Incorporation of $^{18}$O$_2$ into Thymidine 5'-Aldehyde in Neocarzinostatin Chromophore-damaged DNA*

(Received for publication, April 27, 1984)

Der-Hang Chint$, Steven A. Carr$, and
Irving H. Goldberg‡

From the §Department of Pharmacology, Harvard Medical School and the $Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts 02115

Strand scission of DNA by the chromophore of neocarzinostatin converts the 5'-hydroxyl of deoxyribose to a 5'-aldehyde. The origin of the aldehydic oxygen has now been elucidated by mass spectrometry. DNA-associated thymidine 5'-aldehyde produced by treatment of DNA with neocarzinostatin chromophore in $^{18}$O$_2$ or in $^{18}$O was reduced, liberated by nuclease treatment, permethylated, and analyzed by gas chromatography-mass spectrometry. The data clearly show that molecular oxygen is the only source of the 5'-aldehydic oxygen. The addition of molecular oxygen at C-5', possibly via a reactive form of neocarzinostatin chromophore, must be involved; a carbocation intermediate at C-5' is ruled out.

The nonprotein chromophore of the antitumor antibiotic NCS produces single-stranded scissions in DNA due to the oxidation of the 5'-carboxyl of nucleosides in DNA to the 5'-aldehyde (1, 2). Oxidation at C-5' is also involved in the formation of NCS chromophore-DNA adducts covalently linked to DNA strands (3, 4). The mechanism of this reaction, which requires a reducing agent such as thiol and utilizes 1 mol of O$_2$/mol of chromophore remains obscure (5-7). It is not known whether oxygen is involved in the generation of an active species which attacks DNA or in the fixation of nascent DNA lesions, or both (7). It is possible that a radical form of the drug generated by thiol addition to the chromophore abstracts a hydrogen from the 5'-carboxyl of deoxyribose in DNA to form a carbon-centered radical which undergoes subsequent reactions leading to 5'-aldehyde formation and strand breakage (1, 7). Two possible general mechanisms for 5'-aldehyde formation can be envisaged, one involving electron removal from the 5'-carbon to form a carbocation ion which after hydration forms the aldehyde and a strand break with a phosphate at the 3'-end (8), the other due to the addition of O$_2$ to the carbon-centered radical to form a peroxy radical which subsequently degrades to the aldehyde (7). In the former mechanism the carbonyl oxygen comes from H$_2$O, while in the latter it comes from molecular O$_2$. These mechanisms have now been distinguished by mass spectrometric analysis of the oxidized thymidine product formed in the presence of either H$_2$O or O$_2$.

**MATERIALS AND METHODS**

Sonicated calf thymus DNA (1.7 mM nucleotide) was prepared as described (9) and dialyzed against 20 mM sodium citrate buffer at pH 4. Sodium borodeuteride (98 atom % $^2$H) was from Aldrich; $^1$H$_2$O (99.8 atom % $^1$H), $^1$H$_2$O (99 atom % $^1$H, 99 atom % $^18$O), and $^1$H$_2$O (99 atom % $^18$O) were from Stohler Isotope Chemicals. Chemically synthesized thymidine 5'-aldehyde was a gift from Dr. J. G. Moffatt, Syntex Research.

**NCS Chromophore—Clinical NCS (gift of Dr. W. T. Bradner, Bristol Laboratories) was dialyzed against distilled water and lyophilized. The nonprotein chromophore was extracted with 0.1 M trichloroacetic acid in methanol (0.2 ml/1.3 mg of NCS) at 0 °C for 2 h. The protein precipitate was pelleted by centrifugation, and the colorless supernatant solution (approximately 0.3 mM chromophore) was decanted and stored at -70 °C.

**Isolation of Deuterium-labeled Thymidine Produced by Reduction of Thymidine 5'-Aldehyde Generated by DNA-NCS Chromophore Reaction**—The following components were mixed and lyophilized in the dark (standard reaction): 65 μl of DNA, 150 μl of 10 mM EDTA, and 185 μl of 0.32 mM NCS chromophore solution. To the dried material, 23.1 mg of Tris powder, pH 7.2, (made by premixing 7.02 g of Trizma HCl (Sigma) and 0.67 g of Trizma base (Sigma)) and 31.1 mg of sodium borodeuteride were added, and oxygen was flushed through the septum cap into the vessel for approximately 1/2 h. Five hundred μl of 1 mM AET in $^2$H$_2$O was injected into the vessel to initiate the reaction. The final concentration of each ingredient was as follows: DNA (1 μM), sodium citrate (6 mM), EDTA (3 mM), NCS chromophore (0.125 mM), Tris buffer (300 mM), and sodium borodeuteride (150 mM). The final pH of the mixture was 8.0. To ensure complete reduction, the reaction was maintained at room temperature for 1 h before digestion of the DNA with DNase I (Worthington) and S$_1$ endonuclease (New England Nuclear) at 37 °C overnight as described (1). The released thymidine was isolated by reverse phase HPLC using a Bondapak C18 column (Waters Associates) at 10 μm, 0.39 × 30 cm as described above (1), except that distilled water was the eluent at a flow rate of 2 ml/min. Thymidine eluted as a single symmetrical peak at approximately 22 min. These fractions were rechromatographed on a Microsorb C18 column (Rainin Instrument Co., Inc.) with a flow rate of 1 ml/min of distilled water and elution of the thymidine peak at 80 min. The lyophilized samples were extracted with methanol, and the thymidine was isolated by evaporation of the methanol under reduced pressure.

**Test for Nonspecific $^18$O/$^16$O Exchange**—Standard reaction conditions were used except that the sodium borodeuteride (3.1 mg) was not present during the drug scission reaction but was added as solid to the lyophilized reaction mixture after the reaction. $^1$H$_2$O (0.5 g) was then introduced to initiate the reduction of 5'-aldehyde groups and to permit the measurement of the extent of $^18$O exchange into the carbonyl oxygen prior to the reduction. After lyophilization, the DNA was digested by nuclease in H$_2$O and the thymidine product was isolated by HPLC as described above.

**Preparation of Thymidine from NCS Chromophore-DNA Reaction Carried Out in $^2$H$_2$O**—The method is essentially the same as that in $^1$H$_2$O except that 0.5 g of $^2$H$_2$O was used. After lyophilization, the DNA was digested in normal water before isolation of the thymidine by HPLC.

**Preparation of Thymidine from NCS Chromophore-DNA Reaction Carried Out in $^2$H$_2$O**—Standard reaction components were mixed in a 5-ml pear-shaped flask and lyophilized in the dark. The residue was
dissolved in 500 μl of 1 mM AET in 1H218O. After three cycles of freezing and thawing under high vacuum (<10⁻⁴ torr) to expel dissolved 18O, 23.1 mg of Tris powder, pH 7.2, and 3.1 mg of sodium borodeuteride were transferred into the flask while the solution was frozen at 77 K. The vacuum was reapplied for another hour to the frozen solution, and 18O was introduced. The solution was thawed and shaken to initiate the reaction. After remaining at room temperature for 1 h, the DNA was digested and analyzed for released thymidine as described above.

Derivatization and GC-MS—Samples of thymidine (0.25–2 μg) were permethylated as described (10). The resulting derivatives were dissolved in 25 μl of CHCl₃, and aliquots of between 1 and 5 μl were analyzed by gas chromatography on a 30-m DB-1 fused silica capillary column (J & W Scientific) using a Hewlett-Packard model 5840A gas chromatograph equipped with a splitless injector and flame ionization detector. The temperature was held at 90 °C for 2 min then linearly programmed to 320 °C at 10 °C/min with a carrier flow (He) of 5 ml/min. The GC-MS computer system consists of a Varian model 3700 gas chromatograph (same conditions as above) modified for direct coupling of the capillary column to the ionization source of a Finnigan-MAT 312 double-focusing mass spectrometer operating in electron impact mode with an ionization potential of 70 eV. A Finnigan-Mat SS-200 data system controls the instrument and acquires, processes, and stores the data. For these experiments, the mass spectrometer was scanned from m/z 50 to 500 at a repetition rate of approximately 1.4 s. Short scans over the molecular ion region (m/z 270–300) were done at a scan rate of 2 s/decade.

RESULTS

GC-MS of Permethylated Thymidine—The electron impact mass spectrum of permethylated thymidine (5'-O-, 5'-O-, N²-trimethylthymidine) obtained during a GC-MS experiment (Fig. 1a) exhibits an abundant molecular ion at m/z 284 as well as fragment ions uniquely related to the sugar and base portions of the molecule. von Minden and McCloskey (11) have demonstrated that the base peak at m/z 145 corresponds to the deoxyribose sugar moiety formed by simple cleavage of the glycosidic bond, while the ions at m/z 140 and 141 correspond to the base moiety + H (formally equivalent to free dimethylthymine) and base + 2H, respectively.

GC-MS of Reduced Synthetic and NCS Chromophore-generated Thymidine 5'-Aldehyde—The source of the 5'-aldehydic oxygen in damaged DNA was determined with stable isotopes by reacting DNA and NCS chromophore in 1H216O/18O2, and 2H216O/18O2 and examining the mass spectra of the corresponding permethyl derivatives for shifts upward in mass of fragment and molecular ions. Hydration of the aldehyde and other possible reactions (see “Control Studies,” below) were minimized by reducing the aldehyde, as it formed during the drug-scission reaction, to a primary alcohol which is incapable of further oxygen exchange. The reduced damage product was then removed from the 5'-phosphate end of the break by digestion with DNase I and S₁ endonucleases. Unfortunately, these enzyme preparations are contaminated with phosphatases which catalyze the release of variable amounts of oxidized thymidine from DNA. Reduction with NaB₂H₄ enabled these two sources of thymidine to be distinguished, since only thymidine originating from NCS chromophore-generated nucleoside 5'-aldehyde will incorporate deuterium. Conditions for reduction and subsequent analysis by GC-MS were evaluated using synthetic thymidine 5'-aldehyde. The mass spectrum of the reduced and permethylated material clearly shows by the shift of m/z 145 to 146 that the deuterium is specifically incorporated in the ribose moiety (Fig. 1b). The mass spectrum of the reduced and permethylated DNA-NCS chromophore reaction product (1H216O/18O2, Fig. 1c) is identical to that obtained for the authentic aldehyde, further substantiating the structure of the strand scission product (1). Thymidine released by contaminant phosphatases gives rise to the ion at m/z 284 and the increase in the relative abundance of m/z 145 (Fig. 1c). In this experiment (experiment 1, Table I), labeled thymidine accounted for approximately 78% of the total thymidine recovered.

18O Incorporation Studies—The results of the 18O incorporation studies are summarized in Table I. Relative mole percents of unlabeled thymidine, (5'-2H,18O)thymidine and (5'-H,18O)thymidine are represented by the summed relative per cent abundances of m/z 284, 285, and 287, respectively, which have been corrected for the natural abundance of 13C (12). Only m/z 285 and 287 represent thymidine 5'-aldehyde originally present in the reaction mixture. Reaction of DNA and NCS chromophore in an atmosphere of 18O2 (solvent = 2H216O) results in 18O labeling of more than 95% of the reducible thymidine (experiment 4, Table I), clearly establish-
ment indicates that oxygen from solvent water is incorporated (experiments 1 and 2). The extent of exchange under these conditions was examined by comparing the relative mol percents of (5'-H, 18O)trimethylthymidine to (5'-1H, 18O)trimethylthymidine for NCS chromophore-DNA reactions performed in 18O2 or H2O2, with reducing agent present during the scission reaction (experiments 1 and 3, Table I) versus reaction in H2O2 and reduction of the lyophilized reaction mixture in H2O2 in a subsequent step (experiment 2, Table I). The latter experiment indicates that oxygen from solvent water is incorporated, since the relative mol percent of (5'-2H, 18O)trimethylthymidine has increased from 0 mol % to 10.4 mol % of the total reducible thymidine, as reflected by the ratio of m/z 287 to 285 (Table I, experiments 1 and 2, respectively). In the actual incorporation studies, the amount of hydrolytic exchange was only half of this value (see experiment 3, Table I), possibly because the mole ratio of NaBH4 to aldehyde is kept very large by reduction of the aldehyde substrate essentially as it is formed. In agreement with earlier experiments in which DNA damage was assayed by the generation of acid-soluble material (5), the formation of thymidine 5'-aldehyde was not affected by the presence of metal chelators, such as diethylenetriamine pentaacetic acid, or by scavengers of reduced forms of O2, such as superoxide dismutase or catalase.

**DISCUSSION**

Cleavage of DNA by NCS chromophore results in strand breaks having a phosphate at the 3'-end and a nucleoside 5'-aldehyde at the 5'-end. Previous work has shown that the formation of these breaks involves molecular oxygen (5-7). This reaction may proceed by a mechanism (e.g. Scheme 1) analogous to those postulated to account for the ability of various electron affinic compounds (including O2) to sensitize DNA to damage by ionizing radiation (13). Although details of the reactions leading to the formation of such a radical centered on C-5' remain to be elucidated, certain facts are known: 1) 1 mol of thiol is consumed during NCS chromophore activation under anaerobic conditions (7); 2) one molecule of thiol adds to the highly unsaturated portion of the NCS chromophore molecule, and this is probably accompanied by a molecular rearrangement to form an activated (possibly free radical) form of the drug (14). Although oxygen is consumed during drug activation even in the absence of DNA (7), it is not known whether oxygen is indispensable for the initial activation. Although there was no evidence for the incorporation of 18O into NCS chromophore treated with thiol in the presence of 18O2 (14), the possibility that an unstable oxygenated form of NCS chromophore is the activated species

![Scheme 1](https://example.com/scheme1.png)

**TABLE 1**

| Reaction conditions                  | (5'-2H, 18O) (m/z 284) | (5'-1H, 18O) (m/z 285) | (5'-1H, 18O) (m/z 287) |
|-------------------------------------|------------------------|------------------------|------------------------|
| 1. 18O2, 2H2O*                      | 22.3                   | 77.7                   | 0.0                    |
| 2. i) 18O2, 2H2O*                   | 17.2                   | 74.2                   | 8.6 (10.4)*            |
| ii) 18O2, 2H2O*                     | 27.8                   | 68.2                   | 4.6*                   |
| 3. 18O2, 2H2O*                      | 77.4*                  | 1.0                    | 21.6 (>95)*            |

* Reducing agent present during reaction of NCS chromophore and DNA.

Reduction performed in 1H2O subsequent to reaction of DNA and NCS chromophore in 18O2 and 2H2O.

Relative mole per cent of total reduced thymidine 5'-aldehyde (i.e., \( \sum_{i=283}^{i=287} = 100 \) mol % of reduced thymidine 5'-aldehyde).

A larger amount of unlabeled thymidine is present because phosphatase inhibitors were not added to this reaction (see text).
cannot be excluded. In this case, X in Scheme 1 might be such a species, e.g. thiol-NCS chromophore-OO or thiol-NCS chromophore-O. In any case, some radical form of the NCS chromophore, bound to DNA by an intercalative mechanism (15), may be viewed as abstracting a hydrogen from C-5' of mainly thymidylate residues in DNA (1, 2) to form a carbon-centered radical on deoxyribose in DNA, although other mechanisms not involving radicals have not been eliminated.

While various oxidizing species may convert ionizing radiation-induced carbon-centered radicals on the DNA to carbonium ions (16, 17) with subsequent aldehyde formation at the C-5' position, the main reaction between our data show clearly that the oxygen of the 5'-aldehyde moiety comes from molecular oxygen, an addition mechanism analogous to that shown in Scheme 1 appears to be involved in the NCS chromophore-induced DNA cleavage reaction. Furthermore, our results eliminate a mechanism involving an unstable enol phosphate intermediate (16, 21). Such an enol intermediate might be formed either by elimination of HOO from a peroxyl radical at C-5' or by the abstraction of a proton from C-4' via a disproportionation mechanism as described by Kochetkov et al. (22). On conversion of the enol phosphate intermediate to the aldehyde in \( \text{H}_2\text{O} \), a deuterium atom would be incorporated into the product at C-4', contrary to our findings. The absence of evidence for \( ^{18}\text{O} \) incorporation into the 5'-aldehyde from \( ^{18}\text{O}_2 \) solvent also precludes this possibility.

If a peroxyl radical adduct were formed in the NCS chromophore-DNA reaction, it presumably would be reduced to the aldehyde by the thiol present in the reaction. In addition, several possibilities for peroxyl radical decay have been proposed for ionizing radiation-induced DNA damage (17, 23), one of which may lead to the formation of formate from the 5'-carbon, a minor product of NCS-induced DNA damage (24). It is also attractive to postulate that an NCS chromophore-bound form of O2 participates in the concerted abstraction of a hydrogen and donation of oxygen as part of a ternary complex that is cleaved to form the hemiacetal precursor of the 5'-aldehyde group. Such a mechanism would also explain why only 1 mol of O2 is consumed (per mol of NCS chromophore) (7), even though it would be involved in both NCS activation and DNA damage fixation. It is possible that the thiol, present at high concentration in the NCS chromophore reaction, is involved in the cleavage reaction. Consistent with this possibility are our findings that nucleoside 5'-aldehyde formation is increased, while spontaneous base release is decreased, with increasing thiol level, and that some mole of thiol is consumed following the consumption of 1 mol of O2 (7). On the other hand, the similarity between hydroxyl group formation in the NCS reaction and that produced in cytochrome P-450 systems, as already noted (7), is further supported by the finding that in both cases the hydroxyl group is derived from O2. Finally, the involvement of oxygen in the formation of an aldehyde at C-5' appears to be unique, for although other electron affinic compounds such as the nitroaromatic radiation sensitizers can substitute for oxygen in DNA strand scission by NCS chromophore, nucleoside 5'-aldehyde is not a product (25). Instead, a gap bounded by phosphate at both the 3' and 5'-ends is produced. No evidence implicating a metal-catalyzed reduction of \( \text{O}_2 \) to a diffusible form of DNA-damaging agent has been found in either reaction.

Acknowledgment—We wish to thank Dr. V. N. Reinhold, Department of Nutrition, Harvard School of Public Health, for his interest and use of the Mass Spectrometry Laboratory.

REFERENCES

1. Kappen, L. S., Goldberg, I. H., and Leisch, J. M. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 744-748
2. Kappen, L. S., and Goldberg, I. H. (1983) Biochemistry 22, 4872-4878
3. Povirk, L. F., and Goldberg, I. H. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 369-373
4. Povirk, L. F., and Goldberg, I. H. (1982) Nucleic Acids Res. 10, 6255-6264
5. Kappen, L. S., and Goldberg, I. H. (1978) Nucleic Acids Res. 5, 2953-2967
6. Burger, R. M., Peisach, J., and Horwitz, S. B. (1978) J. Biol. Chem. 253, 4830-4832
7. Povirk, L. F., and Goldberg, I. H. (1983) J. Biol. Chem. 258, 11763-11767
8. Raleigh, J. A., Greenstock, C. L., and Koenens, W. (1973) Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med. 23, 457-467
9. Povirk, L. F., and Goldberg, I. H. (1980) Biochemistry 19, 4773-4780
10. DeLeenheer, A. P., and Gelliékers, C. F. (1976) Anal. Chem. 48, 2203-2206
11. von Minden, D. L., and McCloskey, J. A. (1973) J. Am. Chem. Soc. 95, 7480-7490
12. Biemann, K. (1962) Mass Spectrometry: Organic Chemical Applications, pp. 223-227, McGraw-Hill, Inc., New York.
13. Ward, J. F. (1975) Adv. Radiat. Biol. 5, 181-239
14. Hensens, O. D., Dewey, R. S., Lisch, J. M., Napier, M. A., Reamer, R. A., Smith, J. L., Althers-Schönberg, G., and Goldberg, I. H. (1983) Biochem. Biophys. Res. Commun. 113, 538-547
15. Povirk, L. F., Dattagupta, N., Warf, B. C., and Goldberg, I. H. (1981) Biochemistry 20, 4007-4014
16. Stelter, L., Von Sonntag, C., and Schulte-Frohlinde, D. (1976) Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med. 29, 255-269
17. Von Sonntag, C., and Schulte-Frohlinde, D. (1978) Mol. Biol. Biochem. Biophys. 27, 204-251
18. Theard, L. M., Peterson, F. C., and Myers, L. S., Jr. (1971) J. Phys. Chem. 75, 3815-3821
19. Willson, R. L. (1970) Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med. 17, 349-358
20. Simic, M. and Hayon, E. (1973) Biochem. Biophys. Res. Commun. 50, 364-369
21. Stelter, L., Von Sonntag, C., and Schulte-Frohlinde, D. (1975) Z. Naturforsch. 30b, 609-615
22. Kochetkov, N. K., Kudrjashov, L. I., Chlenov, M. A., and Grineva, L. P. (1974) Carbohydr. Res. 31, 235-241
23. Isidir, M., Schuchmann, M. N., Schulte-Frohlinde, D., and Von Sonntag, C. (1982) Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med. 41, 525-533
24. Hatayama, T., and Goldberg, I. H. (1980) Biochemistry 19, 5890-5898
25. Kappen, L. S., and Goldberg, I. H. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 3312-3316

2 L. S. Kappen and I. H. Goldberg, unpublished data.