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Apurinic/Apyrimidinic Endonuclease Sensitive Sites as Intermediates in the in Vitro Degradation of Deoxyribonucleic Acid by Neocarzinostatin†

Kallol K. Bose, Kouichi Tatsumi,‡ and Bernard S. Strauss*

ABSTRACT: Neocarzinostatin (NCS) induces alkali-labile sites in DNA which are stabilized by $NaBH_4$ reduction. The stabilized sites are sensitive to an AP endonuclease from human lymphoma cells. NCS-induced degradation of supercoiled Col E1 DNA proceeds in stepwise fashion with apurinic/apyrimidinic (AP) sites as intermediates. Degradation is increased when reaction occurs in the presence of AP endonuclease, and DNA reacted with NCS can be shown to have numerous AP endonuclease sensitive sites.

The antitumor antibiotic neocarzinostatin (NCS) is a small acidic protein of defined sequence with a molecular weight of 10 700 (Ishida et al., 1965; Meienhofer et al., 1972). NCS cytotoxicity and antitumor activity are thought to be due to its interaction with, and subsequent degradation of, cellular DNA (Ono et al., 1966; Beerman & Goldberg, 1974; Sawada et al., 1974) resulting from reaction at the sites of dT and dA residues in DNA with dT being the preferred substrate (Hatayama et al., 1978; D'Andrea & Haseltine, 1978). Sulfhydryl agents are required for NCS activity (Beerman & Goldberg, 1974), and free thymine and to a lesser extent adenine are released in an amount correlated with the number of strand scissions (Poon et al., 1977; Ishida & Takahashi, 1976). It has been suggested that NCS first produces single-strand breaks in a double-stranded DNA and that the double-strand breaks result from two independent single strand break events (D'Andrea & Haseltine, 1978). These authors suggested that the strand breaks might be produced by a two-stage reaction involving base removal, followed by cleavage of phosphodiester bonds. We were interested in this possible mechanism because of the previous studies from this laboratory on the pathway of degradation of alkylated DNA via apurinic sites. In this communication we show that apurinic/apyri-

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‡ Fellow of the Leukemia Society of America. Present address: Department of Internal Medicine, Division of Hematology and Immunology, Kanazawa Medical University, Uchinada-machi, Kahoku-gun, Ishikawa 920-02, Japan.

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midinic (AP) sites are intermediates in the DNA breakage pathway.

Materials and Methods

Neocarzinostatin (Lot 730859) was obtained from the National Cancer Institute. Stock solutions from materials kept at −20 °C were stored in the dark at 0–4 °C in sodium acetate buffer (0.015 M; pH 5.0) at a concentration of 1 mg/mL. Required dilutions were made in 50 mM Tris-HCl buffer, pH 7.5, prior to use.

Enzymes and Substrates. The human apurinic/apyrimidinic (AP) endonuclease was prepared from the human lymphoma line, Daudi, as previously described (Bose et al., 1978). This AP endonuclease preparation can break 1.5×10^{13} AP sites per μg of protein per 30 min and has an associated 5′–3′-exonuclease activity (Bose et al., 1978).

Preparation and Alkylation of Phage T7 DNA. Coli phage T7 DNA was prepared as previously described (Bose et al., 1978). [^{3}H]DNA was prepared by the addition of [^{3}H]thymidine (Schwarz Bioresearch Inc.) to a final concentration of 1 mCi/mL, 2 min after infection. DNA was prepared by exhaustive phenol extraction of a phage suspension purified through a CsCl gradient. Alkylation of the T7 DNA was done as follows. T7 DNA (1.2–1.5 mg/mL; 11 000–12 000 cpm/μg) was incubated at 37 °C with an equal volume of 12.5 or 25 mM MMS in 50 mM potassium phosphate buffer, pH 7.5. After the desired reaction time, the reaction mixture was diluted 1:10 with ice-cold 50 mM potassium phosphate buffer, pH 7.5, and dialyzed overnight against the same buffer. The DNA solution was then concentrated with 2-butanol to ~300–500 μg/mL (Stafford & Bieber, 1975) and dialyzed against 10 mM potassium phosphate buffer containing 100 mM NaCl and 0.1 mM EDTA.

Depurination of Alkylated T7 DNA. T7 DNA alkylated as above was heated at 70 °C for 30 min to depurinate the alkylated bases. Since the exact level of alkylation is difficult to control, we measured breaks and apurinic sites directly by alkaline sucrose gradient centrifugation as described (Lindahl & Andersson, 1972).

NCS Treatment of T7 DNA and Reduction of DNA with Sodium Borohydride. ^{3}H-Labeled T7 DNA (either depurinated as above or native) was treated separately in a 300–350-μL reaction mixture, containing 10 mM potassium phosphate buffer, pH 7.5, 100 mM NaCl, 0.1 mM EDTA, and 5 mM 2-mercaptoethanol, with 5 μg/mL NCS. The mixtures were incubated at 30 °C for 30 min, after which period 1 M potassium phosphate buffer, pH 6.6, was added to a final concentration of 300 mM. The samples which were to be reduced were treated with 5 M $NaBH_4$ solution added in three aliquots at 0, 15, and 30 min to a final concentration of 0.25 M, and the reaction was allowed to proceed at room temperature for 1 h. The samples which were not to be reduced received similar volumes of H_2O instead of $NaBH_4$ solution at similar time intervals and were incubated similarly. After the reaction, each sample was dialyzed in separate bags against 50 mM Tris-HCl, pH 7.5, at 0–4 °C for 16 h (Hadi & Goldthwait, 1971).

AP Endonuclease Induced Strand Break in T7 DNA. ^{3}H-Labeled T7 DNA, either native, depurinated, depurinated and reduced, or NCS treated and reduced, was treated separately with AP endonuclease in a 100-μL reaction mixture containing 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 2 μg/mL bovine serum albumin. The reaction mixtures were incubated at 37 °C for 30 min, after which 400 μL of H_2O, followed by 100 μL of 0.3 M NaOH containing 0.5 M NaCl and 10 mM EDTA, was added to each. The mixtures were allowed to stand at room temperature for 10 min, and then 100 μL of each mixture was layered on the top of 5–20% sucrose gradients containing 0.3 M NaOH, 0.5 M NaCl, and 10 mM EDTA.

Sucrose Gradient and Determination of Molecular Weight. Alkaline sucrose gradient centrifugation was as described (Lindahl & Andersson, 1972). Centrifugation was done at 4 °C except for a 10-min incubation at room temperature to hydrolyze apurinic or apyrimidinic sites (Lindahl & Andersson, 1972). Number-average molecular weights (Charlsby, 1954) were calculated from the relationship $M_n = \sum C_i / \sum (C_i/M_i)$, in which C_i is the radioactivity in the ith fraction and M_i is calculated from the relationship $M = k[d/(\omega^2 t)]^{1/\alpha}$ by using the value of α determined by Studier (Studier, 1965) and k, a constant determined by calibration of a gradient with intact T7 DNA. The number of breaks per T7 strand was estimated from the relationship: $n = M_n(\text{T7})/M_n(\text{treated}) - 1$. The ratio of M_n's is very sensitive to error at high levels of DNA degradation, and differences of less than 10% between samples are probably not significant (Crine & Verly, 1976).

Assay for Strand Breaks and AP Endonuclease Sensitive Sites in DNA. ^{3}H-Labeled double-stranded supercoiled Col E1 DNA was prepared from *Escherichia coli* JC411 (Bose et al., 1978). Our preparation contained 90–92% of the molecules in the intact, supercoiled form. ^{3}H-Labeled Col E1 DNA (6 μg; 4000–6000 cpm/μg) was incubated in a reaction mixture (total volume 50 μL) containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 40 μg of bovine serum albumin (Sigma Chemical Co.) per mL. Wherever indicated, 5 mM 2-mercaptoethanol, 1–5 μg/mL NCS, and 1.2 μg of AP endonuclease were added to the reaction mixture. After a 30-min incubation at 37 °C, 100 μL of "stop solution" containing 40 mM Tris–acetate (pH 7.8), 2 mM EDTA, 60% (w/v) sucrose, and 0.03% bromphenol blue was added to the reaction mixture. An aliquot (10 μL) of this reaction mixture containing 0.4 μg of DNA was electrophoresed in 0.8% agarose gels. At the end of a 3–5-h electrophoresis at 5 mA/gel current density, the gels were stained in ethidium bromide and photographed (Sharp et al., 1973). For determination of the extent of DNA degradation, the bands were cut out from the gel, placed in 2 mL of water, and boiled, and 12 mL of Aquasol (New England Nuclear) was added to each tube. The radioactivity was determined and the percent of DNA remaining supercoiled was calculated.

For kinetic study of DNA degradation, ^{3}H-labeled Col E1 DNA was incubated at 37 °C with 4 μg/mL NCS in a final volume of 100 μL in a similar reaction mixture. At the end of the designated time periods, 10-μL aliquots were withdrawn and diluted with stop solution containing 60% sucrose. A volume containing 0.45 μg of DNA was analyzed by gel electrophoresis as described above. The time intervals are the time elapsed between addition of NCS to the reaction mixture and placement of the final diluted mixture on top of the gel.

Results

We decided to test for the production of AP sites in NCS-treated DNA by looking for alkaline-sensitive sites which lost their sensitivity after reduction with sodium borohydride. Reduction after base removal stabilizes AP molecules to alkali (Hadi & Goldthwait, 1971) and hence permits a distinction between AP sites which are stabilized by reduction and strand interruptions which remain alkali sensitive. We first standardized our reaction conditions using for this purpose MMS-reacted phage T7 DNA since we had previously shown that this system could be used for quantitative studies (Bose et al., 1978).

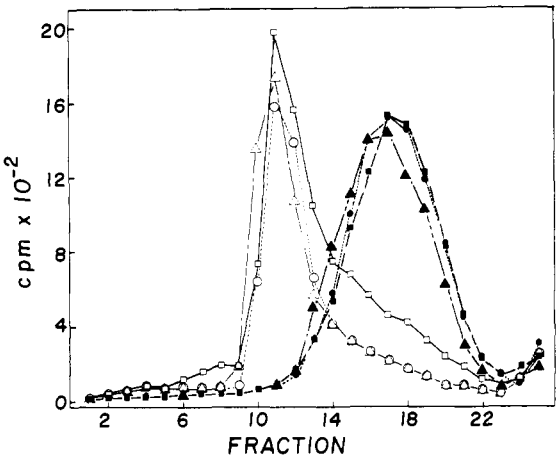

FIGURE 1: Effects of alkali and AP endonuclease on the size of native, depurinated, and depurinated and reduced T7 DNA. The reactions were carried out in two stages. In the first stage, 125 μg of ^{3}H-labeled T7 DNA (11000 cpm/μg), treated with 12.5 mM MMS, was heated to 70 °C in a 300-μL reaction volume containing 10 mM potassium phosphate buffer, pH 7.5, 100 mM NaCl, and 0.1 mM EDTA. After 30 min the reaction mixtures were cooled, and 150 μL (1 M) of potassium phosphate buffer, pH 6.6, was added to them. $NaBH_4$ (5 M) was added to one reaction mixture at 0-, 15-, and 30-min intervals to a final concentration of 0.25 M, while the other reaction mixture received an equal volume of water at same intervals of time. The mixtures were incubated at room temperature for 1 h after which they were dialyzed against 50 mM Tris-HCl, pH 7.5, overnight at 0–4 °C. In the second stage of reaction, 8 μg of depurinated DNA, 9 μg of depurinated and reduced DNA as prepared above, and 8 μg of native ^{3}H-labeled T7 DNA were incubated separately in a 100-μL reaction mixture containing 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 2 μg/mL bovine serum albumin, with or without 1.2 μg of endonuclease. After 30 min at 37 °C, 400 μL of H_2O, followed by 100 μL of 0.3 M NaOH containing 0.5 M NaCl and 0.01 M EDTA, was added to each reaction mixture and they were allowed to stand at room temperature for 10 min. 100 μL of each reaction mixture was layered on a 5–20% sucrose gradient containing 0.3 M NaOH, 0.5 M NaCl, and 0.01 M EDTA. The gradients were centrifuged at 45 560 rpm for 2.5 h at 5 °C. Samples were collected from the bottom of the tube on filter paper disks (Whatman 3MM), dried, washed with 5% Cl_3AcOH, 95% ethanol, and finally acetone, and dried. Radioactivity was determined by using a toluene-based scintillation cocktail. Open circles, native T7 DNA (0.56); open triangles, native T7 DNA plus AP endonuclease (0.47); open squares, depurinated and reduced T7 DNA (0.99); filled circles, depurinated T7 DNA (7.6); filled triangles, depurinated T7 DNA plus AP endonuclease (6.1); filled squares, depurinated and reduced T7 DNA plus AP endonuclease (7.9). The numbers in the parentheses indicate the strand breaks per T7 DNA molecule.

Incubation of T7 DNA with 12.5 mM MMS for 10 min, followed by heating for 30 min at 70 °C in pH 7.5 buffer, gave a substrate with about eight alkali-labile (AP) sites per strand (Figure 1). Reduction of this substrate with $NaBH_4$ as described by Hadi & Goldthwait (1971) produced a substrate containing only one alkali-sensitive site per strand. Incubation of the reduced depurinated substrate with the AP endonuclease preparation previously described (Bose et al., 1978) resulted in about eight breaks per strand. Addition of enzyme to native T7 DNA produced about 0.5 break per strand (Figure 1). Addition of enzyme to the depurinated but not reduced T7 DNA produced 6.1 breaks per strand.

The correspondence between the loss of alkali-labile sites by reduction with borohydride and their quantitative reappearance on treatment with AP endonuclease, along with the observed resistance of native T7 DNA to enzyme, shows the specificity of the enzyme for AP sites. We therefore studied the effect of sodium borohydride reduction and of enzyme treatment on DNA after reaction with NCS. When T7 DNA with about 0.5 alkali-labile site per strand was treated with

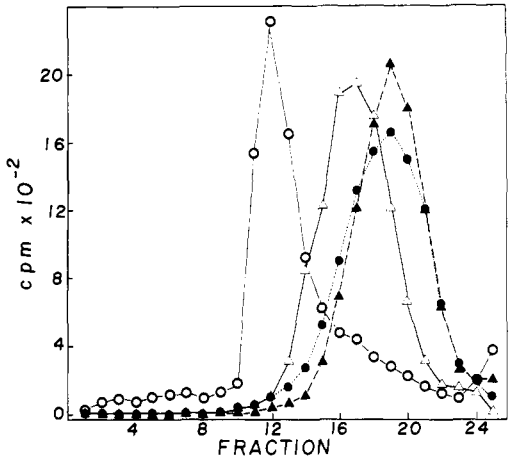

FIGURE 2: NCS-catalyzed degradation of T7 DNA and enzyme degradation of NCS-treated and reduced DNA in alkaline sucrose gradient. The reactions were carried out in two stages as in Figure 1. In the first stage ^{3}H-labeled T7 DNA was incubated in two separate reaction mixtures 300 μL each, containing 50 mM potassium phosphate buffer, pH 7.5, 10 mM NaCl, 5 mM 2-mercaptoethanol, and 44 μg of DNA with 5 μg/mL NCS. After 30 min at 37 °C, 1 M potassium phosphate buffer, pH 6.6, was added to final concentrations of 350 mM. One reaction mixture received 5 M $NaBH_4$ at 0-, 15-, and 30-min intervals to a final concentration of 0.25 M, while the other received the same volume of water at the same time intervals. The mixtures were incubated at room temperature for 1 h and were dialyzed against 50 mM Tris-HCl, pH 7.5, at 0–4 °C overnight as before. In the second stage of reaction 8 μg of native T7 DNA, 5 μg of NCS-treated T7 DNA, 5 μg of NCS-treated and reduced T7 DNA, and 5 μg of NCS-treated and reduced plus 1.2 μg of AP endonuclease were incubated in a 100-μL reaction mixture containing 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 2 μg/mL bovine serum albumin. After 30 min at 37 °C, 400 μL of H_2O, followed by 100 μL of 0.3 M NaOH containing 0.5 M NaCl and 0.01 M EDTA, was added to each reaction mixture, and then it was further incubated for 10 min at room temperature. 100-μL aliquots of each reaction mixture were layered on a 5–20% alkaline sucrose gradient as before, and gradients were centrifuged at 45 120 rpm for 2.5 h at 0–5 °C. The fractions were collected and the radioactivity was determined as before. Open circles, native T7 DNA (0.46); filled circles, NCS-treated T7 DNA (11.1); open triangles, NCS-treated and -reduced T7 DNA (5.28); filled triangles, NCS-treated and reduced T7 DNA plus AP endonuclease (11.1). The numbers in the parentheses indicate the strand breaks per T7 DNA molecule.

5 μg/mL NCS in the presence of 2-mercaptoethanol, about 11 alkali-labile sites per strand were observed (Figure 2). Upon $NaBH_4$ reduction of the NCS-treated DNA prior to alkali treatment, the number of alkali-labile sites was reduced to about 5.3 per strand. Treatment of the $NaBH_4$-reduced, NCS-treated DNA with AP endonuclease preparation once again resulted in a product with about 11 alkali-labile sites per strand. These data indicate that there were five strand breaks and six AP sites in the NCS-treated DNA, and the correspondence of the alkaline gradients of NCS-treated DNA and of NCS-treated, $NaBH_4$-reduced DNA after treatment with AP endonuclease indicates that the enzyme preparation is a quantitative probe for AP sites in DNA.

These observations indicate that AP sites are produced by NCS action. In order to determine whether they are likely to be necessary intermediates in the degradation, we decided to study the kinetics of the reaction. In order to analyze the numerous samples that would be required, we decided to carry out these studies using the small circular supercoiled Col E1 DNA molecule. The use of supercoiled DNA molecules for such investigations has been described before (Bose et al., 1978). We were attracted to the system because of the ease with which supercoiled and relaxed circles and linear forms can be distinguished from each other by gel electrophoresis.

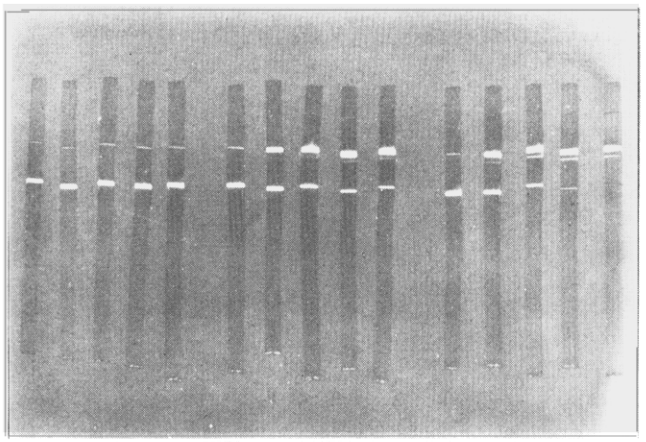

FIGURE 3: Treatment of Col E1 with NCS and mercaptoethanol. ^{3}H-Labeled Col E1 DNA (6 μg) (4000 cpm/μg) was incubated for 30 min with 0, 1, 2.5, 4, and 5 μg/mL NCS. Left to right: 1–5, without mercaptoethanol; 6–10, plus 5 mM mercaptoethanol; 11–15, plus 5 mM mercaptoethanol plus 1.2 μg of AP endonuclease in a 50-μL reaction mixture. Stop solution was added and 10 μL (0.4 μg of DNA) was electrophoresed (5 h at 5 mA/gel). The gels were stained with ethidium bromide as described under Materials and Methods.

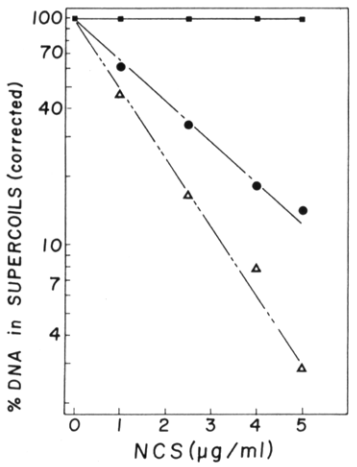

FIGURE 4: Effect of NCS on supercoiled DNA. The bands shown in Figure 3 were cut out, placed in 2 mL of water, and boiled, and 12 mL of Aquasol was added. The radioactivity was determined and the percent remaining supercoiled was calculated. Filled squares, no mercaptoethanol; filled circles, plus mercaptoethanol; open triangles, plus mercaptoethanol plus AP endonuclease.

In the absence of 2-mercaptoethanol, no additional strand breaks occur in the supercoiled fraction (bottom band of the gel) of the DNA even at very high NCS doses (Figure 3). The supercoiled fraction diminishes progressively as the dose of NCS is increased in the presence of 5 mM 2-mercaptoethanol, and at the same time the fraction of relaxed circles (top band) and linear molecules (middle band) increases. When these reactions were carried out in the presence of 1.2 μg of AP endonuclease, the extent of conversion of supercoiled DNA to relaxed circle and linear forms was much greater at all doses of NCS (Figures 3 and 4). In the presence of 2-mercaptoethanol, ~2.35 μg/mL NCS was necessary to produce an average of one strand break per molecule (37% of the DNA remaining supercoiled) in the existing supercoiled fraction. In the presence of AP endonuclease, twice as many strand breaks occurred at this same NCS concentration (only 18% of DNA remaining in the supercoil state). An enhanced amount of DNA degradation was seen throughout the range of NCS concentrations tested (1–5 μg/mL) in the presence of AP endonuclease.

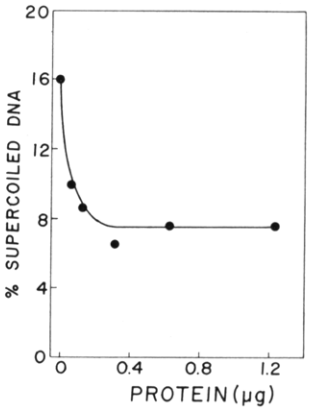

FIGURE 5: Effect of AP endonuclease concentration on NCS-treated Col E1 DNA. 6 μg of ^{3}H-labeled Col E1 DNA (4000 cpm/μg) was incubated in a 50-μL reaction volume with 4 μg/mL NCS in the presence of 5 mM 2-mercaptoethanol and 0–1.2 μg of AP endonuclease as described under Materials and Methods and in the legend to Figure 1. The reactions were stopped by the addition of 100 μL of stop solution to each tube after 30 min at 37 °C. Aliquots (10 μL) were analyzed for the proportion of supercoiled DNA remaining in the reaction mixtures by agarose gel electrophoresis as described in the legend to Figure 4. Controls without NCS or AP endonuclease and without NCS but with 1.2 μg of AP endonuclease showed 90.6 and 90.9% of the DNA in the supercoiled band.

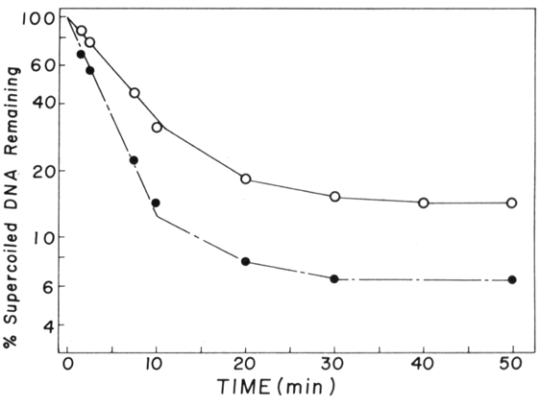

FIGURE 6: Kinetics of the breakdown of supercoiled DNA reacted with NCS. ^{3}H-Labeled Col E1 DNA (13.7 μg) was incubated at 37 °C with 4 μg/mL NCS and 5 mM 2-mercaptoethanol in a final volume of 100 μL in the absence or presence of 3.1 μg of AP endonuclease as described in the legend to Figure 3. At the indicated time periods, 10 μL of reaction mixture was withdrawn and mixed with 20 μL of a stop solution containing 60% (w/v) sucrose and buffer as described under Materials and Methods. 10 μL of this mixture containing 0.45 μg of DNA was analyzed by agarose gel electrophoresis, followed by radioactivity measurement as described in the legend to Figure 4. The time intervals are the time elapsed between addition of NCS to the reaction mixture and placement of the final diluted mixture on top of the gel. Open circles represent reaction without AP endonuclease; closed circles indicate reaction in the presence of 3 μg of AP endonuclease per 100-μL reaction mixture.

The concentration of AP endonuclease used in these experiments (1.24 μg) was saturating. We found (Figure 5) that 0.3 μg of the enzyme preparation was sufficient to degrade DNA treated with 4 μg/mL NCS in the presence of 2-mercaptoethanol (16% DNA remaining in the supercoiled form) to its limit (7.5% supercoils), and no further degradation was obtained by the addition of up to 1.24 μg of enzyme.

We studied the kinetics of DNA degradation induced by NCS in the presence and absence of AP endonuclease using supercoiled DNA. The data (Figure 6) indicate that the reaction proceeds at an exponential rate for about the first 10 min and then slows down and stops at the end of 30 min. The exponential decay constant for the first part of the reaction calculated as $k = -1/t \ln (N/N_0)$ was 0.12 min^{-1} in the absence

Table I: Degradation of NCS-Treated Col E1 DNA by AP Endonuclease[a]

treatment of DNA	% of DNA in supercoil form
(A) control Col E1 DNA	90.9
(B) Col E1 DNA plus NCS, 30-min incubation	15.6
(C) Col E1 DNA plus NCS plus AP endonuclease, 30-min incubation	7.3
(D) reaction mixture from (B) after a further 10-min incubation with AP endonuclease	8.0
(E) reaction mixture from (C) after a further 10-min incubation without further additions of AP endonuclease	7.6

[a] ^{3}H-Labeled Col E1 DNA (12.3 μg) was incubated at 37 °C with 4 μg/mL NCS plus 5 mM 2-mercaptoethanol in a 100-μL reaction mixture as in Figure 1. A similar reaction mixture was incubated in the presence of 2.5 μg of AP endonuclease. After a 30-min incubation period, a 50-μL sample was withdrawn from each reaction mixture for electrophoretic analysis. AP endonuclease (1.2 μg) was then added to the remaining portion of the reaction mixture initially incubated without enzyme, and both mixtures were incubated for an additional 10 min at 37 °C. At the end of this 10-min period, the reactions were stopped as before and the mixtures were analyzed for the amount of supercoiled DNA remaining.

of AP endonuclease and 0.20 min^{-1} in its presence.

At the end of the 30-min reaction period, ~15% of the DNA remained in the supercoiled fraction in the absence of AP endonuclease whereas only ~7% was intact when enzyme was included in the reaction mixture. This observation suggested that part of the DNA migrating in the supercoiled band after incubation with NCS should contain sites sensitive to AP endonuclease as would be expected from the data of Figure 2. We therefore incubated Col E1 with NCS for 30 min in the absence of AP endonuclease (Table I). At the end of the reaction period, a portion of the reaction mixture was taken for gel electrophoresis, and another portion (50 μL) was incubated with 1.2 μg of AP endonuclease for 10 min and then analyzed by gel electrophoresis along with a control reaction mixture, incubated with AP endonuclease for 30 min plus an additional 10 min. About 16% of the DNA remained in the supercoiled fraction when no AP endonuclease was present, and this amount was reduced to 8% by the addition of endonuclease for 10 min, compared to a final level of 8% when AP endonuclease was present for a 30-min incubation period plus a 10-min additional incubation as a control.

Discussion

Although it has been known for some time that NCS produces strand breaks in DNA (Ono et al., 1966; Beerman & Goldberg, 1974; Sawada et al., 1974), the mechanism of the reaction has been incompletely understood. The antitumor drug bleomycin has been reported to induce alkaline-labile sites in DNA which are converted to single-strand breaks in the presence of –SH reagents (Ross & Moses, 1978). Thiol groups are required also for the action of neocarzinostatin (Beerman & Goldberg, 1974; Figures 1 and 2), and the active material produced by the interaction of NCS with 2-mercaptoethanol has a half-life of ~5–10 min in the absence of DNA under conditions similar to those in our experiments (Ishida & Takahashi, 1978; Kappen & Goldberg, 1978). In the presence of 5 mM 2-mercaptoethanol, the NCS reaction with DNA is complete in ~30 min (Figure 6). The reaction is also highly specific for thymine and adenine residues (Hatayama et al., 1978; D'Andrea & Haseltine, 1978).

Our data indicate that the reaction proceeds in two separate stages and that AP (apurinic/apyrimidinic) sites are intermediates in the reaction. The qualitative observation that T7 DNA treated with NCS has numerous AP sites at the conclusion of the reaction (Figure 2) as well as the demonstration that the rate of loss of supercoiled molecules is greater in the presence of endonuclease (Figures 3 and 4) is sufficient to establish the role of the AP sites. Furthermore, the finding that AP endonuclease can increase the rate of degradation even in the presence of active NCS (Figures 3–5) shows that the enzyme can gain access to the reaction site. NCS therefore participates in at least two separate reactions. One might suppose that NCS reacts with a thiol group to produce an active radical which then diffuses to the reaction site except that special constraints must still be employed to account for the base specificity of the reaction.

AP DNA is not particularly susceptible to NCS-induced degradation. We prepared AP DNA by MMS treatment as described above (legend of Figure 1) and treated this material with NCS plus 2-mercaptoethanol. The mixture was then reduced with $NaBH_4$ and compared for alkali-labile sites with NCS-treated nondepurinated DNA. We found 7.1 breaks per T7 DNA strand in depurinated, NCS-treated reduced DNA, 6.3 breaks in NCS-treated reduced DNA, 1 break per T7 strand in reduced depurinated DNA (compared to 5.3 breaks in alkali before reduction), and 0.6 break per strand in our unreacted T7 preparation. The results are what would be expected if NCS attacked the bases preferentially and ignored preexisting AP sites since the 7.1 breaks obtained in the depurinated, NCS-treated preparation minus the 6.3 breaks per strand in the preparation treated only with NCS equals 0.8 compared to the 1.0 residual break per strand observed in the depurinated, reduced preparation. Unfortunately, the results are not conclusive since without sequencing experiments, it is impossible to distinguish breaks at AP sites produced by depurination (which would occur preferentially at G's) from those produced by NCS action [which would occur mainly at T's (D'Andrea & Haseltine, 1978; Hatayama et al., 1978)]. The hypothesis that NCS prefers unreacted bases to AP sites helps explain how an AP endonuclease can compete with NCS and increase the rate of strand breakage (Figures 4 and 5).

Some idea of the stoichiometry of the reaction can be obtained from the data of Figure 6. In the absence of AP endonuclease, we found 15.5% residual supercoiled DNA at the conclusion of the reaction corresponding to 1.86 breaks per molecule. Since 13.7 μg of Col E1 DNA (molecular weight 4.2×10^6) corresponds to 2×10^{12} molecules, there were 3.65×10^{12} breaks produced by $(0.4 \times 10^{-6}$ g/10 700)$(6.023 \times 10^{23}) = 2.25 \times 10^{13}$ molecules of NCS or 6 molecules per break, in good agreement with the ratio of drug to strand scissions reported by Beerman et al. (1977). Since there are two reactions involved in producing a break and since there must be a nonproductive loss of NCS without reaction with DNA (Ishida & Takahashi, 1978; Kappen & Goldberg, 1978), the data support the hypothesis that one or two NCS molecules are required for each step of the reaction. NCS does not behave in a catalytic fashion.

Our data can also suggest an answer to the question: are *all* NCS-induced breaks produced with AP sites as an intermediate? The data of Figure 6 can be used to construct kinetic curves showing the loss of unreacted supercoils (measured as the proportion of remaining supercoils in the presence of AP endonuclease), the accumulation of DNA with apurinic sites (measured as the difference in the amounts of supercoiled DNA in the presence or absence of AP endonuclease), and the production of DNA with single-strand breaks (measured as 1.0 minus the proportion of supercoils remaining in the

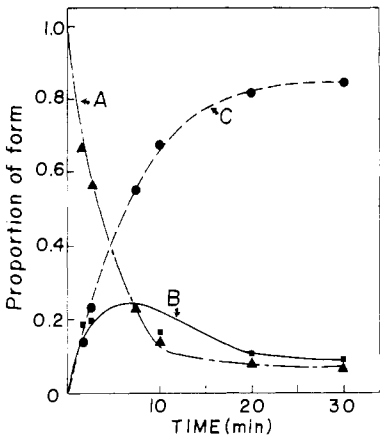

FIGURE 7: Accumulation of AP endonuclease sensitive sites in NCS-treated Col E1 DNA as a function of time. (A) Proportion of unreacted supercoils remaining = reaction with NCS in the presence of AP endonuclease; (C) proportion of Col E1 DNA with nicks = 1.0 − (fraction of supercoils remaining after reaction with NCS in the absence of AP endonuclease); (B) accumulation of AP endonuclease sensitive sites = (A) − (C). Data points are from Figure 6. Curves were calculated from the data of Figure 6 as drawn.

Table II: Reaction Rate Constants for NCS-Induced Degradation for Col E1 DNA Assuming Two Sequential First-Order Reactions

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

reaction time (min)	k_1 (min^{-1})	k_2 (min^{-1})
1.5	0.27	0.60
2.5	0.23	0.30
7.5	0.20	0.063
10.0	0.19	0.037
20.0	0.13	
30.0	0.09	

absence of AP endonuclease). This curve (Figure 7) has the expected features of a chain reaction of the type

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

(Strauss & Hill, 1970). Owing to the rapid decay of the NCS–thiol reaction product, this kinetic formulation is overly simplified, and the decay of apurinic sites must be at least a second-order reaction. The calculated first-order reaction rate constants (Table II) therefore change with time, although k_1 remains reasonably constant at early stages of the reaction. The half-life for the reaction yielding apurinic sites is ~3.2 min^{-1} at 4 μg/mL NCS, and the finding that apurinic sites do accumulate indicates that the second reaction proceeds at about the same rate. Although these data are insufficient to demonstrate that all NCS degradation proceeds via apurinic sites, this remains the best working hypothesis. The half-life for AP sites in the presence of AP endonuclease is of the order of a fraction of a second (Bose et al., 1978). The spontaneous half-life of AP sites at 37 °C and neutral pH is ~190 h (Lindahl & Nyberg, 1972). Our estimate of a half-life of 1.2 min for apurinic sites at the start of the NCS reaction indicates this reaction to be much faster than the spontaneous reaction but still much slower than that catalyzed by an AP endonuclease.

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