Schwartz, R., & Rothblum, K. (1980) *Biochemistry 19*, 2506.
Southern, E. (1975) *J. Mol. Biol. 98*, 503.
Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866.
Storti, R. V., Coon, D. M., & Rich, A. (1976) *Cell (Cambridge, Mass.) 8*, 521.
Sutcliffe, J. G. (1978) *Nucleic Acids Res. 5*, 2721.
Tobin, S. L., Zulauf, E., Sanchez, F., Craig, E. A., & McCarthy, B. J. (1980) *Cell (Cambridge, Mass.) 19*, 121.
Ullman, J. S., & McCarthy, B. J. (1973) *Biochim. Biophys. Acta 294*, 405.
Vandekerchove, J., & Weber, K. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1106.
Whalen, R., Butler-Browne, G. S., & Gros, F. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2018.
Woo, S. L. C., Haris, S. E., Rosen, J. M., Chan, L., Sperry, P. L., & O'Malley, B. W. (1974) *Prep. Biochem. 4*, 555.

Deoxyribonucleic Acid Sugar Damage in the Action of Neocarzinostatin†

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ABSTRACT: Neocarzinostatin (NCS), an antitumor protein antibiotic which causes base release and both true and alkali-labile, single-strand breaks almost exclusively at thymidylic acid and, to a lesser extent, at deoxyadenylic acid residues in deoxyribonucleic acid (DNA) in the presence of mercaptans and oxygen in vitro, produces gaps which are not the result of simple splitting of the phosphodiester bond. We show that sugar damage plays an important role in the NCS-induced DNA strand scission reaction. The release of ^{3}H-labeled compounds, mainly as formic acid and H_2O from [5′-^{3}H]-thymidine-labeled λDNA, accounts for 50–80% of the thymine released without or with postincubation alkaline treatment, respectively. The release of these labeled sugar degradation, products correlates well with the increase in both thymine release and 5′-phosphate ends due to postincubation alkaline treatments. In addition, a malonaldehyde-like substance (characterized by its reaction with thiobarbituric acid and its chromatographic properties), containing tritium label derived from the 1′,2′ carbons of the deoxyribose moiety of thymidylate in DNA, is produced in a bound form concomitantly with the strand breaks and thymine release; its production is dependent on the presence of mercaptans and oxygen, is stimulated by 2-propanol, and is inhibited by α-tocopherol. Labeled malonaldehyde-like material and H_2O are released from DNA only after alkaline treatment and account for ~40% of the corresponding thymine released. The correlation between thymine release and strand breaks is examined to elucidate the nature of the gaps produced by NCS. The number of thymines released by various postincubation treatments is consistent with the number of 5′-phosphate ends generated at the DNA gaps as determined by the combined use of alkaline phosphatase and polynucleotide kinase; after alkaline treatment (0.3 N NaOH, 37 °C, 30 min) both values are in good agreement with the number of single-strand breaks, as estimated by alkaline sucrose gradients. Taken together, these data indicate that a significant fraction of the DNA damage caused by NCS requires alkaline treatment for removal of both the DNA base and a sugar fragment from the 5′-phosphate end of the DNA gap.

There is a substantial body of evidence [reviewed in Goldberg et al. (1980)] that cellular deoxyribonucleic acid (DNA) is the primary target in the action of the antitumor protein antibiotic neocarzinostatin (NCS).[1] In addition to placing single-strand breaks in DNA in vivo, NCS causes single-strand breaks in DNA in vitro in a reaction that is markedly enhanced by mercaptans (Beerman & Goldberg, 1974; Beerman et al., 1977). The breaks in the DNA are not simple phosphodiester nicks (Kappen & Goldberg, 1977; Poon et al., 1977) but consist of gaps that bear 3′- and 5′-phosphoryl termini (Kappen & Goldberg, 1978a). Cleavage of the DNA occurs almost exclusively at thymidylic acid and, to a lesser extent, at deoxyadenylic acid residues (Hatayama et al., 1978; D'Andrea & Hazeltine, 1978) and is associated with the release of the corresponding bases (Poon et al., 1977; Ishida & Takahashi, 1976). Evidence suggesting a free-radical mechanism in the DNA cleavage event has been presented (Kappen & Goldberg, 1978b; Sim & Lown, 1978).

While the primary sequence of the NCS protein has been known for some time (Meienhofer et al., 1972), we have only recently discovered a nonprotein chromophore to be associated with the protein (Napier et al., 1979) and have found that it possesses the cytotoxic and in vitro DNA strand scission activities of the parent compound (Kappen et al., 1980). The apoprotein stabilizes the chromophore and controls its release (Kappen et al., 1980). Fluorescence, dichroism, and other studies indicate that the chromophore binds tightly and specifically to the target DNA, by an intercalative mechanism (Povirk & Goldberg, 1980; L. F. Povirk, N. Dattagupta, B. C. Warf, and I. H. Goldberg, unpublished data). A partial structure of the chromophore (M_r 661) has recently been

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[1] Abbreviations used: NCS, neocarzinostatin; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; TBA, 2-thiobarbituric acid; DNase, deoxyribonuclease; ATP, adenosine 5′-triphosphate.

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reported (Albers-Schönberg et al., 1980).

In this paper, we examine the chemical nature of the cleavage products and the relation between these products and the generation of breaks caused by NCS in an effort to elucidate the primary site in DNA attacked by NCS.

Materials and Methods

[*methyl*-^{3}H]Thymidine, [*methyl*,1′,2′-^{3}H]thymidine, [5′-^{3}H]thymidine, [γ-^{32}P]ATP, [^{14}C]formic acid, [^{14}C]acetic acid, [^{14}C]glycolic acid, [^{14}C]oxalic acid, and [^{3}H]H_2O were purchased from New England Nuclear. [U-^{14}C]Thymidine was from Amersham Corp. Calf thymus DNA (type I) was from Sigma Chemical Co. Sephadex G-10 was from Pharmacia Fine Chemicals. Scintiverse (for liquid scintillation counting) was from Fisher Scientific Co. Bacterial alkaline phosphatase was from Worthington Biochemical Corp. Polynucleotide kinase was from New England Biolabs. μC_{18} Bondapak column was from Waters Associates. NCS was provided by Dr. W. T. Bradner (clinical form) or Dr. T. S. A. Samy (purified form).

Preparation of λDNA. Various radioactive thymidine-labeled λDNAs were prepared by the method of Hedgpeth et al. (1972) using the different radioactive forms of thymidine listed above. Specific activities of [*methyl*-^{3}H]thymidine-labeled λDNA, [5′-^{3}H]thymidine-labeled λDNA, [*methyl*-,1′,2′-^{3}H]thymidine-labeled λDNA, and [U-^{14}C]thymidine-labeled λDNA were 4×10^4–8×10^4 cpm/μg, 2.8×10^4 cpm/μg, 5×10^4–6.5×10^4 cpm/μg, and 5×10^2 cpm/μg, respectively.

NCS Reaction Condition and Various Treatments. The standard NCS reaction mixture containing 50 mM Tris-HCl (pH 8.0), 10 mM 2-mercaptoethanol, 10 μg/mL λDNA, and 200 μg/mL NCS was incubated at 37 °C for 1 h. After incubation, the samples were subjected to various treatments: 0.1 or 0.3 M NaOH at room temperature for 10 min, 0.3 M NaOH at 37 or 90 °C for 30 min, or 65 °C for 30 min in the above Tris buffer. After alkaline treatment the samples were neutralized with an equivalent amount of HCl.

2-Thiobarbituric Acid (TBA) Reaction. Estimation of TBA-reactive material was essentially based on the procedure described by Waravdekar & Saslaw (1959) for determination of malonaldehyde. After incubation of the NCS reaction mixture (0.3 mL), 0.6 mL of 0.6% 2-thiobarbituric acid, pH 2.0, was added, and the contents were heated in a capped test tube in boiling water for 20 min. After cooling for 5 min in water at room temperature, the contents were spun at 10 000 rpm for 10 min to remove a precipitate. The absorption spectrum (450–600 nm) of the supernatant liquid was determined in a Cary spectrometer (Model 118) against a reference solution containing water. The amount of malonaldehyde-like material was estimated by using an E^M_{532} of 1.53×10^5 (Waravdekar & Saslow, 1959).

Gradient Analysis. Single- or double-strand breaks of λDNA were analyzed by sucrose gradient centrifugation as described before (Poon et al., 1977). Aliquots (50–100 μL) of the NCS reaction mixture were loaded on either alkaline sucrose gradients (5 mL of 5–20% sucrose in 0.7 NaCl, 0.3 M NaOH, and 0.01 M EDTA) or neutral sucrose gradients (5 mL of 5–20% sucrose in 0.7 M NaCl, 0.01 M EDTA, and 0.01 M Tris-HCl, pH 8.0) and were spun in a SW50.1 rotor at 49 000 rpm for 2 h at 20 °C. Seven-drop fractions (0.2 mL) were collected from the top of the gradient, and the radioactivity was determined in a Packard liquid scintillation counter after the fractions were mixed with either 1 mL of water for the neutral gradient or 1 mL of 0.06 M HCl for the alkaline gradient and 10 mL of Scintiverse. The number of single- or double-strand breaks was calculated by using the equation of Abelson & Thomas (1966).

Column Chromatography. Sephadex G-10 column chromatography was performed to quantitate the products released from DNA by NCS. The sample, to which was added thymine (50 μg) as a marker and glycerol (final 5%), was applied to a Sephadex G-10 column (1.5 × 68 cm) equilibrated with 0.01 M Tris-HCl, pH 8.0, 1 mM NaCl, and 0.1 mM EDTA, and eluted with the same buffer at a flow rate of 30–36 mL/h at room temperature. After measurement of the A_{260} to determine the elution position of thymine, the radioactivity of each fraction (1–1.2 mL) was counted after addition of 10 mL of Scintiverse.

Reverse-phase high-pressure liquid chromatography (Waters Associates) was also used to separate formic acid, acetic acid, malonaldehyde, and thymine. Separation was performed with 0.1 M $NH_4H_2PO_4$, pH 2.5, at a flow rate of 2 mL/min at room temperature by using a μBondapak C_{18} column (0.4 × 30 cm).

Paper Chromatography. Descending paper chromatography was performed to identify the products from NCS-treated DNA. Separation of formic acid and acetic acid was done by using Whatman 3 MM paper (3.8 × 60 cm) with the solvent system of either 95% ethanol-concentrated (28–30%) NH_4OH (100:1) or the alcoholic phase of 1-butanol–ethanol–3 N NH_4OH (4:1:5) for 20–40 h. For rapid separation of thymine from DNA, paper chromatography with ethanol–1 M sodium acetate (7:3) was performed for 2 h. After the paper was dried, the position of the thymine marker was marked under a UV lamp. Then the paper strip was cut into 1-cm pieces, and they were put into vials to which was added 1 mL of water. After sitting for 1 h, the radioactivity was counted after mixing with 10 mL of Scintiverse.

Paper Electrophoresis. Paper electrophoresis was carried out by using Whatman 3 MM paper (3.8 × 50 cm) with 0.1 M ammonium carbonate, pH 8.9, at 1000 V for 2 h. After electrophoresis, the radioactivity was measured as mentioned above. Under this condition, formic acid moved 34 cm, whereas acetic acid moved 24 cm and glycolic acid moved 23 cm from the origin.

Thin-Layer Chromatography. To determine whether thymine derivatives were generated during the reaction with NCS, we performed two-dimensional thin-layer chromatography (silicagel 254 TLC plate, Merck) with the following solvent system: first run with chloroform–methanol–water (4:2:1) and second run with ethyl acetate–2-propanol–water (75:16:9) as described by Teoule et al. (1974).

Phosphatase–Kinase Assay. To quantify and identify the 5′-terminal end of the break generated in NCS-treated DNA after various treatments, we followed its availability for phosphorylation by polynucleotide kinase by the method of Weiss et al. (1968), as modified by Poon et al. (1977). The NCS reaction mixture (50 μL) containing [*methyl*-^{3}H]thymidine-labeled λDNA was incubated at 37 °C for 1 h. After addition of water (50 μL), the sample was subjected to various treatments. In the case of alkaline treatment, the NaOH was neutralized by adding an equivalent amount of HCl, and 0.2 M Tris was added to adjust the pH to 8. Each sample was then divided into two tubes (50 μL each). To one alkaline phosphatase (0.24 μg) was added, and both samples were incubated for 30 min at 37 °C. After incubation with or without phosphatase, 60 μL of the following mixture was added to each tube: 0.02 M $MgCl_2$, 0.02 M 2-mercaptoethanol, 3 mM potassium phosphate (pH 7.0), 2 units of polynucleotide kinase, and 2.6 μCi of [γ-^{32}P]ATP (sp act. 1 Ci/mmol).

Table I: Summary of Percent Release of Thymine and Sugar Fragments from NCS-Treated λDNA by Various Treatments[a]

treatment	[*methyl*-^{3}H]-thymidine-labeled λDNA, thymine	[*methyl*,1′,2′-^{3}H]thymidine-labeled λDNA					[5′-^{3}H]thymidine-labeled λDNA				
		malonaldehyde region	H_2O	malonaldehyde plus H_2O	thymine	peak IV	peak I, formic acid	peak II	peak III, H_2O	peak IV	total
none	3.7	0	0	0	3.8[b] (2.3)[c]	0	0.95	0.42	0.42	0	1.79
0.1 N NaOH, RT,[d] 10 min	4.1	0	0.85 (0.34)	0.85 (0.34)	4.60 (2.76)	0	1.30	0	1.70	0	3.00
0.3 N NaOH, RT, 30 min	5.5	0.43 (0.17)	1.18 (0.47)	1.61 (0.64)	5.60 (3.35)	0	1.40	0	2.30	0.16	3.86
0.3 N NaOH, 37 °C, 30 min	7.7	1.00 (0.40)	1.95 (0.78)	2.95 (1.18)	8.00 (4.80)	(0.20)	1.30	0	4.30	0.73	6.33
0.3 N NaOH, 90 °C, 30 min	12.9	1.00 (0.40)	4.50 (1.80)	5.50 (2.20)	13.20 (7.90)	(0.20)	3.00	0	5.00	0.70	8.70
65 °C, 30 min	3.9	0	0.65 (0.26)	0.65 (0.26)	4.20 (2.50)	0	1.00	0.50	0.97	0	2.47

[a] These values were obtained from the data of Sephadex G-10 column chromatography of incubations containing [*methyl*-^{3}H]thymidine-labeled λDNA, [*methyl*,1′,2′-^{3}H]thymidine-labeled λDNA, or [5′-^{3}H]thymidine-labeled λDNA. [b] This value represents a correction of the value in the parentheses (*c*) to take into account the distribution of ^{3}H between thymine and deoxyribose in [*methyl*,1′,2′-^{3}H]thymidine. 60% is in thymine at the methyl moiety and 40% is in deoxyribose at 1′ and 2′ positions. [c] The value in the parentheses is the uncorrected percent of the total radioactivity. In the absence of drug, there was no significant release of radioactivity (as thymine or sugar fragments) under any of the treatment conditions. [d] RT, room temperature.

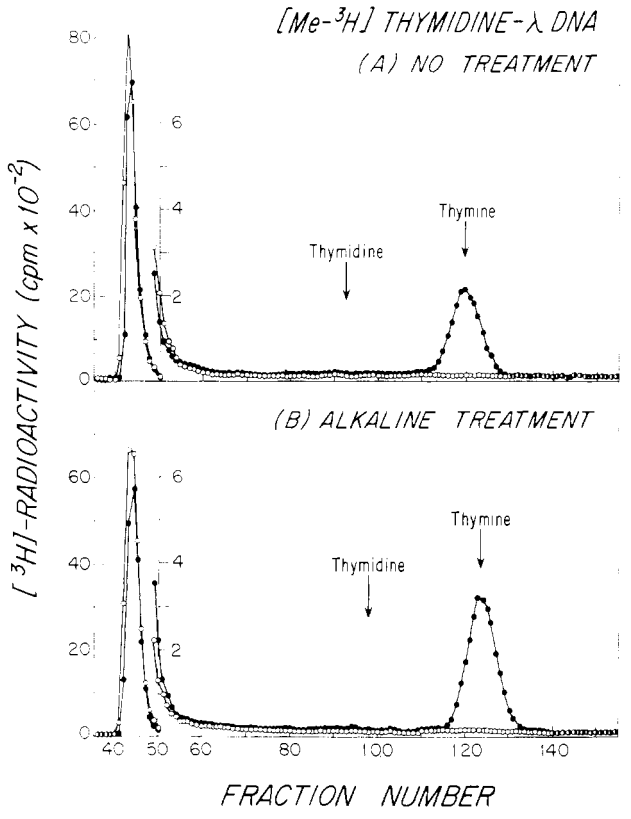

FIGURE 1: Sephadex G-10 column chromatography of NCS-treated [*methyl*-^{3}H]thymidine-labeled λDNA. The standard NCS reaction mixture (100 μL) with [*methyl*-^{3}H]thymidine-labeled λDNA incubated at 37 °C for 1 h was applied to Sephadex G-10 columns after (A) no treatment or (B) alkaline treatment (0.3 N NaOH at 37 °C for 30 min). (●) Complete NCS reaction; (○) no NCS reaction. Arrows indicate the position of elution of authentic thymine and thymidine.

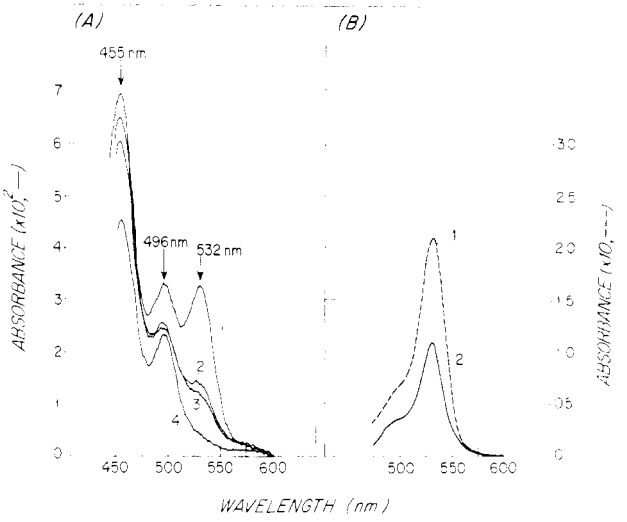

FIGURE 2: Absorption spectrum of TBA reaction of NCS incubations with DNA. (A) NCS reaction mixture (0.3 mL) with 50 mM Tris-HCl, pH 8, 10 mM cysteine, calf thymus DNA (500 μg/mL), and purified NCS (500 μg/mL) incubated at 37 °C for 1 h was reacted with TBA reagent as described under Materials and Methods. (1) Complete NCS reaction; (2) after preincubation of NCS with cysteine for 1 h at 37 °C, DNA was added and incubated at 37 °C for 1 h; (3) NCS reaction without cysteine; (4) reaction without NCS. (B) (1) TBA reaction of standard malonaldehyde; (2) difference spectrum between (A3) and (A1).

Following incubation for another 1 h at 37 °C, the reaction was stopped by addition of cold 0.5 M trichloroacetic acid and 10 mM sodium pyrophosphate. After 10 min in ice the trichloroacetic acid precipitate was collected on a Millipore filter whic was then washed with 0.5 M trichloroacetic acid and 10 mM sodium pyrophosphate, dried, and counted in a toluene-based scintillator. The incorporation of ^{32}P into λDNA was corrected for DNA recovery by determining the ^{3}H radioactivity of λDNA retained on the filter, taking account of the loss of thymine due to release by NCS. The same result was obtained by using highly purified alkaline phosphatase (Weiss et al., 1968).

Results

Thymine Release. Incubation of NCS with DNA in the resence of a mercaptan results in the release of mainly thymine and 15% as much adenine (Poon et al., 1977). Using various separation methods, including two-dimensional thin-layer or paper chromatography and Sephadex G-10 chromatography, we confirmed that thymine itself, and no modified forms, including those bearing sugar fragments, was released. Also, we found that the amount of thymine released was increased on postincubation alkaline treatment (Figure 1 and Table I). In the absence of NCS no thymine was released even after strong alkali treatment (0.3 M NaOH, 90 °C, 30 min).

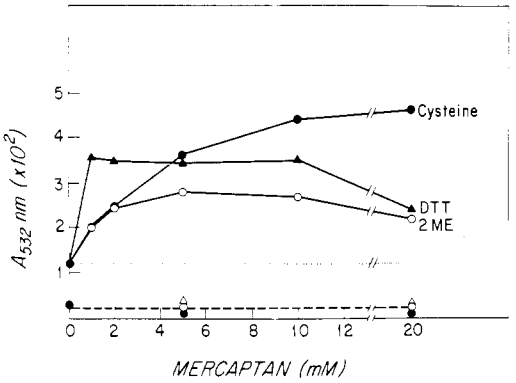

FIGURE 3: Mercaptan dependency of production of TBA-reactive material. NCS reaction mixture (0.3 mL), as described in Figure 2 except for various concentration of mercaptans, was incubated at 37 °C for 1 h and then subjected to TBA reaction as described under Materials and Methods. (●) Cysteine; (▲) dithiothreitol; (○) 2-mercaptoethanol. (—) NCS plus mercaptan; (···) without mercaptan; (- - -) without NCS.

Table II: Effect of 2-Propanol, α-Tocopherol, or N_2 on Production of TBA-Reactive Material[a]

addition	A_{532} (×10³)	%
none	26	100
2-propanol, 1 M	65	250
in air	23	100
in N_2	0	0
2% ethanol	33	100
α-tocopherol (+2% ethanol)		
0.5 mM	21	64
2 mM	8	24
10 mM	0	0

[a] NCS reaction mixture (0.3 mL) as described in Figure 2 except for the DNA concentration (100 μg/mL) was incubated at 37 °C for 1 h in the presence of 2-propanol, α-tocopherol, or N_2 gas and subjected to TBA reaction as described under Materials and Methods. The A_{532} of the TBA reaction with the incubation lacking cysteine was subtracted from that with the complete reaction.

Production of TBA-Reactive Materials. As shown in Figure 2A, TBA-reactive material was produced when DNA and NCS were incubated in the presence of a mercaptan. Visible absorption spectra revealed peaks at 532, 496, and 455 nm. The peaks at 496 and 455 nm have been previously observed upon reaction of DNA with the TBA reagent (Kapp & Smith, 1970). The peak at 532 nm which is characteristic of the interaction of malonaldehyde and TBA was completely dependent on both NCS and mercaptan (cysteine). Preincubation of NCS with cysteine, which inactivates the antibiotic for DNA strand breakage (Kappen & Goldberg, 1978b), also results in loss of its ability to generate the 532-nm material. The difference spectrum obtained between the complete reaction and that lacking cysteine was the same as that of standard malonaldehyde (Figure 2B); the ratio of A_{532} to A_{500} was 3.16 compared to 3.20 for standard malonaldehyde. While the production of TBA-reactive material is dependent on the presence of mercaptans, the amount of TBA-reactive material produced in the presence of cysteine is about twice as much as that produced in the 2-mercaptoethanol reaction at a concentration of 10 mM (Figure 3), as also has been reported in the case of acid-solubilization of DNA by NCS (Kappen & Goldberg, 1978b). As shown in Table II, the production of TBA-reactive material is also inhibited by α-tocopherol and by the absence of oxygen but is enhanced 2.5-fold by 2-propanol (1 M). The requirement of a high concentration of α-tocopherol for inhibition of this reaction is due to the high concentration of DNA in the reaction. Inhibition of the reaction by α-tocopherol is overcome by adding excess of DNA (T. Hatayama, L. S. Kappen, and I. H. Goldberg, unpublished data). EDTA (10 mM) and NaCl (100 mM) do not affect the reaction.

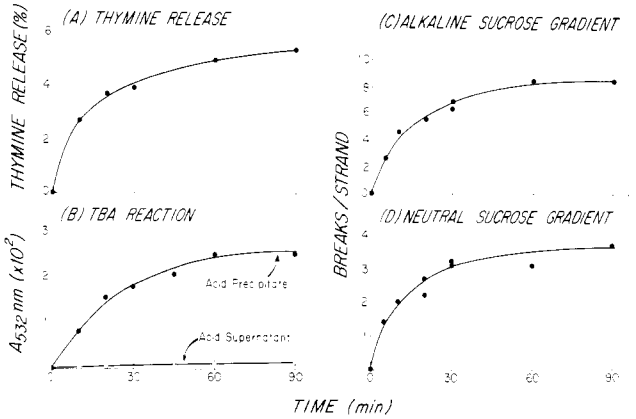

FIGURE 4: Time course of thymine release, production of TBA-reactive material, and strand breaks. (A) For thymine release, standard NCS reaction mixture (300 μL) containing [*methyl*-³H]thymidine-labeled λDNA was incubated at 37 °C. At each time point, aliquots (45 μL) were spotted on Whatman 3 MM paper, and paper chromatography was done as described under Materials and Methods. (B) For production of TBA-reactive material, the NCS reaction mixture (4.5 mL) as described in Table II was incubated at 37 °C. At indicated times, aliquots (0.3 mL) were withdrawn to a tube, and trichloroacetic acid was added to a final concentration of 5%; mixtures were kept in ice for 30 min, and then spun at 2000 rpm for 10 min. The supernatant fluid was removed, and the precipitate was washed twice with 5% trichloroacetic acid and finally suspended in 300 μL of 50 mM Tris-HCl (pH 8). 200-μL aliquots of the supernatant fluid or the precipitate fraction to which was added 100 μL of water were then reacted with 0.6 mL of the TBA reagent as described under Materials and Methods. The presence of 5% trichloroacetic acid did not affect the TBA reaction of authentic malonaldehyde. (C) For single-strand breaks and (D) double-strand breaks, the standard NCS reaction mixture (250 μL) containing [*methyl*-³H]thymidine-labeled λDNA, except for the NCS concentration of 1 μg/mL (C) or 5 μg/mL (D), instead of 200 μg/mL, was incubated at 37 °C. At the indicated time, aliquots (45 μL) were withdrawn to a tube containing 45 μL of 2 mM α-tocopherol (Kappen & Goldberg, 1978b) in ice; then 80-μL aliquots of each tube were analyzed on alkaline (C) or neutral (D) sucrose gradients as described under Materials and Methods.

Time Course. To determine the relationship between the production of TBA-reactive material, thymine release, and strand breaks due to NCS, we analyzed the time course of the reaction. As shown in Figure 4, production of TBA-reactive material, thymine release, and single- and double-strand breaks all showed similar patterns; the reactions reached completion between 30 and 60 min. The TBA-reactive material remained attached to the DNA, as revealed by its acid insolubility (Figure 4B). Further, the NCS-induced material does not react with 2-methylindole which, unlike TBA, does not require heat for production of its characteristic chromophore with soluble malonaldehyde (Scherz et al., 1967).

Identification of TBA-Reactive Material. To determine the carbon source of the TBA-reactive material, we used [*methyl*,1′,2′-³H]thymidine-labeled λDNA as the substrate in the NCS-reaction. As shown in Figure 5B, in the absence of subsequent alkaline treatment, radioactivity was found only in the DNA and thymine peaks, and all the TBA-reactive

Alkaline treatment (0.3 M NaOH, 37 °C, 30 min) did not show any increase in the total production of TBA-reactive material. On the other hand, we have found that for the formation of TBA-reactive material from depurinated DNA, alkaline treatment was required (D. Cox and I. H. Goldberg, unpublished data).

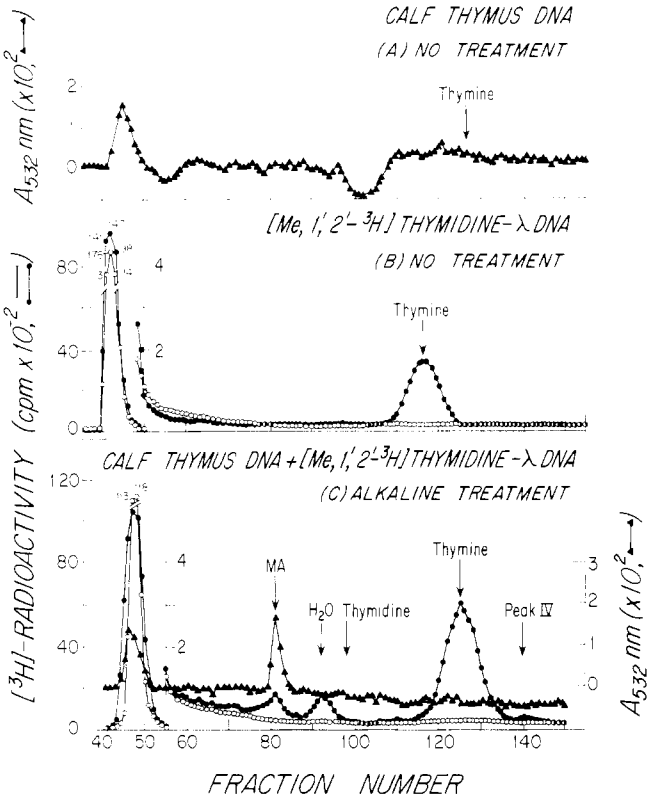

FIGURE 5: Alkaline release of TBA-reactive material from NCS-treated DNA. (A) The standard NCS reaction mixture (3 mL) with calf thymus DNA (100 μg/mL), instead of λDNA, and a NCS concentration of 500 μg/mL, was incubated at 37 °C for 1 h, before applying onto a Sephadex G-10 column. To each eluted fraction (1.2 mL) was added 0.6 mL to TBA reagent (×2 concentrated), and the A_{532} of the TBA reaction was determined as described under Materials and Methods. (B) The standard NCS reaction (100 μL) with [*methyl*,1′,2′-^{3}H]thymidine-labeled λDNA was applied to a Sephadex G-10 column, and the radioactivity of each fraction was counted. (●) Complete reaction; (○) reaction without NCS. (C) The NCS reaction mixture (4 mL) with calf thymus DNA as in (A) and the standard NCS reaction mixture (0.5 mL) with [*methyl*,1′,2′^{3}H]thymidine-labeled λDNA were incubated separately at 37 °C for 1 h. After the two reaction mixtures were mixed, they were subjected to alkaline treatment (0.3 N NaOH, 37 °C, 30 min) and then applied to a Sephadex G-10 column. Radioactivity was measured by using 200-μL aliquots of each fraction mixed with 0.8 mL of water and 10 mL of Scintiverse as described under Materials and Methods. The remainder of each fraction (1.0 mL) was subjected to the TBA reaction after being mixed with 0.5 mL of TBA reagent (×2 concentrated) (▲) A_{532}. The closed and open circles represent ^{3}H radioactivity from reactions containing or lacking NCS, respectively. The position where marker malonaldehyde elutes is indicated by the arrow designated as MA.

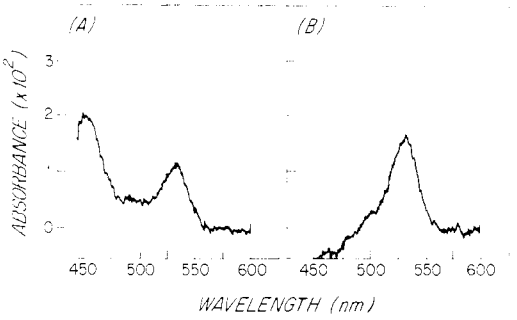

FIGURE 6: Visible absorption spectra of TBA reactions with Sephadex G-10 column chromatography fractions. (A) Fraction 46 and (B) fraction 81 of Figure 5C.

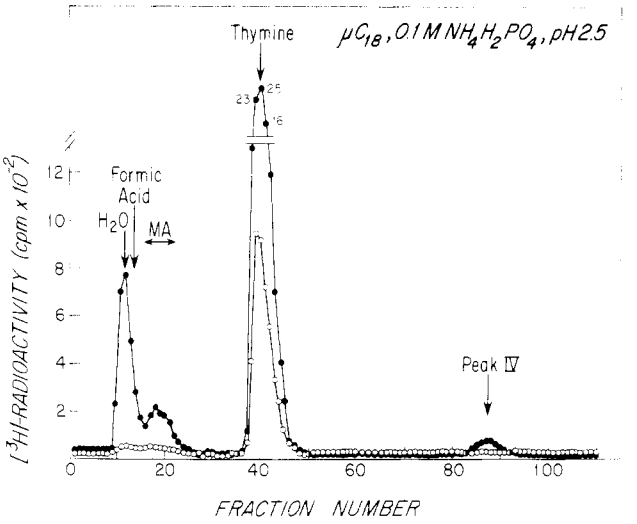

FIGURE 7: High-pressure liquid chromatography profile of [*methyl*,1′,2′-^{3}H]thymidine-labeled λDNA treated with NCS. The standard NCS reaction mixture (200 μL) with [*methyl*,1′,2′-^{3}H]thymidine-labeled λDNA was incubated at 37 °C for 1 h; then [^{14}C]formic acid (2 μL of 1 μCi/mL solution) was added. Following alkaline treatment (0.3 N NaOH, 90 °C, 30 min) of half the reaction, the samples with or without alkaline treatment were mixed with an equal volume of 0.1 M $NH_4H_2PO_4$, pH 2.5, spun at 2500 rpm for 10 min to remove precipitate, and applied to high-pressure liquid chromatography, and eight-drop fractions were collected as described under Materials and Methods. No treatment, (○); alkaline treatment, (●). Arrows indicate the positions where authentic [^{3}H]H_2O, [^{14}C]formic acid, [^{3}H]thymine, and malonaldehyde eluted.

material was found under the DNA peak (Figure 5A). Reactions lacking NCS produced neither thymine nor TBA-reactive material. After alkaline treatment of the reaction mixture, however, two additional radioactive peaks appeared. One peak was eluted at the same position as the TBA-reactive material released by alkaline treatment of calf thymus DNA treated with NCS (Figure 5C). The other peak was eluted at the bed volume of the column where [^{3}H]H_2O elutes. This material was volatile. The first peak cochromatographed with authentic malonaldehyde which was made by acid hydrolysis of malonaldehyde tetramethyl acetal. Comparison of the visible absorption spectrum of the two TBA-reactive materials in Figure 5C showed (Figure 6) that the 455-nm peak found with DNA (Figure 2A) is lacking in the fraction eluting at the same position as authentic malonaldehyde, and its spectrum is identical with that of standard malonaldehyde (Figure 2B). To characterize these products further, we employed reverse-phase high-pressure liquid chromatography (Figure 7). The radioactive H_2O, malonaldehyde, and thymine peaks were found to comigrate with authentic compounds [the malonaldehyde peak was broad and moved between fractions 16–22, possibly due to a tendency to aggregate or polymerize (Kwon & Olcott, 1966)]. There was also a small unidentified peak (IV) with a long retention time as had been noticed on Sephadex G-10 column chromatography at the position immediately after the thymine peak (peak IV). The presence of 2-propanol (1 M), which causes an enhancement of thymine release and a corresponding increase in H_2O plus malonaldehyde release, change only slightly the ratio of H_2O plus malonaldehyde to thymine, i.e., 0.38 and 0.46, control and 2-propanol-containing samples, respectively. EDTA (10 mM) had no effect.

Characterization of the Product Derived from [5′-^{3}H]-Thymidine-Labeled λDNA. To determine the fate of the sugar fragment generated at the 5′ end of the thymidylate residue in NCS-treated DNA, we used [5′-^{3}H]thymidine-labeled λDNA as the labeled substrate. Direct Sephadex G-10 column chromatography of the NCS reaction gave three peaks (peaks

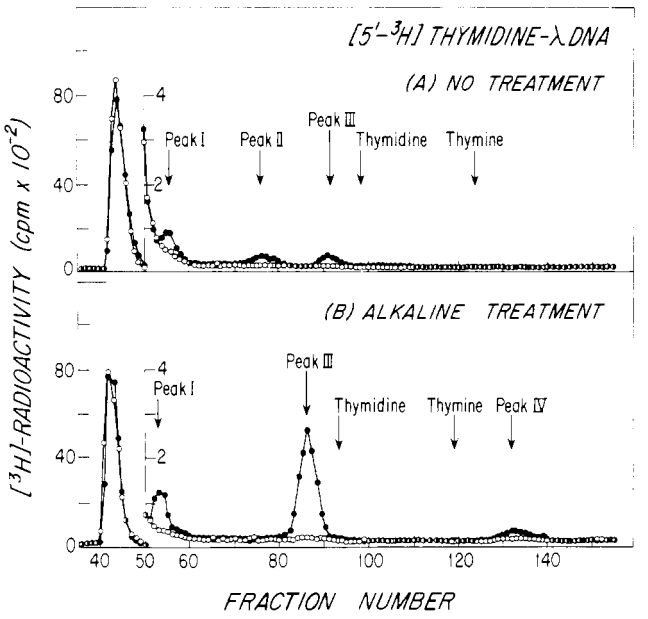

FIGURE 8: Sephadex G-10 column chromatography of NCS-treated [5'-^{3}H]thymidine-labeled λDNA. The standard NCS reaction (150 μL) with [5'-^{3}H]thymidine-labeled λDNA was applied to the Sephadex G-10 column as described under Materials and Methods. (A) No treatment; (B) alkaline treatment (0.3 N NaOH, 37 °C, 30 min). (●) Complete reaction; (○) reaction without NCS.

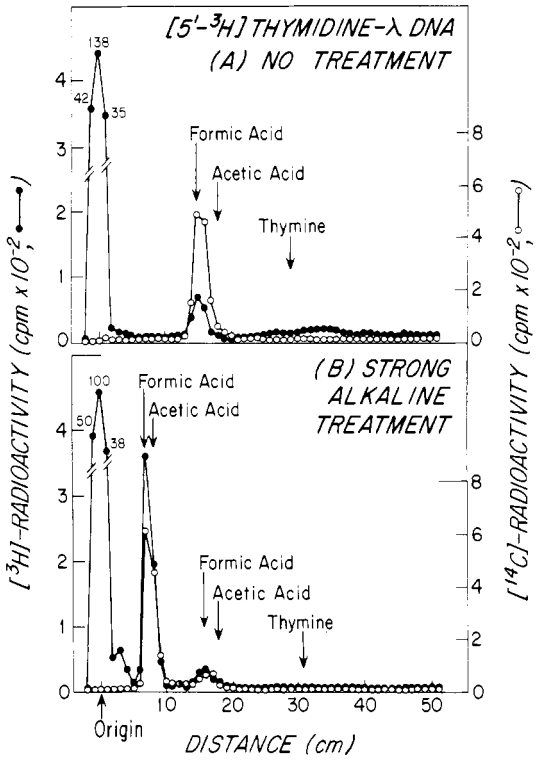

FIGURE 9: Paper chromatography of products from NCS-treated [5'-^{3}H]thymidine-labeled λDNA. The standard reaction mixture (150 μl) of [5'-^{3}H]thymidine-labeled λDNA was incubated at 37 °C for 1 h; 2 μL of [^{14}C]formic acid (1 μCi/mL) was then added. The samples with or without alkaline treatment (0.3 N NaOH, 90 °C, 30 min) were analyzed by paper chromatography using 95% ethanol–concentrated NH_4OH solvent system as described under Materials and Methods. [^{14}C]Oxalic acid remained at the origin in this system. (A) No treatment; (B) alkaline treatment. The migration positions of various labeled markers are shown.

I, II, and III) other than that due to DNA (Figure 8A). After alkaline treatment, peaks I and III increased, peak II disappeared, and another small peak IV appeared (Figure 8B). Peak I cochromatographed with [^{14}C]formic acid, whereas [^{14}C]acetic acid eluted a little earlier. Peak III is at the bed volume of the column at which [^{3}H]H_2O elutes. This material was volatile. Formic acid was further identified as the putative product of the reaction by means of paper chromatography. As shown in Figure 9, ^{3}H radioactivity from [5'-^{3}H]thymidine-labeled λDNA cochromatographed exactly with marker [^{14}C]formate, both before and after alkaline treatment. The two peaks of formate found on chromatography after alkaline treatment of the sample are due to the formation of the sodium salt (slow moving peak in Figure 9B) in addition to the ammonium salt. Similarly, on paper electrophoresis, the ^{3}H radioactivity comigrated identically with [^{14}C]formic acid (data not shown). While reverse-phase high-pressure liquid chromatography showed little separation between H_2O and formic acid (Figure 7), the ^{3}H radioactivity from the [5'-^{3}H]thymidine-labeled λDNA sample was exclusively at these positions (data not shown).

The production of [^{3}H]H_2O from NCS treatment of [*methyl*,1',2'-^{3}H]thymidine-labeled λDNA or [5'-^{3}H]thymidine-labeled λDNA was also consistent with results of an experiment with [U-^{14}C]thymidine-labeled λDNA. Due to the low specific activity of [U-^{14}C]thymidine compared to ^{3}H-labeled thymidine we could not use it for quantitative study. But no peak appeared at the position of the bed volume of the column at which the ^{3}H peak appeared from the sample of [*methyl*,1',2'-^{3}H]thymidine-labeled λDNA and [5'-^{3}H]thymidine-labeled λDNA.

Summary of Thymine and Sugar Fragment Release. The release of thymine and deoxyribose fragments from NCS-damaged DNA by various postincubation treatments as analyzed by Sephadex G-10 column chromatography is summarized in Table I. Without treatment, 3.7% of the thymine was released from [*methyl*-^{3}H]thymidine-labeled λDNA and 1.79% of ^{3}H-labeled compounds from [5'-^{3}H]thymidine-labeled λDNA. No ^{3}H-labeled compounds other than thymine were released from [*methyl*,1',2'-^{3}H]thymidine-labeled λDNA; furthermore, since the radioactivity under the thymine peak can be entirely accounted for as free thymine (3.8% thymine release compared with 3.7% from [*methyl*-^{3}H]thymidine-labeled DNA), there are no species under this peak in which a sugar fragment from the first two carbons of the deoxyribose remains attached to the thymine. Under the same standard drug treatment conditions, malonaldehyde-like material (TBA-reactive) production (with or without alkaline treatment) was 1.04% of total thymidylic acid in the DNA.

Thymine release was increased up to 3.5-fold by strong alkaline treatment (0.3 M NaOH, 90 °C, 30 min). The release of ^{3}H-labeled compounds from [5'-^{3}H]thymidine-labeled λDNA, whose main products were identified as formic acid and H_2O, accounted for 50–80% of the thymine released without or with alkaline treatment, respectively. While the main ^{3}H-labeled products, other than thymine, from [*methyl*,1',2'-^{3}H]thymidine-labeled λDNA behave chromatographically like malonaldehyde and H_2O, these compounds were only recovered after alkaline treatment and accounted for up to 42% (5.50/13.20, with 0.3 N NaOH, 90 °C, 30 min) of the thymine released by corresponding alkaline treatment. Heating at 65 °C for 30 min at pH 8, which increased the release of thymine and 5'-^{3}H-labeled compounds only slightly, also produced only a small amount of [^{3}H]H_2O from [*methyl*,1',2'-^{3}H]thymine-labeled λDNA.

Correlation between Thymine Release and Strand Breaks. Thymine release, measured by Sephadex G-10 chromatography, and DNA strand breakage, determined by the phosphatase–kinase method and by alkaline sucrose gradient

Table III: Relation between Thymine Release and Strand Breaks

treatment	Sephadex G-10, thymine released/ single-stranded λDNA[a]	phosphatase–kinase expt: pmol of ^{32}P incorpd/ 600 pmol of λDNA[b]		phosphatase–kinase expt: no. of ^{32}P incorpd/ single-stranded λDNA[c]		alkaline sucrose gradient, no. of breaks/ single-stranded λDNA
		−phosphatase	−phosphatase	+phosphatase	−phosphatase	
none	444	1.93	0.76	154	61	
0.1 N NaOH, RT,[d] 10 min	492	6.33	1.20	506	96	
0.3 N NaOH, RT, 10 min	660	7.45	0.96	596	77	
0.3 N NaOH, 37 °C, 30 min	924	11.93	1.05	954	84	1088
65 °C, 30 min	468	6.48[e]	1.47	518	118	

[a] These values were calculated from the percent of thymine release in Table I, on the basis of the fact that the GC content of λDNA is 49%. [b] Kinase experiment was done as described under Materials and Methods. These values were obtained by subtracting the values of the no drug control (0.68 or 0.51 pmol, with or without phosphatase, respectively) from that of the complete incubation. [c] These values were calculated from (*b*) using 4.8×10^4 nucleotides/single-stranded λDNA. [d] RT, room temperature. [e] Without pretreatment, the phosphatase reaction was carried out at 65 °C for 30 min.

centrifugation, were compared (Table III). With the phosphatase–kinase procedure the total number of 5′ termini generated in the DNA by NCS can be determined. Since alkaline phosphatase released phosphate from internal sites of double-stranded DNA at 37 °C only poorly (Weiss et al., 1968), the ^{32}P incorporation found in the sample without treatment must be mainly at the 5′ ends of λDNA fragments produced by double-strand breaks. On the other hand, alkaline phosphatase at 65 °C releases the internal 5′-phosphates at sites of single-strand breaks without prior denaturation of the DNA. As thymine release is not significantly increased by treatment at 65 °C for 30 min, the amount of ^{32}P incorporated under this condition can be used as a measure of the number of 5′ termini generated by NCS without subsequent treatment. This condition was used to avoid the possiblity that mild alkaline denaturation of the DNA (0.1 M NaOH, room temperature, 10 min) might cause an increase in 5′ termini by releasing sugar fragments from this site. That this was not the case is seen by the fact that both types of conditions gave essentially identical results. These results are in agreement with our earlier findings that denaturation of the DNA by treatment with formamide or dimethyl sulfoxide was as effective as mild alkali denaturation in the release of inorganic phosphate by alkaline phosphatase from the ends of the gaps of NCS-treated DNA (Kappen & Goldberg, 1978a).

Enhancement of ^{32}P incorporation into NCS-treated DNA by alkaline phosphatase treatment was observed, as reported before (Poon et al., 1977). ^{32}P incorporation without alkaline phosphatase treatment, which is not increased by alkaline treatment, shows the production of free 5′-hydroxyl ends by NCS, accounting for 10–20% of the total 5′ ends produced by alkaline phosphatase treatment. The total amounts of ^{32}P incorporated into DNA after various alkaline treatments were comparable to that of the thymine released from λDNA by the corresponding treatments.

When the number of single-strand breaks is also analyzed by the alkaline sucrose gradient method, only very low doses of NCS can be used. To calculate the single-strand breaks at 200 μg/mL concentration of NCS, we compared the NCS dependency of thymine release, malonaldehyde-like substance production, ^{32}P incorporation into DNA by the phosphatase–kinase method, and single-strand breaks by alkaline sucrose gradient analysis. As shown in Figure 10A–C, the amount of product generated at a concentration of 200 μg/mL of NCS was 70–75% of that determined by a simple extrapolation of the line at the low concentrations of NCS. On the basis of a normalization value of 75%, calculation revealed that 1088 breaks per strand were produced by 200 μg/mL NCS (as analyzed on alkaline sucrose gradients, Figure 10D) which is close to the thymine release (924 per single strand, Table III) of the sample treated with 0.3 N NaOH at 37 °C for 30 min and is in good agreement with the number of radioactive phosphates (954, Table III) incorporated per single strand of DNA.

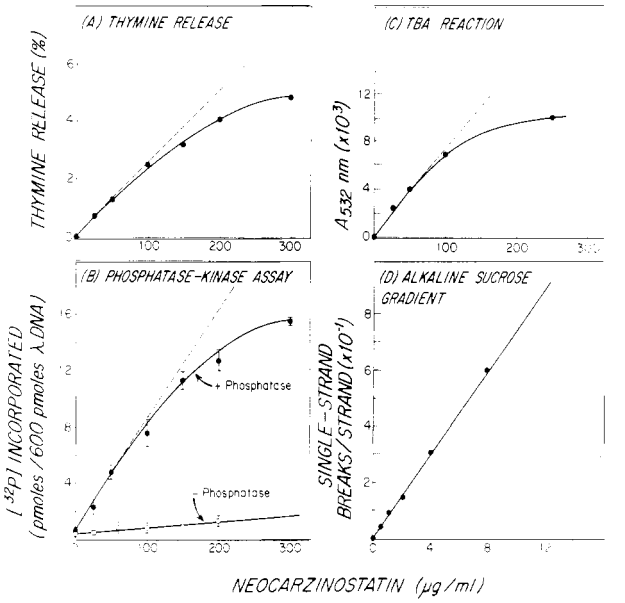

FIGURE 10: NCS-dependency of thymine release, ^{32}P incorporation into DNA by kinase, production of malonaldehyde-like substance, and single-strand breaks of λDNA. (A) Thymine release was measured as indicated in Figure 4A by using various concentrations of NCS and 1-h incubation. (B) The NCS reaction with various concentrations of drug was treated with 0.3 N NaOH at 37 °C for 30 min, and then ^{32}P incorporation into DNA by kinase was measured as described under Materials and Methods. (C) NCS reaction (0.3 mL) with calf thymus DNA (10 μg/mL), cysteine (10 mM), and various concentrations of NCS was incubated at 37 °C for 20 min and then subjected to the TBA reaction as described under Materials and Methods. The A_{532} was obtained by subtracting the absorbance of the reaction without cysteine from that of the complete reaction. (D) The number of single-strand breaks was obtained by alkaline sucrose gradient analysis of the standard NCS reaction with [*methyl*-^{3}H]thymidine-labeled λDNA and various concentrations of NCS incubated at 37 °C for 1 h, as described under Materials and Methods. The broken line represents the extrapolation of the values at lower concentrations of NCS.

Discussion

Earlier work from this laboratory has partially characterized

the breaks in DNA caused by NCS in vitro. We have shown that NCS causes single-strand breaks specifically at positions of thymidylic acid and to a lesser extent at deoxyadenylic acid residues with base release (Poon et al., 1977; Hatayama et al., 1978), that the breaks bear 5′- and 3′-phosphate groups at each end with loss of the intervening nucleoside (Kappen & Goldberg, 1978a) and are not repairable by polynucleotide ligase (Poon et al., 1977), and that NCS-treated DNA binds *Escherichia coli* DNA polymerase I in an inactive state unless first treated with alkaline phosphatase or exonuclease III to remove the 3′-phosphate from the DNA fragment (Kappen & Goldberg, 1977). While NCS can cause true strand breaks, alkali-labile breaks are also observed (Kappen & Goldberg, 1978a; Goldberg et al., 1980). These several results indicate that the strand breaks caused by NCS are not the result of simple splitting of phosphodiester bonds. Instead, they are compatible with two other possible mechanisms for strand breakage: (1) pyridimine–purine base release followed by β-elimination reactions to release the deoxyribose moiety or its degradation products; (2) direct attack on the sugar with ring opening and subsequent release of base and sugar fragments.

Although the release of thymine from NCS-treated DNA was observed in the absence of any additional treatments, as reported before (Ishida & Takahashi, 1976; Poon et al., 1977), we also find that there is about an equal amount of alkali-labile thymine release (Figure 1, Tables I and III). While values obtained by the phosphatase–kinase method with undenatured DNA do not represent the true number of free 5′ ends of the DNA, as discussed earlier, the number of thymines released by the various treatments is in excellent agreement with the number of free 5′ gap ends determined by this method. Furthermore, after alkaline treatment these values are in good agreement with the number of breaks measured on alkaline sucrose gradients. These data indicate that alkaline treatment is required to completely remove the thymine and sugar fragment so as to uncover the 5′-phosphate end of the DNA strand at the point of the break.

The NCS-induced release of formic acid (plus H_2O) from the 5′ carbon of the deoxyribose of thymidylic acid in DNA and the good correlation of the sum with thymine release (and strand breaks), especially after alkaline treatment, presumably result from direct sugar damage. Similarly, the generation of a malonaldehyde-like product, as judged by the TBA reaction, and the release of labeled products with chromatographic properties similar to malonaldehyde and H_2O after alkaline treatment are compatible with primary sugar damage. That the sugar damage is not secondary to the simple generation of an aldehyde apyrimidinic–apurinic site and attendant alkali-catalyzed β-elimination reactions is also indicated by the finding that $NaBH_4$ does not block the formation of NCS-induced single-strand breaks on alkaline gradients (Goldberg et al., 1980) under conditions where those of depurinated DNA are prevented (Kirtikar et al., 1975). However, if base release is associated with oxidation of the 1′ carbon to form deoxyribonic acid, such as occurs with H_2O_2 or NH_2OH treatment (Rhaese & Freese, 1968), an effect of $NaBH_4$ would be absent. Also, these breaks cannot be directly attributed to the mercaptan, since we found that incubation of depurinated DNA with mercaptan does not result in strand breaks (Goldberg et al., 1980). Further, no evidence could be found that NCS modifies the DNA bases in a manner similar to alkylating agents so as to produce alkali-labile breaks after splitting of the *N*-glycosidic bond by heating. Thus, in contrast to methyl methanesulfonate treated DNA, heating (54 °C, 30 min) of NCS-treated DNA produced no additional breaks on alkaline sucrose gradients (L. S. Kappen and I. H. Goldberg, unpublished data). These results agree with our present finding that no modified forms of thymine could be detected.

TBA-reactive material is produced from DNA by X-ray irradiation (Krushinskaya & Shal'nov, 1967; Kapp & Smith, 1970) or by bleomycin treatment (Kuo & Haidle, 1973; Sausville et al., 1978), and in the latter case the product has been shown to be malonaldehyde, presumably originating from the 1′,2′,3′ carbons of deoxyribose (Takeshita et al., 1978; Takeshita & Grollman, 1979). In the mechanism of cleavage of DNA by bleomycin, primary cleavage of the 3′–4′-carbon bond of deoxyribose, followed by release of base and of malonaldehyde leaving a 5′-phosphate at the end of the DNA, has been proposed (Takeshita & Grollman, 1979). In addition to different base (and sequence) specificities, the DNA fragments produced by bleomycin behave differently on polyacrylamide gel electrophoresis from those produced by NCS, indicating that a sugar fragment remains attached to the 3′ end of the break in the former case (Takeshita et al., 1978; D'Andrea & Hazeltine, 1978). The release of free malonaldehyde by bleomycin apparently does not require alkaline treatment of the DNA (Takeshita et al., 1978). As reported for X irradiation of DNA (Ullrich & Hagen, 1971; Payes, 1974), the malonaldehyde-like product from a NCS reaction is recovered in the trichloroacetic acid insoluble fraction, and there is no obvious reaction with 2-methylindole. The TBA-reactive compound is released only after alkaline treatment, cochromatographs with authentic malonaldehyde, and possesses an identical absorption spectrum after reaction with TBA. $[^3H]H_2O$ is also released with the free [^{3}H]malonaldehyde-like material by using [*methyl*,1′,2′-^{3}H]thymdine-labeled λDNA. The production of $[^3H]H_2O$ is explained by an alkali-catalyzed exchange of ^3H at the 2′ position of the generated malonaldehyde (derived from the 1′,2′,3′ carbons of deoxyribose) with H_2O. The production of TBA-reactive material is dependent on the presence of mercaptans and oxygen, is stimulated by 2-propanol, which facilitates release of the chromophore from the apoprotein for interaction with the DNA (Kappen et al., 1980), and is inhibited by α-tocopherol as previously reported for NCS-induced acid solubility of DNA (Kappen & Goldberg, 1978b, 1979). Also, this compound is produced concomitantly with strand breaks and thymine release (Figure 4).

In spite of extensive efforts (including thin-layer, paper, and high-pressure liquid chromatography), recovery of the sugar products released from NCS-treated [*methyl*,1′,2′-^{3}H]thymidine-labeled λDNA was poor (~40%) compared to that from [5′-^{3}H]thymidine-labeled λDNA (up to 80% after alkaline treatment). Similarly, only 30–40% of the theoretical amount of malonaldehyde could be recovered in a reaction between bleomycin and DNA (Takeshita et al., 1978). Likewise, Ward & Kuo (1976) reported that the correlation between strand-break production and release of low molecular weight substances by X-ray was not simple, making it difficult to determine the mechanisms by which ionizing radiation causes strand breaks in DNA. We did not observe any release of free malonaldehyde-like product from NCS-treated DNA without alkaline treatment (Figure 5). Yet, from the phosphatase–kinase experiments, the 5′ ends of the gaps appear to be free of sugar fragments (Table III). It is possible that the malonaldehyde-like substance released during the NCS reaction reacts instantaneously with DNA (Brooks & Klamerth, 1968) or protein (NCS) (Crawford et al., 1967), so

as to remain in a bound form, and that after alkaline treatment additional malonaldehyde-like substance is released and detected as the free form, corresponding to the alkali-induced increase in thymine release and in 5′-phosphate end group formation. Evidence suggesting that protein binding of released malonaldehyde-like material occurs was obtained from experiments in which, after drug treatment, the reaction was treated exhaustively with DNAse I and phosphodiesterase, and acid-precipitable radioactivity (derived from [*methyl*,1′,2′-^{3}H]thymidine-labeled λDNA) was determined. At least 1.5% of the total radioactivity in the deoxyribose of the DNA remained acid insoluble, whether or not the reaction had been treated with alkali before enzymatic digestion of the DNA.

While the radioactive fragments released from the 1′,2′ positions in the deoxyribose have been referred to as malonaldehyde-like, this is based mainly on their chromatographic properties. Malonaldehyde, as determined by the TBA reaction, however, accounted for only ~30% of the thymine release without alkaline treatment and still less after alkaline treatment since the TBA-reactive material was not increased by alkali. These results suggest that true malonaldehyde may not be a major product of the DNA scission reaction. Further, the generation of formic acid from the 5′ carbon raises the possibility that the other sugar fragment contains four rather than three carbons. It is of interest in this regard that in the presence of oxygen another DNA single-strand breaking agent, γ irradiation, produces formic acid and a four-carbon dialdehyde, 2-deoxytetrodialdose, due to splitting between carbons 4′ and 5′ of the sugar moiety (Dizdaroglu et al., 1975). On the basis of the available fragmentation data, however, it is not possible to propose a mechanism for the NCS-induced sugar damage at this time. Further, it must also be considered that the mercaptan or other components in the DNA scission reaction react secondarily with the region of sugar damage to generate some of the observed products.

References

Abelson, J., & Thomas, C. A., Jr. (1966) *J. Mol. Biol. 18*, 262–291.

Albers-Schönberg, G., Dewey, R. S., Hensens, O. D., Leisch, J. M., Napier, M. A., & Goldberg, I. H. (1980) *Biochem. Biophys. Res. Commun. 95*, 1351.

Beerman, T. A., & Goldberg, I. H. (1974) *Biochem. Biophys. Res. Commun. 59*, 1254–1261.

Beerman, T. A., Poon, R., & Goldberg, I. H. (1977) *Biochim. Biophys. Acta 475*, 294–306.

Brooks, B. R., & Klamerth, O. L. (1968) *Eur. J. Biochem. 5*, 178–182.

Crawford, D. L., Yu, T. C., & Sinnhuber, R. O. (1967) *J. Food Sci. 32*, 332–335.

D'Andrea, A. D., & Haseltine, W. A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3608–3612.

Dizdaroglu, M., Schulte-Frohlinde, D., & von Sonntag, C. (1975) *Z. Naturforsch., C: Biosci. 30*, 826–828.

Goldberg, I. H., Hatayama, T., Kappen, L. S., & Napier, M. A. (1980) in *Molecular Actions and Targets for Cancer Chemotherapeutic Agents*, Academic Press, New York (in press).

Hatayama, T., Goldberg, I. H., Takeshita, M., & Grollman, A. P. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3603–3607.

Hedgpeth, J., Goodman, H. M., & Boyer, H. W. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3448–3452.

Ishida, R., & Takahashi, T. (1976) *Biochem. Biophys. Res. Commun. 68*, 256–261.

Kapp, D. S., & Smith, K. C. (1970) *Radiat. Res. 42*, 34–49.

Kappen, L. S., & Goldberg, I. H. (1977) *Biochemistry 16*, 479–485.

Kappen, L. S., & Goldberg, I. H. (1978a) *Biochemistry 17*, 729–734.

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

Kappen, L. S., & Goldberg, I. H. (1979) *Biochemistry 18*, 5647–5653.

Kappen, L. S., Napier, M. A., & Goldberg, I. H. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1970–1974.

Kirtikar, D. M., Slaughter, J., & Goldthwait, D. A. (1975) *Biochemistry 14*, 1235–1244.

Krushinskaya, N. P., & Shal'nov, M. I. (1967) *Radiobiol. (Engl. Transl.) 7*, 36–39.

Kuo, M. T., & Haidle, C. W. (1973) *Biochim. Biophys. Acta 335*, 109–114.

Kwon, T. W., & Olcott, H. S. (1966) *Nature (London) 210*, 214–215.

Meienhofer, J., Maeda, H., Glaser, C. B., Czombos, J., & Kuromizu, K. (1972) *Science 178*, 875–876.

Napier, M. A., Holmquist, B., Strydom, D. J., & Goldberg, I. H. (1979) *Biochem. Biophys. Res. Commun. 89*, 635–642.

Payes, B. (1974) *Biochim. Biophys. Acta 336*, 251–260.

Poon, R., Beerman, T. A., & Goldberg, I. H. (1977) *Biochemisty 16*, 486–493.

Povirk, L. F., & Goldberg, I. H. (1980) *Biochemistry 19*, 4773–4780.

Rhaese, H. J., & Freese, E. (1968) *Biochim. Biophys. Acta 155*, 476–490.

Sausville, E. A., Stein, R. W., Peisach, J., & Horwitz, S. B. (1978) *Biochemistry 17*, 2746–2754.

Scherz, H., Stehlik, G., Bancher, E., & Kaindl, K. (1967) *Mikrochim. Acta 2*, 915–919.

Sim, S.-K., & Lown, J. W. (1978) *Biochem. Biophys. Res. Commun. 81*, 99–105.

Takeshita, M., & Grollman, A. P. (1979) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, 502.

Takeshita, M., Grollman, A. P., Ohtsubo, E., & Ohtsubo, H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5983–5987.

Teole, R., Bonicel, A., Bert, C., Cadet, J., & Polverelli, M. (1974) *Radiat. Res. 57*, 46–58.

Ullrich, M., & Hagen, U. (1971) *Int. J. Radiat. Biol. Relat. Stud. Phys., Chem. Med. 19*, 507–517.

Waravdekar, V. S., & Saslaw, L. D. (1959) *J. Biol. Chem. 234*, 1945–1950.

Ward, J. F., & Kuo, I. (1976) *Radiat. Res. 66*, 485–498.

Weiss, B., Live, T. R., & Richardson, C. C. (1968) *J. Biol. Chem. 243*, 4530–4542.