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# Poly(deoxyadenylic-deoxythymidylic acid) Damage by Radiolytically Activated Neocarzinostatin†

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ABSTRACT: The anaerobic reaction of poly(deoxyadenylic-deoxythymidylic acid) with neocarzinostatin activated by the carboxyl radical $CO_2^-$, an electron donor generated from $\gamma$-ray radiolysis of nitrous oxide saturated formate buffer, has been characterized. DNA damage includes base release and strand breaks. Few strand breaks are formed prior to alkaline treatment; they bear 3′-phosphoryl termini. In contrast, most (66%) of the base release occurs spontaneously. DNA damage is highly (95%) specific for thymidine sites. Neither DNA–drug covalent adduct nor nucleoside 5′-aldehyde, which are major products in the DNA-nicking reaction initiated by mercaptans and oxygen, is formed in this reaction. Data are presented to show that the $CO_2^-$-activated neocarzinostatin intermediate is a short-lived free radical able to abstract hydrogen atoms from the C-1′ and C-5′ positions of deoxyribose. Attack occurs mostly (68%) at the C-1′ position, producing a lesion whose properties are consistent with those of (oxidized) apyrimidinic sites.

Neocarzinostatin (NCS),[1] a protein antibiotic isolated from cultures of a *Streptomyces* species (Ishida et al., 1965), has recently been characterized as a complex in which a non-protein chromophore is bound reversibly to the protein ($M_r$ 10 700) component (Napier et al., 1979; Hensens et al., 1983). The isolated chromophore binds reversibly to DNA by an intercalative mechanism (Povirk et al., 1981) and exhibits the full biological activity of NCS (Kappen et al., 1980). DNA strand breaks constitute the main lesions induced by NCS in vivo (Beerman & Goldberg, 1974, 1977; Ohtsuki & Ishida, 1975; Hatayama & Goldberg, 1979; Moustacchi & Favaudon, 1982). DNA damage is also observed in vitro in the presence of mercaptan and oxygen (Beerman & Goldberg, 1974; Ishida & Takahashi, 1976; Beerman et al., 1977; Kappen & Goldberg, 1978; Burger et al., 1978) or oxygen substitutes such as nitroaromatic compounds (Kappen & Goldberg, 1984). Mercaptan addition to the NCS chromophore triggers the reaction (Napier & Goldberg, 1983) whose products in the presence of $O_2$ include spontaneous base release, formation of a nucleoside 5′-aldehyde esterified to the 5′ end of the breaks (Kappen et al., 1982), and formation of a covalent DNA–NCS chromophore adduct, presumably on the same oxidized 5′-carbon of the deoxyribose of DNA (Povirk & Goldberg, 1982).

Short-lived radical species that nick DNA have been proposed as intermediates in the mercaptan-induced reaction of NCS (Kappen & Goldberg, 1978; Edo et al., 1980; Sheridan & Gupta, 1981). Although no direct evidence for involvement of a radical form of the NCS chromophore has been presented, we have recently reported that thiol-activated chromophore abstracts a hydrogen atom from the 5′-carbon of deoxyribose in DNA (Charnas & Goldberg, 1984). Furthermore, the incorporation of $^{18}O$ from molecular oxygen into the 5′-position of nucleoside 5′-aldehyde in DNA (Chin et al., 1984) suggests that, among other possibilities, hydrogen atom abstraction generates a carbon-centered radical at the 5′-carbon to which molecular oxygen can add. It was therefore, of interest to study in detail the DNA-damaging action of a characterizable free radical species of NCS chromophore produced by a

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[1] Abbreviations: $PF_{5.0}$, 20 mM potassium hydrogen phosphate and 100 mM sodium formate buffer, pH 5.0; poly(dA-dT), alternating poly(deoxyadenylic-thymidylic acid) copolymer; NCS, neocarzinostatin; apo-NCS, protein component of NCS; holo-NCS, NCS–chromophore–protein complex; HPLC, high-performance liquid chromatography; DNase II, deoxyribonuclease II; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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different mechanism and to compare it with the thiol-dependent reaction.

It has been shown recently (Favaudon, 1983) that the carboxyl radical $CO_2^-$, a good one-electron donor [$E^{0\prime}(CO_2^-/CO_2) \simeq -1.1$ V, $pK_a(HCO_2/CO_2^-) = 1.4$] (Lilie et al., 1971; Buxton & Sellers, 1973; Schiffrin, 1973) generated upon $\gamma$-ray radiolysis of formate buffer, reacts quantitatively with the NCS chromophore to form an activated species of NCS that causes DNA strand breaks as revealed by alkaline sucrose gradient centrifugation. We report here that the $CO_2^-$-activated intermediate is a free radical and that DNA damage proceeds under anaerobic conditions. The DNA-damage products and the pattern of the reaction are very different from those observed in the mercaptan-dependent process.

## Experimental Procedures

*Enzymes.* The enzymes used in this study and their suppliers are as follows: *Escherichia coli* DNA polymerase I and *E. coli* exonuclease III, New England Biolabs; acid phosphatase (from potato, grade II) and alkaline phosphatase (from calf intestine, grade I), Boehringer-Mannheim; nuclease $S_1$ (from *Aspergillus oryzae*), Calbiochem; DNase II (from bovine spleen, type V), Sigma.

*Neocarzinostatin.* Holo-NCS (clinical ampules from Kayaku Antibiotics provided by Dr. W. T. Bradner of Bristol-Meyers Co.) was filtered on Sephadex G-50 at pH 4.0, dialyzed against water, and lyophilized. This preparation showed only traces of the type D degradation product (Napier et al., 1981). Solutions of holo-NCS were titrated spectrophotometrically with $\epsilon_{273} = 35.4$ $mM^{-1}$ $cm^{-1}$ and $\epsilon_{340} = 10.9$ $mM^{-1}$ $cm^{-1}$ (Napier et al., 1979; Povirk et al., 1981).

*Preparation of Poly(dA-dT).* Copolymerization of poly(dA-dT) primed with poly(dA-dT) was performed at 37 °C essentially as described by Richardson et al. (1964). *E. coli* DNA polymerase I was added (5 units/mL) to a solution containing 2 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 10 $\mu$M poly(dA-dT) (Collaborative Research), and 200 $\mu$M each of TTP and dATP. One of the following labeled nucleotide triphosphates, [*methyl*-$^3$H]TTP, [8-$^3$H]dATP, [$\alpha$-$^{32}$P]TTP, [$\alpha$-$^{32}$P]dATP (ICN), [*methyl*,1′,2′-$^3$H]TTP (New England Nuclear), or [5′-$^3$H]TTP (Amersham), was added subsequently and the reaction followed by monitoring the hypochromic effect (262 nm) resulting from polymerization. The reaction was stopped at 90% completion by addition of one equivolume of phenol followed by chloroform extraction and precipitation of DNA with ethanol at −20 °C after addition of sodium acetate (500 mM). The precipitate was redissolved in a small volume and dialyzed exhaustively against 10 mM Tris-HCl buffer until complete elimination of residual free nucleotides.

For $\gamma$-ray irradiation experiments, poly(dA-dT) was dialyzed overnight against $PF_{5.0}$ buffer. Its concentration was determined by using $\epsilon_{262} = 6.6$ $mM^{-1}$ $cm^{-1}$.

*$\gamma$-Ray Irradiations.* $\gamma$-ray irradiations were performed at room temperature with a Gammacel-220 (Atomic Energy of Canada, Ltd.) $^{60}$Co source at a dose rate of 43.2 Gy/min (4.32 krd/min), unless otherwise stated. Radiation doses were calibrated by Fricke's procedure based on the oxidation of ferrous ammonium sulfate (1 mM in air-saturated 0.8 N $H_2SO_4$) using a $G$ value of 15.6 for the $Fe^{2+} \rightarrow Fe^{3+}$ radiochemical transformation (Scharf & Lee, 1962). Experiments with NCS were carried out in nitrous oxide saturated 20 mM dipotassium hydrogen phosphate–100 mM sodium formate buffer adjusted to pH 5.0 with formic acid ($PF_{5.0}$ buffer). A slightly acidic buffer was chosen for better stability of native holo-NCS since, as shown previously (Favaudon, 1983), the radiation-induced reaction of NCS does not depend on pH throughout the pH 4.7–7.0 range. Under these conditions, hydroxyl radicals OH·, hydrogen atoms H·, and hydrated electrons $e_{aq}^-$ are quantitatively converted into the carboxyl radical $CO_2^-$ with a radiolytic yield $G = 5.95$, through the following reactions:

$$e_{aq}^- + N_2O \rightarrow OH\cdot + OH^- + N_2 \qquad (0.9 \times 10^9\ M^{-1}\ s^{-1}) \quad (1)$$

$$HCOO^- + OH\cdot \rightarrow CO_2^- + H_2O \qquad (3.5 \times 10^9\ M^{-1}\ s^{-1}) \quad (2)$$

$$HCOO^- + H\cdot \rightarrow CO_2^- + H_2 \qquad (0.5 \times 10^9\ M^{-1}\ s^{-1}) \quad (3)$$

The $CO_2^-$ radicals formed at a dose rate of $\gamma$-rays of 43.2 Gy/min amount to 27.5 nmol $min^{-1}$ $mL^{-1}$. Samples to be irradiated were contained in 5-mL round-bottom flasks fitted with a septum inlet adapter containing a glass stopcock; 99.9% $N_2O$ with less than 5 ppm of $O_2$ was introduced by using stainless steel tubings and equilibration allowed to proceed for 60–70 min under permanent stirring. The whole procedure was performed in dim light to prevent photodecomposition of NCS.

Residual oxygen in the irradiated samples was estimated as follows. A 2-mL aliquot of a solution of methylviologen (50 $\mu$M) in $PF_{5.0}$ buffer was put into a 57-mL flask, saturated with $N_2O$, and irradiated for short periods (10–15 s), resulting in the formation of the blue methylviologen radical ($G = 5.95$). The color was stable for minutes but disappeared on agitating vigorously to trap residual $O_2$ in the gas phase. Further irradiation was made until the blue color was no longer bleached after 2 min of agitation; this happened for irradiation times of 30–35 s (average over three experiments). Therefore, 55 mL of the gas phase does not contain enough $O_2$ to reoxidize 32 nmol of the methylviologen radical. Considering that 1 mol of $O_2$ will oxidize 2 mol of the one-electron-reduced methylviologen radical, one may calculate an upper limit of the residual oxygen concentration in these experiments (<7 ppm in the gas phase, <10 nM in the buffer at equilibrium).

*Activation of NCS with Thiols.* Poly(dA-dT) damage by thiol-activated NCS was assayed in aerated 100 mM Tris-HCl buffer at pH 8.0. Solutions of DNA (127 or 200 $\mu$M) were heated to 37 °C; 2-mercaptoethanol (10 mM) was then introduced and the reaction allowed to proceed to completion (1 h at 37 °C).

*Borohydride Reduction.* Samples were supplemented with an equivolume of 2 M Tris-HCl buffer pH 7.0, and two aliquots of 1 M $NaBH_4$ in 1 M NaOH were introduced under stirring at 15-min intervals. The final $NaBH_4$ concentration was 200 mM. DNA was subsequently precipitated with cold ethanol, evaporated to dryness in a Speed-Vac concentrator, and submitted to hot alkaline treatment or redissolved in the proper buffers for further characterization.

*Thiobarbituric Acid Assay.* Reaction with thiobarbituric acid for the determination of malondialdehyde-like chromogens was performed essentially as described by Buege & Aust (1978).

*Enzyme Digestions.* Digestion of poly(dA-dT) for characterization of products by HPLC was performed (37 °C, 2 h) with a mixture of DNase II (200 units/mL) and acid phosphatase (2 units/mL) in $PF_{5.0}$ buffer supplemented with 1 mM $ZnCl_2$, with or without nuclease $S_1$ (5 units/mL).

Alkaline phosphatase digestion of $\alpha$-$^{32}$P-labeled poly(dA-dT) was carried out at 56 °C in 100 mM Tris-HCl–1 mM $MgCl_2$–100 $\mu$M $ZnCl_2$ buffer, pH 9.0, by addition of three aliquots (20 units/mL) of the enzyme at 30-min intervals. Assays with exonuclease III (400 units/mL) were performed

Table I: Thymine Release from [*methyl*-$^3$H]Thymidine-Labeled Poly(dA-dT)[a]

| | γ-ray irradiation | | | | | | |
|---|---|---|---|---|---|---|---|
| | with NCS (A) | | | without NCS (B) | | 2-mercaptoethanol | |
| | neutral | $OH^-$ | $NaBH_4/OH^-$ | neutral | $OH^-$ | neutral | $OH^-$ |
| cpm | 10779 | 16988 | 16754 | 48 | 161 | 15385 | 41231 |
| | 10957 | 16628 | | | | | |
| | 10924 | 16538 | | 46 | 155 | | |
| | 10531* | 15986* | | | | | |
| cpm | 10798 ± 194 | 16535 ± 414 | 16574 | 47 ± 1 | 158 ± 3 | | |
| difference (A − B) (cpm) | 10752 ± 195 | 16377 ± 417 | | | | | |
| pmol of [$^3$H]thymine | 72.1 ± 1.3 | 109.9 ± 2.9 | | | | 103.3 | 276.7 |
| [thymine]/[NCS] (%) | 3.01 ± 0.05 | 4.58 ± 0.12 | | | | 4.30 | 11.53 |
| ratio $OH^-$/neutral | 1.52 ± 0.06 | | | | | 2.68 | |

[a] DNA (127 μM, specific radioactivity 149 cpm/pmol of thymine) was irradiated with γ-rays in $N_2O$-saturated $PF_{5.0}$ buffer in the absence or in the presence of NCS (24 μM). Results from a thiol-activated reaction are shown for comparison. Thymine was determined on 100-μL aliquots of the irradiated samples by DEAE-Sephacel elution or by HPLC (*), either directly (neutral) or after hot alkali treatment preceded ($NaBH_4/OH^-$) or not ($OH^-$) by treatment with sodium borohydride. Background radioactivity (24–50 cpm) is subtracted.

in 50 mM potassium phosphate–10 mM $MgCl_2$–15 mM 2-mercaptoethanol buffer, pH 7.0, for 35–40 min at room temperature, at which time the enzyme was inhibited by addition of $ZnCl_2$ (10 mM). Free [$^{32}$P]$P_i$ released by either enzyme was determined as follows. One milliliter of a 1% solution of ammonium molybdate was added to the digested samples (70–100 μL), and 100 μL of 10 N $H_2SO_4$ was added under vortexing. The mixtures were allowed to stand for about 2 min at room temperature, then 1 ml of a 1:1 benzene–2-propanol mix was added for extraction of the phosphomolybdate complex under vigorous vortexing (3 × 15 s) followed by centrifugation (8000*g*, 3 min). A 500-μL sample of the upper organic phase containing all the phosphomolybdate complex was withdrawn for scintillation counting in Hydrofluor (National Diagnostics).

*Chromatography.* Reverse-phase HPLC was performed at room temperature with a Waters Associates Model 660 instrument using a μBondapak $C_{18}$ column (0.39 × 30 cm) preceded by a 2-cm guard column packed with Porasil $C_{18}$. Samples were eluted at 1 mL/min with a 20–30-min wash of aqueous buffer (10 mM ammonium acetate, pH 5.0) eventually followed by a linear gradient of methanol (0–90% for 120 min or 0–70% for 93 min, unless otherwise specified) containing the same buffer. Eluates were monitored by absorbance and by fluorescence (excitation 340 nm, emission ≥ 418 nm) for visualization of the NCS chromophore fractions. Radioactivity in the fractions collected was assayed after addition of 10 volumes of Hydrofluor.

Chromatography of thymine released from poly(dA-dT) by $CO_2^-$-activated NCS was also performed on an analytical 5-μm Ultrasphere-ODS column (0.46 × 25 cm) with water as eluant, according to Frenkel et al. (1981).

*Sucrose Gradient Centrifugation.* DNA strand breaks were analyzed on neutral (700 mM NaCl, 50 mM potassium phosphate, 10 mM EDTA, pH 7.0) and alkaline (300 mM NaOH, 700 mM NaCl, 10 mM EDTA) 5–20% sucrose gradients spun in a SW-41 rotor (39 000 rpm for 4.5 h) at 20 °C. Thymidine [*methyl*-$^3$H]phage λ DNA, prepared as described (Kappen & Goldberg, 1977) was used in these experiments. Gradients were analyzed by collecting 400-μL fractions with the aid of an ISCO Model 640 density gradient fractionator.

*Base Release.* [*methyl*-$^3$H]Thymine release from poly(dA-dT) by activated NCS was determined by HPLC or, for routine experiments, by a DEAE-Sephacel elution technique. Small DEAE-Sephacel columns (ca. 1 mL of gel) were made in Pasteur pipets over a glass wool plug and equilibrated with 25 mM Tris-HCl buffer, pH 7.0. Samples adjusted to the same pH and ionic strength were applied and eluted with four successive 1-mL aliquots of buffer. The eluates collected were counted in 10 volumes of Hydrofluor.

For determination by HPLC, DNA was precipitated by cold ethanol. The supernatant fraction was evaporated to dryness, redissolved in 200 μL of 10 mM ammonium acetate buffer, pH 5.0 (or 200 μL of $H_2O$), applied to a μBondapak $C_{18}$ (or Ultrasphere-ODS) column, and run as described above. Due to slow but significant exchange with water of the $^3$H label at the C-8 position of adenine under conditions for alkali treatment, determination of [8-$^3$H]adenine was made by HPLC only.

DNA was treated with hot alkali by addition of 0.2 volume of 1 M NaOH and heating to 90 °C for 15 min in a water bath. Samples were neutralized after cooling by addition of 0.2 volume of 1 M HCl and 0.1 volume of 1 M Tris-HCl buffer, pH 7.0. The alkali-dependent release of thymine from NCS-treated DNA has been shown to be mainly due to the formation of thymidine 5′-aldehyde (Kappen & Goldberg, 1983). Under these conditions poly(dA-dT) was found to renature completely as determined by measurement of hyperchromicity.

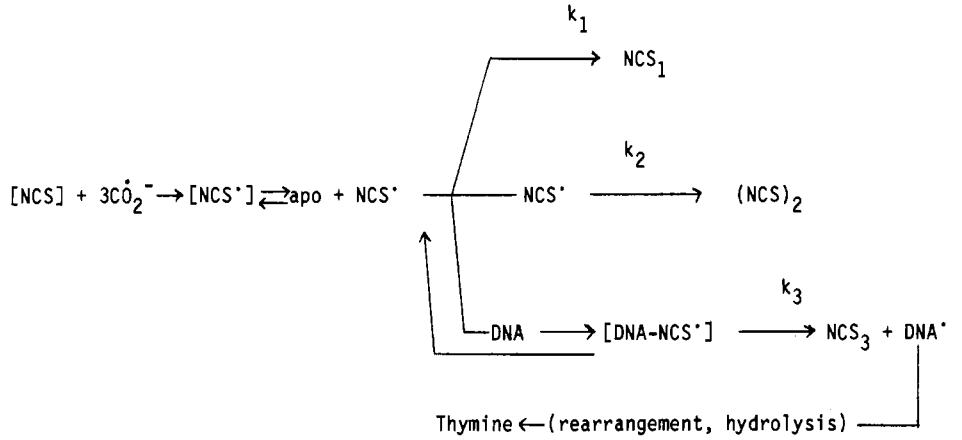
## Results

*Base Release.* Mixtures containing holo-NCS (24 μM) and [*methyl*-$^3$H]thymidine-labeled poly(dA-dT) (127 μM) in $PF_{5.0}$ buffer were saturated with $N_2O$ and irradiated with γ-rays for 3 min, i.e., for a time slightly in excess of that required for complete NCS reaction (see next paragraph). Aliquots of the irradiated solutions were withdrawn and treated in one of three ways: no treatment, borohydride reduction and/or hot alkaline solution, and then analyzed for thymine release (Table I). Thymine was released in relatively good yield in the presence of NCS and was increased further upon alkaline treatment. In the absence of NCS γ-ray irradiation released very little thymine. The same values were obtained when irradiation was performed in $PF_{5.0}$ buffer degassed with argon instead of $N_2O$, giving nearly equivalent amounts of $CO_2^-$ ($G$ = 3.25) and $e_{aq}^-$ ($G$ = 2.70). Borohydride did not afford protection against alkali-promoted base release (Table I).

The thymine recovered either from DEAE-Sephacel eluates or from the supernatant of cold ethanol-precipitated samples, with or without alkaline treatment, showed a single peak and comigrated with pure thymine on HPLC using μBondapak $C_{18}$ or Ultrasphere-ODS columns according to Frenkel et al. (1981). This shows that the base released from poly(dA-dT) by $CO_2^-$-activated NCS is intact, suggesting that the reaction involves deoxyribose sugar rather than base attack.

The amount of adenine released from [8-$^3$H]adenine-labeled poly(dA-dT) irradiated with NCS under the same conditions as in Table I was low compared with that for thymine, yielding,

Scheme I: Simplified Reaction Scheme for the Competition between Dimerization ($k_2$), Inactivation by Hydrolysis ($k_1$), and Reaction on DNA ($k_3$) of the NCS Chromophore Activated by $CO_2^-$, at Apo-NCS Concentrations Low Enough ($\leq 50$ $\mu$M) Not To Interfere with the System[a]




[a] NCS, NCS·, and $(NCS)_2$ are the native, $CO_2^-$-activated, and dimeric forms of the chromophore, respectively. The products $NCS_1$, $(NCS)_2$, and $NCS_3$ are inactive but able to bind to DNA and to apo-NCS (Favaudon, 1983). Complexes are indicated by square brackets.

on a molar ratio to NCS, 0.19 and 0.25% prior to and after alkaline hydrolysis, respectively. Poly(dA-dT) damage by $CO_2^-$-activated NCS is thus very specific for thymidine sites.

*Irradiation Time Dependence of the Reaction.* The time-dependence of thymine release from poly(dA-dT) irradiated in the presence and absence of NCS is shown in Figure 1. Both the yield of thymine release and the increase in fluorescence of the NCS chromophore, characteristic of its reaction with the $CO_2^-$ radicals (Favaudon, 1983), increased linearly with the time of irradiation for up to 3.2 min at which time a plateau was obtained for the fluorescence intensity while released thymine still increased slowly, at the same rate as in the blank irradiated in the absence of NCS. The rate of reaction of NCS in this system is 9.4 $\mu$m/min, corresponding to a radiolytic yield $G = 2.0$. This $G$ value is exactly one-third of that for the overall production of $CO_2^-$. In other words, the activation of the NCS chromophore requires uptake of 3 equiv of $CO_2^-$. Figure 1 also shows that DNA damage is related directly to this activation process.

*Effect of the NCS Apoprotein.* The yield of DNA damage as measured from total thymine release is low compared to that observed for the reaction initiated by thiols in the presence of oxygen, starting from a poly(dA-dT)–NCS chromophore complex. Such complexes are made simply by mixing the DNA with NCS apoprotein-free solutions of the NCS chromophore extracted with acidic methanol and have been found to be relatively stable (Povirk & Goldberg, 1983). We tried to prepare such complexes in cold phosphate–formate buffer by addition of a solution of NCS chromophore in methanol containing 0.1 N formic acid followed by lyophilization to remove methanol (which is very reactive under $\gamma$-ray irradiation). Lyophilization in this medium invariably led to complete inactivation of the NCS chromophore, as measured by both the $\gamma$-ray and thiol assays. There was, therefore, no other possibility than using mixtures of holo-NCS and DNA for the $\gamma$-ray irradiation experiments, necessitating a study of the possible effects of the NCS apoprotein on the DNA-nicking reaction.

Pure apo-NCS was introduced at various concentrations in samples containing fixed amounts of DNA and holo-NCS. The reaction was analyzed after complete irradiation by measurement of thymine release (Figure 2A). Apo-NCS had no effect below 50 $\mu$M, but a marked decrease of thymine release was observed at higher concentrations, reaching 50% inhibition at 200 $\mu$M apo-NCS. This effect results from

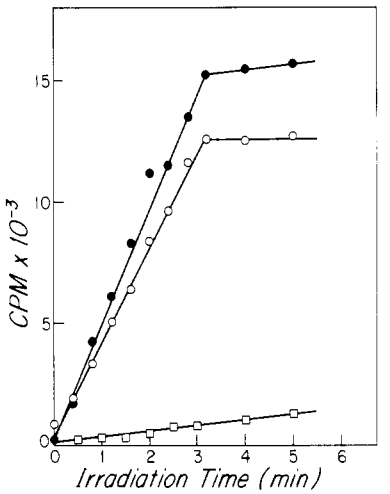



FIGURE 1: Time dependence of [*methyl*-$^3$H]thymine release after alkaline hydrolysis from poly(dA-dT) (200 $\mu$M) irradiated in the absence (□) and in the presence (●) of holo-NCS (30 $\mu$M). The effect of irradiation on the fluorescence emission (relative units) of the NCS chromophore at 440 nm ($\lambda_{excit}$ 340 nm) in the same experiment (○) was determined after dilution of the samples to 5 $\mu$M NCS.

competition between DNA and the NCS apoprotein for binding of a short-lived activated intermediate able to decay via self-recombination (dimerization) in the absence of DNA (Favaudon, 1983).

*Effect of the Poly(dA-dT) Concentration.* Thymine release at fixed NCS concentrations is very dependent on the poly-(dA-dT) concentration. The pattern observed (Figure 2B) indicates that the yield of DNA damage by the $CO_2^-$-activated intermediate is controlled kinetically by competition between reaction on DNA and decay via other routes. The pathways for inactivation correspond to dimerization of the activated transient (Favaudon, 1983) and to hydrolytic processes. It has to be considered that, as usually found in $\gamma$-ray irradiation experiments generating highly reactive compounds at low dose rates, there is a low steady-state concentration of the activated intermediate as long as consumption of "native" NCS is not complete. Therefore, the inactivation reactions are intrinsically more rapid than those producing DNA damage, since, although DNA is present in large excess, the yield of thymine release is low compared to total NCS. Considering that the apo-NCS concentration under conditions for Figure 2B is low enough not to interfere with other processes, the yield of DNA

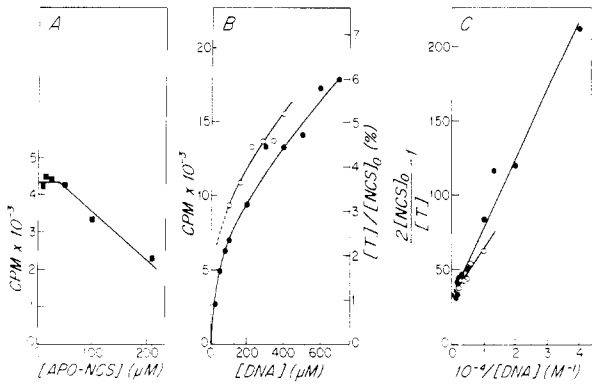



FIGURE 2: Effect of apo-NCS (A) and poly(dA-dT) (B) concentrations on the yield of thymine release. (A) Samples containing 10 μM holo-NCS and 100 μM [*methyl*-$^3$H]thymidine-labeled poly(dA-dT) were adjusted to the indicated concentration of NCS apoprotein and irradiated for 1.5 min after $N_2O$ saturation; 100-μL aliquots were withdrawn, treated with hot alkali, and applied to DEAE-Sephacel for thymine release determination. (B) Samples contained a fixed amount of holo-NCS (20 μM). γ-ray irradiation was for 2.5 min at 43.2 Gy/min (○) or 1.8 min at 60.1 Gy/min (●). Free thymine (T) was determined after alkaline hydrolysis. Background counts due to DNA damage by irradiation after complete reduction of NCS (2.1 and 1.5 min at 43.2 and 60.1 Gy/min, respectively) were subtracted. (C) Reciprocal plot for eq 4 (see text). The dose rate of γ-rays was 43.2 (○) or 60.1 Gy/min (●).

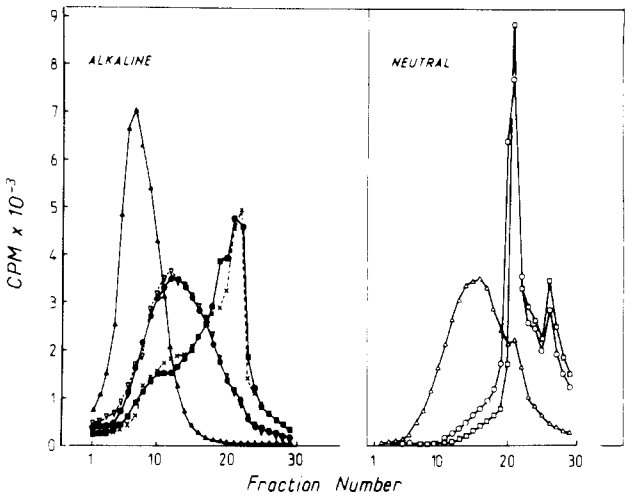



FIGURE 3: Alkaline and neutral sucrose gradient profiles of phage λ DNA treated with γ-rays (43.2 Gy/min) for 1.7 min in the presence (▲, △) or in the absence (●, ○) of NCS in $N_2O$-saturated $PF_{5.0}$ buffer. Native (untreated) DNA is shown as control (■, □). Incubation with NCS (2 h, room temperature) without activating agents (no thiols, no γ-ray irradiation) does not cause DNA damage (×). The effect of hot alkali treatment (90 °C for 15 min) on native DNA is also shown for comparison (▽). [*methyl*-$^3$H]Thymidine-labeled phage λ DNA (specific radioactivity 11 000 cpm/μg) was 200 μM. When present, NCS was 15 μM; 3 μg of DNA was layered at the top of each gradient tube. Sedimentation is from left to right.

damage should be controlled by competition between the reactions shown in Scheme I. The treatment of the steady state leads easily to the equation

$$2[NCS]_0/[T] - 1 = [k_1 + [(k_1 + k_3[DNA])^2 + 8ak_2]^{1/2}]/(k_3[DNA]) \quad (4)$$

where $[NCS]_0$ is the total (initial) NCS concentration and $a$ the rate of formation of the intermediate NCS· generated upon three electron equivalents uptake by the NCS chromophore ($1.53 \times 10^{-7}$ and $2.13 \times 10^{-7}$ M s$^{-1}$ at 43.2 and 60.1 Gy/min, respectively). The linearity of the plot in Figure 2C indicates that under the square root member in eq 4 the term containing the DNA concentration is much smaller than the other ones below 500 μM DNA; it follows that the lines drawn for best fit with the experimental data at two different dose rates of γ-rays should extrapolate to the same ordinate intercept, as actually observed. Moreover, the slopes in the linear portion of the plot are exactly in the same ratio (0.69) as that of the related dose rates of γ-rays, indicating that the dimerization reaction dominates over other decay processes and we can calculate $k_2^{1/2}/k_3 = 3.3 \pm 0.3$. It follows that $k_3$ has an upper limit of $10^4$ M$^{-1}$ s$^{-1}$ for which $k_2 = 10^9$ M$^{-1}$ s$^{-1}$, close to the diffusion control limit. This is consistent with DNA binding [$k_{on} \simeq 10^6$ M$^{-1}$ s$^{-1}$ (D. Dasgupta, personal communication)] not being the rate-limiting step in the DNA-nicking reaction.

*Search for Aldehydes and Adducts.* DNA damage by holo-NCS activated by mercaptans in the presence of oxygen involves a covalent adduct between DNA sugar and the nonprotein chomophore of NCS (Povirk & Goldberg, 1982) and breaks associated with spontaneous base release on the one hand or bearing a thymidine 5′-aldehyde residue esterified at the 5′-termini on the other hand (Kappen et al., 1982). The adduct and the thymidine 5′-aldehyde are both alkali labile (Kappen & Goldberg, 1983) but can be quantitatively excised by enzymic digestion of DNA at acidic pH. Under the conditions used for thiol assays (see Experimental Procedures), the adduct represented 3.7 mol % NCS.

In contrast, neither adduct nor thymidine 5′-aldehyde was found from HPLC analysis of enzyme-digested poly(dA-dT) that had been reacted with $CO_2^-$ and NCS. Absence of nucleoside 5′-aldehyde was also demonstrated by sequence analysis using high-resolution polyacrylamide gel electrophoresis of a DNA restriction fragment of plasmid DNA containing the *lacI* gene of *E. coli* labeled with $^{32}$P at its 3′ end (L. F. Povirk, unpublished results). Also as shown in Table I, the ratio of spontaneous vs. alkali-induced thymine release is totally different from that seen with thiols and is apparently independent of the DNA and NCS concentrations. Moreover, the alkali-labile thymine release in poly(dA-dT) irradiated anaerobically with NCS is, in fact, heat labile. This was demonstrated by heating neutralized (pH 7.0, Tris buffer) samples to 90 °C from times ranging from 7 min to 2 h. Thymine release proceeded slowly ($t_{1/2}$ = 24 min) compared with alkaline conditions (where the release is complete after a few minutes), reaching completion in about 2.5 h. Interestingly, the pattern of heat-induced thymine release was biphasic and appeared to result from the superimposition of two exponentially time-dependent processes with apparent rate constants of 0.145 min$^{-1}$ ($t_{1/2}$ = 4.8 min) and 0.0185 min$^{-1}$ ($t_{1/2}$ = 37.5 min) and relative amplitudes of 0.22 and 0.78, respectively. Another difference between the thiol-dependent reaction and that due to radiolytically activated NCS is that the base attack site specificity for strand breakage (T > A ≫ C > G) of the former (Kappen and Goldberg, 1983), as determined by gel electrophoresis of 3′-$^{32}$P- or 5′-$^{32}$P-end-labeled restriction fragments of plasmid DNA, was lacking in the latter (L. F. Povirk, unpublished results). While there was heterogeneity in the attack sites for a particular base, T's were not favored over C's as sites of strand breakage.

Traces only (ca. 0.5% with respect to NCS of thiobarbituric acid reacting chromogens were found in the supernatant fraction of cold ethanol-precipitated poly(dA-dT) treated with hot alkali after complete reaction with $CO_2^-$-activated NCS under conditions [30 μM NCS, 300 μM poly(dA-dT)] where thymine release amounts to ca. 4.5% relative to NCS.

*Strand Breaks.* [*methyl*-$^3$H]Thymidine-labeled phage λ DNA (200 μM) treated with NCS (15 μM) and irradiation (1.7 min) was analyzed by neutral and alkaline sucrose gradient centrifugation (Figure 3). It appears from the profile

of the neutral gradients that irradiation with NCS produces a few double-strand breaks only, probably resulting from the coincidence of single-strand breaks at a few nucleotide intervals. By use of the method of Abelson & Thomas (1966), the number of breaks in DNA irradiated in the presence of NCS was estimated at 1.0–1.2 on neutral gradients (double-strand breaks) and at 18–20 on alkaline gradients (single-strand breaks). DNA irradiated for the same length of time in the absence of NCS showed no damage under neutral conditions and only 3.9–4.4 breaks on alkaline gradients; hot alkaline treatment (90 °C for 15 min) of nonirradiated DNA produced the same amount of breaks as $\gamma$-ray irradiation in the absence of NCS (Figure 3).

*Enzymatic Analysis of Termini at Nicked Sites.* The nature of the amount of 3′- and 5′-termini present in poly(dA-dT) at sites nicked by $CO_2^-$-activated NCS have been determined by the release of $[^{32}P]P_i$ from thymidine [$\alpha$-$^{32}$P]triphosphate and deoxyadenosine [$\alpha$-$^{32}$P]triphosphate labeled poly(dA-dT) upon digestion with exonuclease III or alkaline phosphatase.

Exonuclease III catalyzes the hydrolysis of several types of phosphoester bonds in double-stranded DNA; namely, it has a 3′→5′-exonuclease activity, an apurinic/apyrimidinic-endonuclease activity, and a DNA 3′-phosphatase activity. Only the latter, releasing inorganic phosphate from 3′-phosphoryl ends found at free termini or at internal breaks, will produce inorganic phosphate under the conditions used in our assay. As seen in Table II, the amount of $[^{32}P]P_i$ released by exonuclease III from thymidine [$\alpha$-$^{32}$P]triphosphate labeled poly(dA-dT) treated with $CO_2^-$-activated NCS increases significantly after alkaline treatment. The yield of free $[^{32}P]P_i$ after treatment with hot alkali correlates well with that of total thymine release (Figure 2). In contrast, the amount of spontaneously released $[^{32}P]P_i$ is lower by a factor of 4.3. Therefore, only 23% of the sites damaged by NCS give rise to spontaneous breaks bearing 3′-phosphate ends.

Deoxyadenosine [$\alpha$-$^{32}$P]triphosphate labeled poly(dA-dT) shows much less phosphate group excision by exonuclease III, as expected from the low yield of adenine release, yet it is ca. 2-fold larger than the amount of adenine release determined by HPLC or DEAE-Sephacel elution and does not show exactly the same ratio of spontaneous vs. alkali-dependent base release. This has also been repeatedly observed in the analysis of poly(dA-dT) damage by thiol-activated NCS. The reason for this is uncertain, but contamination of the exonuclease III preparation by some phosphatase able to excise slowly 5′-phosphoryl termini at adenosine sites is a likely possibility.

Digestion with alkaline phosphatase produces $[^{32}P]P_i$ release consistent with the yield of base release (Table II). Despite some imprecision inherent in this enzyme assay, it is clear that $[^{32}P]P_i$ release is not dependent on strong alkali treatment and that borohydride reduction, which does not protect against alkali-induced thymine release (Table I), has no effect whatsoever. Finally, even though spontaneous strand breaks represent only a minor part of the total damage caused by $CO_2^-$-activated NCS at pH 5, the bulk of the damaged sites are labile under the conditions (Tris buffer with 1 mM $Mg^{2+}$, pH 9.0, 56 °C) used for the alkaline phosphatase assay and are recognized as gaps by the enzyme.

*Reaction with [methyl,1′,2′-$^3$H]Thymidine-Labeled Poly(dA-dT).* Poly(dA-dT) (250 $\mu$M) labeled in the methyl group (82%) and at the C-1′ (9%) and C-2′ (9%) positions of deoxyribose at thymidine sites was irradiated in the presence of NCS (50 $\mu$M) for a time sufficient to reach completion of the NCS reaction (5.4 min at 43.2 Gy/min). The irradiated solution was loaded directly on the HPLC column and eluted

Table II: Determination of $[^{32}P]P_i$ Released by Enzymic Digestion of Thymidine [$\alpha$-$^{32}$P]Triphosphate and Deoxyadenosine [$\alpha$-$^{32}$P]Triphosphate Labeled Poly(dA-dT) Cleaved by $CO_2^-$-Activated NCS[a]

| | exonuclease III | | | | calf intestinal phosphatase | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | [$\alpha$-$^{32}$P]dT | | [$\alpha$-$^{32}$P]dA | | [$\alpha$-$^{32}$P]dT | | | | [$\alpha$-$^{32}$P]dA | | | |
| | neutral | $OH^-$ | neutral | $OH^-$ | neutral | $OH^-$ | $NaBH_4$/neutral | $NaBH_4$/$OH^-$ | neutral | $OH^-$ | $NaBH_4$/$OH^-$ |
| cpm with NCS (A) | 782 ± 48 | 3278 ± 202 | 294 ± 38 | 548 ± 36 | 5636 ± 212 | 6168 ± 160 | 5402 ± 219 | 5559 ± 206 | 2562 ± 80 | 2224 ± 24 | 2754 ± 98 |
| cpm without NCS (B) | 21 ± 2 | 32 ± 3 | 24 ± 1 | 30 ± 2 | 1206 ± 38 | 1198 ± 50 | | | 645 ± 10 | 571 ± 5 | |
| difference (A − B) (cpm) | 761 ± 50 | 3246 ± 205 | 270 ± 39 | 518 ± 38 | 4430 ± 250 | 4970 ± 210 | 4196 ± 431 | 4361 ± 256 | 1917 ± 90 | 1653 ± 29 | 2183 ± 103 |
| pmol of $[^{32}P]P_i$ | 3.09 ± 0.20 | 13.19 ± 0.83 | 2.01 ± 0.29 | 3.87 ± 0.28 | 18.01 ± 1.02 | 20.20 ± 0.85 | 17.06 ± 1.75 | 17.73 ± 1.04 | 14.31 ± 0.67 | 12.34 ± 0.22 | 16.89 ± 0.77 |
| $[[^{32}P]P_i]/[NCS]$ (%) | 0.77 ± 0.05 | 3.30 ± 0.21 | 0.50 ± 0.07 | 0.97 ± 0.07 | 4.50 ± 0.26 | 5.05 ± 0.21 | 4.27 ± 0.44 | 4.43 ± 0.26 | 3.58 ± 0.17 | 3.09 ± 0.06 | 4.07 ± 0.19 |

[a] $\gamma$-ray irradiation of the solutions of NCS (20 $\mu$M) and DNA (200 $\mu$M) was for 2.5 min (43.2 Gy/min). DNA damage by irradiation in the absence of NCS was determined in parallel. To allow for buffer changes, irradiated samples were precipitated with cold ethanol, desiccated in vacuo, and redissolved in the convenient buffers. Each enzyme assay was performed by using 20-$\mu$L aliquots as described under Experimental Procedures. Data are corrected for the loss of DNA occurring upon ethanol precipitation. The specific radioactivity of thymidine [$\alpha$-$^{32}$P]triphosphate labeled poly(dA-dT) and deoxyadenosine [$\alpha$-$^{32}$P]triphosphate labeled poly(dA-dT) was of 246 and 134 cpm/pmol of $[^{32}P]P_i$, respectively. Each value given is an average of three experiments. Alkaline hydrolysis and borohydride reduction were performed as described under Experimental Procedures.

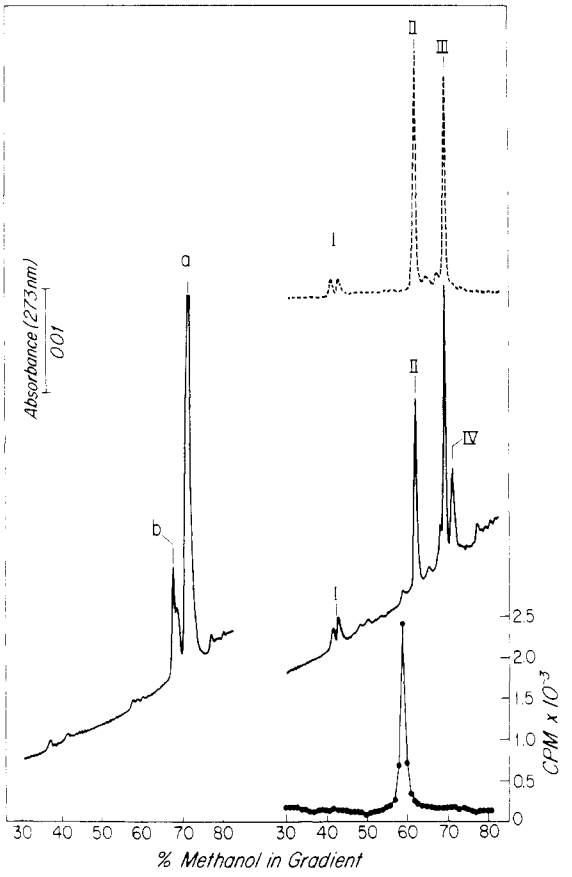



FIGURE 4: Reverse-phase ($\mu$Bondapak $C_{18}$) HPLC elution profiles of the NCS chromophore from native NCS (left) and from a sample irradiated with $\gamma$-rays in the presence of 2 mM poly(dA-dT) (right). Elution was monitored by optical absorption at 273 nm (—) (total NCS injected 30 nmol) and by fluorescence (---) (excitation 340 nm, emission > 418 nm, total NCS injected 5 nmol). (a) and (b) are the type A and type B components of the native NCS chromophore (Napier et al., 1981). The radioactive peak found after reaction with [*methyl*,1′,2′-$^3$H]thymidine-labeled poly(dA-dT) (●) (specific radioactivity 11 cpm/pmol of thymidine at C-1′) is shown at bottom right. The radioactive fraction formed on reaction with [5′-$^3$H]-thymidine-labeled poly(dA-dT) (specific radioactivity 49 cpm/pmol of thymidine) had exactly the same elution profile and contained 2670 cpm under the peak. Fractions were collected at 1-min intervals (1 mL).

using conditions described under Experimental Procedures. Free thymine in the eluate amounted to 2.6% of the NCS chromophore, in good agreement with the value (2.8%) predicted from Figure 2B at this DNA concentration. No $^3H_2O$ was found in the HPLC eluate. A radioactive peak came off from the column in the region of the gradient (58% methanol) corresponding to the NCS chromophore (Figure 4). The molar ratio of this product to spontaneously released thymine was 72%. Since in experiments with DNA labeled with tritium only in the methyl group of thymine there is no transfer of radioactivity to the chromophore, it is clear that tritium atoms are abstracted from the C-1′ and/or C-2′ position of deoxyribose and are covalently linked to the chromophore at a position which does not exchange with solvent.

*Reaction with [5′-$^3$H]Thymidine-Labeled Poly(dA-dT).* [5′-$^3$H]Thymidine-labeled poly(dA-dT) was irradiated under the same conditions of NCS and DNA concentration as in the preceding experiment. A peak containing radioactivity was found exactly at the same position in the HPLC methanol gradient where the above-described labeled chromophore is eluted. The molar ratio of this product to spontaneously released thymine was 34% (assuming an equal specific radioactivity for both protons at the C-5′ position of deoxyribose).

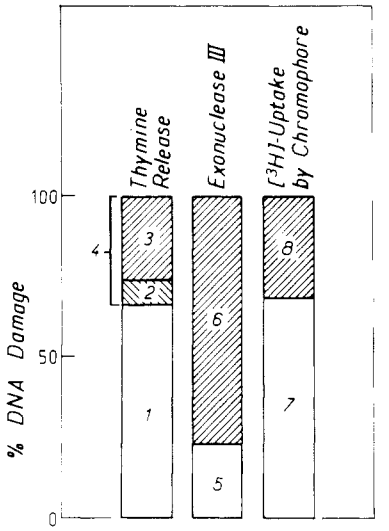



FIGURE 5: Summation of the relative yields of thymine release, of 3′-$PO_4$ groups excised from thymidine [$\alpha$-$^{32}$P]triphosphate labeled poly(dA-dT) by exonuclease III, and of $^3$H uptake by the activated chromophore from labeled positions of deoxyribose at thymidine sites in poly(dA-dT). Total damage in each assay is taken as 100%. (1) Spontaneous thymine release; (2 and 3) heat-induced thymine release [(2) rapid phase; (3) slow component]; (4) alkali-induced thymine release; (5 and 6) amount of 3′-[$^{32}$P]$P_i$ excised before (5) and after (6) alkaline treatment; (7 and 8) $^3$H uptake by $CO_2^-$-activated NCS chromophore from [*methyl*,1′,2′-$^3$H]thymidine-labeled (7) and [5′-$^3$H]thymidine-labeled (8) poly(dA-dT).

Therefore, the $CO_2^-$-activated chromophore is also able to perform hydrogen atom abstraction at the position C-5′, though with a lower efficiency than at C-1′/C-2′ (Figure 5).

## Discussion

The reductive activation of NCS can be brought about by a variety of reducing agents such as mercaptans (Beerman & Goldberg, 1974), to some extent by borohydride and dithionite (Napier & Goldberg, 1983), and by one-electron donors like the carboxyl radical (Favaudon, 1983). However, the pattern of NCS activation by thiols is very different from that induced by $CO_2^-$, and, as shown here, the same is true for DNA damage by the related activated intermediates.

Thiol addition, formally a two-electron process, is the first step in the mercaptan-induced activation of the NCS chromophore (Napier & Goldberg, 1983). This process occurs independently of oxygen, but DNA degradation under these conditions requires oxygen with 1 mol of $O_2$ consumed per mol of NCS chromophore (Povirk & Goldberg, 1983); DNA degradation involves single-strand breaks coupled with spontaneous base release or esterified at their 5′ ends with a nucleoside 5′-aldehyde residue (Kappen et al., 1982) and a covalent deoxyribose–chromophore adduct (Povirk & Goldberg, 1982). Both the adduct and the nucleoside 5′-aldehyde eliminate the attached base upon alkaline hydrolysis, and the latter lesion is borohydride protectable (Kappen & Goldberg, 1983).

The ratio of total (i.e., after alkaline treatment) to spontaneous thymine release from poly(dA-dT) attacked by $CO_2^-$-activated NCS is 1.52 (Table I). This value is low relative to that obtained with NCS activated by mercaptans in the presence of oxygen (in control experiments using 10 mM 2-mercaptoethanol, a ratio of total vs. spontaneous thymine release of 2.7 was measured). It is not modified by borohydride treatment, and neither adduct nor nucleoside 5′-aldehyde is a product of the reaction. Furthermore, the reaction is very specific for thymidine sites. Few sites bearing 3′-phosphoryl termini susceptible to dephosphorylation by exonuclease III are formed spontaneously (Table II). It ap-

pears, therefore, that different types of lesions occur at thymidine sites in the reaction of poly(dA-dT) with $CO_2^-$-activated NCS (Figure 5). The high specificity for thymine and the inhibition of DNA damage by intercalators (Favaudon, 1983) indicate that DNA damage involves complexation of the activated chromophore to the poly(dA-dT) duplex with a rather specific configuration. The major lesion corresponds to attack at the C-1′ position of deoxyribose, as demonstrated by tritium-transfer experiments using [*methyl*,1′,2′-$^3$H]thymidine-labeled poly(dA-dT). Accordingly, it can be proposed that most of the damaged sites giving rise to spontaneous thymine release are simply apyrimidinic sites that are unstable under the conditions used for digestion with alkaline phosphatase (Tris buffer with $Mg^{2+}$, pH 9, 56 °C) (Hadi & Goldthwait, 1971). Gaps at these sites may result from a $\beta$-elimination reaction involving a carbonyl function at C-1′ susceptible to mild alkaline hydrolysis or to Schiff base formation with the Tris amino groups. After borohydride reduction, the alkali lability is brought about by the free OH group at C-4′ (Dizdaroglu et al., 1977a,b).

In pure formate buffer, the decay of the carboxyl radical occurs through dimerization yielding oxalate

$$2CO_2^- \rightarrow C_2O_4^{2-} \qquad (2k_5 = 1.2 \times 10^9\ M^{-1}\ s^{-1}) \quad (5)$$

in such a way that the steady-state concentration of $CO_2^-$ in the absence of NCS is approximately 20 nM at the dose rates of $\gamma$-rays used in our experiments. The observation of a linear time dependence until complete exhaustion of "native" NCS (Figure 1) indicates (i) that the reaction between $CO_2^-$ and NCS is much faster than $CO_2^-$ dimerization, (ii) that the rate constant for any of the three one-electron steps of NCS reduction should be greater than $10^8\ M^{-1}\ s^{-1}$, and (iii) that the availability of $CO_2^-$ is the rate-limiting factor. In contrast, the yield of DNA damage by irradiation in the absence of NCS is very small ($G \simeq 3.7 \times 10^{-3}$) so that the extent of this reaction is negligible as some native NCS is still present. The pattern of the reaction also indicates that the residual oxygen concentration (<10 nM; see Experimental Procedures), is too low to interfere with the radiolytically induced process. In fact, the one-electron reduction of oxygen by the carboxyl radical

$$CO_2^- + O_2 \rightarrow CO_2 + O_2^- \quad (6)$$

is very rapid ($k_6 = 4.2 \times 10^9\ M^{-1}\ s^{-1}$). Consequently, oxygen, when present in sufficient amounts, lowers the bulk rate of NCS reduction due to trapping of $CO_2^-$ (e.g., 1% $O_2$ in $N_2O$ lowers the rate of NCS reduction by a factor of 3.4). We should, therefore, observe a lag time for the NCS reaction if the actual rate of $O_2$ reduction were in the range of that of NCS or if residual $O_2$ reacted with $CO_2^-$-activated NCS as well as with the primary lesions introduced by activated NCS in DNA. Since the amount of oxygen in the buffer at equilibrium is much smaller (<1%) than total DNA damage, this provides direct evidence that the DNA-nicking reaction observed here is an anaerobic process.

The activated NCS chromophore intermediate is a free radical. This is conclusively shown from four lines of evidence: (i) the reaction involves uptake of 3 mol of $CO_2^-$/mol of NCS chromophore, which is quite unusual, (ii) the activated species undergoes rapid dimerization in the absence of DNA, (iii) the reaction is temperature independent (Favaudon, 1983), and further (iv) DNA damage is completely inhibited by low concentrations of thiols (Favaudon, 1983). Three electron equivalents uptake by the NCS chromophore was already recognized (Favaudon, 1983) but was incompletely understood for lack of data showing clearly that NCS chromophore reduction and subsequent DNA damage are absolutely parallel (Figure 1). The data of Figure 1 also indicate that dissociation of the chromophore from the NCS apoprotein occurs only after complete reaction. As a matter of fact, the fluorescence yield of the naphthoate moiety in the NCS chromophore is mostly controlled by its interaction with the apoprotein or by intercalation into double-stranded DNA. Also the affinity of the native form of the chromophore for apo-NCS complexation is about 3 orders of magnitude higher than for DNA (Povirk & Goldberg, 1980; Povirk et al., 1981; Napier & Goldberg, 1983). The apo-NCS and DNA equilibrium binding constants of the chromophore derivative $NCS_3$ (see Scheme I) isolated from $\gamma$-ray irradiated samples have been measured previously (Favaudon, 1983) and have been shown to be higher for apo-NCS ($K_d \simeq 20$ nM) than for DNA ($K_d \simeq 100$ nM per base pair). The related affinities of the $CO_2^-$-activated transient are not known, but they should still be in favor of apo-NCS binding in view of the data of Figure 2A which indicate that inactivation of the intermediate within the apo-NCS complex is kinetically favored over reaction with DNA.

As is well documented from radiolytic studies, radical reactions on DNA may affect both the base and sugar moieties. Hydrated electrons only react on the bases. To the best of our knowledge, DNA damage by the $CO_2^-$ radical has not been reported before, but this reaction (in the absence of NCS) has a very low yield with poly(dA-dT); we did not analyze it in any detail. In any case, the fact that thymine released from poly(dA-dT) is unaltered makes deoxyribose the unique target of $CO_2^-$-activated NCS. As this cannot result from a simple electron-transfer process, we are faced therefore with the hypothesis that DNA damage is initiated by hydrogen atom abstraction from deoxyribose. The incorporation of tritium in a fraction of the NCS chromophore upon reaction with [5′-$^3$H]thymidine- and [*methyl*,1′,2′-$^3$H]thymidine-labeled poly(dA-dT) (Figure 4) provides support for this scheme. This may appear paradoxical considering that the carboxyl radicals which bring about NCS chromophore activation are essentially good one-electron donors. However, rearrangement of the chromophore molecule may occur following the uptake of three electrons and give rise to a species able to perform hydrogen atom abstraction. We also took into consideration the possibility of $CO_2^-$ addition to the NCS chromophore. This is not the case, as evidenced from the unchanged rate of NCS reduction on irradiation in argon-deaerated formate buffer where the reducing equivalents are constituted of $CO_2^-$ (55%) and $e_{aq}^-$ (45%). Poly(dA-dT) damage is also the same in argon-deaerated and $N_2O$-saturated formate buffer; hence, it can be stated that $CO_2^-$ radicals do not recombine with DNA radicals produced by activated NCS.

The reason why three major fractions of the chromophore are found by chromatography after reaction with $CO_2^-$ (Figure 4) may appear unclear considering the data of Fig. 2A. These data are consistent with chromophore dimerization being the main pathway of inactivation competing with reaction on DNA (Scheme I). However, the observation that a short incubation at pH 9 of the irradiated material produces a complete change of the elution pattern and gives rise to as many as 12 fluorescent fractions suggests that the reduced forms of the chromophore are prone to hydrolysis. On the other hand, the fraction of the chromophore, which incorporates radioactivity upon reaction with poly(dA-dT) labeled at C-5′ or at C-1′/C-2′, is nonfluorescent. This indicates that the radioactive product no longer contains the naphthoate group which is part of the structure of the native chromophore (Hensens et al., 1983). Lack of the naphthoate residue further explains the weakness of the absorbance of the related fraction since most

of the UV light absorption in the NCS chromophore is due to the naphthoate residue (Napier & Goldberg, 1983). It appears, therefore, that $CO_2^-$-induced activation of the chromophore may induce elimination of the naphthalene group at some stage of the reaction. However, the elution profile of $\gamma$-ray irradiated NCS provides evidence that the group eliminated is not released as free naphthoic acid but is still esterified to an apolar residue. In fact, the elution profile of irradiated NCS shows four major fractions with regard to absorption and/or fluorescence (Figure 4). Fraction I consists of one to three peaks eluted at a position close to that expected for the so-called type D product formed by decomposition of the NCS chromophore (Napier et al., 1981). Fraction I was already present in nonirradiated NCS; its yield was not increased by irradiation, but its elution pattern depended much on the time and the conditions (temperature) of storage of the irradiated samples and is thus related to hydrolytic degradation. In contrast, the elution profile of the other three fractions was very reproducible. Peak IV is not fluorescent. No gross changes occurred in the elution pattern whether DNA was present or not during irradiation. At high DNA concentration (2 mM), however, where a significant part of the $CO_2^-$-activated intermediate reacts with DNA (Figure 2B), we observed that peak III grew significantly at the expense of both peaks II and IV. It is thus likely that, after reaction with DNA, the chromophore is fragmented into two pieces, one bearing the naphthoate group (peak III) and the other one corresponding to the radioactive peak. Finally, considering the marked regioselectivity for poly(dA-dT) attack by the $CO_2^-$-activated chromophore, elimination of the naphthoate residue is not likely to occur from the activated intermediate NCS· prior to reaction on DNA (Scheme I) since the naphthoate group is involved in the binding of the chromophore to DNA by intercalation (Povirk et al., 1981). We would rather propose that naphthoate elimination proceeds slowly in the course of decomposition reactions, starting mostly from the dimer species $(NCS)_2$ and giving three major fractions at pH 5.0.

Rearrangement of carbon-centered radicals of deoxyribose in the absence of oxygen may include disproportionation reactions, ring opening, base elimination, and hydrolysis of phosphoester bonds; alkali lability at these sites can be brought about by free hydroxyl groups at positions C-1′, C-2′, or C-4′ (Dizdaroglu et al., 1977a,b). The results in terms of the final lesion should then depend primarily on the position of deoxyribose attack. Site specificity, depending on the way the activated chromophore binds to the DNA duplex, is indicated by the large preference for thymine compared to adenine sites in poly(dA-dT). The regioselectivity for sugar attack might also be controlled by the free enthalpy change associated with hydrogen atom abstraction, but in this particular case, the reaction proceeds without a noticeable kinetic isotope effect as observed in other systems (Westheimer, 1961). Binding of the activated portion of the NCS chromophore to the major or the minor groove of the poly(dA-dT) helix will also control the reaction. In this respect, equilibrium studies indicating that the NCS chromophore binds to the minor groove only (D. Dasgupta, personal communication), as do all DNA-nicking drugs, precludes C-3′ attack. Moreover, due to the stereospecificity inherent in the reaction used for labeling deoxyribose simultaneously at positions C-1′ and C-2′, the related labels are in an anti configuration. Consequently, the C-2′ label is in the major groove, and only the C-1′ label should be involved in the formation of a tritium-labeled fraction of chromophore on reaction with [*methyl*,1′,2′-$^3$H]thymidine-labeled poly(dA-dT). However, the heterogeneity of DNA damage (Figure 5) indicates that attack occurs at several positions of deoxyribose. With radiolytically activated NCS hydrogen atom abstraction is observed at both C-1′ and C-5′, in contrast with the thiol-dependent reaction in which hydrogen atom abstraction is found only from C-5′ (Charnas & Goldberg, 1984; L. S. Kappen and I. H. Goldberg, unpublished data). The ratio of these abstraction reactions (C-5′:C-1′ = 0.47) determined from $^3$H incorporation into the NCS chromophore is close to the ratio of alkali-induced vs. spontaneous thymine release (0.51). This suggests that the base is still attached to the modified deoxyribose residue resulting from C-5′ attack and that part of these sites are not breaks, since the yield of C-5′ attack with respect to total damage is larger than that of spontaneous nicks bearing 3′-phosphate ends excised by exonuclease III (Table II; Figure 5). In fact, deoxyribose radicals that are not $\beta$ to a phosphate ester cannot eliminate phosphate and may undergo rearrangement via other routes, leaving the sugar fragment bound to the polynucleotide strands.

Finally, it appears that attack at C-1′ is the main lesion induced by $CO_2^-$-activated NCS. It remains to be shown, however, that the radiolytically activated chromophore binds to the minor groove of DNA or that the reactive part of the chromophore does not project into the major groove. It has been reported that $\gamma$-ray irradiation in the absence of oxygen may produce deoxyribonolactone, the cyclic ester of deoxyribonic acid bearing a carbonyl group at C-1′, by recombination of the C-1′ radical with an OH· radical and elimination of the base (Dizdaroglu et al., 1977a; Isildar et al., 1981). However, OH· radicals are quenched in formate buffer. On the other hand, the properties (e.g., the alkali lability) of the sites resulting from C-1′ attack in our system are consistent with the presence of a carbonyl group at C-1′. It must also be considered that rearrangement of the primary C-1′ radical involves ionic elimination of the base followed by reaction with $OH^-$ ion and reoxidation by formate to yield a normal apyrimidinic site. Such sites can be excised by exonuclease III, but this would not produce any inorganic phosphate under the assay conditions (Weiss, 1976). Were this scheme to be followed, it would require that the primary C-1′ radical be sufficiently strongly oxidizing to react with the formate ion.

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**Registry No.** Poly(dA-dT), 26966-61-0; NCS, 9014-02-2; carboxyl radical, 14485-07-5; nitrous oxide, 10024-97-2; 2-mercaptoethanol, 60-24-2; thymine, 65-71-4; adenine, 73-24-5; formic acid, 64-18-6; oxygen, 7782-44-7; exonuclease III, 9037-44-9; alkaline phosphatase, 9001-78-9.

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