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Activation of neocarzinostatin chromophore and formation of nascent DNA damage do not require molecular oxygen

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ABSTRACT

Thiol-activated neocarzinostatin chromophore abstracts tritium from the 5', but not from the 1' or 2' positions of deoxyribose in DNA and incorporates it into a stable, non-exchangeable form. The abstracted tritium remains covalently associated with the chromophore or its degradation product after treatment with acid or alkali, respectively. Drug activation and the consequent hydrogen abstraction reaction, presumably generating a carbon-centered radical at C-5', do not require molecular oxygen but have a dose-dependent relation with thiol. Under aerobic conditions, where base release and DNA strand breaks with nucleoside 5'-aldehyde at the 5'-ends are produced, hydrogen abstraction from C-5' parallels these parameters of DNA damage. It is possible to formulate a reaction scheme in which the carbon- centered radical at C-5' is an intermediate in the formation of the various DNA damage products found under both aerobic and anaerobic conditions.

INTRODUCTION

The precise roles of thiol and O_2, the two cofactors required (1) in DNA strand breakage by the nonprotein chromophore of the antitumor antibiotic neocarzinostatin (NCS) (2-5) remain incompletely understood. DNA strand breakage occurs only if all the reaction components are simultaneously present, indicative of the extreme lability of the intermediates involved. DNA damage is manifested mainly by the production of single strand breaks which bear a nucleoside 5'-aldehyde at their 5'-termini (6,7), alkali-labile breaks (7,8) and the release of free base (9,10). Labile DNA-NCS chromophore adducts with covalent linkage to the DNA sugar (11,12) appear as minor products and have properties consistent with their being precursors of the strand breaks in DNA. Oxidation at the C-5' of nucleosides in DNA is involved both in the production of the 5'-aldehyde (7) and the labile adducts (13). On the other hand, in the absence of O_2 a stable DNA-NCS chromophore adduct is formed (12). Nitroaromatic radiation sensitizers such as misonidazole can substitute for O_2 in DNA strand cleavage, but a strand gap is produced with phosphate at both ends (14).

While there is ample evidence that thiol is involved in drug activation it

is not known whether O_2 is involved in the generation of an active species of the drug that attacks DNA, in addition to its role in "fixing" the nascent DNA lesion. It has been proposed that thiol addition to the chromophore (15) generates a reactive species, possibly a free radical, that abstracts a hydrogen from the 5'-carbon of deoxyribose in DNA to form a carbon-centered radical that in the presence of O_2 undergoes subsequent reactions leading to 5'-aldehyde formation and a strand break (6,16). Support comes from the recent findings that 1) in an aerobic reaction in which DNA strand breaks are produced, thiol-activated NCS chromophore abstracts a 5'-hydrogen from 5'-[^{3}H]thymidine-labeled poly(dA-dT).poly(dA-dT) and incorporates it in a non-exchangeable form (17), 2) dioxygen is the source of the oxygen incorporated into thymidine 5'-aldehyde at the DNA strand break (18), and 3) kinetic studies show that in the DNA-degrading reaction, reaction of the chromophore with thiol precedes the reaction with O_2 (16). The experiments presented in this paper show that oxygen is not required in the activation of the drug for the hydrogen abstraction reaction and suggest a reaction scheme, involving the intermediacy of a carbon-centered radical at C-5', that is consistent with the various reaction products formed under both aerobic and anaerobic conditions.

MATERIALS AND METHODS

NCS (clinical form) kindly supplied by Dr. W.T. Bradner of the Bristol-Myers Company was dialyzed against distilled H_2O and lyophilized after the addition of sodium citrate, pH 4.0 required to give a final level of 0.02 M prior to extraction of the chromophore with 100% methanol. The chromophore solution (0.3-0.4 mM) was stored in the dark at -70°.

λ DNA with radiolabels at various positions (5'[^{3}H]; [5-methyl-^{3}H]; 5-methyl, 1',2'[^{3}H]; [U-^{14}C]) of thymidine was prepared as previously described (7). The specific activities of the DNAs, cpm per μg, are: [5-methyl-^{3}H], 16540; 5'[^{3}H], 33172; [U-^{14}C], 990; 5-methyl, 1',2'[^{3}H], 5434 of which 40% radioactivity is equally distributed in the 1' and 2' positions. Misonidazole was from Hoffmann-La Roche.

Drug Treatment

The standard reaction (0.3-0.75 ml) contained 10 mM sodium citrate, pH 4.0, 1 mM diethylenetriaminepentaacetic acid, 100 mM Tris-HCl, pH 9.0, 1 mM glutathione, and DNA and NCS chromophore at levels indicated in the legends. The anaerobic reactions were performed in micro Warburg vessels. The components of the reaction were split such that the chromophore was bound to DNA at the acidic pH and following removal of O_2 from the system thiol and pH 9.0

buffer were added (which raises the pH to 8.0) to initiate the reaction. The side arm thus contained in 12% of the total reaction volume Tris-HCl, pH 9.0, and glutathione; the rest of the components (chromophore being added last) were placed in the main chamber. The contents of the flask were frozen in liquid N_2 and were evacuated at $<10^{-3}$ torr in a Kontes high vacuum setup. Freeze-evacuate-thaw procedure was repeated six times before the thawed contents of the two chambers were mixed. The reaction was then allowed to proceed in the dark for 30 min at room temperature. Misonidazole, when present, was in the main chamber. Aerobic reactions to be compared with anaerobic reactions were similarly treated, but at the end of evacuation the flasks were opened and flushed with O_2 for 1 min before mixing the components of the two chambers. Regular aerobic reactions were carried out in test tubes following the same order of addition of the components as described above, but the samples were not frozen or evacuated. Inactivation of NCS chromophore was done by preincubation with the thiol in 20 mM Tris-HCl, pH 8.0 at room temperature for 20 min.

Product Analysis

^3H abstracted from the DNA into NCS chromophore was measured by reverse phase high pressure liquid chromatography (HPLC) using a μBondapak C_{18} column (Waters Associates). The reaction was loaded onto the column either directly or after removal of DNA. In the latter case 3 M Na acetate was added to aliquots (200 μl) of the reaction to the final level of 0.3 M, followed by three volumes of ethanol. After chilling the mixture at -70° for 1 hr, DNA was pelleted by centrifugation at 10,000 x g for 15 min. The supernatant which contained the chromophore was evaporated in a Speed Vac Concentrator (Savant Instruments). The residue was taken up in H_2O containing 10% methanol and loaded onto the column. The column was eluted with an 80 min convex gradient of 0-80% methanolic ammonium acetate (10 mM, pH 4.8) at 1 ml/min at ambient temperature. Absorbance (254 nm) and fluorescence (excitation at 340 nm, emission > 418 nm) were constantly monitored. Radioactivity in the fractions was determined after addition of 10 ml hydrofluor (National Diagnostics). HPLC profiles of chromophore absorbance, fluorescence and radioactivity obtained with the chromophore recovered in the supernatant (after removal of DNA) were qualitatively and quantitatively identical to those from the whole reaction. HPLC on the Altex column (Rainin Instruments) was performed under conditions described above. In some cases the column was eluted initially for 20 min with aqueous 10 mM ammonium acetate pH 4.8 followed by the 80 min gradient. In experiments where the ^3H-containing peaks were to be isolated for further

studies, the HPLC fractions comprising the peak of interest were pooled and concentrated as above. The residue was dissolved in H_2O.

In order to estimate total releaseable thymine the drug-treated DNA was made 0.3 M in NaOH. The samples were then heated at 90° for 45 min and neutralized with HCl. After the addition of 0.5 µg of non-radioactive thymine as marker, thymine was separated by HPLC (C_{18} column) using an isocratic elution with 10 mM ammonium acetate, pH 4.8.

RESULTS

λ DNA radiolabeled at various positions of its thymidine was treated with NCS chromophore in the presence of a thiol and O_2, and the reaction mixtures were analyzed for chromophore-bound radioactivity. Preliminary experiments using λ DNA labeled with H^3 in the 1', 2', 5' or 5-CH_3 positions of thymidine or uniformly with ^{14}C in thymidine as substrate showed that transfer of radioactivity to the chromophore occurred only from 5'[^{3}H]thymidine-labeled DNA, confirming and extending previous findings (17). In subsequent experiments in order to measure both the 5'[^{3}H] abstracted into the chromophore and the DNA damage produced in the same reaction tube an equimolar mixture of 5'[^{3}H]- and [methyl-^{3}H]thymidine-labeled λ DNA was used as substrate. Since most of the DNA damage can be accounted for by single strand scissions with 5'-terminal thymidine 5'-aldehyde (which decomposes in hot alkali to release thymine (7)) and spontaneous release of thymine, the total thymine released after alkali treatment of the drug-treated DNA was used as a measure of total DNA damage.

In the aerobic reaction (Fig. 1A) spontaneously released thymine radioactivity elutes at 8 min coincident with the non-radioactive marker. The peak of radioactivity associated with the main UV-absorbing and fluorescent peak of the chromophore has a retention time of 45 min. Extensive DNA damage occurred in the reaction (Table I). Under anaerobic conditions where DNA damage was inhibited 88%, as shown by both spontaneous thymine release (Fig. 1B) and total DNA damage (Table I), the amount of ^{3}H abstracted into the chromophore (Fig. 1B) is nearly the same as in the aerobic reaction (Fig. 1A). The lower amount of ^{3}H abstraction than aerobic DNA damage is presumably due to a ^{3}H isotope effect. Addition of misonidazole to the anaerobic reaction restored the DNA damage (Table I), and this is reflected in a marked increase in the spontaneous release of thymine (Fig. 1C), as has been shown earlier (14). In the misonidazole-dependent reaction, ^{3}H radioactivity coincident with the chromophore peak is about 60% higher than that in the aerobic or anaerobic reactions, and there is a corresponding increase in the absorbance and fluorescence. On the

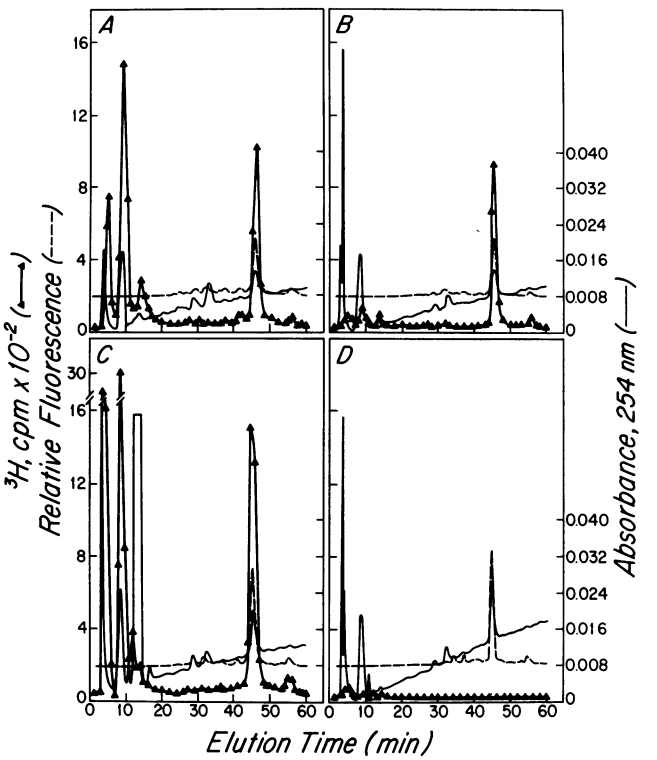


Figure 1. Abstraction of 5'[^{3}H] from DNA into NCS chromophore. An equimolar mixture of 5'[^{3}H]- and [methyl-^{3}H]thymidine-labeled λ DNA (107 μM of each) was reacted with 25 μM NCS chromophore under standard conditions except for the use of 10 mM glutathione in reaction D. After removal of DNA, the chromophore equivalent to 179 μl of the reaction was analyzed by HPLC on a μBondapak C_{18} column. A, aerobic; B, anaerobic; C, anaerobic containing 40 mM misonidazole; D, NCS-chromophore inactivated by preincubation with thiol replaced the active drug in an otherwise complete aerobic reaction. An aerobic reaction similar to D but with active drug gave 2559 cpm of 5'[^{3}H] incorporated into the chromophore peak eluting at 45 min. The absorbance peak at 8 min is due to the non-radioactive thymine marker, and that at 14 min in C represents misonidazole. Elution was continued for 80 min, but no UV-absorbing, fluorescent or radioactive material appeared after 60 min.

other hand, the DNA damage is not significantly different from that in the aerobic reaction. Since the specific activity of ^{3}H in the chromophore peak (cpm per unit absorbance) is the same in all three cases (Table I), it is possible that the increase in the main chromophore peak obtained with misonidazole is due to misonidazole facilitating in some way the recovery of the hydrophobic chromophore. The overall profiles of chromophore absorbance,

TABLE I. Analysis of 5'[^{3}H] Abstracted from DNA into NCS Chromophore and DNA Damage.

	5'[^{3}H] in Chromophore		Thymine Released (pmole)		
Condition	(pmole)	(pmole/unit absorbance)	Spontaneous	Alkali-dependent	Total
Aerobic	156	51	228	362	590
Anaerobic	162	47	22	52	74
Anaerobic + misonidazole	266	51	418	14	432

The data for 5'[^{3}H]abstraction and spontaneous thymine release have been computed from experiments shown in Fig. 1. A second portion of the reaction mixture after treatment with alkali was analyzed for total releaseable thymine as described in Materials and Methods. The values are the mean of triplicates (anaerobic) and duplicates (aerobic and anaerobic + misonidazole). The mean radioactivity in fractions preceding and succeeding the peak fractions (<100 cpm) has been subtracted as background per fraction. The specific activities of 5'[^{3}H] and [methyl-^{3}H]thymine are each 10.7 cpm/pmole. The area under the chromophore absorbance peak was quantitated by cutting it out and weighing it in an analytical balance, and is expressed in arbitrary units.

fluorescence and radioactivity are quite similar in all three cases. In addition to the major 45 min peak there is a minor peak of radioactivity (56 min) also coincident with a minor peak of absorbance and fluorescence. In a reaction (Fig. 1D) where the NCS chromophore was inactivated by preincubation with thiol prior to its binding to DNA the magnitude of the UV absorbing peak eluting at 45 min is the same as that obtained in the complete reaction with the active drug, but there is no ^{3}H radioactivity associated with it and no DNA damage. There are also some early eluting (4-5 min) DNA sugar damage products (Figs. 1A and C), the characterization of which is in progress.

DNA damage by NCS is dependent on thiol as is also 5'[^{3}H] abstraction into the chromophore. Fig. 2 shows that in the aerobic reaction there is a good correlation between the amount of 5'[^{3}H] abstracted into the chromophore and the extent of DNA damage over a wide range of thiol concentrations; at low levels of thiol there is a nearly linear increase both in 5'[^{3}H] abstraction and DNA damage. There is also a corresponding linear increase in the absorbance and fluorescence associated with the 5'[^{3}H] peak (not shown). At the highest level of thiol used there is a small but significant reduction in both DNA damage and ^{3}H-abstraction, probably due to a faster rate of inactivation of the drug prior to its interaction with DNA.

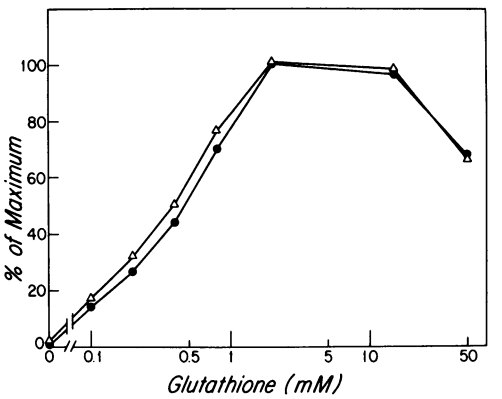


Figure 2. Dependence of 5'[^{3}H] abstraction from DNA into NCS chromophore and DNA damage on thiol. In an aerobic reaction containing DNA and NCS chromophore as in Fig. 1, glutathione levels were varied. One portion of the reaction mixture (179 µl, after removal of DNA) was analyzed by HPLC (C_{18} column) to determine the chromophore-bound ^{3}H. A second aliquot was used to estimate the total thymine released as described in Materials and Methods. One-hundred percent activity found at the optimal glutathione level (2 mM) is 186 pmoles 5'-[^{3}H] abstracted into chromophore and 628 pmoles total released thymine. ●-●, 5'[^{3}H] abstracted; Δ-Δ, total thymine released.

In order to investigate the stability of the chromophore-associated [^{3}H] radioactivity we isolated the 45 min peak by HPLC on the µBondapak C_{18} column and after treatment with alkali or acid reanalyzed it on the Altex column. The original isolated peak is stable under neutral conditions at ambient temperature and has a retention time of 72 min (Fig. 3A). The absorption spectrum (not shown) of the isolated peak was similar to that reported earlier for thiol-treated chromophore (19). On heating in NaOH for short periods (Figs. 3B and C) there is a decrease in the radioactivity, absorbance and fluorescence of the 72 min peak with the concomitant appearance at 84 min of a new radioactive product which is UV-absorbing and fluorescent. On longer heating (Fig. 3D), the 84 min peak degrades into several minor radioactive products including a peak eluting at 4 min that has a significant amount of radioactivity. In addition, two degradation products appear, a major one that is UV-absorbing but nonfluorescent (35 min) and a minor one that is UV-absorbing and fluorescent (56 min), but both without radioactivity. On the other hand, heating the 72 min peak in 0.5 M HCl at 90° for 20 min does not significantly affect its HPLC profile (not shown). These experiments clearly show that the ^{3}H is associated with the chromophore in a stable covalent linkage.

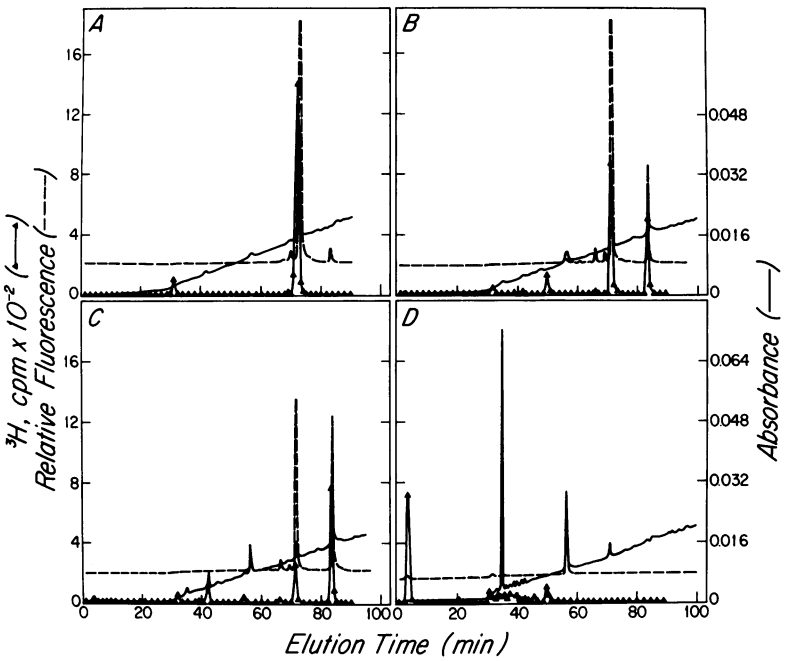
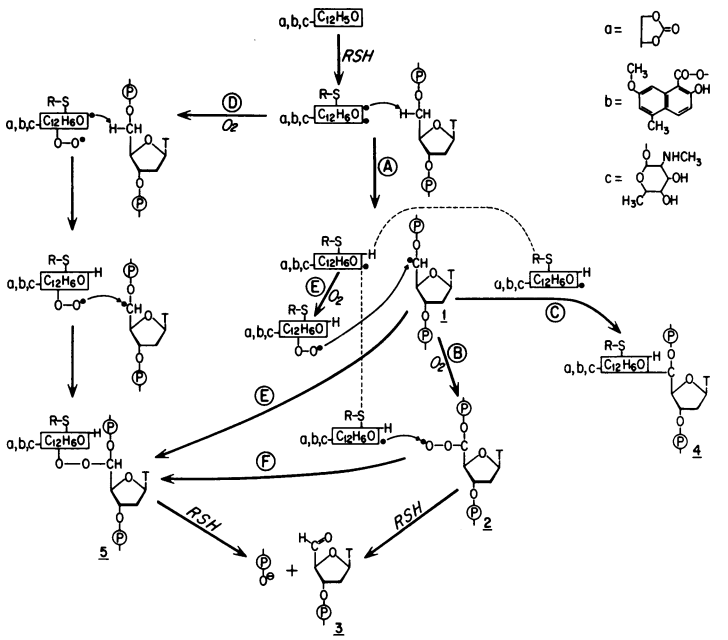


Figure 3. Effect of alkali-treatment on NCS chromophore-bound ^3H radioactivity. From a standard 3 ml aerobic reaction containing 5'[^{3}H]thymidine-labeled λ DNA (255 μm) and NCS chromophore (25 μM) the main chromophore peak containing the incorporated [^{3}H] was isolated by HPLC on the C_{18} column. Equal portions of it were heated in 0.2 M NaOH (B, C, D) at the temperatures and times indicated and were neutralized with HCl. The samples were then analyzed by HPLC on an Altex column. The initial 20 min elution was isocratic with 10 mM ammonium acetate, pH 4.8 followed by the 80 min gradient. A, no treatment; B, 1.5 min, 66°; C, 3 min, 66°; D, 1 hr, 90°.

DISCUSSION

Studies on the kinetics and stoichiometry of the uptake of thiol and O_2 showed that one mole of O_2 and two moles of thiol per chromophore were taken up under conditions of efficient DNA degradation (16). Under anaerobic conditions only one mole of thiol was consumed, but the activation of the drug as judged by fluorescence changes was as complete as in O_2 despite the nearly complete inhibition of DNA degradation as measured by total thymine release. Whether the thiol-activated NCS can directly induce nascent damage on the DNA, or the thiol-activated intermediate has to further interact with O_2 to form a reactive species prior to the attack on the DNA was not clear from these and other studies. The current finding that thiol-activated NCS chromophore, under strict anaerobic conditions, abstracts from the deoxyribose of DNA as much



SCHEME I

5'[^{3}H] as in the presence of O_2 clearly shows that O_2 is not a necessary participant in its primary attack on the DNA. It is very unlikely that the tritium abstraction found under anaerobic conditions is due to trace contaminant O_2. We have calculated that at least 10 times as much DNA damage products are formed under aerobic conditions as there is O_2 potentially available under the anaerobic (10^{-3} torr) conditions used. Since tritium abstraction is the same under both conditions, the aerobic damage should be a quantitative reflection of the hydrogen abstraction reaction.

Although chromophore, inactivated by preincubation with thiol, failed to abstract any 5'[^{3}H] from DNA or to produce DNA damage, it produced the same amount of UV-absorbing and fluorescing material eluting at 45 min (Fig. 1D) as in a parallel reaction containing active drug. This is not surprising since activation of the drug precedes its inactivation. Whatever be the mechanism of inactivation, it is possible that the species that has abstracted the hydrogen atom from DNA and the species representing the inactivated molecules are either structurally the same (e.g., if it accepts a hydrogen from thiol instead of from DNA) or too close to give a different elution and absorbance profile.

Based on the accumulated data we propose the possible sequence of reactions shown in Scheme I. The first step involves the generation of an active

species of the drug (shown as a free radical) upon thiol addition to the highly unsaturated C_{12} subunit of the chromophore (15,16). The activated drug abstracts a hydrogen from the 5'-carbon of deoxyribose (pathway A) to generate a carbon-centered radical (1) on the DNA, the fate of which depends on whether or not O_2 is available. Addition of O_2 to the 5'-carbon-centered radical (pathway B) gives rise to a peroxyl radical (2) (20) which degrades in the presence of thiol to produce DNA strand breaks with 5'-terminal nucleoside aldehyde (3). The involvement of thiol in the degradation of a peroxyl structure to an aldehyde is compatible with experiments in which at least a second molecule of thiol is consumed in the reaction beyond the one used in the initial activation of the drug (16). In the absence of O_2 the carbon-centered radical on the deoxyribose (1) may instead react with the chromophore containing the abstracted hydrogen (pathway C) to form a stable covalent chromophore-DNA adduct (4), possibly by addition of the C-5' radical across a double-bond of the chromophore, or be repaired by abstraction of a hydrogen from thiol. This analysis, involving a common DNA intermediate, is consistent with recent studies (12) showing that there is an inverse relationship between stable chromophore-DNA adduct formation in anoxia and DNA strand breakage and base release in O_2.

While the data reported in this paper clearly show that drug activation and hydrogen atom abstraction from the 5'-position of deoxyribose in DNA can proceed in the absence of O_2, they do not rule out the possibility that in the presence of O_2 the activated chromophore can add O_2 to form a peroxyl derivative of the activated chromophore (pathway D). The peroxyl radical on the drug cannot itself be responsible for hydrogen atom abstraction, since the latter is incorporated into the chromophore in a non-exchangeable form. Nevertheless, a radical elsewhere in the peroxyl form of the drug could abstract the hydrogen atom and then react with the carbon-centered radical on the DNA by way of its peroxyl radical to form a labile adduct, possibly with the structure shown in (5), that may be an intermediate in strand break formation (11-13). Again, this peroxy adduct may be cleaved by thiol to form 3. Consistent with such a second role for thiol in the formation of the 5'-aldehyde are experiments showing that 2-mercaptoethanol accelerates the decomposition of the labile adduct (L.F. Povirk and I.H. Goldberg, unpublished results) and that high thiol concentrations increase both the absolute yield of 5'-aldehyde and the ratio of aldehyde to released free base (L.S. Kappen and I.H. Goldberg, unpublished results).

It is also possible that the activated chromophore adds O_2 <u>after</u> it abstracts the hydrogen atom from DNA (pathway E) and then reacts with 1 to form

the labile adduct, 5; or it may instead react with the peroxyl form of the deoxyribose (2) to form (5) via pathway F. Since the chromophore is anchored on the DNA by intercalation and electrostatic interactions (21), it would not be surprising if it participated in such reactions after the initial hydrogen abstraction. Reaction pathway E, involving a peroxyl form of the drug and a labile chromophore-DNA adduct intermediate, best fits data showing that one molecule of O_2 is consumed for each molecule of chromophore, whereas DNA damage is somewhat less than stoichiometric (16). Furthermore, pathway E is more attractive than D, since it does not require postulating a different hydrogen-abstracting form of the chromophore under aerobic and anaerobic conditions. Finally, it should be noted that it is possible to combine pathways A and E in a concerted mechanism involving a ternary complex of activated chromophore, O_2 and C-5' of deoxyribose.

The proposed scheme focuses on the 5'-carbon of deoxyribose as the predominant site of attack. It is consistent with the findings that over 80% of the DNA strand breaks (major lesion) have nucleoside 5'-aldehyde at their 5'-termini (7) and that C-5' oxidation also occurs in the formation of the labile chromophore-DNA adducts (13). The absence of any ^3H abstraction from the C-1' and C-2' of thymidine in DNA eliminates these positions as targets. It should, however, be noted that C-1' appears to be the primary site of hydrogen abstraction when radiolytically-activated NCS reacts with DNA (22). Furthermore, since the mechanism of spontaneous base release and the chemistry of the alkali-labile lesions are unknown, it is not possible at this stage to invoke attack at C-5' in the generation of these lesions.

When misonidazole substitutes for O_2 in the NCS-DNA reaction, sites of DNA breakage and specificity of base attack are the same as in O_2, but in the presence of misonidazole base release and gap formation (with 3'- and 5'-phosphoryl termini) predominate (14). Despite the difference in final DNA products, abstraction of 5'-hydrogen by thiol-activated drug to form a carbon-centered radical on C-5' of deoxyribose appears to be a common initial step in both the O_2- and misonidazole-dependent reactions; in fact, there appears to be a competition between the misonidazole- dependent and the oxygen-dependent pathways (14). It is likely that after the induction of the same nascent lesion on the DNA participation of misonidazole in the subsequent steps, by a mechanism different from that of O_2, leads to different products.

It should be pointed out that while the two antitumor antibiotics NCS and bleomycin cause hydrogen atom abstraction from deoxyribose in DNA (from the 5'-position with NCS and from the 4'-position with bleomycin), the mechanisms

are fundamentally different. NCS is activated to a species that abstracts the hydrogen atom itself, whereas iron in bleomycin activates oxygen to do the abstraction (23-25).

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