Articles

Deoxyribonucleic Acid Damage by Neocarzinostatin Chromophore: Strand Breaks Generated by Selective Oxidation of C-5′ of Deoxyribose†

Lizzy S. Kappen and Irving H. Goldberg*

ABSTRACT: Among the lesions induced in DNA by neocarzinostatin chromophore are spontaneous and alkali-dependent base release, sugar damage, and single-strand breaks with phosphate (PO_4) at their 3′ ends and PO_4 or nucleoside 5′-aldehyde at the 5′ ends. By measuring alkali-dependent thymine release and decomposition of the 5′-terminal thymidine 5′-aldehyde in drug-cut DNA, we show that the kinetics are the same for each process and that the nucleoside aldehyde is the source of about 85% of alkali-dependent thymine release. Reduction of the 5′-aldehyde ends to 5′-hydroxyls followed by incorporation of ^{32}P from [γ-^{32}P]ATP by polynucleotide kinase permits their selective quantitation. Nucleoside 5′-aldehyde so measured accounts for over 80% of the drug-generated 5′ ends; the remainder have PO_4 termini. Since these techniques also include the contribution of alkali-labile sites in the measurement of PO_4 ends, DNA sequencing was used to measure the ends directly. Using 3′-^{32}P end-labeled DNA restriction fragments as substrates for the drug, it was found that drug attack at a T results in mainly two bands—the stronger one represents oligonucleotide with 5′-terminal nucleoside 5′-aldehyde and may account for over 90% of a particular break. Its structure was verified by its isolation from the sequencing gel, followed by various chemical and enzymatic treatments. In each case, the mobility of the product on the gel was altered in a predictable manner. In addition to spontaneous breaks, neocarzinostatin also causes alkali-labile breaks preferentially at T residues. These sites are heterogeneous in their sensitivity to alkali and are protected by reduction.

It is now well established that DNA is an important target in the action of the antitumor antibiotic neocarzinostatin (NCS)[1] [reviewed in Goldberg et al. (1981)]. The native drug consists of a nonprotein chromophore noncovalently complexed with an apoprotein (Napier et al., 1979). A partial structure of the nonprotein chromophore has been reported (Napier et al., 1981). The NCS chromophore possesses the full cytotoxic and DNA-damaging properties of the parent drug; the apoprotein stabilizes the highly labile chromophore and serves as its carrier (Kappen et al., 1980; Kappen & Goldberg, 1980; Povirk & Goldberg, 1980; Koide et al., 1982). Earlier work showed that the NCS chromophore binds to DNA by intercalation (Povirk & Goldberg, 1980; Povirk et al., 1981), forms novel covalent DNA adducts (Povirk & Goldberg, 1982a,b), and in a mercaptan- and oxygen-dependent reaction (Goldberg et al., 1981) produces single-strand breaks almost exclusively at thymidylic and deoxyadenylic acid residues (Hatayama et al., 1978; D'Andrea & Haseltine, 1978; Takeshita et al., 1981) with the release of the corresponding bases (Ishida & Takahashi, 1976; Poon et al., 1977). The breaks result in gaps bounded by 3′- and 5′-phosphoryl termini (Kappen & Goldberg, 1978a) and in damaged deoxyribose attached to the 5′-phosphoryl ends (Hatayama & Goldberg, 1980; Takeshita et al., 1981). We have recently identified the latter lesion as a nucleoside 5′-aldehyde, resulting from selective oxidation of C-5′ of deoxyribose (Kappen et al., 1982). While the pattern of base release is similar to that of the base specificity of strand breaks, spontaneous base release represents only a small fraction of the DNA damage produced as judged by the ability of alkali to markedly increase base release in drug-damaged DNA (Hatayama & Goldberg, 1980). Since nucleoside 5′-aldehyde at the 5′ end of the DNA strand break would be expected to release its base (and sugar) via β-elimination reactions upon alkaline treatment, it seems reasonable to expect that this represents the main lesion of the strand-breaking reaction. In this paper, we have characterized and quantitated the products of drug-treated DNA by use of enzymatic and DNA sequencing techniques.

Materials and Methods

The enzymes used in these studies and their suppliers are as follows: restriction enzymes and DNA polymerase I, New England Biolabs; nuclease-free bacterial alkaline phosphatase, Enzobiochem; deoxyribonuclease II, Sigma; Deoxyribonuclease I, micrococcal nuclease, and snake venom phosphodiesterase, Worthington; endonuclease S_1, Miles and Calbiochem. Endonuclease IV (*Escherichia coli*) was a generous gift from Dr. T. Lindahl. The radioactive compounds were purchased from New England Nuclear. NCS (clinical form) was kindly provided by Dr. W. T. Bradner of Bristol Laboratories.

DNA Substrates. [*methyl*-^{3}H]Thymidine-labeled λ DNA [(1.5–1.9) × 10^4 cpm/μg] was prepared as described (Kappen & Goldberg, 1977). Plasmid pBR322 DNA was isolated by gentle lysis of *E. coli* (HB101) with lysozyme and detergent followed by centrifugation to remove chromosomal DNA (Clewell & Helinski, 1969). Further treatment included banding in a CsCl gradient containing ethidium bromide; the ethidium bromide was subsequently removed with Dowex-50 resin. After dialysis against 10 mM Tris-HCl, pH 8, and 0.05 mM EDTA, the DNA was concentrated by ethanol precipitation or lyophilization. Calf thymus DNA (Sigma) was sonicated and was purified by phenol extraction followed by

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[1] Abbreviations: NCS, neocarzinostatin; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography; AP, apurinic/apyrimidinic; PO_4, phosphate.

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dialysis against distilled H_2O.

3′-^{32}P end-labeled restriction fragments were prepared from pBR322 DNA. The plasmid was digested with *Bam*HI and then phosphorylated at the 3′ ends by filling in with [α-^{32}P]dGTP and the Klenow fragment of DNA polymerase I by using procedures similar to those reported (Challberg & Englund, 1980). The ^{32}P-labeled DNA was further cut with *Sal*I or *Eco*RI to obtain singly end-labeled 275 and 375 base pair double-stranded DNA fragments, respectively, which were isolated by electrophoresis on preparative low melting agarose gels (1%). The conditions for enzyme digestions were as specified by the suppliers.

Drug Treatment. Preparation of the chromophore from NCS has been described (Kappen et al., 1980). All incubations were with the isolated chromophore as the drug. A standard reaction of the DNA with the drug was performed at 0 °C for 20 min in the dark in 20–50 mM Tris-HCl, pH 8.0, and 1 mM EDTA in the presence of 10 mM 2-mercaptoethanol or other activating agents. The DNA–drug levels varied depending upon the subsequent analysis. In the incubation of ^{32}P-labeled restriction fragment with the drug, the DNA level was at 5 μg/mL as measured by the amount of carrier calf thymus DNA added. The amount of ^{32}P radioactivity per assay varied from 5×10^4 to 50×10^4 cpm. The chromophore was the last component added in the reaction. The concentration of the chromophore is expressed as equivalents of the native holoantibiotic.

Reduction. The reduction of the drug-treated DNA was done in 0.4 M potassium phosphate, pH 6.5, with 0.2 M $NaBH_4$ or in 0.35 M Tris-HCl, pH 7.0, with 0.28 M $NaBH_4$ at room temperature for 90 min. The $NaBH_4$ (1 M) was added in three equal portions at 30-min intervals to the final levels indicated. Excess $NaBH_4$ was then destroyed by the addition of glacial acetic acid (pH to ~5). The reduction in phosphate buffer was followed by exhaustive dialysis of the DNA against 10 mM Tris-HCl, pH 8, and 0.05 mM EDTA containing 0.4, 0.2, and finally 0.01 M NaCl. The unreduced samples to which the same amount of phosphate and inactivated $NaBH_4$ (by acetic acid) had been added were also dialyzed under the same conditions as for the reduced samples. In experiments with the ^{32}P-labeled DNA restriction fragment, reduction was always done in Tris buffer (pH 7), following which the DNA was precipitated with 3 volumes of ethanol after the addition of carrier tRNA (10 μg) and 0.2 M sodium acetate. Ethanol precipitation was repeated twice by redissolving the pellet in 0.3 M sodium acetate and adding 3 volumes of 95% ethanol. The final DNA pellet was freed of salt by rinsing with 95% ethanol.

Oxidation. 5′-Nucleoside aldehyde on the DNA was oxidized with sodium hypoiodite as previously described (Kappen et al., 1982) by the method of Pfitzner & Moffatt (1965).

Enzyme Assays. 5′-Phosphoryl and 5′-hydroxyl termini on drug-treated λ DNA were determined by the incorporation of ^{32}P from [γ-^{32}P]ATP using polynucleotide kinase with or without prior treatment of the DNA with bacterial alkaline phosphatase under conditions similar to those described (Weiss et al., 1968). The drug-cut DNA (reduced or unreduced), after various alkaline treatments, was neutralized by the addition of 0.2 M Tris–1 N HCl. In order to ensure that equal amounts of DNA are present in the reactions to be compared, recovery of the DNA after various stages of handling was checked by determining the ^{3}H radioactivity of aliquots. In the first stage of the assay, DNA (0.1–0.4 μg) was incubated with saturating levels of alkaline phosphatase (generally in 40 μL) at 37 °C for 30 min. This was followed by the addition (in 20 μL) of the rest of the components (containing 2–4 Ci/mmol of [γ-^{32}P]ATP and 1–2 units of polynucleotide kinase) for the kinase reaction. After 45 min at 37 °C, the reaction was terminated and was processed for determination of ^{32}P incorporated into DNA as described (Weiss et al., 1968).

In order to quantitate the 5′-terminal nucleoside aldehyde, drug-treated [^{3}H]thymidine-labeled λ DNA was first digested (2 h, 37 °C) with nuclease S_1 (300 μL containing 0.1 M sodium acetate, pH 4.5, 0.0014 M $ZnCl_2$, 0.133 M NaCl, and 1–2 units of enzyme). Digestion by a combination of deoxyribonuclease I and snake venom phosphodiesterase (Kappen et al., 1982), or deoxyribonuclease II and nuclease S_1 (Povirk & Goldberg, 1982a), gave identical results. Thymidine 5′-aldehyde (or thymidine) in the enzyme digest was quantitated by HPLC on a μBondapak C_{18} column as described (Kappen et al., 1982).

Digestion of DNA with micrococcal nuclease was as described (Richardson & Kornberg, 1964). Depurination of the DNA and digestion with endonuclease IV were by the method of Ljungquist (1977).

DNA Sequencing. The nucleotide sequence of the DNA fragments was determined by the method of Maxam & Gilbert (1977). Unless otherwise stated, the DNA from the drug reactions was precipitated with 3 volumes of 95% ethanol after the addition of carrier tRNA and 0.3 M sodium acetate. The DNA pellet, collected by centrifugation, was rinsed free of salt with 95% ethanol and dried. The DNA pellets from drug treatment and chemical reactions were dissolved in 80% deionized formamide containing 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue. The samples were heated at 90 °C for 30 s and were chilled before being layered onto 8.3 M urea-containing polyacrylamide gels (0.8 mm). The gels were exposed to X-ray film (Kodak XAR5) at −20 or −70 °C when juxtaposed with an intensifying screen. The autoradiographs were scanned in a Schoeffel model spectrophotometer equipped with a density computer and linear recorder. Band intensities were compared in relation to the areas under peaks.

In experiments where specific DNA bands from the sequencing gel were to be isolated for further treatment, the bands were cut out and incubated at 37 °C with 1.5 mL of distilled H_2O containing 25 μg of tRNA for 2 h. After removal of the supernatant, a second incubation was done by resuspending the gel pellet in 1.5 mL of a solution containing 20 mM Tris-HCl, pH 7.5, 10 μg/mL tRNA, and 0.1 mM EDTA. The combined eluates were concentrated by lyophilization, and the DNA was recovered by ethanol precipitation.

Results

Thymidine 5′-aldehyde at the 5′ ends of NCS-induced DNA strand breaks decomposes in alkali, releasing thymine and 2-furaldehyde, but becomes alkali resistant after its reduction to thymidine (Kappen et al., 1982). Thymine is spontaneously released upon drug treatment of DNA (Ishida & Takahashi, 1976; Poon et al., 1977), and if the drug-treated DNA is heated in alkali, thymine release is enhanced 2–3-fold (Hatayama & Goldberg, 1980). In order to determine if the alkali-dependent thymine comes from the decomposition of the 5′-terminal nucleoside aldehyde, we measured thymine and thymidine 5′-aldehyde before and after alkali treatment of [*methyl*-^{3}H]thymidine-labeled λ DNA cut with NCS chromophore. Hot alkali enhanced the thymine release 2.5-fold (Table I). The kinetics of the release of thymine and the disappearance of thymidine 5′-aldehyde are the same (Figure 1). The amount of thymine released in hot alkali at the completion of the reaction (30 min) is slightly more (about

Table I: Quantitation of Thymine Release and 5′-Terminal Thymidine 5′-Aldehyde in DNA Treated with NCS Chromophore[a]

expt	thymine released		thymidine 5′-aldehyde
	spontaneous	alkali-dependent	
1	100	227	193
2	100	274	227
3	100	250	200
3a	100	263	226

[a] [*methyl*-^{3}H]Thymidine-labeled λ DNA (15 μg/mL in experiments 1 and 2 and 10 μg/mL in experiment 3) was treated with the drug (200 μg/mL) under standard conditions. Alkali treatment was in 0.2 M NaOH containing 1 mM EDTA at 90 °C for 30 min in experiment 1 and for 60 min in experiments 2 and 3. In the estimation of spontaneous thymine and thymidine 5′-aldehyde, DNA was not exposed to alkali; the latter was estimated after digestion of the DNA with nuclease S_1 as described under Materials and Methods. Each assay contained 1–3 μg of DNA. Background radioactivity (24–163 cpm) obtained with control DNA not treated with the drug has been subtracted. Experiment 3a is identical with experiment 3 except that the DNA was precipitated with ethanol, prior to treatment with alkali or nuclease S_1. Spontaneous thymine in experiment 3a is based on the unprecipitated sample. The values are normalized to thymine equivalent to a relative value of 100. Spontaneously released thymine in experiments 1, 2, and 3 was 1572, 1788, and 1178 cpm, respectively.

Table II: Determination of 5′-Terminal Thymine-Containing Nucleosides and ^{32}P Incorporation into NCS Chromophore Treated DNA before and after Reduction[a]

	^{32}P incorporated (pmol)		product at 5′ terminus (pmol)	
	−phosphatase	+phosphatase	thymidine 5′-aldehyde	thymidine
−$NaBH_4$	0.6	93.1	66.7	
+$NaBH_4$	81	93.5		56.8 (77.8)

[a] [*methyl*-^{3}H]Thymidine-labeled λ DNA (82.6 μg/mL) was treated with 100 μg/mL NCS chromophore under standard conditions. Half of the DNA was then reduced with $NaBH_4$; 5.77 μg of DNA (reduced and unreduced) was digested with nuclease S_1 to release the 5′-terminal product which was quantitated by HPLC. Prior to the determination of ^{32}P incorporated into the 5′ termini of the breaks by the phosphatase–kinase assay, DNA was heated in 0.1 M NaOH at 90 °C for 1 h; 0.4 μg of DNA was used per assay. The ^{32}P values have been normalized for 5.77 μg of DNA; a background radioactivity (9 pmol) obtained with the control DNA, not treated with the drug, but subjected to the same reactions, has been subtracted in all cases.

15%) than the 5′-terminal nucleoside aldehyde originally present. These data suggest that the source of most of the alkali-dependent thymine is the 5′-terminal nucleoside aldehyde.

In order to further quantitate the 5′-terminal aldehyde, a strategy was devised in which the drug-treated DNA was first reduced by $NaBH_4$ to convert thymidine 5′-aldehyde to thymidine (Table II), and then the incorporation of ^{32}P from [γ-^{32}P]ATP by polynucleotide kinase was followed. As shown in Table II, the drug-dependent thymidine 5′-aldehyde is almost quantitatively recovered as thymidine after reduction. The 5′-hydroxyl groups thus generated incorporate ^{32}P from [γ-^{32}P]ATP linearly with increasing levels of drug (data not shown).

By the combined use of alkaline phosphatase and polynucleotide kinase, one can quantitate both the 5′-hydroxyl and 5′-phosphoryl termini after drug treatment (Poon et al., 1977). Alkaline phosphatase dephosphorylates the phosphoryl ends; the 5′-hydroxyls thus generated are reesterified with ^{32}P from

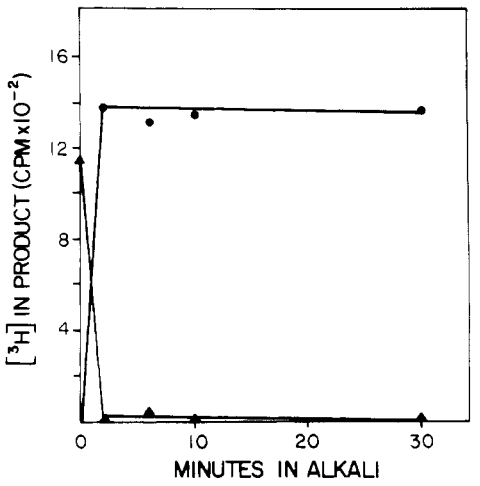

FIGURE 1: Kinetic relationship between content of 5′-terminal thymidine 5′-aldehyde and thymine release in alkali-treated NCS chromophore cut DNA. [*methyl*-^{3}H]Thymidine-labeled λ DNA (10 μg/mL) was treated with 200 μg/mL NCS chromophore. DNA was then precipitated with ethanol and was redissolved in distilled H_2O. Duplicate samples of the control and the drug-treated DNA (0.6 μg) were then heated in 0.2 M NaOH containing 1 mM EDTA (200 μL) at 90 °C for the times indicated. After neutralization with HCl, one set of samples was digested with nuclease S_1 to release the 5′-terminal nucleoside aldehyde. Thymine and thymidine 5′-aldehyde in samples treated and not treated with NaOH were determined by HPLC on a μBondapak C_{18} column. Since spontaneously released thymine had been removed during precipitation, there was only negligible (8 cpm) thymine in the absence of alkali. Background radioactivity (24–130 cpm) with control DNA not treated with the drug has been subtracted. (●) Thymine; (▲) thymidine 5′-aldehyde.

[γ-^{32}P]ATP in the presence of polynucleotide kinase. Hence, the ^{32}P incorporation dependent on both enzymes measures the 5′-phosphoryl termini, while that incorporated in the presence of only polynucleotide kinase estimates free 5′-hydroxyls. In previous measurements of 5′-phosphoryl (and 5′-hydroxyl) termini in NCS-cut DNA by the phosphatase–kinase assay, it was found that prior treatment of the DNA with hot alkali was required to obtain maximal ^{32}P incorporation (Hatayama & Goldberg, 1980). We, therefore, compared ^{32}P incorporation into the reduced and unreduced DNA after treatment with alkali, followed by alkaline phosphatase. The results are summarized in Table II. In agreement with earlier reports with unreduced DNA, maximal ^{32}P incorporation required pretreatment of the DNA in hot alkali followed by alkaline phosphatase. Since decomposition of the 5′-terminal nucleoside aldehyde by alkali generates a 5′-phosphoryl terminus, ^{32}P incorporation under these conditions measures 5′ ends so generated in addition to spontaneously produced 5′-PO_4 ends and those from alkali-labile breaks. In contrast, with the $NaBH_4$-reduced DNA, ^{32}P incorporation is over 80% of the maximum without prior exposure of the DNA to phosphatase, and this selectively represents the 5′-hydroxyl groups resulting from reduction of 5′-aldehyde groups. Unlike the situation with unreduced DNA, the incorporation of ^{32}P into reduced DNA was the same with mild alkaline treatment (10 min at room temperature).

In order to compare the value for ^{32}P incorporation (81 pmol) into the 5′ terminus at the break with the amount of 5′-ended reduced nucleoside 5′-aldehyde (reduction being 85% complete), the value for thymidine (56.8 pmol) in Table II must be corrected for the presence of other nucleosides at the 5′ end. This requires the determination of the distribution among the four nucleotides of the ^{32}P incorporated into the reduced DNA. The reduced DNA into which ^{32}P had been incorporated was digested to the level of mononucleotides with

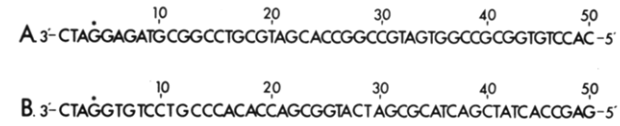

FIGURE 2: Primary sequence of restriction fragments. Double-stranded fragments consisting of (A) 275 and (B) 375 base pairs of pBR322 DNA were prepared as described under Materials and Methods. Only 50 bases of the strand labeled with ^{32}P are shown. The asterisks indicate the position of the ^{32}P label and correspond to positions 379 (A) and 376 (B) from the *Eco*RI site (Sutcliffe, 1978).

nuclease S_1, and the products were analyzed by thin-layer chromatography as described (Poon et al., 1977). TMP accounted for 73%, dAMP for 19%, dCMP for 4.7%, and dGMP for 3.3% of the radioactivity. On the basis of these results, the thymidine value (56.8 pmol) becomes 77.8 pmol for the total nucleoside at the 5′ end, in excellent agreement with the total ^{32}P incorporation of 81 pmol.

Analysis on DNA Sequencing Gels. The data from the enzymatic assays show that the 5′-nucleoside aldehyde constitutes at least 80% of the total 5′ ends in NCS-cut DNA. We sought to confirm and extend these results by analyzing the products of NCS reaction in high-resolution polyacrylamide gels, since preliminary experiments (Takeshita et al., 1981) using a 3′-^{32}P end-labeled restriction fragment indicated that the oligonucleotides produced by NCS have mobilities different from those of the chemically produced markers in the Maxam–Gilbert sequencing analysis. A 3′-^{32}P end-labeled restriction fragment (A) of defined sequence (Figure 2) was incubated with the drug under standard conditions (Figure 3) in the presence of 10 mM 2-mercaptoethanol and 1 mM EDTA (lanes 4 and 5). Previous sequencing studies using 5′-^{32}P end-labeled DNA restriction fragments show that the NCS chromophore breaks DNA at positions of T > A ≫ C > G, with exact correspondence in mobility on the gel between the drug-induced break and the chemically produced marker, indicating the presence of a 3′-PO_4 at the break (Takeshita et al., 1981). By contrast, when a 3′-^{32}P end-labeled DNA restriction fragment was used (Figure 3), the major product of the drug reaction at each T (e.g., T_{20}, T_{32}) does not have the same mobility as that of the marker fragment of comparable length produced by the hydrazine reaction. For example, attack at T_{20} generates two bands—a weak one coincident with the T_{20} standard (bearing a 5′-PO_4 terminus) and a strong band (at least 3 times more intense) migrating slower than the marker and in a position corresponding to a DNA fragment about two nucleotides longer (e.g., G_{22}). Since we already had evidence from other assays for the presence of nucleoside aldehyde at the 5′ ends, the drug-treated DNA was subjected to various reactions that are known to affect the aldehyde; such treatments should selectively alter the mobility of the oligonucleotides with 5′-terminal nucleoside aldehyde. Thus, NaOH treatment (lane 6) shifts the major band to the standard T_{20} position, and on reduction (lane 7), it migrates slightly ahead of its previous position and coincident with the 5′-hydroxyl-bearing oligonucleotide band produced by micrococcal nuclease digestion (lanes 8 and 9) but slower than the T_{20}-PO_4 band.

When EDTA was omitted from the incubation containing 10 mM 2-mercaptoethanol, an extra band of varying intensity appeared in between the T_{20}-PO_4 and T_{20}-aldehyde bands (lanes 2 and 3). Its behavior on various chemical and enzymatic treatments suggests that the 5′-aldehyde moiety has been oxidized to the 5′-carboxylic acid, probably via a metal-catalyzed reaction. Thus, the mobility of this "middle" band is unaltered by treatment with NaOH, $NaBH_4$, or alkaline

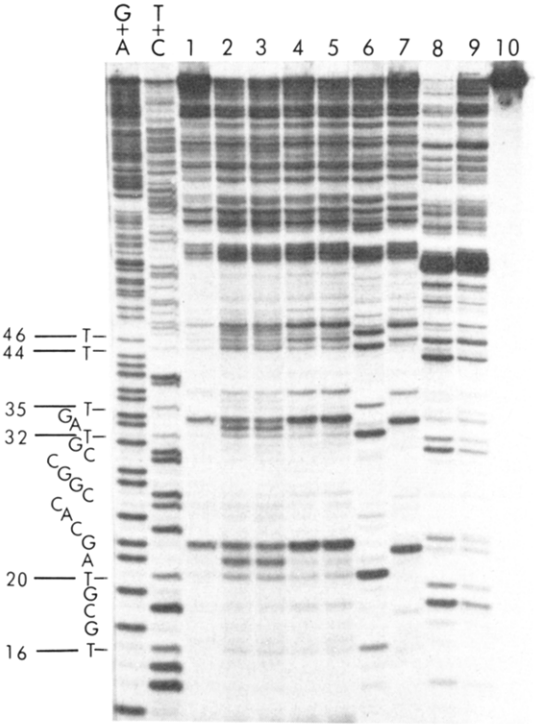

FIGURE 3: Cleavage of the 3′-^{32}P end-labeled restriction fragment A by NCS chromophore. Incubations of the DNA (2.7×10^5 cpm) with the drug (5 μg/mL) under standard conditions contained (lane 1) 150 mM 2-mercaptoethanol, (lanes 2 and 3) 10 mM 2-mercaptoethanol, and (lanes 4 and 5) 10 mM 2-mercaptoethanol + 1 mM EDTA. After the drug treatment, the DNA was precipitated with ethanol (Materials and Methods). (Lane 6) The product from a reaction identical with that from lane 5 was heated in 0.1 M NaOH at 90 °C for 2.5 min. (Lane 7) The products of a reaction identical with that from lane 5 were reduced with $NaBH_4$. The samples of lanes 6 and 7 were reprecipitated to free the DNA of salts. (Lanes 8 and 9) Micrococcal nuclease digested; (lane 10) drug-free control. G + A and T + C represent the pattern obtained after cleavage by chemical methods (Maxam & Gilbert, 1977). These patterns were not altered by reduction. The products were analyzed on a 15% sequencing gel for 8 h at 1000 V. The gel was exposed to the X-ray film for 32 h.

phosphatase but is identical with that of isolated T_{20}-aldehyde (see later) that had been mildly oxidized with sodium hypoiodite, which converts nucleoside 5′-aldehyde to nucleoside 5′-carboxylic acid (Kappen et al., 1982). At high concentrations of mercaptan (lane 1), the middle band was not produced even in the absence of EDTA.

The above results show that the major product of NCS attack at a T residue (or other residue) is an oligonucleotide with a 5′-terminal nucleoside aldehyde and that fragments bearing a 5′-PO_4 are minor products. This is further illustrated in the autoradiographic scan of the T_{20} region in lanes 5 and 6 of Figure 3. The ratio of the intensities of T_{20}-aldehyde and T_{20}-PO_4 in the absence of alkaline treatment is 6.5 (or a maximum of 15% of the breaks have a 5′-PO_4), and the intensity of the PO_4-ended band after the alkali-induced conversion is nearly equal to the sum of the intensities of the T_{20}-PO_4 initially present and the T_{20}-aldehyde. This also shows that there is very little alkali-labile component in the break at the T_{20} position. It should be noted, however, that reduction of the drug-cut DNA is associated with an absence of the 5′-PO_4-ended band (lane 7) at all positions of spontaneous breaks (and a small decrease in the radioactivity appearing in the reduced 5′-OH band compared with the 5′-aldehyde band). This indicates that its precursor has been completely protected from breakdown and suggests that the 5′-PO_4-ended band may not be spontaneously produced in the drug reaction

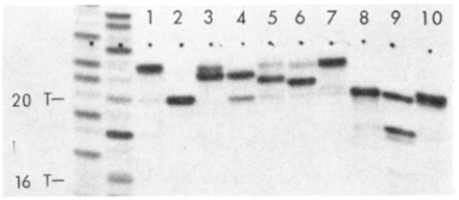

FIGURE 4: Effect of various treatments on isolated T_{20}-aldehyde and T_{20}-PO_4 fragments. T_{20}-Aldehyde and T_{20}-PO_4 oligonucleotides were isolated from a 15% sequencing gel onto which the products of a reaction of 3′-^{32}P end-labeled fragment A ($\sim 4 \times 10^6$ cpm) with 10 μg/mL NCS chromophore had been loaded. The isolated products [(6–9) $\times$ 10^3 cpm] were subjected to various treatments. 5′-End phosphorylation of the reduced aldehyde was done after denaturation in 0.1 M NaOH for 10 min at room temperature using nonradioactive ATP and polynucleotide kinase. Alkaline phosphatase treatment (30 min, 37 °C) was in 50 mM Tris-HCl, pH 8.0. Following all the treatments, the DNA was precipitated before analysis on a 20% sequencing gel (14 h, 1100–1400 V). The gel was exposed to the film for 2 days. Lanes 1–7 represent T_{20}-aldehyde: (1) no treatment; (2) +NaOH; (3) reduced; (4) reduced + NaOH; (5) reduction followed by phosphorylation with ATP and kinase; (6) oxidized; (7) +phosphatase. Lanes 8–10 represent T_{20}-PO_4: (8) no treatment; (9) +NaOH; (10) +phosphatase. The two lanes on the far left are the same as in Figure 3, i.e., G + A and T + C patterns obtained after chemical cleavage.

but results, rather, from breakdown of the 5′-aldehyde or a labile precursor of the break during workup and analysis.

Proof of the structure for the 5′-terminal nucleoside aldehyde termini comes from experiments (Figure 4) in which the bands were isolated from the sequencing gel and were subjected to various chemical and enzymatic treatments. Treatment of isolated T_{20}-aldehyde (lane 1) with alkali (lane 2), $NaBH_4$ (lane 3), and $NaBH_4$ followed by introduction of a PO_4 using polynucleotide kinase (lane 5) and oxidation with hypoiodite (lane 6) all altered the mobility of the isolated band in a predictable manner. While NaOH had no effect on the reduced product (lanes 3 and 4), a small amount of T_{20}-aldehyde remaining from incomplete reduction was converted to T_{20}-PO_4 with alkali. Alkaline phosphatase had no effect on the aldehyde (lane 7). As expected, the migration of the isolated T_{20}-PO_4 (lane 8) was slowed by treatment with alkaline phosphatase (lane 10). Of quantitative importance is the finding that alkali treatment of the T_{20}-PO_4 band results in about half of the radioactivity moving more rapidly on the gel in a position expected for C_{18}-PO_4 (lane 9). This indicates that the so-called T_{20}-PO_4 band contains an equal amount of comigrating C_{18}-aldehyde. This finding requires that the percentage (15%) of the breaks having a 5′-phosphate at the T_{20} break as determined in the scanning experiment be reduced by about half (to 8%). Similar analysis at other sites of cleavage also reveals that 10% or less of the breaks are 5′-PO_4 ended.

When other mercaptans such as dithiothreitol (5 mM) or *S*-(2-aminoethyl)isothiouronium bromide–HBr (0.5 mM) (Kappen & Goldberg, 1978b) were substituted for 2-mercaptoethanol in the reaction of the DNA with the drug, results similar to those shown in Figure 3 were obtained.

Alkali-Labile Sites. Inspection of the position at T_{16} in Figure 3 reveals that both T_{16}-PO_4 and T_{16}-aldehyde bands are weak (lanes 4 and 5). On NaOH treatment (lane 6), the intensity at the T_{16}-PO_4 position is greater than the combined intensity of T_{16}-PO_4 and T_{16}-aldehyde present in the absence of alkali, indicating the presence of alkali-labile breaks at this site. Similar alkali-dependent intensification of bands is seen at T_{44} but not at T_{20}, T_{32}, T_{35}, or T_{46}. We have also examined another 3′-^{32}P end-labeled restriction fragment (fragment B) for potential alkali-labile sites (Figure 5). There is a clear

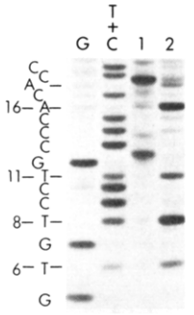

FIGURE 5: Strand scission and expression of alkali-labile breaks in 3′-^{32}P end-labeled fragment B treated with NCS chromophore. After reaction of DNA with drug (5 μg/mL) under standard conditions, aliquots were heated in 0.1 M NaOH at 90 °C for 3 min. Samples were then neutralized and precipitated with ethanol. An equivalent amount of drug-cut (but not exposed to alkali) DNA was also precipitated under the same conditions. Analysis was on a 20% gel (1025 V, 9 h). (Lane 1) Drug treated; (lane 2) drug treated + NaOH. Identical results were obtained when the samples were directly put onto the gel after NaOH treatment without precipitation.

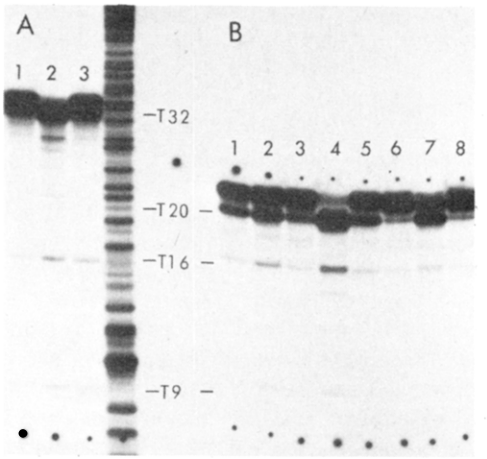

FIGURE 6: Expression of alkali-labile breaks in oligonucleotides isolated from NCS chromophore cut DNA. 3′-^{32}P end-labeled fragment A ($\sim 4 \times 10^6$ cpm) was treated with 20 μg/mL NCS chromophore. The products were analyzed on a 15% sequencing gel. The bands corresponding to T_{20}- and T_{32}-aldehydes were cut out, and the products were isolated as described under Materials and Methods. The isolated fragments, after the following treatments, were rerun on a 20% sequencing gel (8.5 h at 1100 V). In panel A is shown the T_{32} fragment: (lane 1) no treatment; (lane 2) heated in 0.1 M NaOH for 10 min at 90 °C; (lane 3) heated at 90 °C for 10 min at neutral pH. Panel B shows the isolated T_{20} fragment: (lane 3) no treatment; (lane 1) incubated at pH 8.2 for 30 min at 37 °C; (lane 2) heated at 90 °C for 10 min at neutral pH; (lane 4) heated in 0.1 M NaOH at 90 °C for 10 min; (lane 5) same as lane 1. Lanes 6–10 have reduced T_{20}-aldehyde: (lane 6) no treatment; (lane 7) +0.1 M NaOH at 90 °C for 10 min; (lane 8) incubated at pH 8.2 for 30 min at 37 °C. The unmarked lane contains the chemically produced G + A markers.

distinction among T_6, T_8, T_{11}, and A_{16}. Breakage at the T_{11} and A_{16} regions is mostly spontaneous, and the product is mainly 5′-aldehyde which shifts to the 5′-PO_4 position with alkali. Breaks at T_6 and T_8, however, are highly alkali dependent.

In order to visualize the alkali-labile sites without interference by spontaneously produced breaks, we isolated from the sequencing gels the T_{32} and T_{20} regions of drug-treated fragment A, treated them with alkali, and examined the products on sequencing gels (Figure 6). Incubation of isolated T_{20}-aldehyde in 0.1 M NaOH at 90 °C produces breaks at T_{16} and T_9 (Figure 6B, lane 4). In the absence of alkali, heating at 90 °C, but not as 37 °C partially expresses these breaks. With the isolated T_{32} fragment, alkali generates bands at T_{16} and T_9 but not at T_{20} (Figure 6A), showing again that

some but not all thymines are potential alkali-labile sites. Also, some sites, such as T_{44}, are less dependent on alkali for expression of the break than other sites, such as T_{16} or T_9. Furthermore, reduction of the isolated DNA fragments prior to exposure to alkali prevents the expression of the alkali-labile breaks (Figure 6B, lane 7). The same was found for the bands at T_6 and T_8 in fragment B (data not shown).

Discussion

The results presented in this paper are consistent with the 5′-terminal nucleoside aldehyde being the main source of the alkali-dependent thymine release. This conclusion is also consonant with the known alkaline sensitivity of isolated thymidine 5′-aldehyde (Kappen et al., 1982), although a detailed comparison (unpublished experiments) of the susceptibility to alkali of nucleoside 5′-aldehyde in the isolated state with that linked to phosphate in DNA at its 3′-OH position revealed that the latter was more sensitive, in keeping with the fact that phosphate is a better leaving group in the β-elimination reaction than is hydroxyl. The production of thymine by alkali exceeds the amount of thymidine 5′-aldehyde by about 15%. It is possible that some of the excess comes from the decomposition of the alkali-labile DNA–drug covalent adducts characterized earlier (Povirk & Goldberg, 1982a,b). In fact, is has been estimated that there may be as many as one such adduct for every ten strand breaks (Povirk & Goldberg, 1982a). Conversely, stabilization by reduction of the 5′-aldehyde termini permits their selective quantitation by measuring the ^{32}P incorporation dependent only on polynucleotide kinase, and this again accounts for 87% of the total ^{32}P incorporated in the unreduced sample after hot alkali and phosphatase treatment. In the latter case, the ^{32}P incorporated includes spontaneously produced 5′-PO_4-containing ends and those generated from the breakdown of 5′-terminal nucleoside aldehyde or alkali-labile sites.

The data obtained from high-resolution gel electrophoresis of the oligonucleotides produced by the action of the NCS chromophore on a 3′ end-labeled restriction fragment are in close agreement with the results from enzymatic assays. While the nucleotide specificity of drug attack is the same as that obtained with a 5′ end-labeled DNA fragment, the electrophoretic pattern of the 3′ end-labeled oligonucleotides is distinctly different from that of the 5′ end-labeled DNA fragments. In the latter case, the oligonucleotides produced by the drug comigrated with the standard markers of comparable length (Hatayama et al., 1978) since the 3′ ends of the breaks, like those of the standards, bear phosphoryl termini (Kappen & Goldberg, 1978a). On the other hand, oligonucleotides from the 3′ end-labeled DNA, by virtue of their modified 5′ terminus, run slower than the standard markers. The slowing of the mobility of the DNA fragment, as though it were two nucleotides longer, is consistent with its having an extra nucleoside (in 5′-aldehyde form, which in itself results in slowing as compared to the 5′-hydroxyl form) and lacking the charge of a phosphate end. The shift of the "aldehyde band" to the standard position on treatment with alkali is in accord with the generation of a 5′-phosphoryl terminus after the splitting of the terminal nucleoside aldehyde to release the base and 2-furaldehyde (Kappen et al., 1982). From the DNA sequencing experiments, we find that 92% of the breaks have nucleoside 5′-aldehyde at the 5′ end, a value close to that (87%) obtained in the phosphatase–kinase experiments. Since the latter assay requires that the DNA be in a denatured state to measure internal breaks, the alkali used for denaturation also cleaves alkali-labile sites and increases the number of 5′-phosphate ends; denaturation by heating at neutral pH gave similar results (unpublished experiments). Even the 92% value may be an underestimate since reduction after drug treatment appears to eliminate most of the 5′-phosphate ends. Since some formic acid has been found to be released by drug from the 5′-carbon of deoxyribose of thymidylic acid in DNA (Hatayama & Goldberg, 1980), 5′-phosphate end groups might be directly produced by a series of reactions similar to those postulated to take place with X-ray-induced damage of DNA (Von Sonntag & Schulte-Frohlinde, 1978). Thus, selective oxidation of the C-5′ of deoxyribose would be an early step in the production of both the major product (nucleoside 5′-aldehyde) and the minor product (phosphate) at the 5′ end of the strand break. Since covalent adducts between the drug chromophore and DNA have also been found at sites of C-5′ oxidation (Povirk & Goldberg, 1982a,b), a similar mechanism may be involved in the formation of these structures. In all cases, abstraction of a hydrogen from C-5′ by a presumed radical form of the drug would be the first step in DNA damage.

Several DNA-damaging agents produce alkali-labile breaks, and NCS is no exception. Bose et al. (1980) and we (Goldberg et al., 1981) have earlier reported evidence for the presence of alkali-labile sites using different substrates under different experimental conditions. Analysis of the DNA fragments on gels permits the assignment of the alkali-labile breaks to specific sites in the DNA sequence. From these studies, it appears that alkali-labile breaks also preferentially occur at thymines, although there is marked heterogeneity in their dependency on alkaline treatment. At some sites, only direct breaks are found; at others, breaks are found only after alkaline treatment; still others are a mixture of the two. As is also the case in the directly produced strand breaks (Takeshita et al., 1981), no clear-cut sequence specificity for the alkali-labile sites could be found, although the analysis was limited in scope. In both cases, it appears that some higher ordered structure of the DNA may be the determining factor.

It is possible that some of the drug-induced alkali-labile sites are AP sites (e.g., resulting from spontaneous base release) and are substrates for the repair enzymes specific for AP sites. Evidence for the susceptibility of such sites to an AP endonuclease has been reported (Bose et al., 1980). In preliminary experiments, we have found that the alkali-labile sites appear to be partially susceptible to the action of *E. coli* endonuclease IV. These experiments are continuing. At least some of these alkali-labile lesions may be sites of DNA–drug adducts; if so, these adducts are likely to be different from those already characterized (Povirk & Goldberg, 1982a,b), as judged from their chemical properties and their stabilization by reduction. Thus, it is possible that the alkali-labile sites are a mixture of the two types of lesions, only one of which is susceptible to the AP endonuclease.

It is interesting that bleomycin, another antitumor antibiotic that interacts with DNA, cleaves the deoxyribose of DNA between the 3′- and 4′-carbons, resulting in oligonucleotides with a modified 3′ end and a 5′-phosphoryl terminus (Burger et al., 1980; Giloni et al., 1981), a situation just opposite to that with NCS.

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Registry No. Neocarzinostatin chromophore, 81604-85-5; thymidine 5′-aldehyde, 6049-39-4.

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Acridine–Psoralen Amines and Their Interaction with Deoxyribonucleic Acid†

John Bondo Hansen, Torben Koch, Ole Buchardt,* Peter E. Nielsen, Michael Wirth, and Bengt Nordén

ABSTRACT: A series of novel compounds in which a 9-acridinyl nucleus is linked to a psoralen nucleus in the 5- or 8-position via polyamines was prepared and examined. Their reversible binding to DNA and their irreversible binding to DNA and DNA cross-linking upon irradiation with UV-A light were examined. It was found that they were all less efficiently photoreactive than 8-methoxypsoralen (8-MOP), both in cross-linking and photobinding to DNA, whereas the ratio between their photobinding and cross-linking was 40–400 times that of 8-MOP. Compounds in which the linker was attached to the 5-position in psoralen showed smaller cross-linking and photobinding efficiencies and larger ratios between photobinding and cross-linking than those of psoralens attached in the 8-position. This strongly indicates that the 9-substituents of the acridines are oriented toward the minor groove. Flow linear dichroism studies showed that the compounds were DNA intercalating with the acridine moiety, whereas the psoralen moiety in no case was clearly intercalating. This conclusion was further supported by viscometry which also strongly indicated monointercalation.

Psoralen derivatives are used clinically in the PUVA[1] treatments of dermatological diseases, e.g., psoriasis (Anderson & Voorhees, 1980), and as probes for the study of the secondary structure of nucleic acids (Song & Tapley, 1979). It is generally accepted that the photobiological effects of psoralens are due to their photoreactions with nucleic acids, which

† From the Chemical Laboratory II, The H. C. Ørsted Institute, University of Copenhagen, DK-2100 Copenhagen Ø, Denmark (J.B.H., T.K., and O.B.), Biochemical Institute B, The Panum Institute, DK-2200 Copenhagen N, Denmark (P.E.N.), and Department of Physical Chemistry, Chalmers Technical University, S-412 96 Göteborg, Sweden (M.W. and B.N.). *Received April 7, 1983.* This work was supported by the Danish Medical Science Research Council, the Danish Natural Science Research Council, and The Swedish Natural Science Foundation.

[1] Abbreviations: 8-MOP, 8-methoxypsoralen; PUVA, photochemotherapy with UV-A light (UV-A = 320–390 nm); LD, linear dichroism; IR, infrared; NMR, nuclear magnetic resonance; Me_2SO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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