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# Inhibition of DNA Replicon Initiation by 4-Nitroquinoline 1-Oxide, Adriamycin, and Ethyleneimine[1]

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## ABSTRACT

**The effects ot three widely differing chemical carcinogens, 4-nitroquinoline 1-oxide, Adriamycin, and ethyleneimine, on DNA replication were studied by pulse labeling of DNA with [$^3$H]thymidine and sedimentation analysis with alkaline sucrose gradients. At doses that reduced the rate of DNA synthesis to 30 to 60% of control values, only ethyleneimine produced damage that resulted in lower molecular weights of parental DNA. All three chemicals inhibited replicon initiation, but to differing extents. Inhibition of replicon initiation was the first clearly identified effect of 4-nitroquinoline 1-oxide and was the main cause of inhibition of DNA synthesis. Ethyleneimine caused severe inhibition of replicon initiation, but blocks to chain elongation also contributed significantly to the inhibition of overall DNA synthesis. Adriamycin affected replicon initiation to a small but significant extent; the primary cause of inhibition of DNA synthesis by this drug was a slowing of the rate of chain elongation. These results indicate that inhibition of replicon initiation is an important mechanism for the action of DNA-damaging agents in mammalian cells and strengthen the concept that control of DNA replication depends on the structural integrity of a chromosomal subunit that consists of several replicons.**

## INTRODUCTION

Much attention has been directed toward mechanisms of repair of drug-induced DNA damage, with less devoted to the effects of drugs on normal semiconservative DNA replication. Yet, it is clear that the consequences of damage are more clearly expressed in disturbances in DNA replication than in events of successful repair. I reported earlier that the first observable effect of methyl methanesulfonate on HeLa DNA synthesis was an inhibition of replicon initiation, with suppression of chain elongation occurring later (21). These results were not totally unexpected, because X-irradiation of the HeLa cells also causes an inhibition of replicon initiation (17, 24, 30), and methyl methanesulfonate is often referred to as a radiomimetic agent.

In this report I describe the results of experiments on HeLa DNA synthesis with other mutagenic carcinogens. Despite the fact that each of these chemicals exhibits a different pattern of damage and repair and contributes to a different extent to the overall inhibition of DNA synthesis, all of them have a significant effect on replicon initiation.

## MATERIALS AND METHODS

**Cells and Culture Conditions.** HeLa S3 cells, from stocks used for several years in this laboratory, were grown in Petri dishes with Eagle's minimal essential medium supplemented with 15% fetal bovine serum in a humidified 5% $CO_2$-95% air atmosphere. Monthly checks confirmed the absence of *Mycoplasma* contamination. For labeling of parental DNA, all cultures were incubated for 24 hr or more with 0.02 $\mu$Ci [$^{14}$C]dThd[2] per ml (50 mCi/mmol) before irradiation.

**Experiments with Chemicals.** 4NQO, a gift of Dr. James Cleaver, was dissolved in ethanol at 100 to 1000 times the concentration used in the experiments. Adriamycin (Adria Laboratories, Inc., Wilmington, Del.) and ethyleneimine (a gift of Dr. Bruce Ames) were both dissolved in water for stock solutions 100 to 1000 times the concentration used in the medium for cell exposure. All procedures were carried out at 37°. The medium containing the chemical under study was prepared and immediately added to culture dishes containing monolayers of HeLa cells. The cells were incubated with each chemical for 30 min, after which the medium was removed and the cells were washed twice with regular medium and then incubated with regular medium.

**Velocity Sedimentation Analyses.** At various times after treatment of the cells with chemicals, the medium was removed from the cultures and replaced with medium containing 20 $\mu$Ci [$^3$H]dThd per ml to label nascent DNA. After a 5-min incubation the radioactive medium was rapidly removed, and ice-cold Saline G (28) containing 1 mM unlabeled dThd was added to the cells. The cells were washed once with this solution, scraped into it, and counted with a hemacytometer. The cells were then X-irradiated with 1000 rads, and an aliquot containing about $10^5$ cells (in less than 0.5 ml) was added directly to 0.5 ml of lysis solution (0.5 M NaOH-0.02 M EDTA-0.2% Nonidet P-40) on top of 30 ml of preformed 5 to 20% alkaline sucrose gradients (0.1 M NaOH-0.9 M NaCl-0.01 M EDTA). The X-radiation releases the DNA from a "complex" so that only a 15-min lysis is required to obtain parental DNA with molecular weights of about $5 \times 10^8$ (31). The gradients were centrifuged in the short buckets of an SW 27 rotor (Beck-

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[2] The abbreviations used are: dThd, thymidine; 4NQO, 4-nitroquinoline 1-oxide.



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man Instruments, Inc., Palo Alto, Calif.) at 27,000 rpm for 3 hr or at 12,000 rpm for 15 hr. This gradient system has been calibrated with phage λ DNA and phage T-4 DNA as described elsewhere (3). A hole was made in the bottom of the centrifuge tube and about 25 equal-weight fractions were collected. Calf thymus DNA (100 μg) was added to each 1.2 ml fraction, and all of the DNA was precipitated by addition of 1 ml solution of ice-cold 6 M HCl and 6% sodium pyrophosphate. Each fraction was then filtered through Whatman GF/C filters that had been soaked with 4% perchloric acid. The filters were washed sequentially with ice-cold 4% perchloric acid, 70% alcohol, and 100% alcohol and dried. The $^{14}C$ and $^{3}H$ on each filter was determined by liquid scintillation spectrometry (Packard Instrument Co., Downers Grove, Ill.). DNA's labeled with $^{14}C$ alone and $^{3}H$ alone were used to determine channel corrections. The $^{3}H/^{14}C$ ratios calculated from the sums of total $^{3}H$ and $^{14}C$ in the gradients were used as measures of specific activity, from which comparisons between controls and treated samples were used to estimate total inhibitions of DNA synthesis. At the levels of DNA synthesis inhibition caused by the treatments used in the work reported here, the contribution of repair synthesis to total DNA synthesis is negligible (19).

## RESULTS

When cells were incubated for 10 min with [$^{3}H$]dThd at various times after a 30-min incubation with $2 \times 10^{-7}$ M 4NQO, there was a steady decrease in the incorporation of the tracer into DNA (22). Sedimentation analysis with alkaline sucrose gradients showed that the major effect of 4NQO, immediately after removal of the drug, was a great decrease in radioactivity in the regions of the gradients containing low-molecular-weight DNA (Chart 1). This decrease in low-molecular-weight nascent DNA is diagnostic for inhibition of replicon initiation (17, 24, 30). At later times the sucrose gradient profiles showed lowered amounts of radioactivity in all size classes of DNA, indicating that inhibition of chain elongation became a major effect at these times. At the concentration of 4NQO used in these experiments ($2 \times 10^{-7}$ M), there was no detectable effect on the molecular weight of parental DNA; *i.e.*, DNA labeled for 24 hr or more with [$^{14}C$]dThd, before incubation with the drug, sedimented to the bottom of the gradient and exhibited the same profile as did the [$^{14}C$]DNA in control cells (data not shown).

HeLa cells were pulse labeled with [$^{3}H$]dThd for 10 min, 30 min after a 30-min incubation with Adriamycin. (At 0 time there was little difference between control and treated samples.) The resulting alkaline sucrose gradient profiles of the DNA showed a small inhibition of replicon initiation (Chart 2) but primarily an effect on the rate of chain elongation. This inhibition of initiation caused only a small deficit in radioactivity at low molecular weight, compared to that in the rest of the gradients. The remainder of the overall inhibition of DNA synthesis seems to be the result of a reduced rate of chain elongation, because the profiles of DNA from control and drug-treated cells are otherwise very similar (11), except that the total radioactivity is reduced in the treated cells. The method of Povirk and Painter (26)

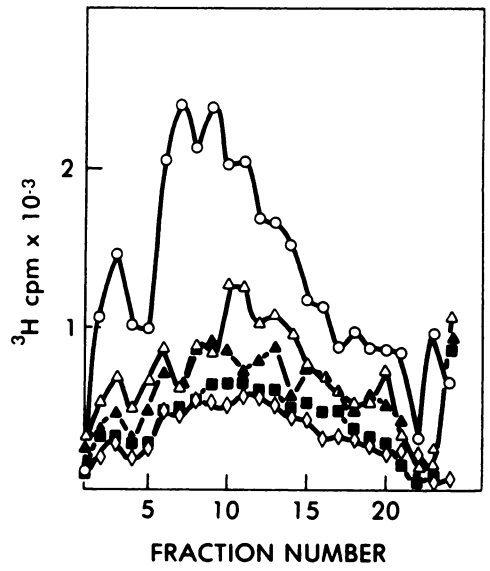


Chart 1. Alkaline sucrose gradient profiles of $^{3}H$-labeled nascent DNA of $10^{6}$ HeLa cells incubated with $2 \times 10^{-7}$ M 4NQO for 30 min and then incubated for 10 min with 20 μCi [$^{3}H$]dThd per ml at 0 (△), 30 (▲), 90 (■), and 150 (◇) min after removal of the drug from the medium. ○, untreated control cells. Sedimentation is from *left* to *right*. Inhibition of DNA synthesis by 4NQO was 48% at 0 time, 58% at 30 min, 66% at 90 min, and 73% at 150 min. Bottom fractions containing 10 to 15% of the counts in each case, because of wall effects, are not included in these charts. $^{14}C$-Labeled parental DNA sedimented in a manner similar to that shown in Chart 2.

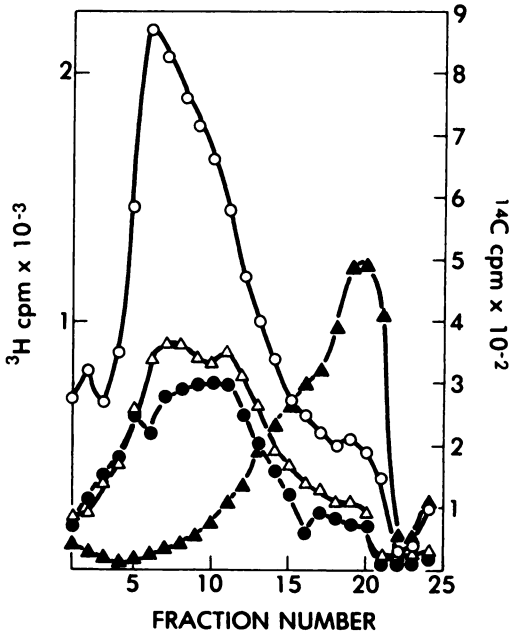


Chart 2. Alkaline sucrose gradient profiles of $^{3}H$-labeled nascent DNA from $10^{6}$ HeLa cells incubated with 2 μM (●) or 1 μM (△) Adriamycin for 30 min, washed, and then incubated for 30 min in drug-free medium before incubation with [$^{3}H$]dThd for 10 min. ○, untreated control cells; ▲, parental DNA in control cells labeled by incubation of cells for 24 hr with 0.02 μCi [$^{14}C$]dThd per ml before drug treatment. Parental DNA from drug-treated cells sedimented almost identically to controls. Sedimentation is from *left* to *right*. Inhibition of DNA synthesis was 46% for 1 μM and 60% for 2 μM.

confirmed the inference that Adriamycin reduced the rate of chain elongation (data not shown).

The effects of ethyleneimine differed from those of the other drugs studied in one important respect. When cells



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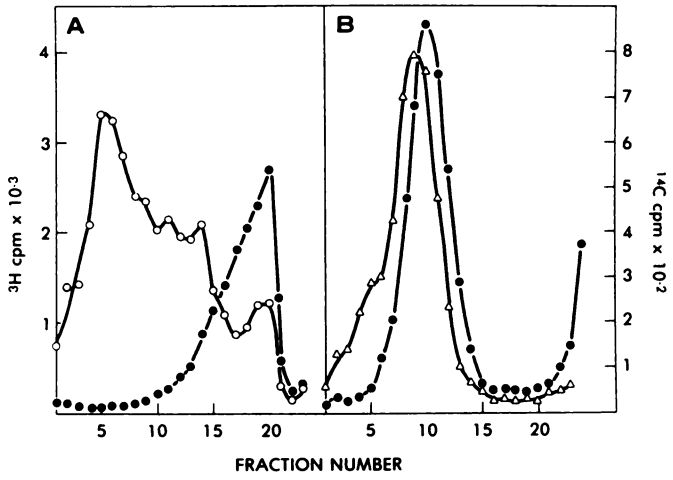


Chart 3. Alkaline sucrose gradient profiles of $^3$H-labeled nascent DNA and of $^{14}$C-labeled parental DNA from HeLa cells incubated with $5 \times 10^{-4}$ M ethyleneimine for 30 min. *A:* ○, DNA from untreated control cells; ●, $^{14}$C-labeled parental DNA. *B:* DNA from cells treated with ethyleneimine for 30 min and immediately pulse labeled with [$^3$H]dThd. △, $^3$H-labeled nascent DNA; ●, $^{14}$C-labeled parental DNA. Sedimentation is from *left* to *right*. Inhibition of DNA synthesis at 0 time was 37%.

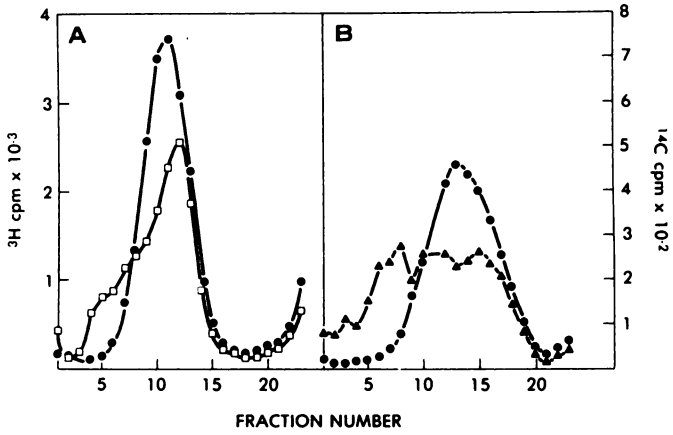


Chart 4. Same as Chart 3, except for HeLa cells pulse labeled with [$^3$H]dThd at 30 min (*A*) and 90 min (*B*) after removal of ethyleneimine from the medium. □, [$^3$H]DNA from cells pulse labeled at 30 min; ▲, [$^3$H]DNA from cells pulse labeled at 90 min; ●, $^{14}$C-labeled parental DNA. Sedimentation is from *left* to *right*. Inhibition of DNA synthesis was 51% at 30 min and 50% at 90 min.

were incubated with concentrations of ethyleneimine that inhibited DNA synthesis to about the same extent (30 to 60%) as the other drugs, the molecular weight of parental DNA, analyzed on alkaline sucrose gradients, was greatly reduced from control values (Chart 3). These breaks were probably primarily due to lesions that resulted in alkali-sensitive bonds rather than frank breaks. The X-irradiation used to enhance lysis had no effect on the incidence of these breaks (data not shown). Accompanying the formation of these lesions was a severe inhibition of replicon initiation immediately after drug treatment. The lesions causing the parental strand breaks also blocked chain elongation, resulting in a narrow range of molecular weights in the newly synthesized DNA. The inability of newly synthesized DNA to extend past the lesion in the parental DNA was more dramatically observed at 30 min after drug treatment (Chart 4*A*). The inhibition of initiation was also more evident, and the profile of the nascent DNA was skewed sharply, with a peak of radioactivity at a molecular weight just slightly lower than that of the parental DNA. At 1.5 hr after treatment with ethyleneimine, a recovery of initiation seemed to occur (Chart 4*B*). There was slow repair of parental DNA; at 1.5 hr after treatment, its molecular weight was about $7.6 \times 10^{-7}$ (Chart 4*B*) compared to $3.8 \times 10^{-7}$ immediately after treatment (Chart 3*B*).

## DISCUSSION

Although each of the agents studied in this investigation inhibited the initiation of replicons, the action of each on DNA replication was unique. With 4NQO, inhibition of replicon initiation was the major effect at early times after treatment and was the main contributor to overall inhibition of DNA synthesis. With Adriamycin, inhibition of initiation was not as important as the effect on the rate of chain elongation. With ethyleneimine, inhibition of initiation was accompanied by what appears to be an equally effective block to chain elongation.

There has been a tendency to classify DNA-damaging agents as "UV-like" or "X-ray-like." 4NQO is generally called a UV-like agent because the DNA damage it induces is not repairable by human cells deficient in excision repair (*i.e.*, cells from xeroderma pigmentosum patients) (4) and because, in cells that can excise 4NQO-induced damage, the patch size is about 100 nucleotides (29), similar to the patch size occurring during excision repair of UV-induced damage (5, 29). The primary effect of 4NQO on DNA replication, however, is more like that of X-rays because it inhibits replicon initiation, whereas UV induces blocks to chain elongation (6). Thus, the classifications UV-like and X-ray-like, derived from DNA repair studies, seem to break down when an agent is tested for its effect on DNA replication.

Ethyleneimine is similar to X-rays in its action; it induces lesions that are registered as single-strand breaks under alkaline conditions and strongly inhibits initiation. The lesions induced by ethyleneimine, however, are repaired much more slowly than are those induced by X-rays (15) and thus are probably not simple single-strand breaks of the kind induced by X-rays.

Thus, the effects of both 4NQO and ethyleneimine resemble those of X-rays and UV. When the effects of these agents on DNA synthesis are compared with each other, the following generalizations can be made: (*a*) when an agent (such as UV) induces DNA damage with no or very few strand breaks, blocks to chain elongation are the principal or only cause of inhibition of DNA synthesis, and therefore relatively large amounts of damage are required for inhibition; (*b*) with certain agents, like X-rays, relatively small amounts of damage (probably strand breaks) are effective in causing large inhibitions of DNA synthesis because they block the initiation of clusters of replicons; a single hit by X-radiation will block replication in a unit of DNA of about $10^9$ daltons (23); (*c*) drugs like 4NQO produce damage of both kinds; one kind of lesion blocks replicon initiation, whereas a second kind inhibits chain elongation; (*d*) chemicals like ethyleneimine produce either one kind of damage with 2 effects or 2 different kinds of damage. One lesion is

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registered as a strand break after exposure of DNA to alkali; this lesion also blocks chain elongation. Another lesion, or another effect of the first lesion, blocks replicon initiation. The lesion resulting in the alkali-sensitive bond is not the same as the X-ray-induced lesion that inhibits replicon initiation because it is repaired much more slowly [X-ray-induced strand breaks are repaired with a half-time of 8 to 10 min (15)] and is a much less effective inhibitor of replicon initiation: the large number of strand breaks found in these experiments still inhibit overall DNA synthesis by only about 50%. If one-half of this initiation is due to initiation blocks, one can calculate that about 25 ethyleneimine-induced breaks have about the same effect on initiation as one X-ray-induced strand break.

The results with Adriamycin show that a known mutagen can induce its major inhibitory action on DNA synthesis by slowing the rate of chain elongation. However, Adriamycin is an intercalator and does not form a covalently bound adduct. Böhner and Hagen (2) recently showed that intercalating agents, including daunomycin (which differs from Adriamycin only in substitution of an OH group for H in the acetyl radical of the molecule), reduce the rate of DNA polymerization in an *in vitro* DNA-synthesizing system. The intercalation of Adriamycin into DNA *in vivo*, however, must also induce enough conformational changes in chromatin to cause the effect on initiation reported here.

The mechanism of inhibition of replicon initiation awaits full biochemical analysis, but Povirk and Painter (27) showed that exposure to 313 nm light of mammalian cells, the DNA of which contained 20% substitution of thymine by bromouracil, inhibited replicon initiation; these results imply that DNA damage alone is sufficient to inhibit initiation. Calculations of the frequency of lesions occurring in both the 313 nm-irradiated bromouracil-labeled DNA (27) and the DNA of X-irradiated cells (23, 25) led to the concept that a single lesion within a DNA segment of about $10^9$ daltons blocks the initiation of DNA synthesis in that entire segment. Because this segment is very much larger than a single mammalian replicon [see reviews by Edenberg and Huberman (10) and Painter (20)] and because replicons are known to initiate parasynchronously in clusters (13, 14), our group hypothesized that a single lesion (probably a single-strand break) within a replicon cluster alters the conformation of DNA so that no replicon within the cluster can be initiated (18, 25, 27).

The data presented here with 4NQO and Adriamycin indicate that replicon initiation can also be blocked by chemically induced damage that causes fewer than 1 strand break/2 × $10^8$ daltons in $^{14}C$-labeled parental DNA.

Recent evidence for supercoiling and supersupercoiling within the eukaryotic chromosome (1, 7, 32) offers a structural model for the action of DNA-damaging agents on replicon initiation. The data of Cook and Brazell (7, 8) and Cook *et al.* (9), which furnish evidence for a supercoiled subunit with a molecular weight of about $10^9$ in HeLa and other mammalian cells, are especially pertinent. A single X-ray-induced nick in this structure "relaxes" it and markedly alters its sedimentation characteristics. Supercoiling is necessary for replication of λ and Col $E_1$ DNA (12), and binding of a relaxation protein to supercoiled DNA precedes initiation of the R6K plasmid replicon (16). Supercoiling is probably required for initiation of DNA replication in mammalian cells as well. It seems likely that many DNA-damaging agents are capable of altering the conformation of the mammalian supercoiled subunit (a replicon cluster), thereby blocking DNA replication.

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