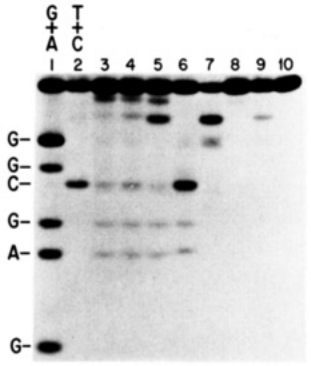

FIGURE 8: Cleavage of alkali-labile C by endonuclease IV. 5′-^{32}P-end-labeled GGAGCGG annealed to CCGCTCC was cut with NCS (20 μM). Portions of the reaction mixture were subsequently treated with endonuclease IV (Ljunquist, 1977) for 5.5 h at room temperature or with alkali as described under Materials and Methods. Half of the alkali-treated sample was incubated with polynucleotide kinase (37 °C, 1 h) under conditions reported by Cameron and Uhlenbeck (1977). Control reactions containing no drug were subjected to similar treatments. Analysis of the samples was on a Hepes-containing gel. Lanes 3 and 4 have NCS-treated oligomer. Lane 5, NCS + endonuclease IV; lane 6, NCS + alkali; lane 7, NCS + alkali, followed by incubation with polynucleotide kinase. Lanes 8–10 have (–) drug controls: lane 8, endonuclease IV; lane 9, alkali + polynucleotide kinase; lane 10, alkali.

use of a 3′-^{32}P-end-labeled substrate (data not shown).

In contrast to its lability at alkaline pH, the lesion at C was found to be stable to mildly acidic pH. Heating an NCS-treated oligomer (^{32}P-GGAGCGG) containing an alkali-labile C site at 60 °C in sodium acetate buffer (pH 4.5) for 2 h did not cause any strand breakage at the C.

Reaction with Endonuclease IV. Previous studies using restriction fragments have shown that the abasic site produced by NCS at a C residue is more resistant than an acid-generated abasic site to cleavage by several apurinic/apyrimidinic endonucleases such as endonuclease IV (Povirk & Goldberg, 1985). Although strand scission at the NCS-induced abasic sites of C residues was obtained at higher (5-fold) concentrations of endonuclease IV, an unambiguous characterization of the products at the breaks (whether hydroxyl or phosphate ended) based on their mobility in the gel was difficult because of the poor resolution obtained with the large-sized restriction fragments (43 bases).

In the present study, the 5′-^{32}P-end-labeled 7-mer (GGAGCGG) was used as the substrate (Figure 8). Products of the drug reaction were digested with endonuclease IV or heated in alkali. Endonuclease IV digestion generates a band with mobility much slower (lane 5) than that of the product (C–PO_4) of alkali treatment (lane 6). Since endonuclease IV is known to cut at the 5′-side of an abasic site to generate a 3′-hydroxyl end (Lindahl, 1982), the slower band most likely represents the 3′-hydroxyl-terminated product (^{32}P-GGAG-OH) as a result of strand cleavage at the abasic site of the C residue. Further confirmation comes from experiments in which the alkali-generated product was reacted with polynucleotide kinase under conditions where it acts as a phosphatase (Cameron & Uhlenback, 1977). Polynucleotide kinase alters the mobility of the alkali-generated product (^{32}P-$GGAG_p$) to correspond to that of the product of endonuclease IV reaction (lane 7). These results confirm that 3′-hydroxyl and 3′-phosphoryl ends are produced by endonuclease IV and hot alkali, respectively.

$NaBH_4$ Reduction. In order to test whether $NaBH_4$ affords protection against alkali-dependent strand breakage at the NCS-induced abasic sites, as it does for acid-generated abasic sites which have a C-1′ aldehyde, a 5′-^{32}P-end-labeled oligomer

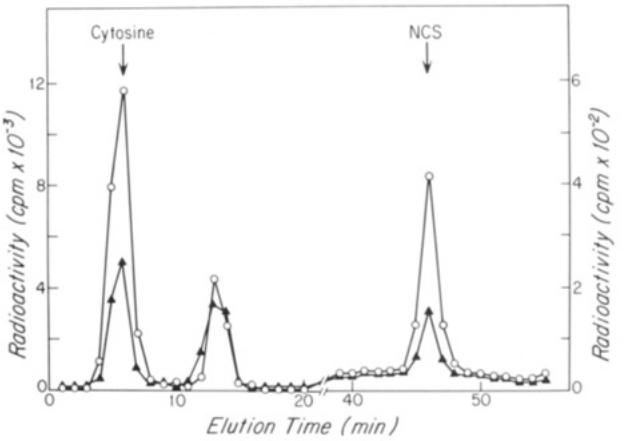

FIGURE 9: Cytosine release and abstraction of ^{3}H from deoxyribose of the substrate. [1′,2′,5-^{3}H]Cytidine-labeled AGCGAGC*G [(*) denotes the target having the ^{3}H label], annealed to $(CGCT)_3$ or CGCTCICTCGCT, was the substrate for NCS (66 μM) in standard reactions. The reaction mixture was analyzed by HPLC as described under Materials and Methods. The times at which cytosine and NCS chromophore elute are shown by the arrows. A control reaction containing no drug (not shown) gave a total of 1175 cpm in the cytosine region, but there was no radioactivity in the chromophore region. The absorbance and fluorescence profiles of the drug are not shown. $(CGCT)_3$ and CGCTCICTCGCT were the (–) strands in (▲) and (○), respectively.

[^{32}P-$(AGCG)_3$], after drug treatment or acid depurination, was reduced with $NaBH_4$ prior to heating in alkali. Unreduced samples were similarly treated. The products were then analyzed on a sequencing gel. The results show (data not presented) that the depurinated oligomer was fully protected, as expected, against strand breakage by reduction; there was very little, if any, protection in the case of NCS-induced abasic sites. In addition, when $NaBH_4$ was used as an activator for the drug (Figure 2, lane 10), alkali-dependent breakage at the C residue was not prevented.

^3H Abstraction. In the induction of direct strand breaks NCS primarily attacks the 5′-carbon by abstracting a hydrogen atom into the drug (Kappen & Goldberg, 1985). In order to determine if ^{3}H abstraction is also involved in the alkali-labile lesions at C residues, an 8-mer containing a single target C (AGCGAGC*G) in which C* carries the ^{3}H label at various positions of the sugar and/or the base as in (a) 1′,2′,5-^{3}H, (b) 5′,5-^{3}H, and (c) 5-^{3}H was used as substrate for the drug. The reaction mixtures were analyzed for cytosine release and abstraction of ^{3}H into the drug.

In Figure 9 is presented an HPLC profile of a drug reaction containing [1′,2′,5-^{3}H]cytidine-labeled substrate. Of the three peaks of radioactivity, the one at 6 min is due to cytosine. The product(s) eluting at 13 min has not been characterized; it most likely represents oligonucleotides resulting from direct strand scission at A residues. The peak of radioactivity at 46 min is coincident with the main UV-absorbing and fluorescent peak of NCS chromophore and represents the ^{3}H abstracted from the deoxyribose of the substrate into the drug. In accord with the stimulation of strand breakage obtained on placement of I opposite a C residue (see Figure 4), there is also a stimulation of cytosine release and concomitant increase in ^{3}H abstraction into the drug. Cytosine release and ^{3}H abstraction into the drug were then compared in reactions containing [1′,2′,5-^{3}H]cytidine- or [5′,5-^{3}H]cytidine-labeled substrates. The results presented in Table IV show that ^{3}H abstraction into NCS chromophore from the former is about 5 times greater than that from the latter substrate at comparable cytosine release. In addition, there was no ^{3}H abstraction into the drug, but only cytosine release, when [5-^{3}H]cytidine-la-

Table IV: Cytosine Release and Abstraction of ^3H from Deoxyribose of the Substrate into NCS Chromophore[a]

substrate	% cytosine released (*a*)	% ^3H abstracted (*b*)	*a*/*b*
AGCGAGC*G (5′,5-^3H)	52.5	0.44	119
AGCGAGC*G (1′,2′,5-^3H)	60.7	2.6	25

[a] The reaction conditions are similar to those in Figure 9. The ^3H-labeled (+) strand [(*) denotes the target C having the ^3H label] was annealed to $(CICT)_2$ in standard reactions containing 66 μM NCS. The reaction mixture was analyzed by HPLC to quantitate the released cytosine and the ^3H abstracted into the drug. Cytosine estimated by paper chromatography was in good agreement with that by HPLC. Given are the mean values from two experiments which differed from each other by less than 10%. Control reactions containing no drug gave 507 and 1161 cpm in the cytosine region of (1) and (2), respectively, and these have been subtracted as background in obtaining the drug-dependent cytosine release. In both (1) and (2), control (−NCS) reactions did not give any radioactivity in the region of NCS chromophore. Following are the 100% radioactivities: (1) cytosine, 84 872 cpm, and 5′-hydrogen, 69 237 cpm; (2) cytosine, 35 133 cpm, and 1′-hydrogen, 29 644 cpm.

beled oligomer (c) was used as the substrate (data not shown).

Discussion

Provided that the substrate is at least five nucleotides long and the putative target C residue in AGC is at least four residues in from the 5′-end and not at the 3′-terminus, oligonucleotides provide good sites for NCS-generated abasic lesions. Fraying at the ends of the duplex DNA may be responsible for the decreased attack by drug at these locations. The failure of the trimer and the tetramer to be substrates for the drug is likely due to their inability to form duplex structures under the conditions used and/or to inadequate size of the binding site for drug binding. Several features of the reaction including sequence specificity and glutathione preference parallel those reported for longer restriction fragments (Povirk & Goldberg, 1985). The latter result is also consistent with the finding that glutathione is the activator of NCS in intact cells (Kappen & Goldberg, 1987). Since the thiol forms an adduct with the chromophore upon activation (Hensens et al., 1983), the structure of the active species will depend on which activating agent is used, and its interaction with DNA might be expected to vary accordingly. It is of interest that $NaBH_4$ activation of the drug causes abasic site formation at C to a comparable degree as glutathione. While the reason for glutathione preference over other thiols remains unclear, the efficient substitution by $NaBH_4$ precludes an absolute requirement for a thiol, as has also been the case for spontaneous thymine release and direct strand breaks (Goldberg et al., 1981).

The binding of NCS chromophore to DNA is governed by several factors among which are base composition, sequence specificity, and secondary and tertiary structures (Goldberg, 1986). Since the intercalative mode of binding via the minor groove is particularly sensitive to intercalators and other agents that occupy or obstruct the minor groove (Povirk et al., 1981; Dasgupta & Goldberg, 1985), the stimulation of strand breakage at the C residue obtained on placement of I (lacking the 2-amino group) opposite C is not unexpected. This finding is consistent with the results (Dasgupta & Goldberg, 1986) on the binding of the drug to poly(dG-dC) and poly(dI-dC), where poly(dI-dC) was shown to have a greater binding affinity than poly(dG-dC) for the drug. The inhibitory effect of I 5′ to the C in AIC probably also reflects a change in DNA microstructure.

The reason why the AGC sequence is so highly favored in the production of the abasic site at C is not obvious, although AGC-containing DNA polymers are known to have nonclassical structures under certain defined conditions (Saenger, 1983). DNA microstructure associated with the AGC sequence is likely to be a critical factor in determining the site and mechanism of DNA sugar damage. The attack preference for C in AGC is also reflected in the complementary strand, where an alkali-labile break is found at the opposing G (or I). The two alkali-labile lesions appear not to be on complementary strands of the same duplex molecule, since L. F. Povirk (personal communication) has found that endonuclease IV treatment of supercoiled molecules remaining after NCS treatment do not form linear molecules whereas singly nicked (closed circular) molecules do. His results suggest that for the formation of double-strand breaks after cleavage at an abasic site there must be a direct strand break close by on the complementary strand, such as the T (or A) opposite the A of AGC (or the T of GCT, lanes 5–8, Figure 5). Our results do not distinguish whether the opposing lesions on the complementary strands result from the action of a single drug molecule or from two molecules. If the activated chromophore is a biradical, as has been proposed (Kappen & Goldberg, 1985; Myers, 1987), the former is a distinct possibility. In this regard, it may be significant, as discussed below, that in a space-filling model of B-DNA the hydrogen at C-1′ is closest to the C-5′ on the complementary strand directly across the minor groove. On the other hand, not all direct breaks at the T on the complementary strand have opposing abasic sites at C, since experiments using self-complementary oligodeoxynucleotides show that direct cleavage at the T is much stronger than the breakage produced by alkali at the C (unpublished data).

In the present study using an oligomer containing a single target C and appropriate radiolabels, we have established a correlation between cytosine release and alkali-dependent strand breakage at the abasic site so generated (Tables II and III). Furthermore, the experiments with an oligomer containing multiple C sites of attack demonstrated (Figure 6) that a full-length molecule with abasic sites is the product of the drug reaction. The stability properties of this product (Figure 7), lack of its protection against strand breakage by $NaBH_4$, and its previously reported relative resistance to cleavage by endonucleases specific for abasic sites (Povirk & Goldberg, 1985) clearly show that the abasic site generated by NCS at C in AGC is chemically different from the acid-generated abasic site. This modified abasic site, however, yields on endonuclease IV digestion the same product (3′-hydroxyl) as that from an acid-generated abasic site (Figure 8). It should be noted that there may be heterogeneity of structure of the abasic sites produced in DNA by NCS, depending on the nucleotide and sequence involved. Earlier we reported that abasic sites formed at T (and A) residues could be protected from alkali-induced cleavage by reduction (Kappen & Goldberg, 1983), suggesting that such lesions possess a susceptable aldehyde moiety. Further, there was no evidence for tritium abstraction by the drug from DNA labeled with [1′,2′-^3H]-thymidine (Kappen & Goldberg, 1985).

The mechanism of formation of the abasic site at the C residue in AGC appears to be different from that of direct strand scission where abstraction of a hydrogen atom from the 5′-carbon (in amounts consistent with the extent of strand breaks) is the initial step (Charnas & Goldberg, 1984; Kappen & Goldberg, 1985). The amount of ^3H abstracted (Figure 9 and Table IV) from the [5′,5-^3H]cytidine-labeled oligomer appears to be much too low to account for the cytosine release and the alkali-labile strand breaks; it most likely represents,

instead, ^3H abstraction due to direct strand scission (a minor component of the total) at the C residue. Although ^3H abstraction from the [1′,2′,5-^3H]cytidine-labeled substrate is also rather low on a molar basis compared with cytosine release (and strand cleavage), probably reflecting a relatively large isotope selection effect, both processes appear to be related from the findings that (a) the increase in ^3H abstraction is concomitant with the increase in cytosine release, when the C is base paired with I, and (b) ^3H abstraction is absent from the 1′,2′-positions in direct strand scission at T residues (Kappen & Goldberg, 1985). It should be noted that NCS induced the formation of a significant amount of tritiated water from presumably the 2′-position of the [1′,2′,5-^3H]cytidine-labeled substrate, especially at pH 10 and elevated temperature (unpublished data). The mechanism and intermediates responsible for these results will be discussed elsewhere.

The antibiotic bleomycin also releases bases from DNA with the generation of alkali-dependent strand breaks. In addition, in the aerobic reaction base propenals are formed with associated strand cleavage (Burger et al., 1982; Giloni et al., 1981; Wu et al., 1983; Murugesan et al., 1985). The mechanism proposed to account for the formation of free base release and alkali-dependent strand breakage involves the abstraction of the 4′-hydrogen and the intermediacy of a 4′-ketone (Wu et al., 1985a,b; Rabow et al., 1986). A consequence of the oxidation at C-4′ is the labilization of the 3′- and 5′-hydrogens (Wu et al., 1985b). Although 4′-hydrogen abstraction cannot be ruled out as a possible mechanism in the NCS-mediated cytosine release and associated alkali-dependent strand scission, the absence of the generation of a band with a mobility faster than a 3′-phosphate-ended fragment due to the formation of a 3′-terminal phosphoryl 2″-glycolate indicates that attack at C-4′ is an unlikely event. Also, the lability of the phosphodiester bond 5′ to the lesion in the NCS-induced damage reaction appears to be significantly greater than that due to bleomycin (Sugiyama et al., 1985). Direct abstraction of 3′-hydrogen would have resulted in immediate strand breakage. Thus, exclusion of the 5′- (Table IV), 4′-, and 3′-positions leaves C-1′ and/or C-2′ as possible sites of attack.

Precedents for attack at C-1′ in the production of alkali-labile lesions can be found in DNA damage induced by H_2O_2 (Rhaese & Freese, 1968), ionizing radiation (Dizdaroglu et al., 1977a), and 1,10-phenanthroline–cuprous complex (Goyne & Sigman, 1987). In all these cases a carboxyl (or lactone) is generated at C-1′. In this regard it is interesting that in DNA damage by electrolytically activated NCS the strand breaks were predominantly alkali dependent and C-1′ was the main site of attack (Favaudon et al., 1985). Abstraction of a hydrogen from C-2′, on the other hand, would result in a C-2′ aldehyde (Dizdaroglu et al., 1977b); formation of which is not compatible with the previously cited release of tritiated water at pH 10. A definitive conclusion concerning the possibility that C-1′ is the preferred attack site at C in AGC, however, awaits identification of the DNA sugar damage products.

It appears that the structural features of the interaction between NCS chromophore (thiol activated) and the DNA target are critical in determining the molecular mechanism and products of the DNA damage reaction. Ordinarily, the binding complex is such that the activated NCS chromophore attacks C-5′ of deoxyribose of mainly T residues, but in AGC not only is an uncommon site (C) attacked, but the attack is on a different carbon of deoxyribose. As noted above, it seems likely that both direct strand break at T and abasic site at C exist on the complementary strands of the same DNA molecule.

Registry No. NCS, 9014-02-2; NCS chromophore, 81604-85-5; cytidylic acid, 63-37-6.

REFERENCES

Burger, R. M., Peisach, J., & Horwitz, S. B. (1978) *J. Biol. Chem. 253*, 4830–4832.

Burger, R. M., Peisach, J., & Horwitz, S. B. (1982) *J. Biol. Chem. 257*, 8612–8614.

Cameron, V., & Uhlenbeck, O. C. (1977) *Biochemistry 16*, 5120–5126.

Charnas, R. L., & Goldberg, I. H. (1984) *Biochem. Biophys. Res. Commun. 122*, 642–648.

Chin, D. H., Kappen, L. S., & Goldberg, I. H. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7070–7074.

Dasgupta, D., & Goldberg, I. H. (1985) *Biochemistry 24*, 6913–6920.

Dasgupta, D., & Goldberg, I. H. (1986) *Nucleic Acids Res. 14*, 1089–1105.

Dizdaroglu, M., Schulte-Frohlinde, D., & von Sonntag, C. (1977a) *Int. J. Radiat. Biol. Relat. Stud. Phys., Chem. Med. 32*, 481–483.

Dizdaroglu, M., Schulte-Frohlinde, D., & von Sonntag, C. (1977b) *Z. Naturforsch., C: Biosci. 32C*, 1021–1022.

Favaudon, V., Charnas, R. L., & Goldberg, I. H. (1985) *Biochemistry 24*, 250–259.

Fritz, H.-J., Belagaje, R., Brown, E. L., Fritz, R. H., Jones, R. A., Lees, R. G., & Khorana, H. G. (1978) *J. Am. Chem. Soc. 17*, 1257–1267.

Giloni, L., Takeshita, M., Johnson, F., Iden, C., & Grollman, A. P. (1981) *J. Biol. Chem. 256*, 8608–8615.

Goldberg, I. H. (1986) *Basic Life Sci. 38*, 231–244.

Goldberg, I. H., Hatayama, T., Kappen, L. S., Napier, M. A., & Povirk, L. F. (1981) in *Molecular Actions and Targets for Cancer Chemotherapeutic Agents* (Sartorelli, A. C., Bertino, J. R., & Lazo, J. S., Eds.) pp 163–191, Academic, New York.

Goyne, T. E., & Sigman, D. S. (1987) *J. Am. Chem. Soc. 109*, 2846–2848.

Hensens, O. D., Dewey, R. S., Liesch, J. M., Napier, M. A., Reamer, R. A., Smith, J. L., Albers-Schonberg, G., & Goldberg, I. H. (1983) *Biochem. Biophys. Res. Commun. 113*, 538–547.

Kappen, L. S., & Goldberg, I. H. (1978) *Nucleic Acids Res. 5*, 2959–2967.

Kappen, L. S., & Goldberg, I. H. (1983) *Biochemistry 22*, 4872–4878.

Kappen, L. S., & Goldberg, I. H. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3312–3316.

Kappen, L. S., & Goldberg, I. H. (1985) *Nucleic Acids Res. 13*, 1637–1648.

Kappen, L. S., & Goldberg, I. H. (1987) *Biochemistry 26*, 384–390.

Lindahl, T. (1982) *Annu. Rev. Biochem. 51*, 61–87.

Ljunquist, S. (1977) *J. Biol. Chem. 252*, 2808–2814.

Maxam, A., & Gilbert, w. (1980) *Methods Enzymol. 65*, 499–560.

Murugesan, N., Xu, C., Ehrenfeld, G. M., Sugiyama, H., Kilkuskie, R. E., Rodriquez, L. O., Chang, L.-H., & Hecht, S. M. (1985) *Biochemistry 24*, 5735–5744.

Myers, A. G. (1987) *Tetrahedron Lett. 28*, 4493–4496.

Povirk, L. F., & Goldberg, I. H. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3182–3186.

Povirk, L. F., & Goldberg, I. H. (1986) *Nucleic Acids Res. 14*, 1417–1426.

Povirk, L. F., Dattagupta, N., Warf, B. C., & Goldberg, I. H. (1981) *Biochemistry 20*, 4007–4014.

Rabow, L., Stubbe, J., Kozarich, J. W., & Gerlt, J. A. (1986) *J. Am. Chem. Soc. 108*, 7130–7131.
Rhaese, H.-J., & Freese, E. (1968) *Biochim. Biophys. Acta 155*, 476–490.
Saenger, W. (1983) in *Principles of Nucleic Acid Structure* (Cantor, C. R., Ed.) p 221, Springer-Verlag, New York.
Sinha, N. D., Biernat, J., McManus, J., & Koster, H. (1984) *Nucleic Acids Res. 12*, 4539–4557.
Sugiyama, H., Xu, C., Murugesan, N., & Hecht, S. M. (1985) *J. Am. Chem. Soc. 107*, 4104–4105.
Wu, J. C., Kozarich, J. W., & Stubbe, J. (1983) *J. Biol. Chem. 258*, 4694–4697.
Wu, J. C., Kozarich, J. W., & Stubbe, J. (1985a) *Biochemistry 24*, 7562–7568.
Wu, J. C., Stubbe, J., & Kozarich, J. W. (1985b) *Biochemistry 24*, 7569–7573.

NMR Studies of the Interaction of the Antibiotic Nogalamycin with the Hexadeoxyribonucleotide Duplex d(5′-GCATGC)$_2$†

Mark S. Searle,‡ Jon G. Hall,‡ William A. Denny,§ and Laurence P. G. Wakelin*,‡

Molecular Pharmacology Group and NMR Facility, Peter MacCallum Cancer Institute, Melbourne, Victoria 3000, Australia, and Cancer Research Laboratory, School of Medicine, University of Auckland, Auckland, New Zealand

Received August 27, 1987; Revised Manuscript Received February 2, 1988

ABSTRACT: ^{1}H resonance assignments in the NMR spectra of the self-complementary hexadeoxyribonucleoside pentaphosphate d(5′-GCATGC)$_2$ and its complex with the antibiotic nogalamycin, together with interproton distance constraints obtained from two-dimensional nuclear Overhauser effect (NOE) spectra, have enabled us to characterize the three-dimensional structure of these species in solution. In the complex described, two drug molecules are bound per duplex, in each of two equivalent binding sites, with full retention of the dyad symmetry. Twenty-eight NOE distance constraints between antibiotic and nucleotide protons define the position and orientation of the bound drug molecule. Nogalamycin intercalates at the 5′-CA and 5′-TG steps with the major axis of the anthracycline chromophore aligned approximately at right angles to the major axes of the base pairs. The nogalose sugar occupies the minor groove of the helix and makes many contacts with the deoxyribose moieties of three nucleotides along one strand of the duplex in the 5′-TGC segment. The charged dimethylamino group and hydroxyl functions of the bicyclic sugar lie in the major groove juxtaposed to the guanine base, the bridging atoms of the bicyclic sugar making contacts with the methyl group of the thymine. Thus the antibiotic is not symmetrically disposed in the intercalation site but is in close contact in both grooves with atoms comprising the 5′-TGC strand. The intercalation cavity is wedge-shaped, the major axes of the base pairs forming the site being tilted with respect to one another. All base-pair hydrogen-bonding interactions are maintained in the complex, and there is no evidence for Hoogsteen pairing. The free duplex adopts a regular right-handed B-type conformation in which all glycosidic bond angles are anti and all sugar puckers lie in the C2′-endo range. In the complex the glycosidic bond angles and the sugar puckers deviate little from those observed for the duplex alone. The presence of two bound nogalamycin molecules substantially slows the "breathing" motions of the base pairs forming the intercalation cavity, and the observation of two downfield-shifted resonances in the ^{31}P NMR spectrum of the complex suggests a pronounced local helix unwinding at the drug binding site. The footprinting data of Fox and Waring [Fox, K. R., & Waring, M. J. (1986) *Biochemistry 25*, 4349–4356] imply that the highest affinity binding sites of nogalamycin have the sequence 5′-GCA (or 5′-TGC). Our findings show that the major determinants of specificity appear to be hydrogen-bonding interactions between the O6 and N7 atoms of the guanine in the intercalation site and the two hydroxyl groups of the bicyclic sugar of the antibiotic, coupled with hydrogen-bonding/electrostatic interaction between the protonated dimethylamino group and the O6 carbonyl of the terminal guanine.

Nogalamycin (Figure 1) is an anthracycline antibiotic active against Gram-positive bacteria and experimental tumours (Bhuyan & Dietz, 1965; Bhuyan & Reusser, 1970; Li et al., 1979). It is a selective inhibitor of DNA-directed RNA synthesis in prokaryotic and eukaryotic organisms (Fok & Waring, 1972; Li et al., 1979; Ennis, 1981), and its interaction with DNA has been well studied. It has been shown to be an intercalating agent by its capacity to both remove and reverse the supercoiling of circular DNA (Gale et al., 1981) and to increase the viscosity and contour length of linear DNA (Kersten et al., 1966; Das et al., 1974; Sinha et al., 1977). As is typical of intercalators, it decreases DNA buoyant density and sedimentation coefficient (Kersten et al., 1966) and stabilizes DNA toward thermal denaturation (Kersten et al., 1966; Das et al., 1974; Sinha et al., 1977). The intrinsic affinity of nogalamycin for eukaryotic DNAs is of the order

† This work was supported by grants from the Australian Research Grants Scheme, the Auckland Division of the Cancer Society of New Zealand, and the Medical Research Council of New Zealand.
‡ Peter MacCallum Cancer Institute.
§ University of Auckland.

0006-2960/88/0427-4340$01.50/0