

## Nitroaromatic radiation sensitizers substitute for oxygen in neocarzinostatin-induced DNA damage

(misonidazole/radiomimetic drug/base release/gaps with 3'- and 5'-phosphate ends/anaerobiosis)

LIZZY S. KAPPEN AND IRVING H. GOLDBERG\*

Department of Pharmacology, Harvard Medical School, Boston, MA 02115

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**ABSTRACT** The ability of neocarzinostatin (NCS) chromophore to damage DNA, as manifested by strand breaks and base release, is markedly decreased under anaerobic conditions but can be restored by nitroaromatic radiation sensitizers, which by themselves have no effect. The effectiveness of these compounds is correlated with their electron affinity as measured by their one-electron reduction potentials and is inversely related to the concentration of thiol used to activate the NCS. Whereas strand breaks with thymidine 5'-aldehyde at the 5' end and released thymine are the main DNA damage products in O<sub>2</sub>, under anaerobic conditions misonidazole causes a marked increase in the release of thymine and in the formation of breaks with 5'-phosphate ends. In both cases the 3' end of the break carries a phosphate group, and the attack-site specificity of spontaneous and alkali-labile DNA strand breakage and base release are identical. In O<sub>2</sub>, misonidazole does not affect the extent of DNA damage or alter the distribution of DNA damage products found with NCS alone. The data do not distinguish whether the nitroaromatic compounds function by interacting with NCS-induced nascent damage on the DNA, by being converted by activated NCS into a DNA-damaging species, or by participating in the activation of NCS to a DNA-damaging species. The implications of these results for the treatment of hypoxic tumor cells with the combined use of radiomimetic drugs and radiation sensitizers are discussed.

The nonprotein chromophore (1) of the antitumor antibiotic neocarzinostatin (NCS) complexes with DNA by an intercalative mechanism (2, 3) and damages DNA in a reaction dependent on O<sub>2</sub> and mercaptan (4-6). DNA damage is manifested mainly by the formation of single-strand breaks and the release of free thymine. Although all strand breaks contain a phosphate at the 3' end, nucleoside 5'-aldehyde (>80%) or phosphate (<20%) is found at the 5' end (7, 8).

In addition to certain biochemical characteristics of the DNA-damaging reaction, many of the cellular effects of NCS resemble those of ionizing radiation (9-12). It seemed possible, therefore, that O<sub>2</sub> might be playing similar roles in the action of the two agents. If this is the case, then nitroaromatic electron-affinic radiation sensitizers that substitute for O<sub>2</sub> in enhancing x-ray effects on hypoxic cells (13) might serve NCS in a similar capacity. Experiments testing this possibility are reported here.

### MATERIALS AND METHODS

Sources of enzymes, substrates, and NCS have been reported (8). The concentration of isolated NCS chromophore (4) was determined spectrophotometrically ( $\epsilon_{340} = 10.8 \text{ mM}^{-1}\text{cm}^{-1}$ ). Misonidazole (MISO), metronidazole, and nitrofurazone were kindly provided by P. Goldman and were dissolved in distilled H<sub>2</sub>O. [2-<sup>14</sup>C]MISO was a gift of P. F. Sorter (Hoffmann-La Roche).

Preparation of [methyl-<sup>3</sup>H]thymidine-labeled  $\lambda$  DNA ( $2.7 \times 10^4 \text{ cpm}/\mu\text{g}$ ) and 3'-<sup>32</sup>P-end-labeled 275- (bases 375-650) and 375- (bases 1-375) base-pair (bp) double-stranded restriction fragments from pBR322 DNA was as described (8). A 5'-<sup>32</sup>P-end-labeled 375-bp fragment was prepared by end labeling BamHI-digested pBR322 DNA using [ $\gamma$ -<sup>32</sup>P]ATP and polynucleotide kinase followed by a second cutting of the labeled DNA with Sal I. A 68-bp restriction fragment containing bases 887-952 of the *lacI* gene and labeled with <sup>32</sup>P at the 5' end of the coding strand was a gift from L. F. Povirk.

**Drug Treatment.** Anaerobic reactions of DNA with the drugs were carried out in the dark in micro Warburg vessels equipped with an inlet and outlet for gas flushing. The standard reaction mixtures (0.75 ml) contained 10 mM sodium acetate (pH 5.0), 100 mM Tris-HCl (pH 8), 1 mM EDTA, 1 mM glutathione (or 2-mercaptoethanol at levels indicated), NCS chromophore in methanol (final concentration, 10%), and DNA (6-9 mol of nucleotides per mol of drug to ensure nearly complete binding). In experiments with <sup>32</sup>P-labeled restriction fragments, sonicated calf thymus DNA was added as carrier. The components of the reaction were split such that the chromophore was added to DNA at pH 5 (sodium acetate) in the main chamber of the reaction vessel in 84% of the reaction volume. The contents were flushed with N<sub>2</sub> for 10 min with shaking, and the reaction was subsequently initiated by the addition of the mercaptan in Tris buffer (16% of the reaction volume) from the side arm. Radiation sensitizers (the maximal level used was limited by their solubility) were present in the main chamber from the beginning. The reaction was allowed to proceed at room temperature for 30 min. The aerobic reaction mixtures were flushed with N<sub>2</sub> for 9 min followed by a 1-min flush with O<sub>2</sub> before the contents of the two chambers were mixed. Warburg vessels with two side arms were used to study the late addition of any of the components.

**Analysis of DNA Damage.** DNA strand breakage was determined by analysis on a 5-20% neutral sucrose gradient containing 0.7 M NaCl, 50 mM potassium phosphate (pH 7.0), and 10 mM EDTA. Centrifugation was at 39,000 rpm in an SW 41 rotor for 4.8 hr at 20°C. Measurement of DNA damage based on trichloroacetic acid-solubilized radioactivity was as reported (5). Thymine release and 5'-terminal thymidine 5'-aldehyde were estimated by HPLC (7, 8). In the latter case, the drug-cut DNA was digested with S1 nuclease to release the 5'-terminal product. Soluble DNA products were also separated by TLC on silica gel in solvent system 1 (ethyl acetate/isopropanol/H<sub>2</sub>O, 74:17:9) or on cellulose in solvent system 2 (1 M sodium acetate/ethanol, 3:7). Procedures for NaBH<sub>4</sub> reduction of the DNA and estimation of 5' termini by the phosphatase-polynucleotide kinase assay were as described (8). <sup>32</sup>P-end-labeled DNA from the reac-

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Abbreviations: NCS, neocarzinostatin; MISO, misonidazole; bp, base pair.

\*To whom reprint requests should be addressed.

tion mixture was processed for sequence analysis (14) on an 8.3 M urea-containing polyacrylamide gel (15%) as described (8).

## RESULTS

**DNA Damage Dependent on Nitroaromatic Compounds.** Although NCS chromophore generates single-strand breaks in DNA, at the high levels of drug used to emphasize the effect of anaerobiosis double-strand breaks also occur, presumably due to the placement of single-strand breaks within a few nucleotides of one another, and can be measured on neutral sucrose gradients. As shown in Fig. 1, the inhibition of double-strand breakage due to anaerobiosis was reversed by the addition of the 2-nitroimidazole MISO. There were  $\approx 80$  breaks per strand in  $O_2$  or anaerobically with MISO, and 1 break per strand anaerobically without MISO. In reactions lacking NCS chromophore, MISO failed to break the DNA under either aerobic or anaerobic conditions, as analyzed by neutral and alkaline sucrose gradients (data not shown).

Similar results were obtained when DNA damage was measured by the release of radioactivity from [*methyl*- $^3H$ ]thymidine-labeled  $\lambda$  DNA into an acid-soluble form, which is mainly a measure of spontaneous thymine release (Table 1). Under all conditions, in the absence of either NCS chromophore or thiol there was no significant DNA damage. Delayed addition of MISO failed to induce damage. MISO did not significantly affect NCS-induced DNA damage in  $O_2$ . DNA damage increased with the concentration of MISO (Fig. 2) and was nearly over by 1 min. As the level of thiol was increased, however, the ability of MISO to restore DNA damage to the level found with  $O_2$  was decreased. In addition to their role in activating NCS chromophore, thiols block DNA damage (5), presumably by scavenging radical forms of the drug or the DNA. Very high concentrations of thiol are required to detect this effect in  $O_2$  since  $O_2$  is highly efficient in fixing the putative DNA sugar lesion. MISO is much less effective than  $O_2$  as a radiation sensitizer (13) and would be expected to compete with the thiol less efficiently, resulting in less DNA damage at thiol levels where DNA damage under aerobic conditions was still increasing. The more electron-affinic nitroaromatic compounds are better

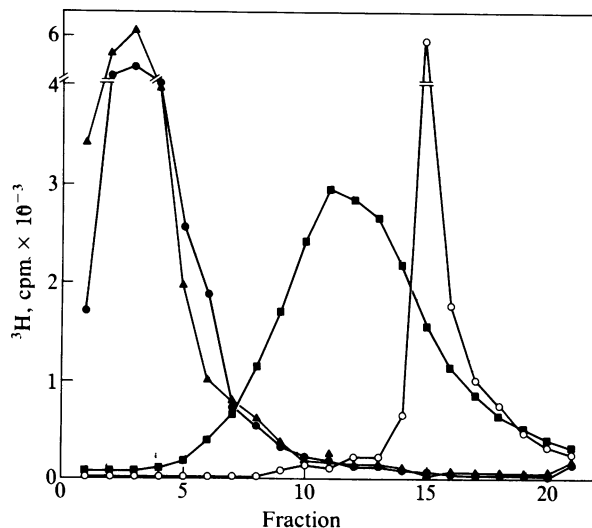


FIG. 1. Enhancement of NCS-induced DNA breakage by MISO under anaerobic conditions. [*Methyl*- $^3H$ ]Thymidine-labeled  $\lambda$  DNA (88  $\mu M$ ) was treated with NCS (12  $\mu M$ ) under standard conditions. Sixty microliters of the samples was analyzed on neutral sucrose gradients. Centrifugation is from left to right.  $\circ$ , No drugs;  $\blacksquare$ , anaerobic reaction with NCS alone;  $\blacktriangle$ , anaerobic reaction with NCS and MISO (40 mM);  $\bullet$ , NCS in  $O_2$ . DNA treated with MISO alone in  $O_2$  or  $N_2$  sedimented coincident with the control DNA (not shown).

Table 1. Effect of MISO on NCS-induced DNA damage

Condition	Gas phase	% radioactivity
NCS	$N_2$	0.91
NCS and MISO	$N_2$	5.50
MISO	$N_2$	0.24
NCS and MISO without glutathione	$N_2$	0.28
NCS	$O_2$	5.20
NCS and MISO	$O_2$	5.00
No NCS or MISO	$O_2$	0.28

[*methyl*- $^3H$ ]Thymidine-labeled  $\lambda$  DNA (88  $\mu M$ ) was treated with NCS chromophore (12.5  $\mu M$ ) under standard conditions. MISO, when present, was at 40 mM. Portions (150  $\mu l$ ) of the reaction mixture were processed for the determination of acid-solubilized radioactivity.

radiation sensitizers (13) and are more effective in substituting for  $O_2$  in the NCS chromophore-induced damage of DNA (Fig. 3 and Table 2). Adventitious  $O_2$  appears not to be involved in MISO action, since the amount of DNA damage produced was at least 20-fold the total amount of potentially available  $O_2$  in experiments performed in an anaerobic glove box equilibrated with  $N_2$  having 0.2 ppm of  $O_2$  or *in vacuo* at  $<5 \times 10^{-4}$  torr.

Under the condition of our experiments, the thiols used did not reduce MISO by themselves, as determined by spectroscopic and chromatographic analysis. There also appeared to be no stable association of [ $^{14}C$ ]MISO with DNA, as determined by either acid-precipitation or dialysis. Dithionite reduced MISO, but we found no evidence for acid-solubilization of thymidine-labeled DNA in the absence of NCS.

**Characterization of DNA Damage Products.** Under aerobic reaction conditions thymidine 5'-aldehyde formation is equal to or exceeds spontaneous thymine release (refs. 7 and 8 and Fig. 4). Both products were inhibited drastically but equally under anaerobic conditions, but the addition of MISO resulted in a selective increase in spontaneous thymine release. In

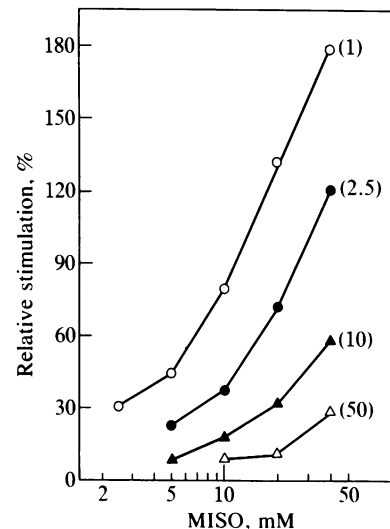


FIG. 2. Effect of various levels of MISO and thiol on the stimulation of NCS-dependent DNA damage in  $N_2$ . Reaction mixtures were similar to those in Table 1, except that 2-mercaptoethanol at concentrations (mM) indicated in parentheses was used and the concentration of MISO was varied as shown. Portions (150  $\mu l$ ) were processed for acid-soluble radioactivity. Acid-soluble radioactivities with NCS alone in  $O_2$  at 1, 2.5, 10, and 50 mM thiol were 3833, 6197, 9076, and 6624 cpm, respectively, and the percentage inhibitions in  $N_2$  were 65%, 80%, 88%, and 80%, respectively. The values with  $N_2$  were subtracted as a background from those obtained with  $O_2$  and MISO. Relative stimulation was calculated by taking the  $O_2$ -dependent reaction as 100%.

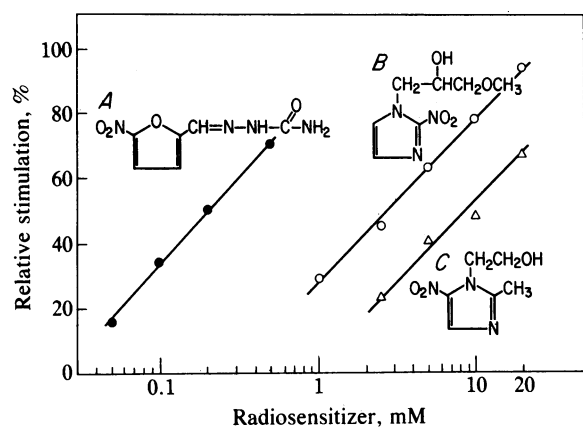


FIG. 3. Comparison of different radiation sensitizers in the stimulation of NCS-dependent DNA damage in  $N_2$ . (A) Nitrofurazone, (B) MISO, and (C) metronidazole. Incubations and analysis were similar to those in Fig. 2 except for 1 mM glutathione. In  $O_2$ , NCS solubilized 4771 cpm; in  $N_2$ , the reaction was inhibited 95%.

$O_2$ , MISO had little effect on the product distribution. Thymine was identified by two TLC systems in addition to HPLC. Neither thymidine nor thymidylic acid was found as products.

The chemical nature of the 5' ends of the strand breaks was first explored by determining the extent to which they could be enzymatically phosphorylated following various treatments, such as reduction of existing nucleoside 5'-aldehyde with  $NaBH_4$  or removal of pre-existing phosphate with alkaline phosphatase, in the manner described earlier (8). These studies showed that  $\approx 90\%$  of the breaks produced by NCS and MISO in  $N_2$  that can be measured by this method had 5'-phosphate termini, with the remainder being nucleoside 5'-aldehyde. Total DNA damage so measured was comparable whether the DNA was treated with NCS in  $O_2$  or in  $N_2$  in the presence of MISO (40 mM) and was inhibited 85% in  $N_2$ . There were no 5'-hydroxyl ends.

These results were confirmed and extended by analysis of four different drug-damaged DNA restriction fragments on DNA sequencing gels, two of which are shown (Figs. 5 and 6). Sequence analysis of a 3'- $^{32}P$ -end-labeled restriction fragment after various drug treatments shows (i) under aerobic conditions (with or without MISO) NCS causes breaks predominantly at T residues with mainly nucleoside 5'-aldehyde at the 5' end (as indicated by slowing of fragment mobility by about two nucleotide positions; see ref. 8 for further discussion); these ends are converted into phosphate ends by treatment with alkali; (ii) anaerobiosis resulted in marked inhibition of DNA fragmentation by NCS; (iii) inclusion of MISO in the anaerobic reaction enhances DNA breakage with 5'-phosphate ends as the main products; alkali increases this product, and treatment with phosphatase converts it to a slower moving band, consistent with the removal of a terminal phosphate (not shown); (iv) the small amount of nucleoside 5'-aldehyde-ended fragment produced under anaerobic conditions (probably due to trace amount of  $O_2$ ) disappears with addition of MISO; (v) the light band between the bands

Table 2. Efficiency of different sensitizers in enhancing NCS-induced DNA damage under anaerobiosis

Radiation sensitizer	$E_7^1/mv$	Concentration for 50% stimulation, mM
Nitrofurazone	-257	0.2
MISO	-389	2.8
Metronidazole	-486	9.0

$E_7^1/mv$  represents the one-electron reduction potential at pH 7.0 (13). The values for 50% stimulation are calculated from Fig. 3.

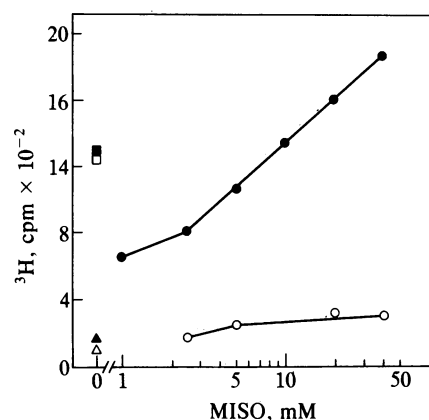


FIG. 4. Analysis of products formed in  $O_2$ - and MISO-dependent DNA damage by NCS. Portions (100  $\mu$ l) of reactions with MISO (in Fig. 3) were analyzed for spontaneous thymine release (closed symbols) and thymidine 5'-aldehyde (open symbols). Circles, anaerobic with NCS and MISO (40 mM); triangles, anaerobic with NCS; squares, in  $O_2$  with NCS. Anaerobic reaction of DNA with MISO (40 mM) alone gave neither product.

representing a 5'-phosphate-ended fragment and a 5'-nucleoside 5'-aldehyde-ended fragment is increased in intensity in anaerobic reactions with MISO (this band is not altered by alkali treatment and its intensity varies in different experiments); (vi) the overall pattern of cleavage (best compared after alkali treatment) is identical under aerobic conditions with NCS chromophore alone or under anaerobic conditions where MISO is also included. In all respects cited above, metronidazole and nitrofurazone were identical to MISO.

Sequence analysis of a similarly treated 5'- $^{32}P$ -end-labeled restriction fragment (Fig. 6) again showed identical cleavage patterns for the various drug-treatment conditions, indicating even more clearly than in Fig. 5 that the sites of base attack remain  $T > A \gg C > G$  for the anaerobic reactions containing MISO as for the aerobic reaction, with certain Ts and As being more favored targets than others. These results also show that *all* breaks have 3'-phosphate ends. In addition, the alkali-labile breaks—e.g., at C-29 and C-43—are also the same in both reactions. Finally, the ratios of spontaneous thymine to adenine released under the two reaction conditions were similar (2.42 in  $O_2$  and 2.30 with 40 mM MISO in  $N_2$ ).

## DISCUSSION

Under aerobic conditions DNA strand breaks result mainly from the selective oxidation of the C-5' of deoxyribose by activated NCS chromophore (8). A single mole of  $O_2$  is consumed during this reaction (15), but from these studies it was not possible to determine whether  $O_2$  is involved in the generation of an active NCS species that attacks DNA or in the fixation of nascent DNA lesions (or both). The latter action is similar to that believed to occur in the  $O_2$  enhancement of ionizing radiation-induced DNA damage (16, 17) and cell killing (13). It is conceivable that rather than  $O_2$  itself adding to a carbon-centered radical at C-5' (presumably generated by hydrogen abstraction by a radical form of NCS) a NCS-bound form of oxygen is involved in a concerted reaction that results finally in the donation of the oxygen that ends up in the aldehyde at C-5'. These reactions might also lead to spontaneous base release and formation of breaks with 5'-phosphate ends (18), although attack elsewhere in the deoxyribose is also possible. Alkali-labile breaks are, at least in part, due to chromophore adducts on the sugar (19, 20).

Nitroaromatic radiation sensitizers resemble  $O_2$  in being both electron affinic and able to form adducts with free radicals. One or both of these properties may account, to a con-

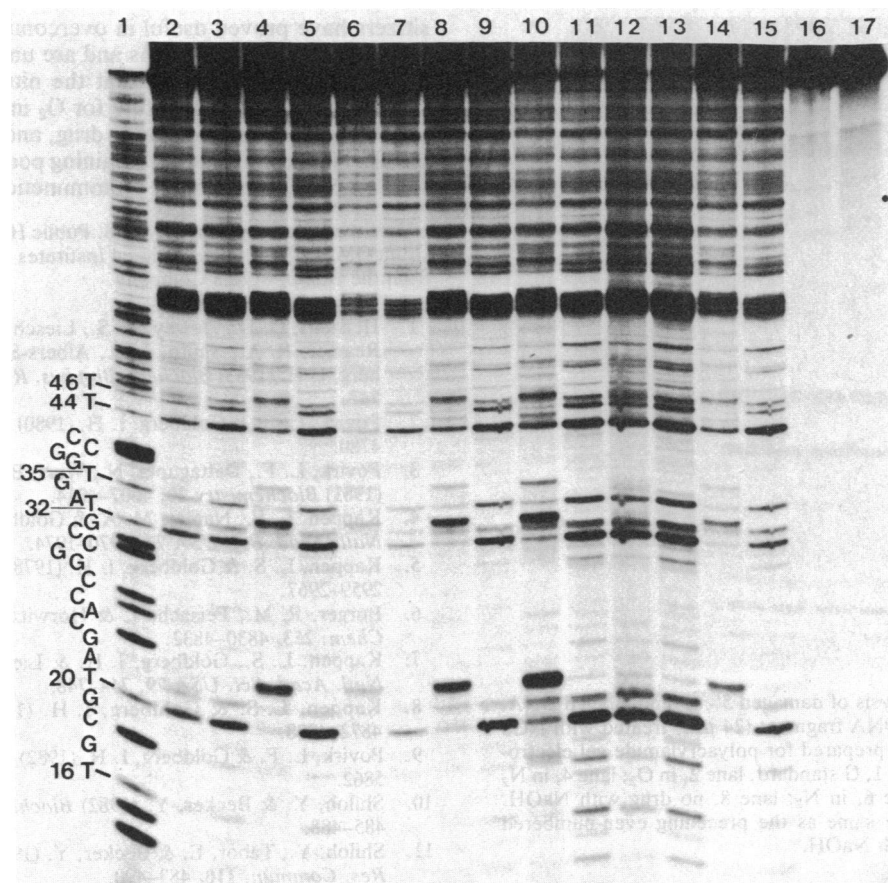


FIG. 5. Sequence analysis of damaged 3'-<sup>32</sup>P-end-labeled DNA. Drug-treated 3'-<sup>32</sup>P-end-labeled 275-bp DNA fragment (24  $\mu$ M) was precipitated with ethanol and redissolved in H<sub>2</sub>O. Half of the sample was heated in 0.1 M NaOH/1 mM EDTA at 90°C for 10 min and neutralized; the other half received an equivalent amount of NaCl/EDTA. The samples were lyophilized and analyzed by polyacrylamide gel electrophoresis. Lanes 3, 5, 7, 9, 11, and 15 are the same as the preceding even-numbered lanes but were treated with NaOH. When present, MISO was at 40 mM. Lane 1, T + C standard. In lanes 2-9, DNA was treated with 2  $\mu$ M NCS. Lane 2, in N<sub>2</sub> with MISO; lane 4, in O<sub>2</sub>; lane 6, in N<sub>2</sub>; lane 8, in O<sub>2</sub> with MISO. In lanes 10-15, NCS was at 5  $\mu$ M. Lane 10, in O<sub>2</sub>; lane 12, in N<sub>2</sub> with MISO; lane 14, in N<sub>2</sub>. Lane 16, no NCS control in N<sub>2</sub> with MISO and NaOH; lane 17, no NCS control with NaOH.

siderable degree, for the ability of these compounds to sensitize hypoxic cells to x-irradiation (13). The effectiveness of the nitroaromatic compounds in potentiating both NCS-induced DNA damage and radiation-induced DNA damage (16, 17) and cell killing (13) is correlated with their one-electron reduction potentials. These results suggest that the mechanism of potentiation is similar in both cases and is probably at least partly due to the electron-affinic properties of the sensitizers. We have not found any evidence for the formation of stable MISO adducts on the DNA in the NCS reaction.

In the case of NCS-induced DNA damage we find that, although the sites of DNA breakage (spontaneous and alkali-labile) and the specificity of base attack are the same for the nitroaromatic compounds and O<sub>2</sub>, base and sugar release (with gap formation), rather than nucleoside 5'-aldehyde formation, predominates with the former. Since DNA attack-site specificity is presumably related to the physical nature of the intercalative complex formed between the NCS chromophore and the DNA, it is not surprising that this specificity is the same in both cases. It is less obvious why the chemical nature of the breaks with the nitroaromatic compounds should be different from that with O<sub>2</sub>. Whether spontaneous base (and sugar) release, with the formation of a gap bounded by 3'- and 5'-phosphate ends, and nucleoside 5'-aldehyde formation are separate offshoots of the same pathway or are totally different pathways of DNA damage is not clear. Furthermore, we do not know if the mechanism that leads to

base (and sugar) release in the presence of O<sub>2</sub> is the same, although less favored, as that involved in radiation sensitizer-dependent DNA damage. It is of interest that 2 mol of thiol are consumed per mol of chromophore in O<sub>2</sub> or anaerobically with MISO (unpublished work), whereas only 1 mol of thiol is used in the absence of both O<sub>2</sub> and MISO (15).

It is conceivable that the nitroaromatic compounds function not by reacting with the site of nascent DNA damage but by being converted into DNA-damaging agents by the activated NCS chromophore, possibly via a one-electron reduction process to form the nitro anion free radical. Such a mechanism might account for the change in product distribution compared with the reaction in O<sub>2</sub>. In preliminary experiments we have found that [2-<sup>14</sup>C]MISO is converted into products provided that anaerobiosis is maintained and that DNA, NCS, and thiol are all present in the reaction. Although this result may be taken as evidence that MISO reacts directly with the nascent DNA damage, it must be interpreted with caution, since it is conceivable that the DNA functions in some passive way—e.g., by binding NCS chromophore the DNA may prevent activated NCS from reacting with itself (21), so that it can react with MISO. Furthermore, since a very low concentration (1.3  $\mu$ M) of [<sup>14</sup>C]MISO was used in these experiments, the conditions differ significantly from those used to study the DNA damage-enhancing activity of MISO (millimolar concentration). Thus, the interpretation of these experiments must await a detailed analysis of the stoichiometry of all the products (MISO and DNA)

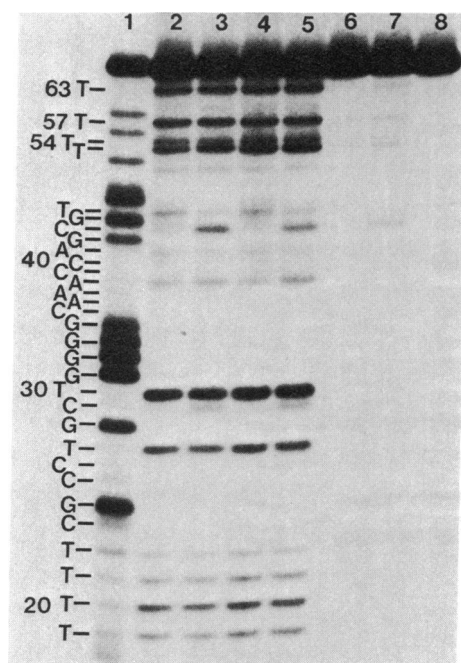


FIG. 6. Sequence analysis of damaged 5'-<sup>32</sup>P-end-labeled DNA. 5'-<sup>32</sup>P-end-labeled 68-bp DNA fragment (24 μM) treated with NCS chromophore (4 μM) was prepared for polyacrylamide gel electrophoresis as in Fig. 5. Lane 1, G standard, lane 2, in O<sub>2</sub>; lane 4, in N<sub>2</sub> with MISO (40 mM); lane 6, in N<sub>2</sub>; lane 8, no drug with NaOH. Lanes 3, 5, and 7 are the same as the preceding even-numbered lanes but were treated with NaOH.

formed. It should also be noted that the DNA products of the nitroaromatic compound-stimulated reaction with NCS under anaerobic conditions differ from those reported with electrolytically reduced nitroimidazoles, where thymidine nucleotides, not thymine, accounted for almost all of the dialyzable DNA reaction products (22).

Inclusion of MISO in the aerobic reaction did not significantly affect either the extent of DNA damage or the formation of thymidine 5'-aldehyde. This result may simply reflect the fact that MISO competes poorly with O<sub>2</sub>, since when O<sub>2</sub> is present only in trace amounts ("anaerobic" conditions in Fig. 5), high levels (40 mM) of MISO resulted in the disappearance of the small amount of thymidine 5'-aldehyde formed. These results do not distinguish whether the competition is at the level of the generation of an active DNA-damaging species or at the level of the nascent DNA damage.

The resistance of hypoxic tumor cells to irradiation limits the effectiveness of this modality of therapy. Radiation sen-

sitizers have proved useful in overcoming this resistance in experimental tumor systems and are undergoing clinical trials (13). Our results show that the nitroaromatic radiation sensitizers can also substitute for O<sub>2</sub> in the DNA-damaging action of NCS, a radiomimetic drug, and suggest their possible use in treating tumors containing poorly oxygenated cells with NCS or other similar radiomimetic agents.

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