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# Nature of DNA Repair Synthesis Resistant to Inhibitors of Polymerase $\alpha$ in Human Cells†

Charles Allen Smith* and Diane S. Okumoto

ABSTRACT: Arabinocytidine and aphidicolin are inhibitors of $\alpha$-DNA polymerase that have been shown to affect both normal DNA replication and repair synthesis in mammalian cells. In contradiction to the prevalent hypothesis that these inhibitors merely slow the polymerization rate at incision sites near lesions, our results suggest that the repair synthesis resistant to inhibitors is mediated by a separate pathway. Repair synthesis in contact-inhibited human cells following UV irradiation was inhibited 75–80% by arabinocytidine or aphidicolin, and most of the repair patches were not ligated into parental DNA, as judged by an enzymatic assay. However, the patches were not demonstrably shorter than those in untreated cells. Even following low-UV doses at which no inhibition of repair synthesis by the inhibitors was observed, a majority of the patches were not ligated. DNA polymerase $\beta$ is implicated in this alternate pathway, both by the known specificity of the inhibitors and by evidence from their sensitivity to S1 nuclease that the patches arise from displacement synthesis. The unligated patches are not degraded in vivo and eventually become ligated into parental DNA, very slowly in the presence of inhibitors but much more rapidly following their removal. Thus, under conditions of $\alpha$-polymerase inhibition, a limited number of normal length repair patches are made, apparently by displacement synthesis, leaving displaced strands that remain substantially undegraded.

Most types of cells can remove damage from their DNA by the process of excision repair (ER),[1] whereby a short stretch of the DNA strand containing the damage is removed and replaced by new DNA, synthesized by using the undamaged complementary strand as template (Hanawalt et al., 1979). This process appears to be responsible for much of the resistance of both prokaryotic and eukaryotic cells to killing by short-wavelength ultraviolet light and a number of chemical damaging agents. Our understanding of ER in *Escherichia coli* is relatively well advanced because of the ease with which both genetic manipulations and biochemical studies may be done with this organism. Although we understand well the broad outlines and overall features of the process in mammalian cells, many of its details at the molecular level remain obscure. Chemicals that inhibit specific molecular processes can offer an alternative to genetic manipulation, and in recent years, inhibitors of DNA synthesis have been used to obtain information about the roles of the various eukaryotic DNA polymerases in ER and to facilitate studies of the incision step in that process (Collins & Johnson, 1984).

The frequencies of incision breaks in the cellular DNA remain small even after high UV doses, presumably because the rate of incision is much slower than the rates of the subsequent steps. Many investigators have used inhibitors of DNA synthesis (usually a combination of hydroxyurea and arabinocytidine) to retard completion of repair events, conditions under which the frequency of DNA single-strand breaks is increased (Collins & Johnson, 1984; Ben-Hur & Ben-Ishai, 1971; Hiss & Preston, 1977; Collins, 1977; Erixon & Ahnström, 1979). When we began this study, it had been reported that these conditions also reduced DNA repair synthesis in UV-irradiated human cells (Dunn & Regan, 1979). Subsequently, a number of investigators have shown that at least in contact-inhibited cells, ara-C or aphidicolin alone can inhibit repair synthesis, although not totally (Johnson et al., 1982; Snyder & Regan, 1982; van Zeeland et al., 1982).

The prevailing hypothesis to explain these effects is that incision proceeds normally (at least to a limited extent) in the presence of the inhibitors but that at each incision site the rate of repair synthesis is so diminished that a single-strand interruption remains, either because a gap made by prior exonuclease action remains or because ligation of a patch cannot take place until it reaches a certain size. We set about to test

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[1] Abbreviations: apc, aphidicolin; ara-C, arabinocytidine (1-$\beta$-D-arabinofuranosylcytosine); ER, excision repair; HU, hydroxyurea; mtDNA, mitochondrial DNA; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $Cl_3CCOOH$, trichloroacetic acid; FdUrd, fluorodeoxyuridine; BrdUrd, bromodeoxyuridine; BrUra, bromouracil.

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this hypothesis directly by measuring the size distribution of the patches made under these conditions and by examining their ligation. The effects of ara-C were studied initially and more completely because it had been the most studied by other investigators. To determine if the incorporation of ara-C into DNA was responsible for the results noted, we also studied the effects of apc, a highly specific inhibitor of polymerase $\alpha$ that cannot be incorporated into DNA (Huberman, 1981). Our results do not support the hypothesis described but support the view that repair synthesis that is resistant to these inhibitors arises from a separate pathway, presumed to involve polymerase $\beta$.

## Materials and Methods

*Cell Culture.* For many of the experiments reported here, we used a human continuous cell line designated T98G, derived from a glioblastoma multiforma tumor (Stein, 1979). These cells have about 3 times the DNA content of diploid cells and grow to high cell densities, but unlike other continuous lines, they exhibit the cell density and serum-dependent G1 arrest (contact inhibition) characteristic of normal human diploid fibroblasts. These properties facilitate the analyses used here. In several instances, we also used normal human diploid fibroblasts.

The T98G cells, GM38 (Institute for Medical Research, Camden, NJ; obtained from E. C. Friedberg, Stanford University Medical Center) and IMR90 normal fibroblasts (ATCC CCL186; obtained from R. Schimke, this department), were cultured in Eagle's basal medium supplemented with 10% fetal bovine serum and antibiotics (Grand Island Biological Co.) at 37 °C in a humidified $CO_2$ atmosphere. Stock cultures of T98G cells were subcultivated at a ratio of 1:4 every 7 days. For prelabeling, they were split at a ratio of 1:4 and grown for 7 days in 0.4 or 0.8 $\mu$Ci/mL [$^{32}$P]-orthophosphate and then subcultivated at a ratio of 1:3 in nonradioactive medium and used for experiments either 1 (growing cells) or 8 (confluent cells) days later. Normal fibroblasts were prepared in the same manner except that they were always subcultivated at a ratio of 1:2.

*Chemicals.* Stock solutions of 1 mM fluorodeoxyuridine plus 10 mM 5-bromodeoxyuridine, 10 mM ara-C, and 50 mM dCyt (obtained from P-L Biochemicals, Milwaukee, WI) were each stored frozen in 1-mL aliquots, which were thawed as needed and stored at 4 °C. Fresh 1 M solutions of HU (Sigma Chemical Co., St. Louis, MO) were prepared just prior to use. Apc was a gift of ICI-UK to A. A. van Zeeland, State University of Leiden, The Netherlands, and was dissolved in dimethyl sulfoxide at 1 mg/mL and stored at 4 °C. Novobiocin (Sigma) and tetrahydrouracil (Vega Biochemicals, Tucson, AZ) were dissolved in water at 50 mg/mL. [$^3$H]-BrdUrd (50 Ci/mmol) and [$^3$H]ara-C (26 Ci/mmol) were obtained from Amersham; [$^3$H]dThd (>50 Ci/mmol) was obtained from New England Nuclear.

*Measurement of Repair Synthesis.* Repair synthesis was measured by using BrdUrd labeling and density gradient centrifugation essentially as described (Smith et al., 1981). Cells prelabeled with $^{32}$P were incubated in BrdUrd–FdUrd for 1 h prior to addition of inhibitors. After 30 min of incubation, they were then UV irradiated or mock irradiated and incubated in medium containing appropriate inhibitors, BrdUrd–FdUrd, and either [$^3$H]BrdUrd or [$^3$H]dThd. When apc was used, dimethyl sulfoxide was added to all cultures at the same concentration, usually 1%. This concentration had no effect on repair synthesis in control cells or cells treated with ara-C. In most experiments, we used 10 $\mu$M BrdUrd, 1 $\mu$M FdUrd, and 2.5–10 $\mu$Ci/mL [$^3$H]dThd, conditions sufficient for the separation of DNA made by normal DNA synthesis from parental DNA containing repair patches by density gradient sedimentation. However, in contact-inhibited cells, increased $^3$H incorporation in repair patches is obtained with [$^3$H]dThd compared to [$^3$H]BrdUrd at the same nominal specific activity (Smith & Hanawalt, 1976), suggesting a slight selectivity for incorporation of dThd over BrdUrd. We have also found the T98G cells require higher concentrations of BrdUrd for complete substitution into repair patches. Therefore, experiments were carried out as required to ensure that none of the results reported are due to any differential effects of inhibitors on these phenomena.

After incubation to allow for repair, cells were lysed with sodium dodecyl sulfate, and the lysates were incubated with proteinase K (Beckman). In most experiments, the DNA was isolated and purified by centrifugation in neutral CsCl gradients. Portions of the fractionated gradients were assayed for radioactivity to provide information about the effects of the inhibitors on normal DNA synthesis and to locate the DNA for further analysis. The DNA of parental density was then centrifuged in alkaline CsCl gradients to determine values for repair synthesis, if needed. In some experiments with contact-inhibited cells, the lysates were centrifuged directly in alkaline CsCl gradients. Values for repair synthesis are expressed as $^3$H/$^{32}$P or $^3$H/microgram of DNA in the parental DNA. The $^{32}$P specific activity was determined from DNA prepared from cells lysed at the beginning of the experiment.

*Analysis of Patch Size.* This was also carried out as described (Smith et al., 1981). The size distribution of DNA fragments after sonication was determined by centrifugation in alkaline sucrose gradients in the SW60Ti rotor. Restriction fragments from digests of *$\phi$X174* DNA with *Hae*III, $^{32}$P end labeled at their 5′ ends, were obtained from Bethesda Research Laboratories and separated by gel electrophoresis. The sucrose gradients were calibrated with suitable combinations of fragments of lengths 72, 118, 194, 271 + 281, 310, and 603 base pairs. Molecular weight averages were calculated as described (Lehmann, 1981).

*Single-Strand Break Frequencies.* These were calculated from the determination of molecular weight averages of DNA from cells lysed directly on alkaline sucrose gradients as described (Ganesan et al., 1981). The sedimentation standard was bacteriophage $\lambda$, whose DNA was assumed to have a single-strand molecular weight of $15.4 \times 10^6$.

*Digestion of DNA by Nucleases.* BAL31 nuclease from *Alteromonas espejiana* (Legerski et al., 1977) was generously supplied by H. Gray, University of Houston, and D. Robberson, M. D. Anderson Hospital and Tumor Institute, Houston, TX. Digestion was carried out in 12 × 75 mm polypropylene test tubes immersed in a water bath at the desired temperature. The reaction buffer was 20 mM Tris-HCl, 1 mM EDTA, 0.1 M NaCl, 12.5 mM $CaCl_2$, and 12.5 mM $MgCl_2$, pH 8. Most assays were at 30 °C with 100 $\mu$g/mL DNA and 1–3 units/mL nuclease. In several early experiments in which the progress of digestion of $^3$H was carefully followed, a single digestion mixture was prepared and equilibrated to temperature, and a time-zero sample (50–100 $\mu$L) was added to a tube containing 10 $\mu$g of carrier DNA in 50 mM EDTA. DNA was then immediately precipitated with cold $Cl_3CCOOH$ and collected on a Millipore filter for assay of radioactivity. Nuclease was then added to the digestion mixture, and samples were withdrawn and processed in this way at desired times. The volumes taken were adjusted to correct for the dilution of the mixture by the nuclease solution so that all samples contained the same fraction of the starting

Table I: Inhibition by Ara-C and Apc of DNA Repair Synthesis in Cells Irradiated with High UV Doses[a]

| cells | UV dose ($J/m^2$) | inhibitor | post-UV incubation (h) | inhibition (%) | $N^b$ |
|---|---|---|---|---|---|
| T98G | 20 | ara-C | 2 | 65 ± 2 | 4 |
| | | | 4 | 63 ± 5 | 11 |
| | | | 6 | 69 ± 5 | 3 |
| | | | 24 | 61 ± 4 | 2 |
| | >30 | ara-C | 4 | 69 ± 4 | 4 |
| | | apc | 4 | 75 ± 4 | 7 |
| GM38 | 20 | ara-C | 4 | 68 | 1 |
| IMR90 | 30 | apc | 4 | 82 ± 3 | 3 |

[a] Repair synthesis was measured as described under Materials and Methods in cells incubated in either $10^{-4}$ M ara-C, 5 or 10 μg/mL apc, or no inhibitor. [b] $N$ indicates the number of experiments from which the average inhibitions and standard deviations were calculated.

material. In several later experiments, in which only the final plateau value for the $^3H/^{32}P$ ratio was desired, equal volumes of DNA samples were placed in tubes containing appropriate volumes of concentrated buffer and water and equilibrated. Enzyme was added to all but one tube, and at appropriate times, tubes were removed, carrier DNA and EDTA were added, and the DNA was precipitated and collected as described. Digestions were usually carried out to a point at which more than 40% of the $^{32}P$ had been rendered acid soluble. The sample not containing enzyme was usually processed midway through the digestion period and its radioactivity compared to that of a sample that was precipitated in a similar manner directly from the stock DNA.

S1 nuclease (24 units/μL) was obtained from Sigma. Digestions were at 30 °C in 0.18 M NaCl, 3 mM $ZnCl_2$, and 60 mM sodium acetate buffer, pH 4.2. The mixtures contained 20 μg/mL heat-denatured salmon sperm DNA and 100 μg/mL test DNA. The second sampling method described above was used.

*Pyrimidine Dimer Frequency.* As measured by thin-layer chromatography, irradiation of mammalian cells under our conditions produces 2.4 pyrimidine dimers per $10^8$ daltons of DNA per joule per meter squared.

## Results

*Characteristics of the Inhibition of Repair Synthesis. (A) Concentration of Inhibitor and Growth State of Cells.* The initial rate of repair synthesis was determined in heavily UV-irradiated T98G cells in the presence of various concentrations of ara-C or apc (Figure 1). The highest concentrations shown inhibited normal DNA replication by greater than 95%, but no inhibition of repair synthesis was observed in exponentially growing cells unless 10 mM HU was also present. This HU concentration alone inhibited normal DNA replication by greater than 95% but had no inhibitory effect upon repair synthesis. In contrast, 60–80% inhibition of repair synthesis was observed in contact-inhibited cells over a wide range of inhibitor concentrations, and the inhibition was only slightly increased by the addition of HU. Inhibition by ara-C was not increased further in 10 mM tetrahydrouracil, which should block degradation of ara-C by deoxycytidine deaminase.

Except where noted, subsequent experiments were with contact-inhibited cells, generally with either $10^{-4}$ M ara-C or 5–10 μg/mL apc. These resulted in similar and reproducible values for the inhibition of repair (Table I).

*(B) Inhibition as a Function of Time.* With ara-C, we observed similar values for inhibition of repair for measurements extended for different times after irradiation (Table I), suggesting that although the overall amount of repair synthesis is greatly reduced by the inhibitor, its time course is nevertheless similar to that in untreated cells. This was confirmed by direct examination (Figure 2). Repair synthesis in the presence of each inhibitor was found to continue at nearly its initial rate for several hours.

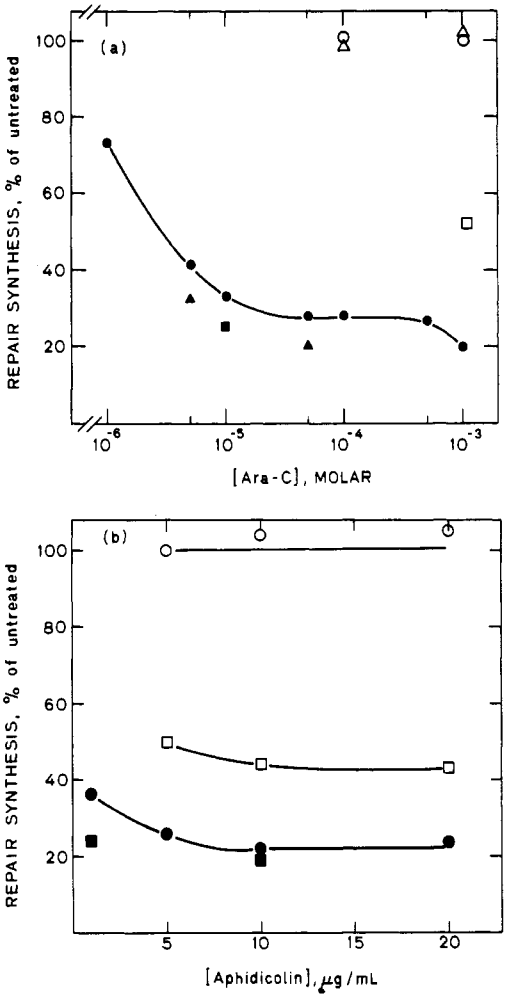


FIGURE 1: Effects of various concentrations of arabinocytidine (a) or aphidicolin (b) on repair synthesis in T98G cells in the first 4 h after irradiation with 20 (a) or 30 (b) $J/m^2$ UV light. Inhibitors were present from 30 min prior to irradiation to the time of cell lysis. [$^3$H]BrdUrd was the repair label in those experiments with exponential-phase cells treated with ara-C; in the other experiments, [$^3$H]dThd was used. Open symbols, cells in the exponential growth phase; filled symbols, cells in the stationary phase. Hydroxyurea concentrations included were 0 (○, ●), 2.5 (△, ▲), and 10 mM (□, ■). "Untreated" refers to irradiated cells incubated in the absence of inhibitor.

*(C) Inhibition as a Function of UV Dose.* In confluent cells, the initial rate of repair in the presence of inhibitors reaches its maximum at a much lower UV dose than in their absence (Figure 3). As expected from the results shown in Figure 3a, we observed no inhibition of repair synthesis in T98G cells treated with ara-C after irradiation with 3.3 $J/m^2$, in two

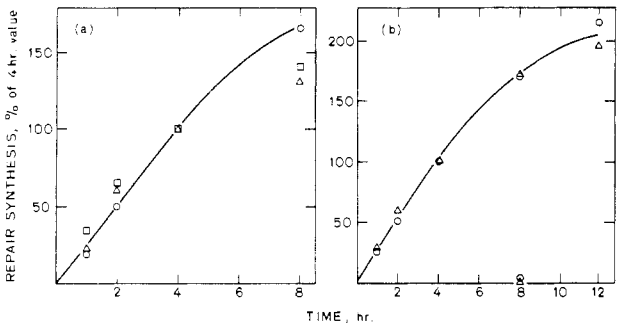


FIGURE 2: Time course of repair synthesis in stationary-phase T98G cells (a) or IMR90 cells (b) irradiated with 30 J/m$^2$ UV light and incubated in no inhibitor (○), $10^{-4}$ M ara-C (□), or 5 μg/mL aphidicolin (△) for the times indicated. The repair label was [$^3$H]dThd. For comparison, each set of data is normalized to the value at 4 h. Points at 8 h near the abscissa in (b) are for unirradiated cells. The actual repair inhibitions at 4 h were 71% (a) for ara-C and 77% (a) and 81% (b) for apc.

separate experiments. Although we did not determine the complete dose response for apc-treated T98G cells in a single experiment, in separate experiments we observed 30% inhibition in cells that received 5 J/m$^2$, and less than 10% inhibition in cells irradiated with 3.3 J/m$^2$, both incubated for 4 h after irradiation.

*Single-Strand Break Frequencies.* To ensure that under our conditions the inhibitors had the expected effects on the frequencies of single-strand breaks in the cellular DNA of confluent cells, we measured its molecular weight after various treatments. Incubation of unirradiated cells in either compound had no effect. DNA in T98G cells irradiated with 5 J/m$^2$ and incubated for 4 h in either $10^{-4}$ M ara-C or 10 μg/mL apc contained about 2.4 breaks/$10^8$ daltons in excess of the frequency of breaks in irradiated untreated cells, which with our technique was not detectably different from that in unirradiated cells. DNA in cells irradiated with 30 J/m$^2$ and incubated as above contained about 3.6 excess breaks/$10^8$ daltons. Similar values were found for IMR90 cells incubated with apc. These values are well within the range reported by others using both alkaline sucrose gradients (Dunn & Regan, 1979; Cleaver, 1981; Snyder et al., 1981; Snyder & Regan, 1982) and strand unwinding techniques (Erixon & Ahnström, 1979; Erixon, 1981.)

*Ligation of Repair Patches after High UV Doses.* We devised an assay for the fraction of repair synthesis in stretches completely ligated into the parental DNA by making use of the nuclease from *Alteromonas espejiana* termed BAL31 nuclease. This enzyme degrades single-stranded DNA endonucleolytically, converts nicks into double-strand breaks, and digests linear duplex DNA from the ends of the molecules in an apparent exonucleolytic fashion, in a slower reaction. Thus, radioactivity located near nicks or ends in duplex DNA should be degraded much more rapidly than the bulk of the DNA. To test these activities, we prepared DNA from exponentially growing cells that had been grown in $^{32}$P and pulse labeled for 90 s with [$^3$H]dThd to label the nascent daughter DNA strands. When digested with the enzyme, the $^3$H was rendered acid soluble much faster than was the bulk, $^{32}$P-prelabeled DNA. In the case of repair patches, one would expect that ligated ones would be digested at the same rate as the bulk of the DNA, while those unligated at their 3′ ends would be digested much more rapidly. An example of the assay (Figure 4) shows that patches made in the presence of ara-C do behave as if a large fraction of them are unligated. Under standard conditions, the bulk DNA from both treated and untreated cells is digested at a slow and nearly linear rate (Figure 4a).

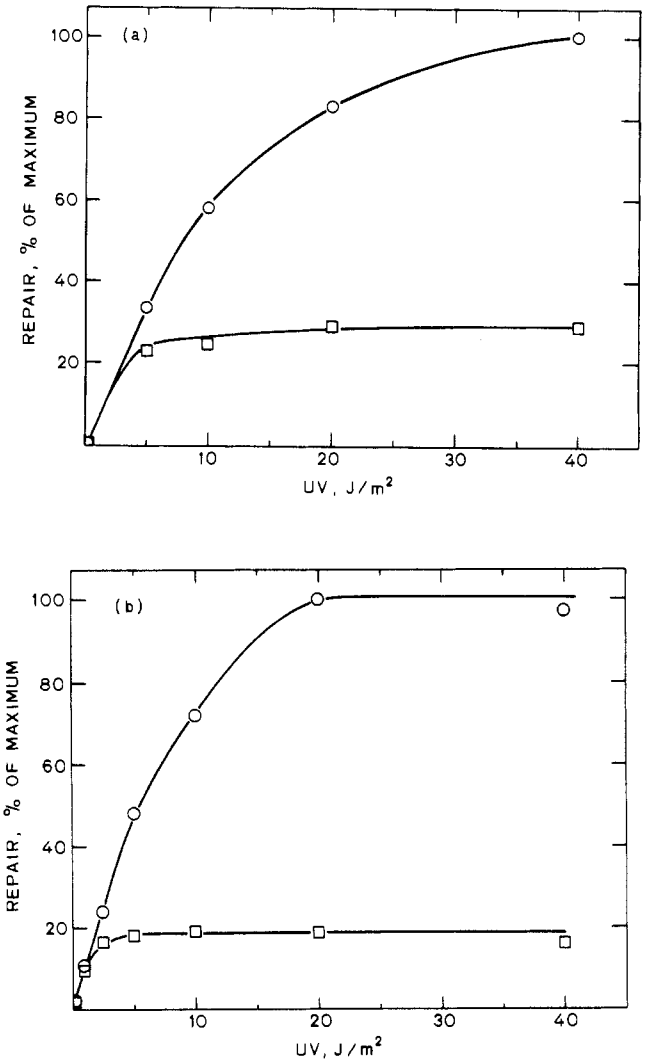


FIGURE 3: Effects of inhibitors on the UV dose response of repair synthesis in the first 4 h after irradiation. In (a), stationary-phase T98G cells were incubated in medium containing $10^{-4}$ M BrdUrd, $10^{-5}$ M FdUrd, and [$^3$H]dThd, in either the presence (□) or the absence (○) of $10^{-4}$ M ara-C. A similar result was obtained in an experiment in which the concentrations of halogenated nucleosides were 10-fold lower. In (b), stationary-phase IMR90 cells were incubated in medium containing $10^{-5}$ M BrdUrd, $10^{-6}$ M FdUrd, and [$^3$H]dThd in the presence (□) or absence (○) of 5 μg/mL aphidicolin.

The radioactivity representing repair synthesis in untreated cells is digested at the same rate. In contrast, the repair label in the DNA from the ara-C-treated cells is digested in a more complex manner: a large fraction is digested very rapidly, after which the remainder is digested slowly. Replotting the data (Figure 4c) to show the ratio of the radioactivity in the repair label to that in the bulk of the DNA as a function of the extent of digestion of the bulk DNA shows that this second component is in fact digested at the same rate as the bulk DNA. This method of plotting also facilitates estimation of the magnitude of the fraction of repair label that is digested at the same rate as bulk DNA, 38% in this example. This radioactivity appears to be in patches like those in the DNA from untreated cells, which is thus presumably ligated. The remainder, 62% in this example, will be termed here "rapidly digestible" and is considered to be in patches that are not ligated at their 3′ ends. Slowing the rate of digestion by lowering the temperature or enzyme concentration did not affect the values calculated for the fraction rapidly digestible (Figure 4b,c).

We used the assay to determine the fraction of repair synthesis in unligated patches in cells treated in various ways

Table II: Ligation State of Repair Patches[a]

| inhibitor | concn | time in repair label (h) | time reincubated (h) | fraction rapidly digestible (%) after no reincubation | after reincubation in inhibitor | after reincubation in dCyt |
|---|---|---|---|---|---|---|
| ara-C | $10^{-4}$ M | 2 | 2 | 65 | 60 | 40 |
| | | 2 | 4 | 70 | 65 | 25 |
| | | 2 | 4 | 70 | 65 | 25 |
| | | 2 | 24 | 70 | 40 | 0 |
| | | 4 | NR[b] | 65 | NR | NR |
| | | 6 | NR | 63 | NR | NR |
| | | 6 | 20 | 72 | ND[c] | 0 |
| | | 8 | NR | 75 | NR | NR |
| | | 22 | 24 | 40 | ND | 15 |
| apc | 5 μg/mL | 2 | 4 | 80 | ND | 35 |
| | | 2 | 24 | 80 | 55 | 0 |
| | | 2 | 24 | 75 | 55 | 0 |
| apc | 10 μg/mL | 4 | NR | 80 | NR | NR |
| | | 4 | NR | 82 | NR | NR |

[a] Confluent T98G cells irradiated with 30 J/m² UV light were incubated in $^3$H-containing medium and then either lysed or reincubated in nonradioactive medium as shown. In most of the experiments involving a reincubation, all cells were first given a 30-min incubation in medium in which the radioactive dThd was replaced by $10^{-4}$ M nonradioactive dThd before being either lysed or reincubated. DNA of parental density was then prepared and analyzed. Each experiment included an untreated control; none of the DNA from these cells was ever rapidly digestible. [b] NR indicates experiments not involving reincubation; cells were lysed immediately at the end of the repair period. [c] ND indicates the value was not determined.

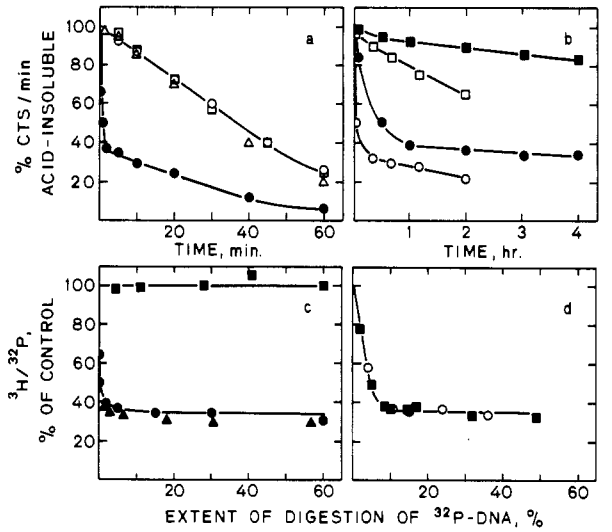


FIGURE 4: Digestion by BAL nuclease of parental-density DNA isolated from cells prelabeled with $^{32}$P and incubated in the stationary phase after UV irradiation (20 J/m²) for 4 h in the presence of [$^3$H]dThd and BrdUrd with and without inclusion of ara-C. Panels a and b depict the loss of acid-precipitable radioactivity as a function of time at different temperatures and enzyme concentrations, at 100 μg/mL DNA: (a) 34 °C at 3 units/mL, $^{32}$P (□) and $^3$H (○) in DNA from cells not incubated with ara-C, $^{32}$P (△) and $^3$H (●) in DNA from cells incubated with $5 \times 10^{-4}$ M ara-C; (b) DNA from cells incubated with $5 \times 10^{-4}$ M ara-C was digested at 20 °C, 3 units/mL (open symbols) or 0.3 unit/mL (filled symbols), (□, ■) $^{32}$P, (○, ●) $^3$H. In panels c and d, the data have been plotted to show the change in $^3$H/$^{32}$P with increasing extents of digestion of the bulk [$^{32}$P]DNA. (c) Data from the two digestions of panel a are shown: (■) DNA from untreated cells; (●) DNA from cells incubated in $5 \times 10^{-4}$ M ara-C; data from a digestion of DNA from cells incubated with $10^{-4}$ M ara-C (▲). Panel d shows the data of panel b; open and closed symbols indicate digestion with 3 and 0.3 units/mL, respectively.

(Table II). These experiments showed that the great majority of the repair label incorporated in the presence of the inhibitors after high UV doses was in an unligated form. The fraction rapidly digestible was about the same for post-UV incubations of 2–8 h and was significantly less for cells incubated 24 h, suggesting that once formed the unligated patches became ligated only very slowly. This was confirmed with a series of experiments in which cells were first incubated in repair label and then incubated in medium containing nonradioactive precursor. Incubation in dCyt to reverse the inhibitory action of apc or ara-C resulted in much more rapid apparent ligation, which was complete by 24 h.

Interpretation of these "chase" experiments requires knowledge of the extent to which unligated patches are degraded during the reincubation period, since this would remove unligated patches from the analysis. In the experiment shown in Table II, row 1, no change in the $^3$H incorporation per unit of DNA was observed after reincubation in ara-C, but a 25% increase was noted after reincubation in dCyt, indicating that the reversal of inhibition was rapid enough to allow significant incorporation of remaining intracellular [$^3$H]dThd into new repair patches. In the subsequent experiments, a 30-min incubation in medium containing inhibitor and dThd was included prior to the start of the chase. In these experiments, no significant changes in the $^3$H incorporation per unit of DNA were observed, indicating that the unligated patches are stable and thus that the decrease in nuclease sensitivity we observed does indeed represent ligation of repair patches into the parental DNA.

We also used the assay to examine the effects of HU and novobiocin. Cells were irradiated with 30 J/m² UV light and incubated for 2 h in 10 mM HU or for 4 h in 500 μg/mL novobiocin. Inhibition of repair synthesis by HU was <10%, and by novobiocin, it was 66%. In neither case, however, was there any indication of unligated repair patches.

*Size Distribution of Repair Patches.* There are a number of possible mechanisms by which the inhibition of a DNA polymerase could result in most of the repair synthesis appearing in an unligated form. Our ignorance of the fundamental details of the repair process becomes apparent when formulating these. In the normal case, we do not know whether excision precedes resynthesis or the two are tightly coupled; what controls the size of the patch is also obscure. It has usually been assumed that the inhibition of synthesis and the appearance of higher frequencies of single-strand breaks in the DNA are manifestations of a greatly decreased rate of DNA synthesis, resulting in incomplete (i.e., shorter than normal) patches that are therefore unligated. Some of the results presented so far are not entirely consistent with this model, and we therefore analyzed directly the sizes of repair

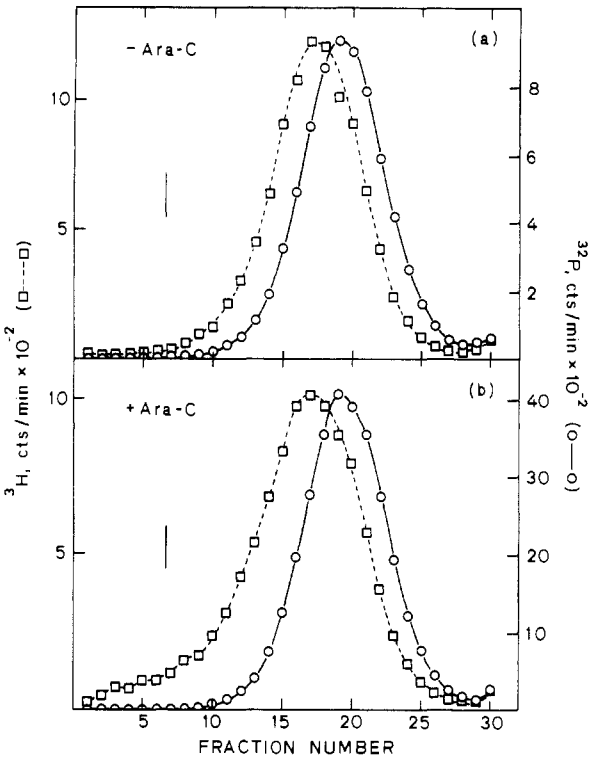


FIGURE 5: Analysis of the average size of the patches synthesized in stationary-phase T98G cells incubated in $10^{-4}$ M BrdUrd, $10^{-5}$ M FdUrd, and 40 $\mu$Ci/mL [$^3$H]BrdUrd for 8 h following 50 J/m$^2$ UV irradiation, with or without inclusion of $10^{-4}$ M ara-C. Parental-density DNA was purified, sonicated, and analyzed in alkaline CsCl gradients, profiles of which are depicted. Bars indicate the position of unsonicated hybrid DNA isolated and purified from actively growing cells incubated in [$^3$H]BrdUrd, relative to the position of light DNA, analyzed in a separate tube in the same run. Density increases to the left.

patches made under inhibitory conditions.

The analysis is an extension of the method used to measure repair synthesis. The parental-density DNA is reduced to fragments small enough that the effect of the BrUra in the repair patches on the density of the fragments carrying them can be measured by density gradient analysis in alkaline CsCl. Parental-density DNA was prepared from cells incubated in [$^3$H]BrdUrd with or without arc-C for 8 h after irradiation with 50 J/m$^2$. Repair synthesis was inhibited 75%, and 75% of it was in unligated patches. The DNA samples were sonicated, and molecular weights were measured on alkaline sucrose gradients. The number-average molecular lengths for both samples were about 175 nucleotides, and in each sample, the profiles for repair label and bulk label were the same. The DNAs were then analyzed in alkaline CsCl gradients (Figure 5). The shift in the density of the fragments carrying [$^3$H]BrUra with respect to the $^{32}$P-labeled bulk DNA is a measure of the length of the patches. Had the inhibition of repair synthesis correlated exactly with a change in the average size of the patches, we would have expected to observe a much decreased density shift in the DNA from the treated cells (Figure 5b). This was not observed. The shift in density of the peak position was the same for the two cases, and the profiles to the light sides of the peaks were identical. The only difference observed was that the dense side of the profile from the treated cells contained slightly more $^3$H than that from the untreated cells; the additional $^3$H amounted to about 18% of the total. It thus appears that in the treated cells, >80% of the incorporation was in normal sized patches, and a small fraction of the incorporation was in slightly longer patches. In this experiment, we used BrdUrd at 100 $\mu$M to ensure full substitution; a previous experiment at 10 $\mu$M gave nearly identical results. We also examined the effect of 5 $\mu$g/mL apc on the sizes of patches made in IMR90 cells irradiated with 30 J/m$^2$ UV light. The repair synthesis was inhibited 80%, and again, the patch size distribution differed from that for untreated cells only by the presence of a small contribution of slightly longer patches.

These results show that the large inhibition of repair synthesis observed is not the result of production of shorter patches under these conditions. The lack of ligation of most of the patches is not therefore a simple consequence of their size, and the inhibition observed appears to be a consequence of the synthesis of fewer patches in the treated cells than in the untreated cells.

*Ligation of Patches after Low UV Doses.* To investigate further the lack of correlation between inhibition of repair synthesis and ligation of patches, we examined ligation in stationary-phase cells irradiated with much lower doses, in which inhibition of repair synthesis is much reduced. In two separate experiments, T98G cells were irradiated at either 3.3 or 33 J/m$^2$ and incubated with or without $10^{-4}$ M ara-C for 4 h. In each case, inhibition of repair synthesis was <10%, but the fraction unligated was 80% as great as that in the DNA from cells irradiated at the high dose. The same result was obtained when comparing cells irradiated with 5 or 20 J/m$^2$, in which case the inhibition was about 50%. In this experiment, the fraction unligated dropped to zero after an 18-h reincubation in inhibitor-free medium.

With cells irradiated with 3.3 J/m$^2$ UV light and incubated with 10 $\mu$g/mL apc, we observed no inhibition of repair, but 80% of the repair synthesis and rapidly digestible. With IMR90 cells under the same conditions, about 50% inhibition was observed (as expected from Figure 3b), but the fraction rapidly digestible was 90%. None of the repair syntheses in any of the untreated cells in these experiments was ever rapidly digestible.

Taken together, all these results may be explained by the hypothesis that a pathway able to operate in the presence of these inhibitors makes full-length patches but is unable to ligate them. At low doses, this system is able to produce patches at the same rate as the system responsible for synthesis in untreated cells, and therefore, overall inhibition of repair synthesis is small. However, the maximum rate of production of patches by the alternate pathway is less than that of the pathway operating in the untreated cells, and at high doses, a large inhibition of overall repair synthesis is observed. Since, in vitro at least, the inhibitors used are specific for $\alpha$ polymerase, it seems reasonable to suggest that some other polymerase, presumably $\beta$, is responsible for synthesis in their presence.

*Displacement Synthesis.* In vitro, DNA polymerase $\beta$ has been shown capable of performing displacement synthesis with nicked DNA primer-templates (Wang & Korn, 1980; Mosbaugh & Linn, 1983). Repair patches made in vivo by such synthesis could not be ligated prior to degradation of the displaced strand. Evidence that displacement synthesis occurs in the presence of the inhibitors in vivo was obtained by analysis of the repaired DNA with S1 nuclease. This nuclease is much more specific for single-stranded DNA than is the BAL nuclease, and it degrades duplex DNA at its ends only at high nuclease concentrations and then only very slowly. The repair synthesis in cells treated with ara-C was also found to be sensitive to S1; e.g., the repair synthesis that was used for the patch size analysis was 75% rapidly digestible by the BAL nuclease, but 30% was degraded by S1 under conditions where 6% of the bulk DNA was digested.

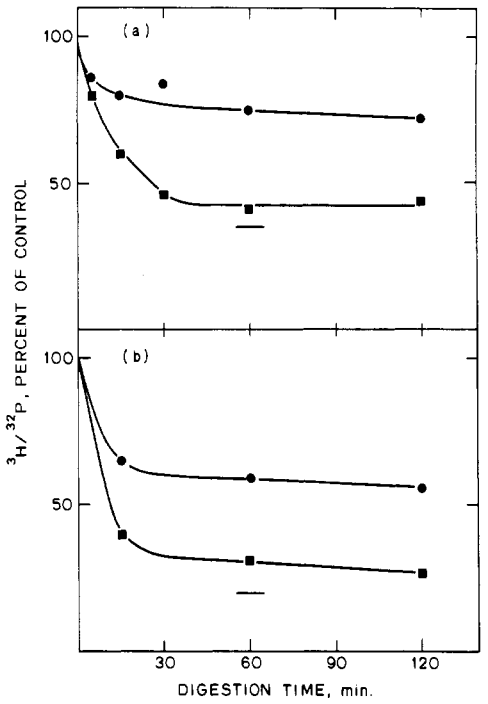


FIGURE 6: Digestions by S1 nuclease of DNA from confluent cells incubated in $^3H$ repair label for 4 h following 30 J/m$^2$ UV irradiation in medium containing $10^{-4}$ M ara-C (a) or 10 μg/mL apc (b). The control value was that obtained for DNA incubated under the same conditions for 60 min but without S1 nuclease. Incubation was at 30 °C with 50 (●) or 500 (■) units/mL S1. At the earliest times shown, about 5% of the $^{32}P$-labeled bulk DNA had been digested. After 120 min, about 10% had been digested. The lines below the lower curves indicate the values after digestion by BAL nuclease.

Examination of the results of digestion of samples from a number of experiments suggested that the extent of digestion increased with increasing S1 concentration. We therefore prepared fresh samples from cells irradiated with 30 J/m$^2$ UV light and incubated in repair label containing ara-C, apc, or no inhibitor. The repair synthesis was inhibited 69% by ara-C and 76% by apc. Analysis of these samples by both nucleases showed that at high concentrations, S1 was able to digest nearly all the rapidly digestible fraction (as defined by digestion with BAL nuclease) but only about half of it at low concentrations (Figure 6). The low S1 concentration used was able to degrade completely authentic single-stranded DNA under the conditions used.

These results are consistent with the presence of displaced parental strands at the sites of the unligated repair patches. Over the whole population, branch migration during purification of the DNA would place roughly half of the $^3H$-labeled nucleotides in a single-stranded form, thus subject to digestion by S1 at low concentrations. Once the displaced strands are degraded, the remaining $^3H$ would be in a double-stranded form and then subject to degradation only by much higher concentrations.

*Incorporation of Ara-C into DNA.* The similarity of results with ara-C and apc makes it unlikely that our observations are related to the actual incorporation of inhibitor into DNA. However, to check this and to examine the fate of any incorporated ara-C residues, we performed experiments similar to those of Table II, but using [$^3H$]ara-C instead of [$^3H$]dThd. For economy, the ara-C was used at $10^{-4}$ M, and 10 mM HU was also included. We did observe incorporation of the labeled ara-C into parental-density DNA dependent upon UV irradiation. In DNA from cells irradiated wiith 20 J/m$^2$ UV light and incubated 4 h, 90% of the incorporation was rapidly digestible. After cells were further incubated 20 h in dCyt, only 5% remained rapidly digestible; thus, the presence of ara-C in internal positions in DNA does not render it rapidly digestible. In this experiment, however, about 50% of the labeled ara-C was lost from the parental-density DNA during the chase.

*Further Controls.* The density shift technique we used is the most reliable method for measuring DNA repair synthesis because it allows physical separation of DNA made by normal replication from the DNA containing repair patches. However, we evaluated the possibility that the use of ara-C or apc might invalidate some of the assumptions underlying the method.

A large increase in the average patch size could be interpreted as repair inhibition because DNA containing repair patches would then band at positions other than that of parental-density DNA. This can be ruled out here since little normal synthesis occurred under these conditions, and no UV-dependent appearance of $^3H$ was observed at any unusual position in the neutral CsCl gradients.

Another possibility is that in the presence of the inhibitors, radioactivity may appear in the parental-density DNA as the result of some other process. Although preincubation in BrdUrd should eliminate any normal synthesis on replicons active at the time of irradiation, any initiation of replicons just prior to or after irradiation might result in the synthesis of very short chains, fully substituted with BrUra. These would not affect the analysis of repair synthesis in alkaline density gradients (confirmed by the results with unirradiated cells), but any such DNA would not be removed by the neutral gradients used to prepare DNA for patch size or nuclease analysis. The contribution of such DNA must be small, because when analyzed directly values for repair synthesis obtained in the neutral gradients were within 10% of those obtained in the subsequent alkaline ones. In addition, a significant amount of such DNA would have been revealed in the chase experiments.

We were also concerned that in the presence of these inhibitors, synthesis of mitochondrial DNA might become a significant fraction of total DNA synthesis in the cell, and the interruption of synthesis on irradiated mtDNA (Clayton et al., 1974) might result in label in parental-density DNA that was not due to repair synthesis. We irradiated cells growing exponentially (to maximize mtDNA replication) with 30 J/m$^2$ and incubated them for 4 h in 5 μg/mL apc and 10 mM HU. DNA was prepared from whole cells, isolated nuclei, and isolated mitochondria (Smith, 1977) and analyzed on alkaline CsCl gradients. The specific $^3H$ incorporation in parental-density DNA was the same for DNA from whole cells as that from nuclei, and the gradient of DNA from mitochondria contained only about 5% as much $^3H$ in the parental-density region as the same region of the gradient of DNA from nuclei. mtDNA synthesis in our experiments was thus not significant.

## Discussion

We have shown that although ara-C and apc cause inhibition of repair synthesis in contact-inhibited cells, the amount of inhibition correlated neither with the size of the repair patches made nor with the extent to which the patches were ligated into the parental DNA. After UV doses that saturate the repair system, the patches were at least as long as those made in untreated cells, but overall repair synthesis was inhibited 70–80%. This indicates that under these conditions, the inhibition results from synthesis of *fewer* rather than *shorter* patches. Nevertheless, the vast majority of such patches were not ligated into parental DNA.

Three independent measurements of the distribution of patch

sizes made under maximal inhibitory conditions indicated that the patches were not shorter than normal. Although calculations have been presented (Cleaver, 1983) suggesting that shorter patches are made under these conditions, our results represent the only direct measurements of their sizes. Our analysis would have readily detected the 4–5-fold decrease in size necessary to account for inhibition of synthesis by a decrease in patch size. In all the analyses, the CsCl gradient profiles of DNA from treated cells showed a component that can be interpreted as patches longer than normal. Subtraction of normalized profiles for treated and untreated cells showed that the average patch size in this component was roughly 3 times the size for untreated cells. Since this component comprised about 15% of the radioactivity, these longer patches were only about 5% of the total. Some of this material may be accounted for as normal DNA synthesis initiated at replicon origins during the experiment. However, radioactivity in the parental DNA in neutral gradients from unirradiated cells never approached 15% of that in the irradiated cells, and we did not observe 15% decreases in the $^3H/^{32}P$ ratios of repaired DNA after alkaline rebanding. Thus, some of this component represents longer stretches of synthesis specific to irradiated cells. If this material were all rapidly digestible, our estimates for the fraction of patches unligated are slightly high.

These results suggest that the repair patches that are made under these conditions are synthesized by a system insensitive to the inhibitors, rather than the consequence of incomplete inhibition of a process sensitive to them. This is also consistent with the relation between inhibition of repair synthesis and concentration of inhibitor (Figure 1) and is strongly supported by the fact that after UV doses so low that the overall amount of repair synthesis is hardly affected by the inhibitors, the patches were still primarily unligated.

The resistance of DNA polymerase $\beta$ to ara-CTP and apc in vitro makes it a logical candidate for the enzyme responsible for the inhibitor-resistant process. Further, its ability to carry out limited strand displacement synthesis in vitro at nicks, single nucleotide gaps, or specific incisions (Wang & Korn, 1980; Nowak et al., 1980; Mosbaugh & Linn, 1983) offers an explanation for the lack of ligation of the repair patches made under inhibitory conditions in vivo. Such an explanation is necessary if we are to discard the hypothesis that such a lack of ligation is merely the result of the patches being incomplete due to inhibition of DNA polymerase. The ligation of patches made by displacement synthesis requires degradation of the displaced strand. This either must exactly match the extent of new DNA synthesis or must proceed further, leaving a gap to the 5′ side that must be filled in by additional synthesis. The evidence obtained for displacement synthesis in vivo requires that such displaced strands remain substantially undegraded during the experiment, since we attribute the partial single-strand character of the repair synthesis to its equilibration with displaced homologous strands in vitro by branch migration. The resistance of these strands to degradation appears to be related to the inhibitors, since following their removal ligation of the patches was relatively rapid. Several 5′–3′-exonucleases in mammalian cells have been described (Bose et al., 1978; Cook & Friedberg, 1978; Hollis & Grossman, 1981) as well as ones that function in either direction in vitro [see Hollis & Grossman (1981)]. Under these conditions, the nucleotides that had been incorporated in the presence of the inhibitors were not degraded; thus, no 3′–5′ activity was apparent. It is conceivable that the exonucleolytic activity responsible for this degradation is normally complexed to DNA polymerase $\alpha$ and thus subject to inhibition by ara-C and apc. The substrate requirements of DNA polymerase $\alpha$ in vitro (Korn et al., 1981) suggest that it would require prior or concomitant exonuclease activity to carry out repair synthesis. Physical association of exonuclease activity with DNA polymerase $\alpha$ during purification has been reported (Chen et al., 1979), and repair of AP sites in vitro by a combination of AP endonuclease, DNA polymerase $\alpha$, and a 5′–3′-exonuclease has been demonstrated (Bose et al., 1978).

Repair activity in vitro mediated by a combination of DNA polymerase $\beta$ and DNase V has recently been demonstrated with a number of substrates (Mosbaugh & Linn, 1983) including UV-irradiated DNA incised with T4 endonuclease V and treated with a class II AP endonuclease to provide a proper 3′-OH terminus. The synthesis resembled nick translation, and the newly synthesized stretches were able to be ligated into the parental DNA and thus did not resemble the synthesis we have observed in vivo. It has not been shown whether ara-C or apc inhibits this combination.

Johnson et al. (1982) incubated human fibroblasts in [$^3$H]dThd for 20 min after UV irradiation and analyzed the labeled DNA by partial alkaline hydrolysis and chromatography on hydroxylapatite. The fraction of radioactivity that behaved as single-estranded DNA was increased in cells treated with ara-C and HU. This is consistent with the persistence of nicks in the vicinity of the repair patches that promote denaturation. At these short times, however, a significant fraction of the repair synthesis appeared to be unligated even in the untreated cells.

The nuclease sensitivity of [$^3$H]dThd incorporated in UV-irradiated cells has also been investigated by Cleaver. In an initial study (Cleaver, 1981) with ara-C and HU, limited sensitivity of repair synthesis to S1 nuclease was taken to suggest displacement synthesis, but not specifically by DNA polymerase $\beta$. In more recent work (Cleaver, 1983), using combinations of inhibitors, emphasis has been placed on the limited sensitivity of repair synthesis to exonuclease III, and a different conclusion was drawn, namely, that most repair synthesis is insensitive to these inhibitors (and thus insensitive to exonuclease III) and that the S1 sensitivity of the remaining patches was due to incorporation of ara-C residues or to degradation of nucleotides at the ends of duplex molecules by S1 nuclease. The origin of the discrepancy with our results is unknown. In a single experiment, we found exonuclease III nearly as effective as BAL nuclease in degrading repair patches made in the presence of inhibitors. Overall, the displacement model appears the most satisfactory explanation of our results.

The lack of an inhibitory effect on repair in exponentially growing cells has also been reported by other authors (Cleaver, 1981; van Zeeland et al., 1982; Johnson et al., 1982; Snyder & Regan, 1982) and is thought to result from the increased levels in these cells of dCTP, a competitor of the polymerase inhibitors. We did observe a small, but significant, amount of repair synthesis in the ligated from (20–30%) in the presence of inhibitors, amounting to 5–10% of the repair synthesis in untreated cells. This fraction was reduced but not eliminated by holding cells in 0.2% serum for 8 days prior to the experiment, or including HU in the medium to reduce the contribution of the small number of cycling cells in our cultures. However, even in the contact-inhibited state, some cells in the population may be less sensitive to the inhibitors due to natural variations among cells in the relevant biochemical parameters.

Despite the large amount of literature concerning the roles of DNA polymerases in repair, the subject remains controversial, and an elaboration of the current evidence is inap-

propriate here. It appears from our studies that under conditions where α polymerase is strongly inhibited, β polymerase is capable of synthesizing DNA at incision sites. Although it is not clear if this activity is related to a role for polymerase β under normal conditions, it is noteworthy that the patches synthesized are of the appropriate size and that after removal of the inhibitor the cell appears capable of processing these patches to the ligated state. It should be noted that polymerase δ has the same sensitivity to these inhibitors as polymerase α (Lee et al., 1981) and might in fact be responsible for the repair synthesis that is inhibited by these agents.

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