Influence of Nitrogen, Oxygen, and Nitroimidazole Radiosensitizers on DNA Damage Induced by Ionizing Radiation†

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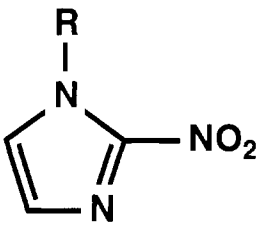
ABSTRACT: Oxygen and nitroaromatic compounds are known to enhance the sensitivity of cells to ionizing radiation. Employing calf thymus DNA and oligo$(dA)_{12}$/poly(dT), we have examined the differences to DNA damage, in particular thymine glycols and the 3′-DNA termini at strand breaks, arising from irradiation under anoxic and oxic conditions and the presence and absence of misonidazole [1-(2-nitro-1-imidazoyl)-3-methoxy-2-propanol]. We show that (i) irradiation under nitrogen generates strand breaks almost exclusively with 3′-phosphate termini; (ii) irradiation under oxic conditions increases the yield of strand breaks 3-fold, and the 3′ termini consist of 3′-phosphoglycolate and 3′-phosphate end groups in a ratio of ~1.6; (iii) the patterns of base and sugar damage detectable by a postlabeling assay [Weinfeld, M., & Soderlind, K.-J. (1991) *Biochemistry 30,* 1091–1097] differ completely between DNA irradiated under oxic vs anoxic conditions; (iv) the presence of misonidazole under anoxic conditions does not increase the level of strand breakage but, like oxygen, significantly enhances the formation of 3′-phosphoglycolate end groups; (v) the presence of misonidazole during anoxic irradiation does not increase the yield of any other type of 'oxic' damage detectable by the postlabeling assay, such as thymine glycols; and (vi) misonidazole at concentrations greater than 50 μM affords significant protection to naked DNA, probably by OH radical scavenging, and both the nitroaromatic ring and methoxyisopropanol side chain contribute to this protective action.

Oxygen has long been known to enhance the sensitivity of cells to killing by ionizing radiation (Gray et al., 1953; Wright & Howard-Flanders, 1957). In the absence of oxygen, cells can be sensitized by nitroaromatic compounds (Adams & Cooke, 1969; Asquith et al., 1974) such as misonidazole [1-(2-nitro-1-imidazolyl)-3-methoxy-2-propanol; see Figure 1]. As a result, several of these compounds have undergone clinical trials directed toward eradication of radioresistant hypoxic tumour cells (Urtasun et al., 1976; Overgaard, 1989). While DNA damage produced by free radicals is considered to be responsible for cell killing induced by ionizing radiation, the molecular processes involved in radiosensitization by either oxygen or nitroaromatic compounds have yet to be fully elucidated (Wardman, 1987).

The influence of oxygen on damage to purines and pyrimidines within naked DNA has been clearly established (Fuciarelli et al., 1990). The yields of several lesions, including thymine and cytosine glycols, increase almost 10-fold when DNA is irradiated in air compared to irradiation under nitrogen. Other lesions, such as formamidopyrimidines, are not significantly affected. Quantitative data for the influence of nitroaromatic radiosensitizers on DNA base damage is limited. A number of groups (Varghese, 1975; Cadet et al., 1976; Nishimoto et al., 1983) have reported enhanced hydroxylation of thymine and uracil when the free bases are anoxically irradiated in the presence of a variety of radiosensitizers, but Remsen (1985) was unable to find any increase of thymine glycol in the DNA of cells irradiated with misonidazole. Assays for base damage in irradiated mammalian cells involving the use of the repair activity in extracts of *Micrococcus luteus* to monitor enzyme sensitive sites—later



Misonidazole R = $CH_2CH(OH)CH_2OCH_3$

Etanidazole R = $CH_2NHCOCH_2CH_2OH$

1-Methyl-2-nitroimidazole R = CH_3

FIGURE 1: Chemical structures of nitroimidazole sensitizers.

shown to be primarily oxidized pyrimidines (Jorgensen et al., 1987)—have provided conflicting results; Skov et al. (1979) found an elevation due to misonidazole of 1.6, while Hentosh (1988) was unable to find a statistically significant enhancement.

The most frequently observed lesions resulting from aerobic radiolysis of deoxyribose groups within DNA are strand breaks with 3′-phosphate and 3′-phosphoglycolate termini (Henner et al., 1982, 1983a,b; Feingold et al., 1988), although a number of other deoxyribose modifications have been reported (von Sonntag, 1987). Oxygen, misonidazole, and other nitroaromatic sensitizers have been shown to enhance the level of DNA strand breaks in hypoxically irradiated cells (Dugle et al., 1972; Skov et al., 1979; Taylor et al., 1987; Hentosh, 1988). The chemical nature of the strand breaks was not investigated in these studies. Indeed, there is relatively little information regarding the nature of strand break termini induced by irradiation under anoxia either in the absence or in the presence of nitroaromatic sensitizers. Beesk et al. (1979)

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showed by gas chromatography/mass spectrometry that aqueous solutions of calf thymus DNA irradiated under oxygen-free nitrous oxide contained 2,5-dideoxypentos-4-ulose bound to 5′-phosphate groups and 2,3-dideoxypentos-4-ulose bound to 3′-phosphate groups at strand breaks, and they further argued that 5′- and 3′-phosphate groups should also be present, although no direct evidence for this was provided. Kappen et al. (1989, 1991) have used the radiomimetic antitumor antibiotic neocarzinostatin, an agent that selectively generates C-4′- and C-5′-centered radicals, to show that misonidazole can substitute for oxygen in the ensuing strand breakage and that reaction at C-4′ gives rise to phosphoglycolate termini.

In this report we have compared the influence of nitrogen, oxygen, and misonidazole on damage to naked DNA irradiated in aqueous solution. A number of assays were employed, including a recently developed postlabeling technique (Weinfeld & Soderlind, 1991) that can measure thymine glycols, phosphoglycolates, and other, as yet unidentified, products, and a 5′-end-labeled oligonucleotide assay that can distinguish 3′-phosphate termini from 3′-phosphoglycolate termini (Henner et al., 1983b).

EXPERIMENTAL PROCEDURES

Chemicals. Oligo(dA)$_{12}$ (1.12 μg/μL), oligo(dT)$_{12}$ (5 AU_{260} units/mL), and poly(dT) (0.06 μg/μL) were purchased from Pharmacia (Baie D'Urfé, PQ); calf thymus DNA was from Sigma Chemical Co. (St. Louis, MO); 1-methoxy-2-propanol was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI); oxygen-free nitrogen and 5% O_2 in nitrogen were from Union Carbide Canada (Edmonton, AB); and [γ-^{32}P]ATP (4500 Ci/mmol) was from ICN Canada (Montreal, PQ). Misonidazole and etanidazole were gifts from Dr. J. D. Chapman (Fox Chase Cancer Center, Philadelphia, PA) and 1-methyl-2-nitroimidazole was generously provided by Dr. M. Tracy of SRI International (Menlo Park, CA). The radiosensitizers were stored frozen as 50 mM solutions in 10 mM sodium phosphate (pH 7.4).

Enzymes. DNase I (10 units/μL) was obtained from BRL (Burlington, ON); snake venom phosphodiesterase (*Crotalus atrox*, type IV, 1 g/mL, 22 units/mL) was from Sigma; T4 polynucleotide kinase (10 unit/μL) was from Pharmacia; and calf alkaline phosphatase (1 unit/μL) was from Boehringer Mannheim Canada (Dorval, PQ). Definitions of the units of these enzymes were those given by the supplier.

Postlabeling Assay. Solutions of calf thymus DNA (0.5 mg/mL) in 10 mM sodium phosphate (pH 7.4) and various concentrations of nitroimidazole were exposed at room temperature to a dose of 100 Gy, as measured by Fricke dosimetry, in a ^{60}Co Gammacell 220 (AECL, Ottawa, ON). Oxygen-free DNA solutions were prepared in glass dishes held in leak-proof aluminum chambers (Koch et al., 1979) by repeated exchanges with nitrogen, and they were subsequently irradiated in these chambers. A 300-μL aliquot of the irradiated DNA was then precipitated by the addition of 30 μL of 2.5 M sodium acetate and 660 μL of ethanol, collected by centrifugation, and redissolved in 150 μL of water to a concentration of 1.0 mg/mL.

Irradiated DNA samples (5 μg) were digested and postlabeled as described by Weinfeld and Soderlind (1991), except that labeling reactions contained 250 ng of digested DNA, 1.1 pmol (5 μCi) of ATP, and 5 units of kinase in 10 μL of 1× One-Phor-All*PLUS* buffer (Pharmacia). Gel electrophoresis equipment and conditions were as previously described (Weinfeld et al., 1990). Radiolabeled products were visualized by autoradiography, excised from the gel, and counted (without addition of scintillant).

3′-Phosphate Assay. The protocol employed was similar to that first described by Henner et al. (1983b). Oligo(dA)$_{12}$ (1.0 μL) was 5′-end-labeled using polynucleotide kinase (0.5 μL) in One-Phor-All*PLUS* buffer and [γ-^{32}P]ATP (0.5 μL). The labeled oligomer was purified by reverse-phase HPLC on a Whatman Partisil 10 ODS-1 (250 × 4.5 mm) column. The product eluted at 35 min using a gradient of 0.1 M ammonium acetate (pH 6.0) to 0.1 M ammonium acetate/methanol (60:40 v/v) over 40 min at a flow rate of 1 mL/min. After the ammonium acetate was removed by repeated lyophilization, the oligomer was resuspended in 40 μL of water. Solutions (400 μL) containing 5.0 μL of labeled oligo(dA)$_{12}$, 5.0 μL of poly(dT), in 10 mM sodium phosphate (pH 7.4), and various concentrations of nitroimidazole were exposed to a dose of 200 Gy under aerobic and anaerobic conditions as described above. A 350-μL aliquot was then lyophilized, and the residue was redissolved in 20 μL of water and divided into two parts. One part (10 μL) was further treated overnight with 1 μL of T4-polynucleotide kinase in 40 μL of 3′-phosphatase buffer (100 mM Tris-HCl, pH 6.5, 10 mM $MgCl_2$, and 5 mM mercaptoethanol). An equal volume of formamide loading buffer (90% formamide, 0.02% bromophenol blue, and 0.02% xylene cyanol in 1 × TBE) was added to each sample, and equivalent quantities of DNA were loaded onto a 20% polyacrylamide/7 M urea gel. Gel electrophoresis equipment and conditions were as previously described (Weinfeld et al., 1990). Radiolabeled products were visualized by autoradiography and quantitated by densitometry on an LKB Ultroscan XL laser densitometer.

Assay for Double-Strand Breaks. Irradiated and nonirradiated DNA samples (2.0 μg of the 1 mg/mL solution described above) were mixed with an equal volume of loading buffer (Maniatis et al., 1982) and run in a 0.8% agarose gel. An exterior lane was loaded with a 1-Kb DNA ladder (BRL, Burlington, ON), and the gel was run in TPE (80 mM Tris–phosphate, pH 8.0, 2 mM EDTA) buffer at 33 V. Following electrophoresis, gels were stained with ethidium bromide, irradiated from below with a UV transilluminator, and photographed with Polaroid Type 665 black and white film.

Assay for Single-Strand Breaks. DNA samples (1.0 μg) were mixed with an equal volume of alkaline loading buffer (50% v/v glycerol, 5× alkaline buffer, 10 mM EDTA, and bromophenol blue) and placed onto a 1.0% agarose gel. An exterior lane was loaded with a 1-Kb DNA ladder, and the gel was run in alkaline buffer (30 mM NaOH, 2 mM EDTA) at 66 V. The gel was later neutralized with two 30-min TBE (90 mM Tris–borate, pH 8.3, 25 mM EDTA) buffer washes, stained with ethidium bromide, and photographed as described above.

RESULTS

Base and Sugar Damage Generated in the Presence of Nitrogen, Air, and Misonidazole. Aqueous solutions of calf thymus DNA were exposed to 100 Gy ^{60}Co γ-radiation under aerobic (air) and anaerobic (nitrogen) conditions and examined by a postlabeling assay (Weinfeld & Soderlind, 1991). Figure 2 is an autoradiogram of the resulting polyacrylamide gel revealing that the pattern of DNA damage is markedly different under these separate conditions. The only bands that co-migrate are nitrogen bands h–j and air bands 6–8. However, the radioactivity in bands h–j is the same as that found in unirradiated controls and so represents damage already present in the commercially obtained calf thymus

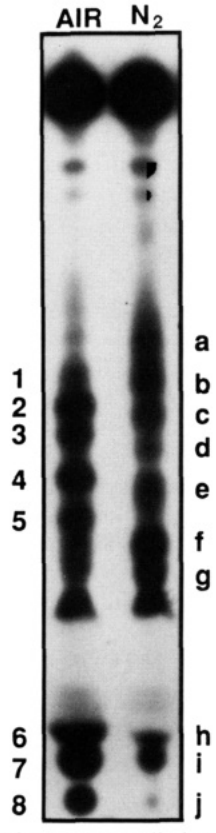

FIGURE 2: Influence of oxygen on radiation-induced DNA damage. Autoradiogram showing patterns of DNA lesions detected by the postlabeling assay (see Experimental Procedures) for calf thymus DNA solutions irradiated with 100 Gy under air and nitrogen. The large band at the top of each lane is oligo(dT)$_{12}$ used to consume the excess ATP after phosphorylation of the radiation products. Bands 1–3 contain dinucleotides with thymine glycols, and bands 6–8 contain phosphoglycolate products. Products in the other bands have yet to be identified.

DNA prior to irradiation. Bands 6–8 have been shown to consist exclusively of "dinucleotides" containing 3′-phosphoglycolates (Weinfeld & Soderlind, 1991). The only other products that have been identified are thymine glycol-containing dinucleotides present in air bands 1–3 (Weinfeld & Soderlind, 1991).

Figure 3A demonstrates the influence of increasing concentrations of misonidazole on DNA irradiated (100 Gy) under nitrogen. Under these conditions, two competitive actions of misonidazole are evident: (i) a sensitization revealed at lower concentrations (5–50 μM) and (ii) a protective effect seen most clearly at high drug concentration (0.05–5 mM). Figure 3B indicates that the sensitization is entirely restricted to the enhanced formation of 3′-phosphoglycolate groups and reaches a maximum of 2.7 ± 0.6 pmol/μg DNA at 100 Gy at a drug concentration of 50 μM, approaching the level of 4.8 ± 0.9 pmol/μg DNA at 100 Gy observed under air in the absence of any compound. Importantly, there is no evidence in Figure 3A for the formation of thymine glycols, i.e., the regions in the misonidazole lanes parallel to air bands 1–3 do not show any qualitative difference from the equivalent region in the N_2 lane. The latter result was confirmed by excising this region from each lane of the gel, eluting the radioactive products, and analyzing them by HPLC, as previously described (Weinfeld & Soderlind, 1991). In comparison to the N_2 lane, there was no increase, due to misonidazole, in the level of radioactivity that coeluted with a UV-detectable marker of synthetically prepared d-pApT^g [5′-phosphorylated 2′-deoxyadenylyl-(3′–5′)-thymidine glycol]. (A small peak

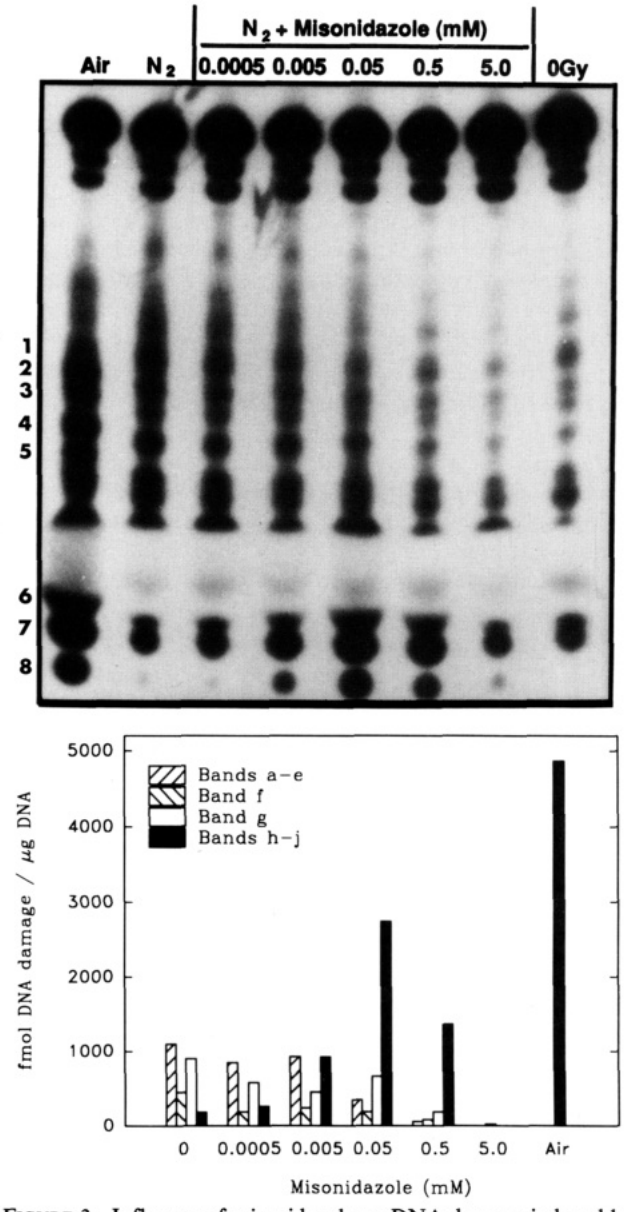

FIGURE 3: Influence of misonidazole on DNA damage induced by irradiation under anoxic conditions. (A, Top) Autoradiogram of a polyacrylamide gel comparing the postlabeled products resulting from DNA irradiation (100 Gy) under nitrogen in the presence of increasing concentrations of misonidazole and under air. Numbers 1–8 refer to the bands in the air lane. Bands arising from inorganic phosphate and pyrophosphate, which migrate well ahead of the phosphoglycolate products, have been omitted from the figure for the sake of clarity, but their radioactivity was taken into account in all calculations. (B, Bottom) Histogram generated by cutting and counting gel bands. Each value represents the mean of three independent determinations.

of radioactivity from the postlabeled DNA irradiated under nitrogen was observed with the same retention time as d-pApT^g. It amounted to approximately one-tenth of the d-pApT^g found in aerobically irradiated DNA.) Similar results were observed with etanidazole (Figure 1), another clinically tested nitroaromatic radiosensitizer (data not shown).

Strand Break Termini. The postlabeling assay cannot be used to detect 3′-phosphate termini, the other major 3′-end group observed at strand breaks induced by oxic irradiation (Henner et al., 1982, 1983a,b). The assay devised by Henner et al. (1983b) was, therefore, employed to determine the relative yield of the two types of end groups. Figure 4 is the autoradiogram of a gel showing 5′-labeled oligo(dA)$_{12}$ that had been annealed to poly(dT) and exposed to 200 Gy of γ-radiation. A ladder of 5′-labeled fragments is generated.

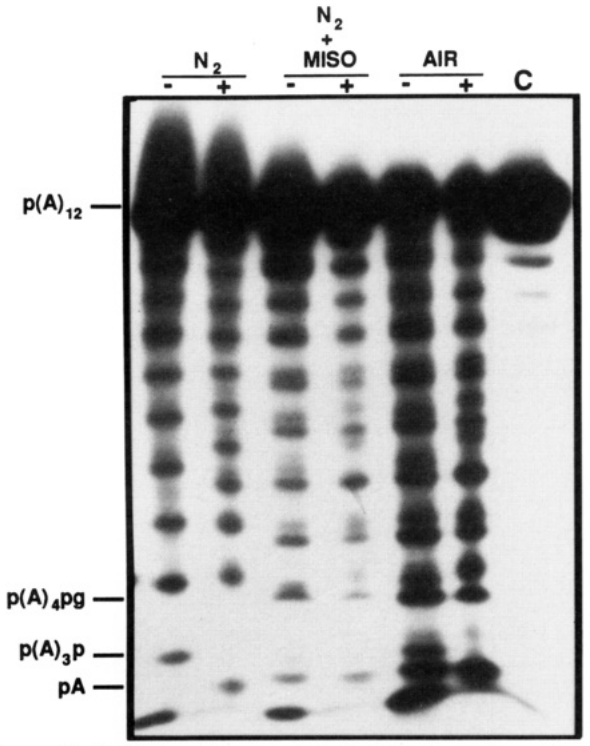

FIGURE 4: Radiation cleavage of 5′-labeled oligo(dA)$_{12}$. The 5′-labeled oligonucleotide, annealed to poly(dT), was irradiated (200 Gy) under various conditions. Before PAGE analysis, half of each sample was treated with the 3′-phosphatase activity of T4 polynucleotide kinase (+) to distinguish fragments with 3′-phosphate termini from those with 3′-phosphoglycolate termini (see Experimental Procedures).

To distinguish between fragments with 3′-phosphoglycolate (pg) and 3′-phosphate (p) termini, the mobilities of the latter were selectively reduced by treatment with the 3′-phosphatase activity of T4 PNK (+ lanes). Thus, in the (–) lanes showing doublets, the faster running band of each pair contains an oligonucleotide with phosphoglycolate termini and the slower band contains an oligonucleotide of the same length, but terminating with a 3′-phosphate. Irradiation under nitrogen produced only 3′-phosphate termini. On the other hand, irradiation under air gave rise to both types of strand breaks, as previously demonstrated by Henner et al. (1982). Densitometry of the bands p(A)$_5$p and p(A)$_5$pg to p(A)$_7$p and p(A)$_7$pg indicated an approximately 3-fold increase in total strand breakage under oxic versus anoxic irradiation and provided a ratio of 3′-phosphoglycolate to 3′-phosphate termini of ~1.6 for the aerobically generated fragments. Anoxic irradiation in the presence of 50 μM misonidazole also generated fragments with both types of termini. However, the sensitizer did not increase the total quantity of fragments of each length when compared with anoxic irradiation in the absence of the misonidazole, i.e., the sum of the activity in each doublet of bands in the N_2 plus misonidazole lane was almost equal to the activity in the single bands of each fragment length in the N_2 lane. The ratios of phosphoglycolate to phosphate termini of the three fragment lengths examined were in the range of 1.5–2.2.

The concentration dependence for misonidazole in this system is shown in Figure 5A. The appearance of doublets, and the relative density of the bands, indicates that 50 μM is the optimal concentration for phosphoglycolate production, in agreement with data obtained with calf thymus DNA. The tailing behind the oligo(dA)$_{12}$ band is suggestive of base damage, and again this appears to decrease with increasing misondiazole concentration. This damage is selectively

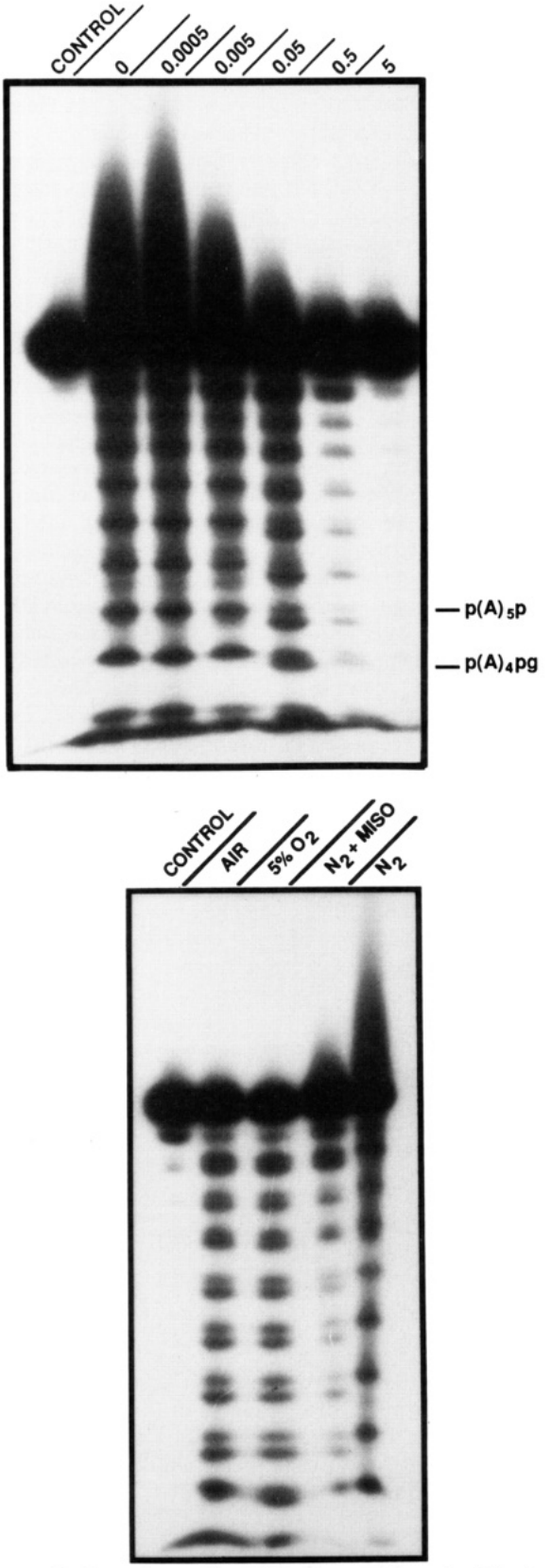

FIGURE 5: Dependence of strand break termini on misonidazole and oxygen concentration. (A, Top) 5′-Labeled oligo(dA)$_{12}$, annealed to poly(dT), anoxically irradiated with 200 Gy in the presence of increasing concentrations (mM) of misonidazole. (B, Bottom) Comparison of strand cleavage by irradiation under air, 5% oxygen/95% nitrogen, and nitrogen + 50 μM misonidazole.

produced by irradiation under anoxic conditions since there is little discernible tailing with oligo(dA)$_{12}$ irradiated under air. (One well-characterized lesion that fulfills these criteria

is the 8,5′-cycloadenosine product (Keck, 1968; Fuciarelli et al., 1988).

In air-saturated solution, the concentration of dissolved oxygen is 200–250 μM (depending on pressure and temperature). To rule out the possibility that the enhanced level of strand breaks following aerobic irradiation, in comparison to anaerobic irradiation in the presence of 50 μM misonidazole, was due to a 4-fold difference in the concentration of oxygen versus misonidazole, we carried out a similar experiment with a DNA solution saturated with 5% O_2/95% N_2. (The free radical scavenging effect of the nitroaromatic compound precluded the alternative of increasing the misonidazole concentration to 200 μM.) It is clear from Figure 5B that reducing the oxygen concentration to one-fourth has little effect.

Radioprotection by Misonidazole. The protective effect of misonidazole seen in Figure 3 was further investigated. If, as has been proposed (Nishimoto et al., 1983), the mechanism of protection is by hydroxyl radical scavenging, then the protection should also be seen when DNA is irradiated in air-saturated solution. Figure 6 shows an autoradiogram of a polyacrylamide gel of labeled products derived from DNA irradiated aerobically in the presence of increasing concentrations of misonidazole and the histogram generated by cutting out and counting individual bands. The figure illustrates that under these conditions misonidazole acts as an efficient radioprotector at concentrations between 50 μM and 5.0 mM. Indeed, at the highest misonidazole concentration virtually no base or sugar damage is detectable. Importantly, the histogram also reveals that all the detectable lesions are equally reduced, a further indication of a mechanism involving OH-radical scavenging.

Damage detected by the postlabeling assay is restricted to base and sugar lesions that hinder or halt the activity of snake venom phosphodiesterase. DNA samples, therefore, were analyzed for single- and double-strand breaks by alkaline (Figure 7A) and neutral (Figure 7B) agarose gel electrophoresis, respectively, in order to examine if the protection by the nitroimidazole was a more general phenomenon. It was observed that the protection seen at the high misonidazole concentrations with the postlabeling assay also applied to single- and double-strand break formation.

Compounds containing hydroxyl groups, such as 2-propanol, are known to protect DNA from the effects of ionizing radiation (von Sonntag, 1987). Misonidazole contains a methoxy-2-propanol group (Figure 1), and it was possible that this side chain was responsible for the observed radioprotection. To test this, the experiments described for misonidazole in air were repeated with *N*-methyl-2-nitroimidazole and 1-methoxy-2-propanol, model compounds for the misonidazole ring and hydroxyl side chain, respectively. The autoradiogram and histogram in Figure 8 show that both moieties contribute toward the protection by misonidazole, but the contribution by the nitroaromatic ring is more pronounced.

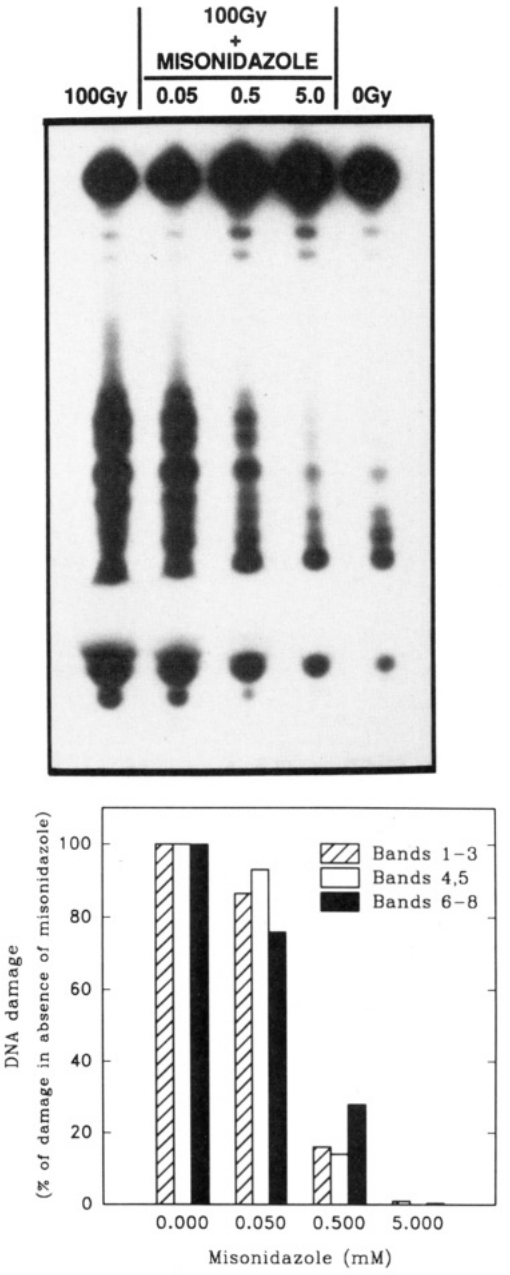

FIGURE 6: Influence of misonidazole on DNA damage induced by irradiation under aerobic conditions. (A, Top) Autoradiogram of postlabeled products derived from irradiation (100 Gy) of DNA under aerobic conditions in the presence of increasing concentrations of misonidazole. (B, Bottom) Histogram to show the extent of radioprotection afforded by misonidazole. The depicted values are the means derived from three independent determinations.

DISCUSSION

Oxidative DNA Damage Induced by Misonidazole and Its Biological Significance. In this study we have compared damage deposition in DNA irradiated in the absence and presence of oxygen and examined the influence of misonidazole under both conditions. The effect of oxygen is clear (Figures 2 and 4); it radically alters the pattern of base damage and significantly enhances the level of strand cleavage, especially strand breaks with phosphoglycolate termini. Nitroaromatic compounds that sensitize radioresistant hypoxic cells to ionizing radiation are believed to mimic oxygen. A primary aim of this study, therefore, was to determine which lesions, if any, are produced in common by oxygen and misonidazole. With native DNA, 3′-phosphoglycolates appear to be the sole detectable oxic-type lesion generated by misonidazole and etanidazole—no oxidative base damage was observed. This differs from the anoxic irradiation of free bases, for which others have shown that the presence of nitroaromatic radiosensitizers enhances the formation of certain radiolytic products, especially thymine glycol (Cadet et al., 1976; Nishimoto et al., 1983). The equal reduction in yields of thymine glycols and phosphoglycolates caused by misonidazole under oxic conditions (Figure 6) rules out the possibility of selective protection of thymine in native DNA by the nitroimidazole. Steric considerations may provide an alter-

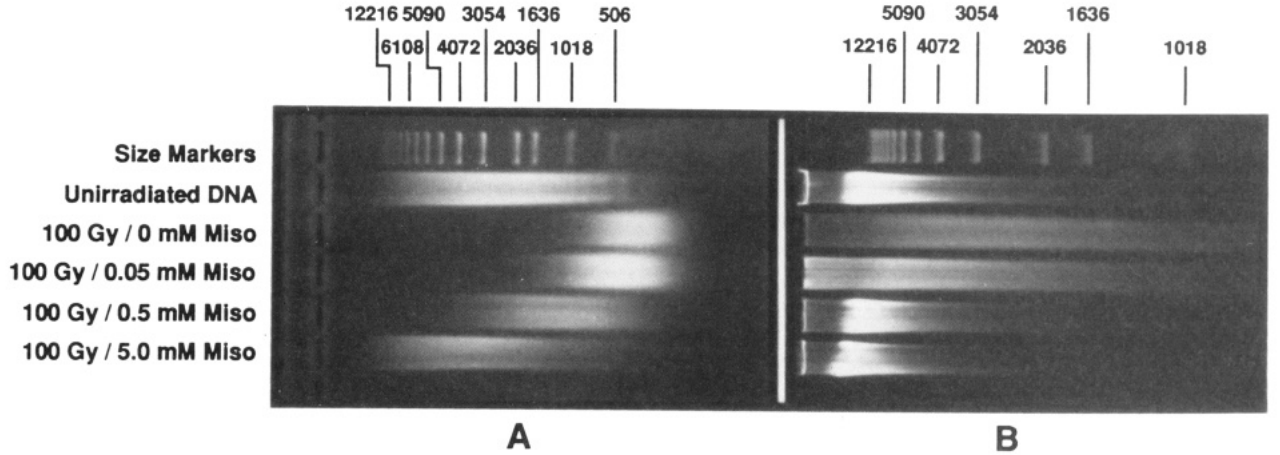

FIGURE 7: Influence of misonidazole on DNA strand breaks induced by irradiation under aerobic conditions. Single- and double-strand breaks were analyzed by alkaline (A) and neutral (B) agarose gel electrophoresis. The sizes of the molecular weight markers are given in base pairs.

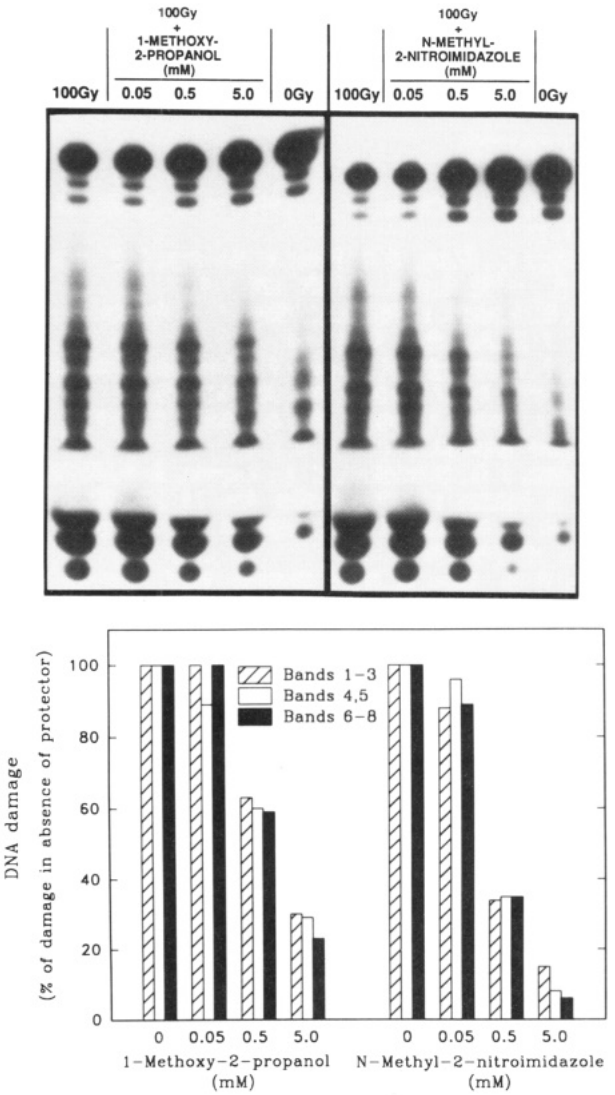

FIGURE 8: Radioprotection by 1-methoxy-2-propanol and *N*-methyl-2-nitroimidazole. These represent model compounds for the side chain and ring components, respectively, of misonidazole. (A, Top) autoradiogram of postlabeled products derived from irradiation (100 Gy) of DNA under aerobic conditions in the presence of increasing concentrations of each compound. (B, Bottom) Histogram showing the extent of radioprotection. The values are derived from three independent determinations.

native explanation for these observations. Steenken and Jagannadham (1985) showed that pyrimidine glycols are formed from the free base as a result of a sequence of reactions in which a hydroxyl radical adds to C-5 to give a 6-yl radical, which can then form a covalent intermediate by reaction with the nitro group on a nitroaromatic compound. This species, in turn, undergoes hydroxide ion-catalyzed heterolysis to yield the oxidized pyrimidine. They confirmed the presence of the nitroxide radical intermediate by ESR. Inspection of a model for the structure of B-DNA reveals that the C-6 of thymidine is held in close proximity to O-1′ and H-5″ and to H-2′ and the base of the adjacent 5′ nucleotide. Thus, in native DNA, radicals centered on C-6 may be too sterically hindered to undergo additon with a relatively bulky nitroaromatic sensitizer but would still be able to react with the much smaller O_2 molecule. The C-4′ position, on the other hand, is well exposed, so that a radical generated here would more easily be able to form a covalent intermediate with misonidazole and subsequently give rise to a strand break with a phosphoglycolate end group.

The presence of nitroaromatic sensitizers during anoxic irradiation of mammalian cells has been shown to increase the initial yield of strand breaks (Dugle et al., 1972; Skov et al., 1979; Taylor et al., 1987; Hentosh, 1988), yet in our systems we did not see such an increase (Figure 4). Even at the dose of misonidazole (50 μM) yielding the maximal level of phosphoglycolate termini, the number of strand breaks generated in oligo$(dA)_{12}$ was only equal to the number produced in the absence of sensitizer. This discrepancy is a manifestation of the considerably lower DNA concentration used for our test tube experiments [$\sim 10^3$-fold for the postlabeling assay and $\sim 10^6$-fold for the oligo$(dA)_{12}$ assay] than is found in mammalian cell nuclei. Under our experimental conditions, hydroxyl radical scavenging by misonidazole is seen at 50 μM–5 mM, while in cells, concentrations of scavenger have to approach 100 mM before any appreciable protection is observed (Chapman et al., 1973). Elevated strand breakage in cellular DNA is observed using 5–15 mM misonidazole in the media, which probably results in an intracellular concentration of the order of 1 mM (Dennis et al., 1985). Thus, the effective sensitizing dose of misonidazole in the cells can be at least 20-fold greater than the optimal concentration observed in either the postlabeling or oligo-$(dA)_{12}$ assays.

Our results provide a physical basis for the conclusion of Remsen (1985) and Hentosh (1988) that misonidazole does not elevate the yield of thymine glycols in cellular DNA and that these lesions are unlikely, therefore, to contribute to the radiosensitization by this agent. On the other hand, they point to the need now to determine whether misonidazole also

changes the termini found at strand breaks of hypoxically irradiated cells and whether a change of termini from phosphate to phosphoglycolate, as well as an increase in strand breaks, contributes to the enhanced toxicity of oxygen and sensitizers. Enzymes recently purified from mammalian sources that remove 3′-phosphoglycolates also harbor 3′-phosphatase activity (Chen et al., 1991), but it has not yet been determined if the enzymes act on the different end groups at the same rate. The 3′-termini of DNA at strand breaks in aerobically irradiated monkey CV-1 cells have been shown to contain phosphate and phosphoglycolate groups (Feingold et al., 1988; Bases et al., 1990). The nature of the termini in anoxically irradiated cells has not been determined, but on the basis of our studies with naked DNA we would anticipate that only 3′-phosphate groups would be found.

3′-End-Group Analysis and Strand Cleavage Mechanisms. Several mechanisms have been proposed for radiation-induced DNA cleavage (von Sonntag et al., 1981; von Sonntag, 1987). Early steps involve hydrogen abstraction, by hydroxyl radicals or by base radicals, from either the 4′ or 5′ carbon atoms of deoxyribose groups. It is thought that strand breaks with 3′-phosphate termini arise from either the C-4′ or C-5′ radicals, but 3′-phosphoglycolate termini result from oxygen addition to the C-4′ radical. We observed that irradiation under nitrogen gave only phosphate termini. Oxic irradiation increased the number of strand breaks, but the absolute quantity with 3′-phosphate termini appears to be the same as produced under anoxic conditions, with all of the additional strand breaks having phosphoglycolate termini. It can, therefore, be asked whether the 3′-phosphate termini under both anoxic and oxic conditions arise by the same free radical and if that free radical is at C-4′ or C-5′. The implication is that if the phoshate termini arise simply from C-5′ radicals, then, under nitrogen, either no C-4′ radicals are generated or all of the C-4′ radicals have to be resolved by an alternative mechanism(s), possibly intramolecular hydrogen abstraction, that does not lead to a strand break. Alternatively, if the phosphate groups stem from C-4′ radicals, then this would imply that under nitrogen there is competition between two reactions—one leading to strand breaks with phosphate termini and a nonstrand breaking reaction. In air this is extended, by potential oxygen addition, to become a three-way competition. Misonidazole can substitute for oxygen at both C-4′ and C-5′ to augment strand cleavage (Kappen et al., 1989, 1991) with phosphoglycolate and phosphate termini, respectively. That the addition of misonidazole under anoxic conditions led to the enhanced formation of phosphoglycolate with a concomitant decrease of phosphate indicates that the phosphate termini in the absence of sensitizer also arise from C-4′ radicals. The greater reactivity of oxygen, due to its small size and high electron affinity (Wardman & Clarke, 1976a,b), would account for the greater total strand cleavage induced by aerobic irradiation.

The observation that the 3′ strand break termini resulting from anoxic irradiation consist almost exclusively of phosphate groups is novel. Unlike Beesk et al. (1979), we saw no evidence for the formation of an end group retaining a deoxyribose fragment. Such an end group would be resistant to the 3′-phosphatase activity of T4 polynucleotide kinase. It remains to be seen if the difference in end groups could be the result of the presence of the nitrous oxide (used to convert solvated electrons to hydroxyl radicals) in the irradiated DNA solution of Beesk and co-workers. In their studies with neocarzinostatin, Kappen et al. (1991) found that under anaerobic conditions in the absence of misonidazole very limited strand cleavage occurred, but instead DNA–neocarzinostatin adducts were formed. Interestingly, 3′-phosphate termini were seen to be the sole type of end group when a 5′-end-labeled oligonucleotide was irradiated in frozen solution at 77 K or as a "dry" film at room temperature (Cullis et al., 1989). These conditions are used to examine direct damage, i.e., damage caused directly to the DNA molecule and not indirectly through the reactions of aqueous radiolysis products such as hydroxyl radicals. The irradiation by Cullis et al. (1989) was performed under aerobic conditions, and yet no phosphoglycolate termini were observed. This would suggest that, in the direct mechanism, strand breaks are not generated through a C-4′ radical intermediate or that, when DNA is irradiated in frozen solution at 77 K or as a "dry" film, molecular oxygen does not react with C-4′ radicals.

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