# Repair of Neocarzinostatin-Induced Deoxyribonucleic Acid Damage in Human Lymphoblastoid Cells: Possible Involvement of Apurinic/Apyrimidinic Sites as Intermediates†

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ABSTRACT: Neocarzinostatin (NCS) induces repair in a xeroderma pigmentosum lymphoblastoid line deficient in the ability to repair DNA damage induced with (acetoxyacetylamino)fluorene. Repair was demonstrated by the induction of repair synthesis and by the disappearance of NCS-induced single-strand breaks and/or alkaline-labile sites in DNA. Estimation of NCS-induced repair patch size, based on the density shift induced in DNA by extensive shear after incubation of treated cells in medium with bromodeoxyuridine or by calculation from the extent of restoration of DNA sedimentation profiles in alkaline sucrose gradients and the amount of repair synthesis measured by the BND cellulose method, indicated that only a few nucleotides were inserted per repaired region. NCS-treated bacteriophage T7 DNA requires incubation with alkaline phosphatase to make it a substrate for DNA polymerase I. NCS-reacted T7 DNA, even after phosphatase treatment, is not a substrate for a DNA polymerase $\alpha$ obtained from human lymphoma cells. NCS-treated T7 DNA did serve as a substrate for the DNA polymerase $\alpha$ when incubated with an apurinic/apyrimidinic (AP) endonuclease with associated 5′–3′-exonuclease activity. The results suggest that NCS-induced AP sites could be intermediates for the in vivo repair synthesis.

The antitumor agent neocarzinostatin (NCS)[1] induces DNA repair synthesis in mammalian cells (Tatsumi et al., 1975; Kappen & Goldberg, 1978a) but the in vivo mechanism of this repair is incompletely understood. NCS treatment inhibits mitosis (Ebina et al., 1975) and DNA synthesis (Beerman & Goldberg, 1974; Tatsumi et al., 1974; Ohtsuki & Ishida, 1975), and it is reasonable to suppose that the repair synthesis starts at the site of the breaks. However, the breaks produced by NCS treatment of DNA have a complex structure with both 3′- and 5′-hydroxyls phosphorylated at the site of the break (Kappen & Goldberg, 1978b) so that any repair synthesis would require at least an initial phosphatase or nuclease action to provide the necessary 3′-OH group for chain elongation. The finding that NCS-induced degradation of DNA occurs via AP (apurinic/apyrimidinic) sites as intermediates (Bose et al., 1980) makes plausible a mechanism in which an AP endonuclease produces 3′-OH groups directly at AP sites as the first step in repair. In this investigation of NCS-induced repair, we use the previously described in vitro T7 bacteriophage DNA system (Bose et al., 1978) as a model to account for our results with human lymphoblastoid lines, particularly those derived from patients with xeroderma pigmentosum. NCS-induced repair has characteristics different from those of nucleotide excision, and we conclude that AP sites are likely to be the intermediate for at least some of the repair observed.

## Materials and Methods

*Chemicals.* Neocarzinostatin (NCS 157365), Lot No. 730849, was supplied by the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. The antibiotic was stored in the dark at −20 °C and was dissolved in PBS just before use. BrdUrd, FdUrd and methyl methanesulfonate (MMS) were obtained from Calbiochem (San Diego, CA) and Eastman Organic Chemicals (Rochester, NY), respectively. (*N*-Acetoxy-*N*-acetyl-2-amino)fluorene (AAAF) was supplied by the NCI Carcinogenesis Research Program. Radioactive dThd ($^{14}$C, 50 mCi/mmol; $^{3}$H, 60, 19, and 3 Ci/mmol) was obtained from Schwarz/Mann (Orangeburg, NY).

*Cells.* The Burkitt's lymphoma line, Raji, was provided by Dr. P. Gerber. Cell line L-33-6-1 derived from a patient with infectious mononucleosis was received from Dr. J. Littlefield. Cell line XPA-3, a lymphoblastoid line derived from a complementation group C xeroderma pigmentosum patient, was provided by Dr. J. Robbins. GM 2253, a line derived from a xeroderma pigmentosum group D individual, and GM 2473, a xeroderma heterozygote (mother of GM 2253), were obtained from the Human Genetic Mutant Cell Repository. All cell lines were maintained in RPMI 1640 medium supplemented with 17% fetal calf serum (Kansas City Biological Inc., Lenexa, KS) heat inactivated for 30 min at 56 °C.

Cell number was determined with a Coulter counter (threshold 10 $\mu$m; amplification 0.25; aperture 1). Viability was determined as follows. Cells were treated with NCS for 1 h at 37 °C and then diluted and plated in quadruplicate by the procedure of Sato et al. (1972), except that no feeder layer was used and 4 mM glutamine, 1 mM $\alpha$-ketoglutaric acid (Sigma Chemical Co., St. Louis, MO), 5% heat-inactivated fetal calf serum, and 10% heat-inactivated horse serum were added to RPMI 1640 medium. Plates were incubated in a humidified incubator at 37 °C in 95% air–5% $CO_2$. The number of colonies was scored after 4 weeks of incubation, and the surviving fraction was corrected for the plating efficiency of nontreated controls. There was little difference in the lethality of NCS at higher doses for L33-6-1, XPA-3, and Raji (Figure 1). Because the different plating efficiency of

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[1] Abbreviations used: NCS, neocarzinostatin; MMS, methyl methanesulfonate; AAAF, (acetoxyacetylamino)fluorene.

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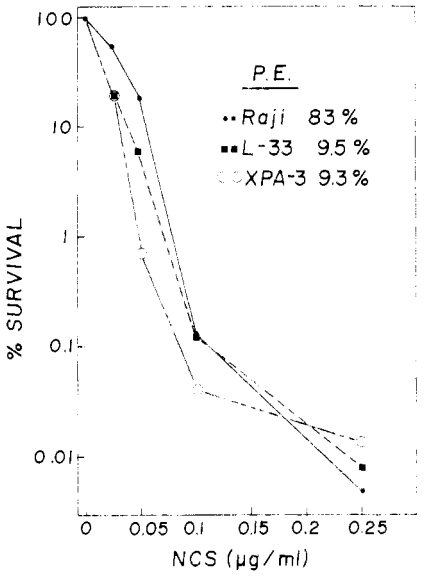


FIGURE 1: Inactivation of Raji, L33-6-1, and XPA-3 lymphoid cells by incubation with NCS. Rapidly growing cells were suspended in RPMI 1640 (without serum) and incubated for 1 h at 37 °C with NCS. The cells were then diluted and plated at three concentrations in soft agar (Sato et al., 1972) but without a feeder layer. Cells were fed at 1 day and then weekly. The plates were scored after 1 month of incubation. The percent survival was corrected for the plating efficiency (P.E.) of control cells on four replicate plates. Cell lines: Raji, closed circles; L33-6-1, closed squares; XPA-3, open circles.

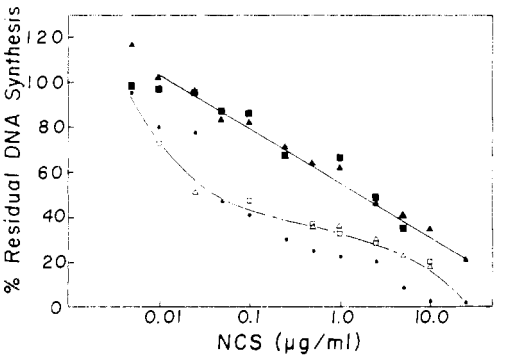


FIGURE 2: Effect of NCS on DNA synthesis. A cell suspension (8 × $10^5$ cells/mL) was incubated for 60 min with antibiotic at the concentrations indicated in the presence of 2 μM FdUrd. The cells were then pulse labeled with [$^3$H]dThd (1 μCi/mL; 3 Ci/mmol) for 30 min. The cells were chilled in ice and filtered with gentle suction onto glass-fiber filters (Whatman GF/C 2.4-cm disk), prewetted with PBS. The filters were washed with cold 5% trichloroacetic acid, dried, and counted in toluene-base scintillation fluid. The values obtained with no NCS served as the control (100%). Raji, closed circles; L33-6-1, closed squares; XPA-3, closed triangles; GM 2473, open triangles; GM 2253, open squares.

the lymphoma and lymphoblastoid cell lines might have some effect on the apparent cytotoxicity, we cannot be sure of the significance of those differences which were observed. GM 2253 and GM 2473 are recently derived lines which grow as clumps, and we did not attempt cloning experiments with these lines. The lines do not differ in any systematic way in the immediate effect of NCS treatment on the rate of DNA synthesis determined by [$^3$H]dThd uptake. Cells were preincubated with NCS for 60 min and then pulse labeled with [$^3$H]dThd for 30 min. NCS inhibited [$^3$H]dThd uptake in a dose-related manner (Figure 2).

*Measurement of DNA Repair Synthesis.* DNA excision repair was determined by a modification of the benzoylated naphthoylated DEAE (BND)-cellulose method developed by Scudiero et al. (1975). Briefly, the column containing 1.5 mL of BND-cellulose (Accurate Chemical and Scientific Co., Hicksville, NY) suspension (0.14 g/mL) was washed with 6 mL of 0.3 M NaCl buffer and eluted with 3 mL of 1.0 M NaCl. DNA was determined by the diphenylamine method (Burton, 1956), and the radioactivity in 600-μL aliquots of the diphenylamine reaction mixture was determined after the addition of 10 mL of Aquasol (New England Nuclear, Boston, MA).

*Equilibrium Centrifugation.* For repair replication studies, DNA was extracted by RNase and Pronase treatment, phenol extraction, and dialysis as previously described (Coyle et al., 1971). DNA samples were sheared by three passages through a 22-gauge needle to give a $M_n$ of 1.5 × $10^7$, diluted to 4.5 mL with 1 × SSC, and added to 5.65 g of CsCl (Varlacoid Chemical Co., Elizabeth, NJ). This mixture was centrifuged at 27 000 rpm for 48–72 h in the SW 50.1 rotor of a Beckman preparative ultracentrifuge at 20 °C. Rebanding and denaturation of selected fractions from the second neutral gradient were performed as follows. The pooled fractions were dialyzed against SSC, diluted to 4.0 mL with SSC, and then denatured by the slow addition of 0.5 mL of 0.5 M NaOH to a pH of 12.5. After 15 min of standing, 5 g of CsCl and 1 g of $Cs_2SO_4$ were added and these gradients were then centrifuged as before. Prior to alkaline denaturation, 1.0-mL aliquots were transferred into plastic tubes and subjected to sonication from a Bronson sonifier (Model LS 75) at maximum power setting, with the microprobe tip. The sonifier was tuned to a resonance at ~1 A, and 15 pulses of 20 s each were applied with 30-s intervals of chilling and mixing between pulses.

*Sedimentation of DNA in Alkaline Sucrose Gradient.* The technique developed by Sawada & Okada (1970) was used with slight modification for analysis of damage in cellular DNA by alkaline sucrose gradient centrifugation. Before treatment with the drug, rapidly growing cultures were incubated for 48 h with [$^{14}$C]dThd (0.02 μCi/mL; 50 mCi/mmol). Labeled cells were chased for at least 2 h by incubation in the presence of 1 μg/mL unlabeled dThd after washing out radioactive precursor. Following exposure to NCS with or without subsequent repair incubation in fresh medium, ~6 × $10^4$ cells in a volume of 0.1 mL were placed in a high alkaline-salt lysing solution (0.3 M NaOH; 0.14 M NaCl; 0.12 M sodium citrate; 1% sodium deoxycholate) layered onto 4.4 mL of a linear 5–20% alkaline sucrose gradient, containing 0.5 M NaCl, 0.4 M NaOH, 0.1 M $Na_2EDTA$, and 0.1% sarkosyl, formed above a 0.5-mL shelf of 60% sucrose saturated with CsCl ($\rho$ = 1.8). Lysis was allowed to continue for 17 ± 5 h at 5 °C in the dark prior to centrifugation in an SW 50.1 rotor for 18 h at 12 000 rpm at 20 °C. About 30 fractions were collected on segments of a single Whatman GF/B glass-fiber filter disk (20 cm in diameter) from each gradient through a hole pierced in the bottom of the tube. The acid-insoluble radioactivity in each fraction was determined by counting dried rectangular segments after washing in cold 5% trichloroacetic acid ($Cl_3AcOH$), ethanol and acetone. The gradients were calibrated by using T7 phage DNA (2.5 × $10^7$ daltons). Number-average molecular weight ($M_n$) was assumed as half of weight-average molecular weight ($M_w$) which was calculated by the use of a computer program based on the Burgi–Hershey equation (Burgi & Hershey, 1963) and Studier's constant (Studier, 1965). The number of breaks per T7 DNA strand was estimated from the relationship $n = M_n(T7)/M_n(\text{treated}) - 1$. 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).

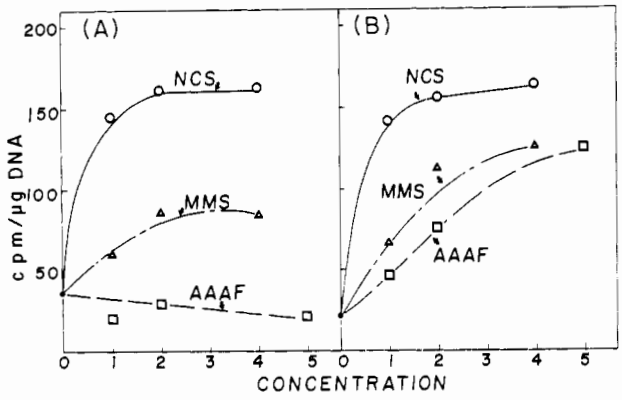


FIGURE 3: Repair synthesis as a function of dose of NCS, MMS, or AAAF by xeroderma pigmentosum and xeroderma pigmentosum heterozygous lymphoblastoid lines. Rapidly growing GM 2253 xeroderma pigmentosum cells (A) and GM 2473 heterozygote cells (B) were incubated with 1 μM FdUrd and 2 mM hydroxyurea for 30 min and then for an additional 60 min with either NCS, MMS, or AAAF in the presence of 1 μM FdUrd, 2 mM hydroxyurea, and [$^3$H]dThd (5 μCi/mL; 15 Ci/mmol). Cells were harvested and lysed, and repair synthesis was measured by the BND-cellulose method (Scudiero et al., 1975). Circles: NCS, concentrations 1, 2, and 4 correspond to 2.5, 5, and 10 μg/mL. Triangles: MMS, concentrations 1, 2, and 4 correspond to 110, 220, and 440 μg/mL. Squares: AAAF, concentrations 1, 2, and 5 correspond to 5, 10, and 25 μg/mL.

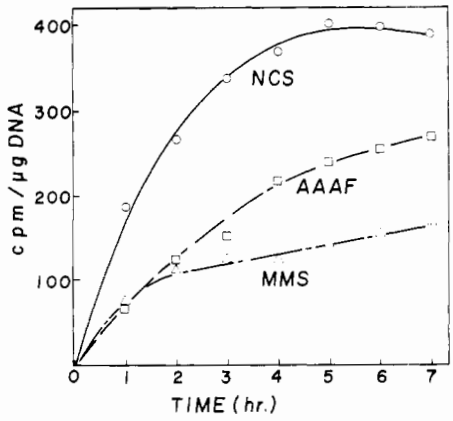


FIGURE 4: Time course of repair synthesis in the xeroderma heterozygote lymphoblastoid line GM 2473. Cells were treated with AAAF, MMS, or NCS for 60 min at 37 °C. After washing and resuspension in fresh medium, repair was measured at hourly intervals by adding 1 μM FdUrd and 2 mM hydroxyurea to incubated cells 15 min prior to the interval in which repair was to be measured and then after the 15 min adding [$^3$H]dThd (5 mCi/mL; 15 Ci/mmol). Repair was allowed to proceed for 1 h and was measured by the BND-cellulose method. The value for the first hour was obtained from separate cultures of cells to which [$^3$H]dThd and inducing compounds were added together at time zero. Cumulative values were calculated after subtraction of the background. Circles, NCS (2.5 μg/mL); triangles, MMS (220 μg/mL); squares, AAAF (10 μg/mL).

*Nucleotide Incorporation into T7 DNA.* DNA polymerase α and an apurinic/apyrimidinic (AP) endonuclease with an associated 5′–3′-exonuclease activity were prepared from the human lymphoma line, Daudi, as previously described (Bose et al., 1978). The α polymerase is devoid of any measurable endo- or 3′–5′- and 5′–3′-exonuclease activity. Phosphatase activity is absent from both enzyme preparations as measured with nitrophenol phosphate and with 5′-$^{32}$P-labeled DNA. T7 DNA, prepared as previously described (Bose et al., 1978) in a reaction mixture containing 50 mM Tris, pH 7.5, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 50 μg of bovine serum albumin per mL and 50 μM each of dAPT, dGPT, dCPT, and dTTP ($^3$H, 125 cpm/pmol each), was incubated with NCS, DNA polymerase α, and/or nuclease at 37 °C. At the end of the reaction period the DNA was precipitated with 17% perchloric acid containing 100 mM sodium pyrophosphate. The precipitate was collected on a Whatman GF/C glass-fiber filter and washed with 25 mL of 5% trichloroacetic acid, followed by 5 mL of ethanol. The filters were dried and the radioactivity was determined by liquid scintillation counting.

## Results

*In Vivo Repair.* The induction of DNA repair synthesis by NCS in the first hour of treatment was measured by the BND-cellulose technique (Scudiero et al., 1975). Results are expressed as the radioactive dThd incorporation per microgram of DNA in the 1 M NaCl eluate from the BND-cellulose column. We compared the ability of NCS, MMS, and AAAF to induce repair in a xeroderma pigmentosum complementation group D lymphoblastoid line with repair induced in a line derived from the patient's mother and therefore heterozygous for the xeroderma trait. NCS, MMS, and AAAF induced an active repair response in the heterozygote, but only MMS and NCS induced repair in the homozygous xeroderma line (Figure 3). These data indicate that at least the initial steps of the nucleotide excision system, required for repair of AAAF- and UV-induced damage and deficient in xeroderma (Tanaka et al., 1977), are not required for the repair of NCS damage. Since the doses at which repair was detected in this experiment are high compared with those at which a lethal effect is first detected (Figure 1) and since xeroderma group D lines have been reported as deficient in a particular AP endonuclease (Kuhnlein et al., 1976), we also measured repair activity at lower NCS doses in a complementation group C xeroderma lymphoblastoid line (XPA-3). XPA-3 has a very active repair system for NCS-induced damage, and we observed repair synthesis in this line at 3 times the background level at a dose of 0.1 μg/mL NCS, a dose which permitted ~10% survival of the XPA line (Figure 1).

Repair synthesis induced by NCS was completed in ~4 h in the xeroderma group D heterozygote with a time variation of ~1 h in different experiments (Figure 4) and was completed in 2 h in the XPA-3 strain. We used doses of MMS, NCS, and AAAF for the time-course experiments (Figure 4) lower than the concentrations required to saturate the repair system. The kinetics of NCS-induced repair are different from those reported for AAAF- and MMS-induced repair (Scudiero et al., 1976) and observed in this experiment. MMS-induced repair has an initial rapid phase, followed by a slow component, whereas AAAF-induced repair continues for many hours. The observation that NCS-induced repair is actually completed at a fairly early time suggests that base damage induced by NCS occurs almost immediately and that in contrast to the action of methylating agents which induce adducts that are slowly depurinated, there is no NCS-induced damage which shows up at later times. Ross & Moses (1978) come to a similar conclusion for bleomycin-induced damage.

*NCS-Induced Change in Sedimentation Profile of Cellular DNA in Alkaline Sucrose Gradients.* Uniformly labeled DNA released from XPA-3 cells by lysis on the top of the gradient was analyzed by alkaline sucrose density gradient centrifugation. The sedimentation profile for DNA from untreated control cells showed a random distribution, and the number-average molecular weight ($M_n$) was estimated as $4.7 \times 10^7$ (Figure 5a). Treatment of XPA-3 cells with 5 μg/mL NCS for 30 min resulted in a shift of the sedimentation peak toward a smaller molecular size (Figure 5b). This change in the profile corresponds to 1.8 breaks and/or alkali labile sites per $4.7 \times 10^7$ dalton DNA. However, resuspension of the treated cells in fresh medium, followed by further incubation for 4 h, resulted in more than 90% rejoining (or removal) of the initial

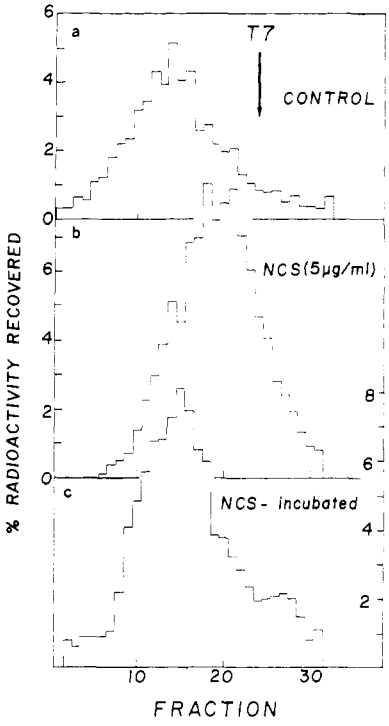


FIGURE 5: Sedimentation profiles of XPA-3 cell DNA in alkaline sucrose density gradients. Experimental details are described under Materials and Methods. (a) Control, without treatment; (b) no postincubation after treatment with 5 μg/mL NCS for 30 min at 37 °C; (c) 4-h postincubation in fresh medium without NCS following the exposure to 5 μg/mL NCS for 30 min. The sedimentation is from right to left. The arrow denotes the position of T7 phage DNA as a reference marker. Total radioactivity recovered in each profile: (a) 4300; (b) 3200; (c) 3600 dpm.

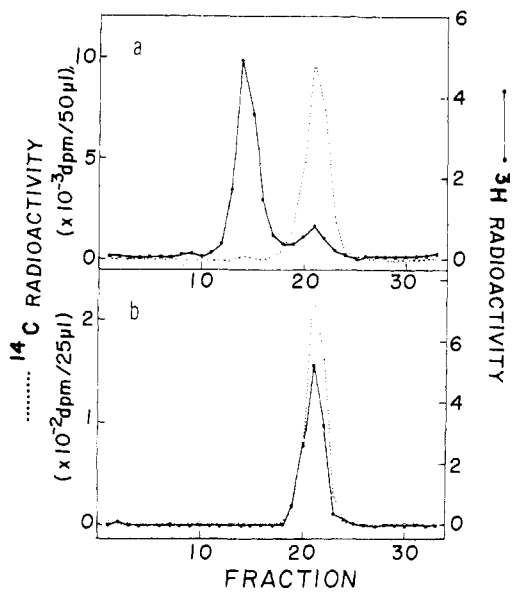


FIGURE 6: Repair replication in XPA-3 cells treated with NCS. A culture was prelabeled with [$^{14}$C]dThd (0.02 μCi/mL; 50 mCi/mmol) for 2 days, washed, resuspended in fresh medium containing 33 μM BrdUrd and 1 μM FdUrd, and then incubated for 2 h. The cells were then treated with 20 μg/mL NCS for 30 min, followed by the addition of [$^{3}$H]dThd (30 μCi/mL; 60 Ci/mmol). After incubation for 34 h in the presence of NCS, BrdUrd, FdUrd, and [$^{3}$H]dThd, the culture was harvested and DNA was extracted as described under Materials and Methods. (a) First banding: 50-μL aliquots of the diluted fractions collected from the first neutral CsCl gradient were processed as described. (b) Second banding: fractions 19–23 of the first gradient were pooled, dialyzed against 1 × SSC, and rebanded in neutral CsCl. 25-μL aliquots of the diluted fractions collected from this gradient were processed as above. Open circles, $^{14}$C; closed circles, $^{3}$H.

breaks and/or alkali labile sites (Figure 5c). This restoration process occurred in the presence of 10 mM hydroxyurea (data not shown) although we do not know whether a shorter time would have been sufficient in the absence of hydroxyurea. An NCS dose related reduction of molecular weight of DNA was also observed in Raji cells.

Although restoration of the integrity of cellular DNA in terms of sedimentation profile may not directly correlate with DNA repair synthesis as measured by the BND-cellulose technique, we attempted to estimate the number of inserted nucleotides per rejoining event. In XPA-3 cells 90.4% of the initial breaks and/or alkaline labile sites (3.9 breaks/$10^8$ daltons) were rejoined in 4 h. Hence, 3.9 × 0.9 = 3.5 breaks/$10^8$ daltons = 2.1 × $10^{10}$ breaks/μg of DNA were repaired. Repair synthesis in 4 h totaled 1.2 × $10^3$ dpm/μg of DNA. At the specific activity of the [$^{3}$H]dThd used (19 Ci/mmol), 1 dpm is equivalent to 1.4 × $10^7$ dThd molecules. Therefore, 1.7 × $10^{10}$ dTMP molecules are incorporated per μg of DNA. Since 2.1 × $10^{10}$ breaks/μg of DNA are rejoined, it appears that 0.81 molecule of dTMP is inserted per one rejoining event. The effective "relative pool size" (Scudiero et al., 1976) of dThd in XPA-3 cells treated with NCS was determined as 2.7 (at 1 μg/mL NCS). Therefore, 0.81 × 2.7 = 2.2 molecules of dTMP are inserted per restoration event.

*Size Distribution of Repair Patches in NCS-Induced Repair Replication (Edenberg & Hanawalt, 1972).* The first neutral isopycnic centrifugation of DNA extracted from XPA-3 cells treated with 20 μg/mL NCS for 30 min and incubated with BrdUrd and [$^{3}$H]dThd showed two peaks corresponding to hybrid-density DNA ($DNA_{HL}$) and light-density DNA ($DNA_{LL}$) (Figure 6). The peak fractions from the $DNA_{LL}$ position were pooled and rebanded in a neutral CsCl gradient (Figure 6b). The tritium found at the $DNA_{LL}$ position in this second neutral CsCl gradient was regarded as NCS-induced repair replication. We found only a marginal incorporation of [$^{3}$H]dThd into $DNA_{LL}$ from the nontreated control. Further analysis of repair replication was performed on the pooled peak fractions of the first reband. One portion was rebanded in alkaline CsCl without shearing; another portion was sonicated to yield smaller DNA fragments.

The size of denatured unsheared DNA from the CsCl gradient was determined as 2.5 × $10^7$ molecular weight units by alkaline sucrose density gradient centrifugation calibrated with T7 DNA. Gel electrophoresis in 0.8% agarose of the nondenatured extensively sheared DNA using *Hae*II restriction fragments of φX174 phage DNA as reference markers gave a relatively homogeneous band corresponding to ~280 base pairs. Determination of molecular weight by alkaline sucrose gradient centrifugation gave a similar figure for the size of sheared DNA. The tritium label coincided with the prelabeled $^{14}$C at light density after alkaline CsCl centrifugation when DNA was not sonicated (Figure 7A). Only a marginal skewing of the [$^{3}$H]dThd peak to the heavier side of the [$^{14}$C]dThd peak was observed in the alkaline CsCl gradient of the sonicated sample (Figure 7B). We estimate this skewing as corresponding to 2.4% (0.35 fraction) of the distance (15 fractions) between light and fully substituted DNA in an alkaline CsCl gradient. We therefore conclude that the length of the NCS-induced repair patch is no greater than about 0.024 × 280 = 6.7 nucleotides, in reasonable agreement with the estimate made above. We also obtained a similar result with Raji cells (~4.8 nucleotides), and it is therefore unlikely that different sizes of the repair patch account for the difference in the NCS-induced repair rates in XPA-3 and Raji cells.

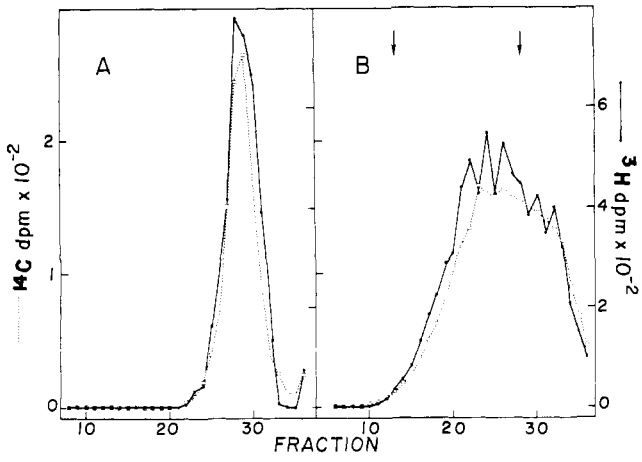


FIGURE 7: Effect of shear on the banding profile of DNA in alkaline CsCl gradients. Fractions 20–23 of the second neutral CsCl gradient (Figure 6b) were pooled and dialyzed against 1 × SSC. A portion was left unsheared. Another portion was sonicated for 5 min. Each portion was diluted to 1.0 mL before sonication, and after the treatment an aliquot of each portion was removed for molecular weight determination. The remainder was denatured in 0.1 N NaOH and rebanded in alkaline CsCl as described under Materials and Methods. The mixture of HL peak and LL peak isolated from the neutral CsCl gradient of nontreated control (profile not shown) was also processed as described above. (A) Unsheared portion of NCS-induced repair replication; (B) sonicated portion of NCS-induced repair replication. The arrows indicate the position of the peaks of heavy and light DNA as determined from the non-NCS-treated samples. Open circles, $^{14}C$; closed circles, $^{3}H$.

*Repair of NCS-Induced Damage in Vitro.* The in vivo studies indicate that NCS induces a short patch repair, that xeroderma cells unable to repair UV- or AAAF-induced damage are competent in NCS-induced repair, and that NCS-induced interruptions in DNA can be demonstrated in vivo. These observations are explicable on the basis of the hypothesis that the repair is of single-strand breaks or, alternatively, in light of the observation of AP sites produced in the course of NCS-induced DNA degradation (Bose et al., 1980), that AP repair occurs. In the absence of the appropriate biochemical mutants, we cannot decide between these hypotheses. We therefore decided to utilize the T7 DNA in vitro system previously used for the study of MMS-induced DNA repair (Bose et al., 1978) as a model.

It had been reported that treatment of DNA with NCS resulted in a product which was not a substrate for DNA polymerase I from *Escherichia coli* unless treated with alkaline phosphatase (Kappen & Goldberg, 1978). T7 DNA treated with NCS behaves in the same way (Figure 8), indicating that NCS-induced DNA degradation produces molecules with 3′-phosphate groups which can be removed by incubation with alkaline phosphatase. Although heating NCS-treated DNA with alkaline phosphatase produces the most drastic effect, an appreciable stimulation of polymerase I catalyzed activity can be obtained by incubation of NCS-treated DNA with alkaline phosphatase at 37 °C. In contrast, NCS-treated DNA treated with alkaline phosphatase is not a substrate for the DNA polymerase isolated from Daudi cells (Figure 10). Addition of the AP endonuclease with associated 5′–3′-exonuclease activity to the reaction mixture during NCS treatment of the DNA resulted in incorporation of nucleotides into DNA by the $\alpha$ polymerase (Figures 9 and 10). This incorporation was NCS-dose dependent. At concentrations higher than 60 $\mu$g of NCS/mL, the incorporation leveled off, probably because sufficient strand breaks were introduced to inhibit the nick translation. The incorporation of nucleotides was linear with time up to 120 min (Figure 10), and the addition of bacterial alkaline phosphatase to the system did not increase the rate.

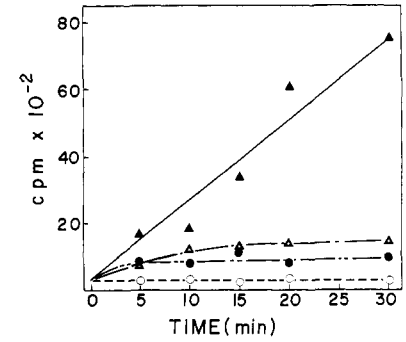


FIGURE 8: Effect of phosphatase treatment on the template activity of NCS-treated T7 DNA. T7 DNA (20.5 $\mu$g) was added to 100 $\mu$L of a reaction mixture containing 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 20 $\mu$g/mL NCS in each of four separate tubes. After 30 min at 37 °C, 0.2 unit of bacterial alkaline phosphatase was added to two of the tubes and the remaining two tubes were stored in the cold. One of the tubes with phosphatase was incubated at 37 °C while the other was incubated at 65 °C for 30 min and then both tubes were cooled. The final volume of the reaction mixture in all four tubes was adjusted to 125 $\mu$L by the addition of a salt and deoxynucleotide triphosphate mixture to a final concentration of 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 50 $\mu$g/mL bovine serum albumin, and 50 mM each of $^{3}H$-labeled dATP, dTTP, dCPT, and dGTP (30 cpm/pmol each). *E. coli* DNA polymerase I (0.1 unit) was added to the reaction mixtures as indicated below and the samples were incubated at 37 °C. Aliquots (20 $\mu$L) were withdrawn from the reaction mixtures at the indicated time intervals to determine the nucleotide incorporation as described under Materials and Methods. Control, no enzyme, open circles; mixture with DNA polymerase but no phosphatase, closed circles; mixture with DNA polymerase and phosphatase treated at 37 °C, open triangles; mixture with DNA polymerase and phosphatase treated at 65 °C, closed triangles.

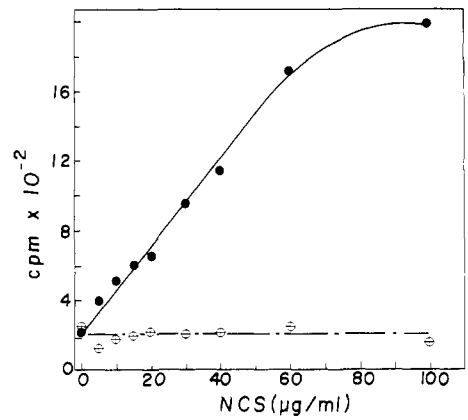


FIGURE 9: Effect of the dose of NCS on the incorporation of nucleotides into T7 DNA. T7 DNA (4.11 $\mu$g) was incubated with NCS at the doses indicated with DNA polymerase $\alpha$ or with DNA polymerase $\alpha$ and AP endonuclease in a 25-$\mu$L total volume under standard reaction conditions as described under Materials and Methods. At the end of a 60-min incubation period, the DNA was precipitated with 17% perchloric acid, and the radioactivity incorporated was determined as described by Bose et al. (1978). Symbols: open circles with cross, DNA polymerase $\alpha$ (2.5 $\mu$g, 1 unit) alone; closed circles, DNA polymerase $\alpha$ (2.5 $\mu$g) plus 1.2 $\mu$g of AP endonuclease.

## Discussion

Our results show that NCS produces DNA damage that can be repaired by a system present in xeroderma cells and therefore not identical with at least the initial step(s) of nucleotide excision. The repair takes place with an accumulation of DNA breaks and with small patches inserted into the repaired DNA as would be found for the repair of X-ray- or

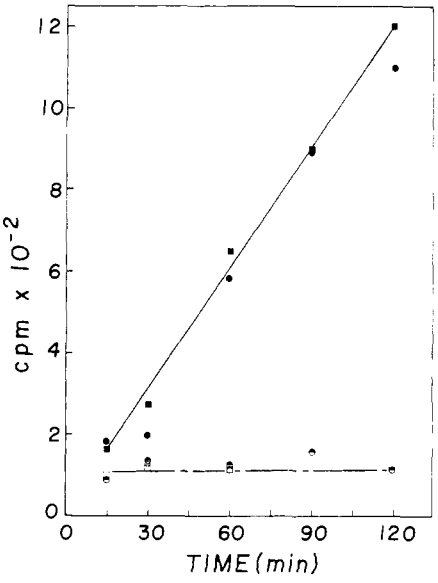


FIGURE 10: Incorporation of nucleotides into NCS-treated T7 DNA as a function of time. T7 DNA (20.5 μg) was incubated in a 125-μL reaction mixture as described under Materials and Methods. At the end of the indicated time intervals, 20-μL reaction mixtures were withdrawn to measure nucleotide incorporation as described in the legend to Figure 9. Symbols: open squares, with no NCS and no enzyme; open circles, with 12.5 μg (5 units) of DNA polymerase α and 3.8 μg of AP endonuclease but without NCS; half-filled circles, with 30 μg/mL NCS, 12.5 μg of DNA polymerase α, and 0.15 unit of bacterial alkaline phosphatase (Worthington); closed squares, with 30 μg/mL NCS, 12.5 μg of DNA polymerase α, and 3.8 μg of AP endonuclease; closed circles, with 30 μg/mL NCS, 12.5 μg of DNA polymerase α, 3.8 μg of AP endonuclease, and 0.15 unit of bacterial alkaline phosphatase.

MMS-induced damage (Regan & Setlow, 1974; Painter & Young, 1972). Repair is over in a relatively short time compared to the time usually observed for nucleotide excision (Edenberg & Hanawalt, 1973). The observation that NCS-induced repair is complete within a few hours indicates that as with bleomycin (Ross & Moses, 1978) there is no base alteration which leads to partial destabilization such as occurs with alkylating agents. The changes produced by NCS are completed in a short time. The characteristics of the repair reactions are those to be expected of base excision or AP repair but also expected of any system which produces breaks.

The finding (Bose et al., 1980) that AP sites are intermediates in the degradation of DNA by NCS makes it possible that a portion of the damage is repaired by a pathway which includes an AP endonuclease. The advantage to the cell of such an action would be the immediate provision of a 3′-OH group on which a patch could be extended. The possibility of such a mechanism is supported by our in vitro studies with T7 DNA. In the presence of the AP endonuclease with associated 5′–3′ activity from Daudi lymphoma cells, NCS-treated T7 DNA is a good template for DNA polymerase α (Figures 9 and 10). Since the AP endonuclease preparation lacks both 3′–5′-exonuclease and phosphatase activity, its 5′–3′-exonuclease activity alone would not permit DNA polymerase α to act at an NCS-induced DNA break. We therefore suppose that the initial action is at AP sites and that both endonuclease and 5′–3′-exonuclease activities are important in making it possible for an enzyme such as DNA polymerase α, which neither strand displaces nor nick translates, to operate. The repair reactions could therefore proceed by using the AP site as an intermediate rather than starting with NCS-broken DNA with terminal 3′-phosphate groups. We cannot be sure what happens in cells on the basis of the data we have obtained. However, considering the ability of AP endonuclease to interact with AP DNA and compete with NCS at AP sites, as well as the high activity of the AP endonuclease in vivo, it would seem likely that at least some of the in vivo activity might proceed directly via the AP pathway.

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