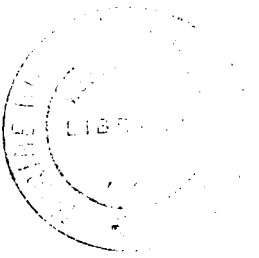

# REPAIR REPLICATION AND DEGRADATION OF BROMOURACIL-SUBSTITUTED DNA IN MAMMALIAN CELLS AFTER IRRADIATION WITH ULTRAVIOLET LIGHT

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ABSTRACT Ultraviolet (UV) light irradiation of HeLa cells in which bromouracil (BU) is substituted for thymine in one strand of the DNA, elicits a number of responses that occur predominantly in the BU strand. A small amount of degradation of both strands occurs, but the BU strand is degraded to a greater extent than the normal strand. Large UV doses (1000 erg/mm$^2$) induce degradation of about 1.7% of the DNA within 6 hr of irradiation of unsubstituted cells; in BU-substituted cells under these conditions about 1.9% of the normal strand is degraded but 17.5% of the BU strand. After irradiation fresh bases are inserted into the BU strands at infrequent intervals throughout the DNA and this is presumed to represent repair of UV damage in the BU strands. After 1000 erg/mm$^2$ the majority (70%) of the thymidine incorporated enters the BU strand. Inhibitors of normal DNA synthesis, hydroxyurea and arabinosyl cytosine, do not appear to inhibit the repair of DNA. The increased sensitivity of mammalian cells that contain BU to irradiation may consequently be due to damage of the BU strand. A specific interference between BU and repair of DNA which leads to large amounts of DNA degradation in bacteria, does not seem to be important in the sensitization of mammalian cells with BU.

## INTRODUCTION

The substitution of bromouracil (BU) for thymine in DNA sensitizes bacteria (1, 2) and mammalian cells (3–5) to killing by ultraviolet (UV) light. The sensitization is due in part to the broad spectrum of photoproducts formed in UV-irradiated BU-substituted DNA (BU-DNA) but not formed in unsubstituted DNA (6). In *Escherichia coli* an additional effect of BU substitution that produces increased killing from UV light is the interference of BU with DNA repair mechanisms (1, 2). After irradiation of BU-substituted *E. coli*, large amounts of DNA are degraded and the cells appear unable to repair their DNA and resume DNA replication (1, 2). Some experiments have indicated that BU substitution does not interfere with

DNA repair mechanisms in mammalian cells in the same way as in bacteria (7, 8). The experiments reported here demonstrate that in HeLa cells, unlike in bacteria, repair replication occurs after UV-irradiation of BU-substituted cells and most of the replication occurs in the BU-containing strand. In addition, the experiments show that only a small amount of DNA degradation occurs after irradiation. This is in contrast to the large amount of degradation that occurs in irradiated bacteria (1, 2, 9) and which has been proposed as a universal phenomenon fundamental to radiation action on living cells (10, 11).

## MATERIALS AND METHODS

### *Cell Lines and Media*

HeLa S3 monolayer cell cultures in asynchronous exponential growth were used throughout the study. They were grown in plastic Petri dishes, glass and quartz flasks at 37°C in water-saturated air containing 5% $CO_2$ using Eagle's basal medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 15% fetal calf serum.

### *Irradiation Methods*

For irradiation, the Petri dishes and quartz flasks were rinsed once in Earle's balanced salt solution and exposed to ultraviolet light at room temperature. The UV light source was a 15 w germicidal lamp which gave light of predominantly 2537 A wavelength at an incident dose rate of 10 erg/mm²/sec. Doses of 200 and 1000 erg/mm² were used throughout the experiments, and the surviving fraction of unsubstituted HeLa cells at these doses are approximately 0.25 and $2 \times 10^{-4}$, respectively, as determined by Lee and Puck (35). After irradiation, the appropriate labeling medium was added to the Petri dishes which were then returned to 37°C. Control cultures were similarly treated except for the irradiation.

### *Labeling Methods*

Cultures were grown in 5 μg/ml bromouracil deoxyriboside (BUdR) for 20–24 hr, approximately one generation time, before irradiation. After this period, most of the DNA in the cultures was hybrid, in which BU substituted for about 60% of the thymine in one strand (12). To label the cultures with radioactive precursors, fresh medium with $^{3}$H-TdR, $^{3}$H-BUdR, or $^{14}$C-BUdR (New England Nuclear Corp., Boston, Mass.) at appropriate concentrations and specific activities was placed in the Petri dishes. In the case of $^{3}$H-BUdR or $^{14}$C-BUdR, the initial nucleoside concentration was always 5 μg/ml. After incubation for the desired period the radioactive medium was removed, the cultures washed twice with 0.15 M saline-0.015 M sodium citrate and the cells processed for density gradient analysis or specific activity determination.

In the experiments designed to measure the release of material from DNA into the medium after irradiation, the specific activity of DNA and the radioactivity in the acid soluble fraction and the medium were measured. The radioactivity in the medium was determined after first precipitating the protein by boiling for 5 min with perchloric acid (PCA, 70% added to a final strength of 10%), and then counting the activity in 0.1 ml of the clear supernatant. The radioactivity in the acid soluble fraction was determined by fixing and extracting the cells with 4% PCA at 4°C for 15 min and counting 0.1 ml of the supernatant. The activity in DNA was determined by digestion of the fixed cells in 1 N NaOH for 1 hr at 37°C, precipitation of

the DNA and protein with 6 N HCl, and extraction of the DNA with 10% PCA for 8 min at 60°C. The concentration of DNA in the PCA extract was measured in terms of the optical density of the solution at 260 mμ. Specific activities were calculated as dpm/μg DNA or cpm/cell. All radioactivity determinations were made with a Packard Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) with a water-miscible counting mixture (14) and the channels so arranged that the tritium contributed insignificantly to the channel used for counting $^{14}C$. The $^{14}C$ contribution to the channel used for tritium was determined with freshly prepared standards. Quenching corrections were determined with $^{3}H$-toluene and $^{14}C$-toluene internal standards.

### *Density Gradient Methods*

The washed cells were suspended in 0.5 ml of saline-citrate to which three drops of sodium dodecyl sulphate (1%) were added. The cells were immediately subjected to three rapid freeze-thaw cycles, by alternating between acetone-dry ice and hot (60°C) water. The lysate was then incubated at 37°C with 20 μg/ml boiled RNase for 30 min followed by 0.5 mg/ml pronase for 2 hr, and deproteinized twice by shaking with an equal volume of chloroform-amyl alcohol (24:1). No attempt was made to avoid shearing, and the final solution contained fragmented DNA with an average molecular weight, in the absence of BUdR, of approximately $10^7$–$10^8$ daltons (12). The solution was dialysed overnight against at least two changes of saline-citrate to remove low molecular compounds and unincorporated radioactivity. For some experiments the lysate was sheared with an omnimixer to a final molecular weight of approximately $5 \times 10^6$ daltons (12).

The final lysate was diluted to about 5 ml with citrate-saline and exactly 4.5 ml of lysate was added to 5.9 g of CsCl (The Harshaw Chemical Co., Cleveland, Ohio optical grade). For rebands at pH 12.5 (single-stranded DNA, reference 15), the CsCl in the pooled fractions from the first gradient was removed by dialysis, the sample diluted to 5 ml with alkaline phosphate buffer to pH 12.5, and exactly 4.5 ml of lysate was added to 6.3 g of CsCl. The solutions were put into 12 ml cellulose nitrate centrifuge tubes, overlayed with mineral oil, and centrifuged in an SW 40 fixed angle rotor at 37,000 rpm for 48 hr at 20°C in a Spinco model L centrifuge (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.). In some rebands the SW 39 swinging bucket rotor was used, and then 3.0 ml of the alkaline lysate was added to 4.1 g of CsCl and centrifuged at 37,000 rpm for 48 hr. At the end of centrifugation the tubes were removed from the centrifuge, a hole made in the bottom of each tube, and 15-drop fractions collected in test tubes with an automatic collecting device. Either continuous absorbance measurements during the drop collection were made at 2600 A, or optical density measurements were made on each fraction. Radioactivity determinations were made with 0.1 ml or 10 μl samples from each fraction.

## EXPERIMENTAL RESULTS

### *DNA Cross-Linking after Irradiation*

In many of the experiments that are to be described, an important step is the alkaline denaturation of DNA harvested from irradiated cells in order to study incorporation of precursors into individual DNA strands. The following experiment was performed as a control to determine whether interstrand cross-linking caused by UV light (16, 17) was an important factor to consider. Cultures were labeled with $^{14}C$-TdR for 90 hr, grown in fresh medium for 2 hr to exhaust labeled nucleo-

tide pools (18), labeled for 20 hr with $^{3}$H-BUdR, and then grown for a further 2 hr to exhaust labeled pools once more. At the end of this sequence, most of the DNA of the culture was hybrid with $^{14}$C-thymine in one strand and $^{3}$H-bromouracil in the opposite strand. The cultures were then irradiated with 1000 erg/mm$^{2}$, incubated for 4 hr in nonradioactive medium, and harvested for an alkaline density gradient. The gradient profile is shown in Fig. 1, and consists of two clearly defined radioactivity peaks corresponding to the denatured complementary DNA strands. If there were significant amounts of cross-linked DNA, one would have expected DNA of intermediate density containing both $^{3}$H and $^{14}$C, or $^{14}$C activity in the $^{3}$H peak region or vice versa. The latter is not seen in Fig. 1 and when DNA

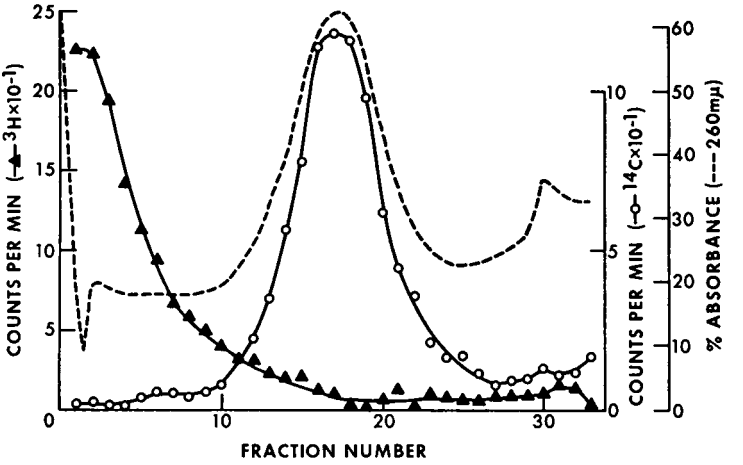


FIGURE 1 Density gradient profile pH 12.5 in SW 40 rotor of HeLa DNA labeled for 90 hr with $^{14}$C-TdR (0.1 μc/ml 50 mc/mmole), followed by 20 hr with $^{3}$H-BUdR (20 μc/ml 5 μg/ml), and then irradiated with 1000 erg/mm$^{2}$, and harvested 4 hr after irradiation. —▲— $^{3}$H, —○— $^{14}$C, ----- absorbance at 260 mμ, nonradioactive HeLa DNA added as normal density absorbance marker.

from the intermediate region (fractions 4–15) was rebanded, the $^{3}$H and $^{14}$C activity showed two distinct peaks in the same respective positions as obtained in Fig. 1. The overlap of activity was consequently due merely to the broad band widths of the original $^{3}$H and $^{14}$C peaks. UV-induced cross-linking was consequently of trivial importance under the conditions of these experiments.

### *Repair in Bromouracil DNA-Strands*

The initial experiments to study the UV response of cells containing BU-DNA were done with cultures in which most of the DNA was hybridized after growth for about one cell cycle in BUdR. The cultures were grown in BUdR for 23 hr, incubated in fresh medium with no BUdR for 2 hr, then irradiated with 200 or 1000 erg/mm$^{2}$, and labeled with $^{3}$H-TdR (10 μc/ml, 17 c/mmole) for 3 hr. The density

gradient profiles obtained from this experiment are shown in Fig. 2. In each profile the main absorbance peak occurs at the position of hybrid DNA with a minor peak at the position of normal density DNA. In the control there is a single $^3H$ peak at the position of normal density but some detectable $^3H$ in the region of hybrid density. This profile is to be expected if the DNA which was not yet substituted by BU after 23 hr in BUdR replicated semiconservatively during the 3 hr of $^3H$-TdR labeling. Semiconservative replication of hybrid DNA would produce

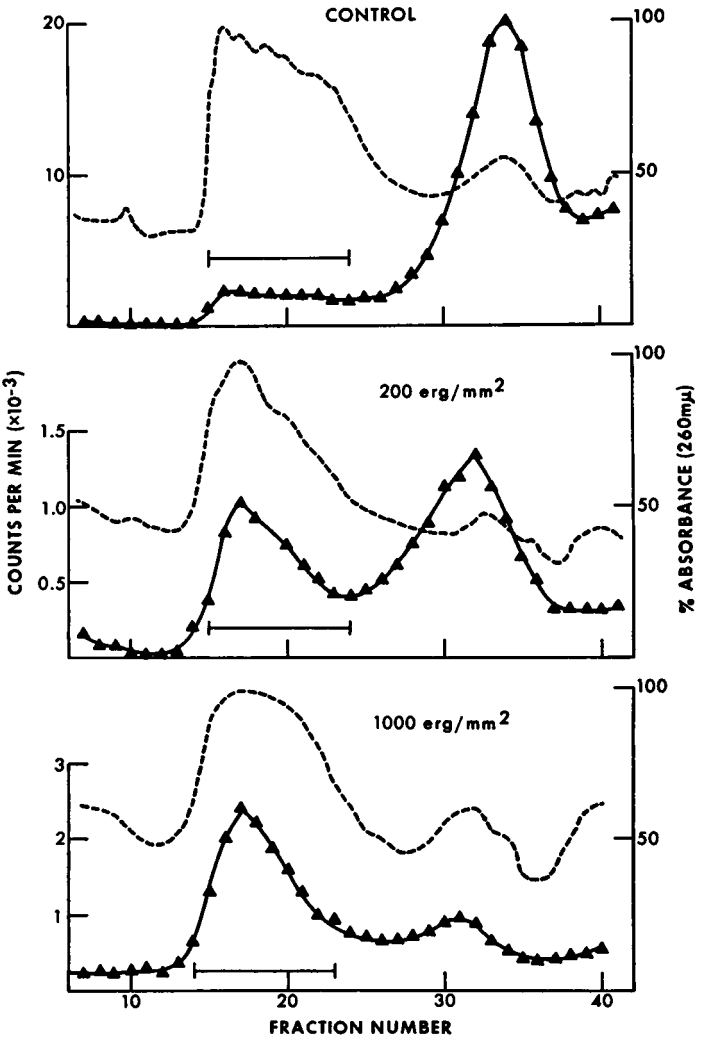


FIGURE 2 Density gradient profiles pH 7.0 in SW 40 rotor of HeLa DNA grown with BUdR (5 $\mu$g/ml) for 23 hr, irradiated with 200 or 1000 erg/mm$^2$, and labeled with $^3H$-TdR (10 $\mu$c/ml 17 c/mmole) for 3 hr. —▲— $^3H$, ----- absorbance at 260 m$\mu$. Bar marks regions harvested for rebanding (see Fig. 3).

equal amounts of $^3$H-labeled hybrid and normal density DNA. The presence of unhybridized DNA in the culture after 23 hr in BUdR, which replicates during the subsequent 3 hr in $^3$H-TdR, may be due either to cells that had not finished one complete cycle of DNA replication in BUdR or to cells in which some of the DNA had not incorporated BUdR (19, 20). These two alternatives were not distinguished in the current experiments.

In the $^3$H profiles from irradiated cultures, the $^3$H-TdR incorporation is reduced in the normal density position but the incorporation at hybrid density appears relatively more prominent. The reduction in the incorporation of $^3$H-TdR into normal density DNA corresponds to the inhibition of DNA replication by UV

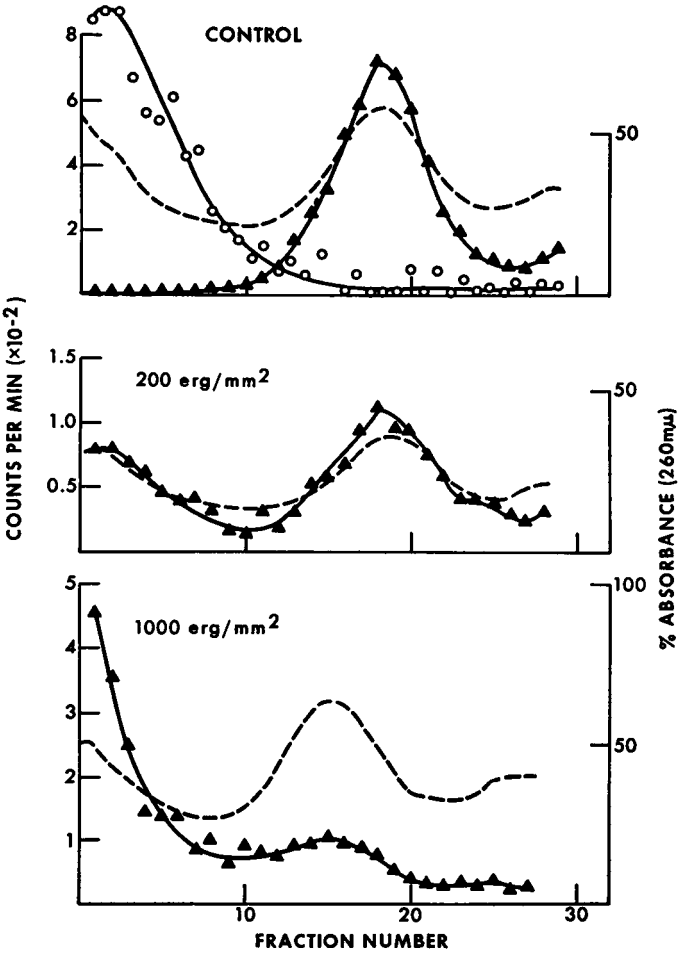


FIGURE 3 Density gradient profiles pH 12.5 in SW 40 rotor of selected regions from profiles in Fig. 2. —▲— $^3$H, —○— $^{14}$C-BUdR-labeled DNA added as marker, ----- absorbance at 260 mμ.

light that has been extensively reported in previous publications (13, 21, 22). The incorporation of $^3$H-TdR into hybrid DNA is, however, unusual because this is increased by the UV light. Particularly at the higher dose, 1000 erg/mm$^2$, the incorporation into hybrid density DNA cannot be due to semiconservative replication because this would have produced equal amounts of $^3$H at normal and hybrid densities. The DNA from the hybrid regions of each gradient was then harvested and rebanded at pH 12.5 to separate the BU and normal strands and determine the distribution of $^3$H between the two strands.

The profiles obtained from the alkaline rebands are shown in Fig. 3. In each profile there are two absorbance peaks corresponding to the BU and the normal strands. In the control, the $^{14}$C-BUdR labeled DNA marker identifies the position of BU strands, and the $^3$H label is confined to the region of normal density. Consequently the $^3$H-TdR incorporation into unirradiated hybrid DNA in the original density gradient is due to semiconservative DNA replication in which the BU strand was the template and $^3$H-TdR was incorporated exclusively into newly synthesized normal density strands. After irradiation, however, there is a different incorporation pattern, as illustrated in Fig. 3. After both 200 and 1000 erg/mm$^2$, there is a significant amount of $^3$H-TdR in the BU strands. At the higher dose of 1000 erg/mm$^2$, incorporation into the BU strand is predominant over incorporation into the normal strand (approximately 70% of the $^3$H activity in the two absorbance peaks is in the heavy region). For $^3$H activity to be found at the position of single-stranded BU-DNA, $^3$H-TdR must have been incorporated into the strands in a way that did not significantly affect the density. This could have occurred, for example, by the incorporation of $^3$H-TdR into small regions of the UV-damaged BU strands during repair, a process that is termed "repair replication" (9, 23). The results shown in Fig. 3 suggest that in HeLa cells containing BU-hybridized DNA, the DNA strand containing BU is responsible for much of the UV response.

Incorporation of $^3$H-TdR into normal density strands still occurred at 1000 erg/mm$^2$ (Figs. 2 and 3). This may have been the result of either a small amount of semiconservative DNA replication or of repair replication of UV damage in the normal strand. The latter is known to occur after irradiation of unhybridized HeLa cells with UV light (23) or high doses of X-rays (24).

### *Repair of Bromouracil Strands in the Presence of Inhibitors of DNA Synthesis*

The survival of HeLa cells irradiated with X-rays is reduced by inhibitors of DNA synthesis (25). To determine whether these observations could be related to a specific effect of inhibitors on repair replication, the experiments described in the previous section were repeated with two inhibitors. A similar experimental protocol was used: 18 hr in 5 $\mu$g/ml BUdR followed by 2 hr in fresh medium and then irradiation with 200 erg/mm$^2$. After irradiation cultures were labeled for 3 hr with $^3$H-TdR in the presence of hydroxyurea ($2 \times 10^{-3}$ M) or arabinosyl cytosine ($2 \times 10^{-5}$

M). On the basis of preliminary experiments using the same protocol, the incorporation of $^3$H-TdR into DNA of unirradiated cultures was reduced to 7% by hydroxyurea and 10% by arabinosyl cytosine. The density gradient profiles obtained from control and irradiated cultures are shown in Fig. 4. The two inhibitors give very similar profiles, and there is an increase in the amount of $^3$H at hybrid

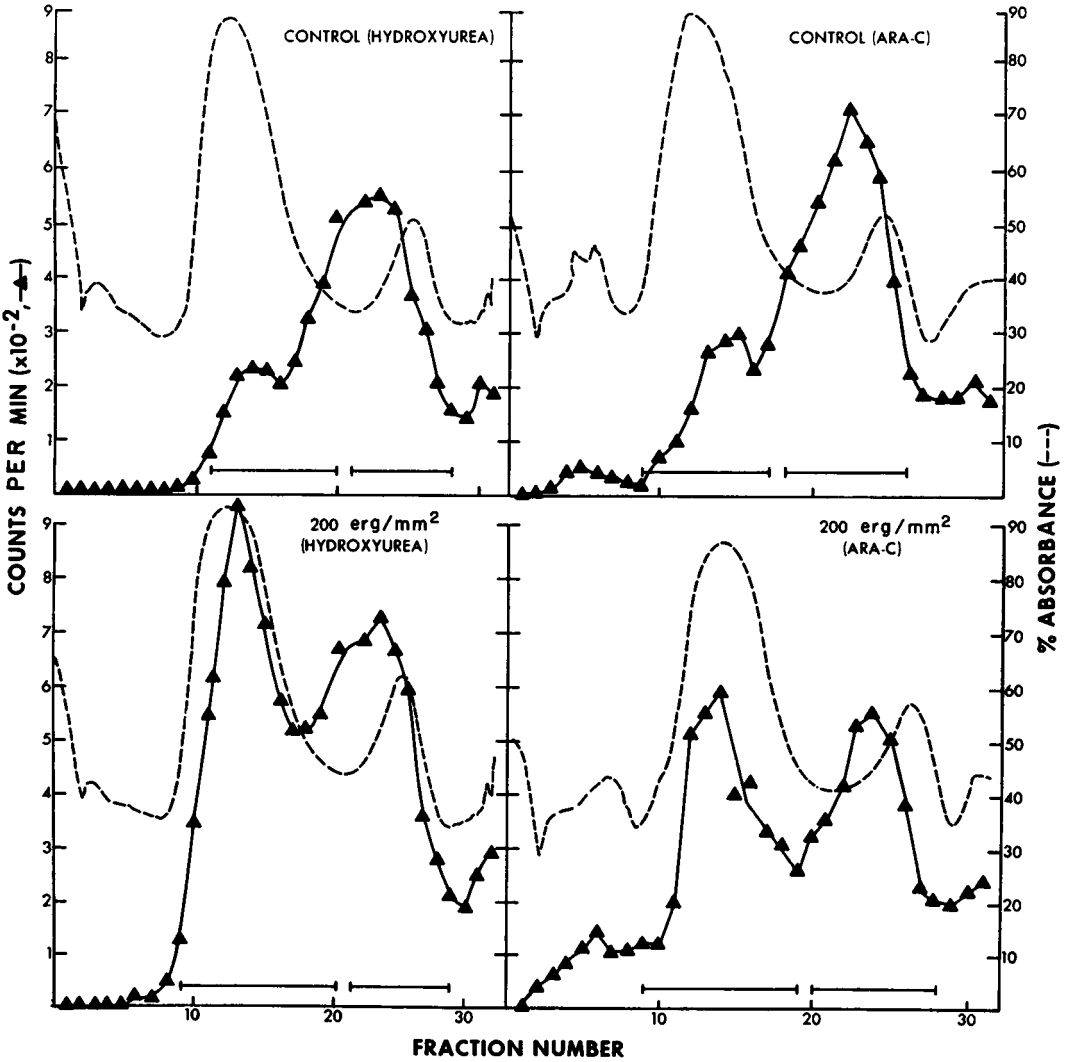


FIGURE 4 Density gradient profiles pH 7.0 in SW 40 rotor of HeLa DNA grown with BUdR (5 $\mu$g/ml) for 18 hr, irradiated with 200 erg/mm$^2$, and labeled with $^3$H-TdR (10 $\mu$c/ml 17 c/mmole) for 3 hr in the presence of hydroxyurea ($2 \times 10^{-3}$ M) or arabinosyl cytosine ($2 \times 10^{-5}$ M). —▲— $^3$H, —— absorbance at 260 m$\mu$. Bar marks regions harvested for rebanding (see Fig. 5).

density positions as a result of the irradiation. In the control profiles, the peaks of $^3H$ activity are not coincident with the absorbance peaks, which indicates that the DNA replicated with $^3H$-TdR in the presence of an inhibitor had a density intermediate between the densities of normal and hybrid DNA. After irradiation, the light density $^3H$ peaks are in the same positions as in the corresponding control profiles, but the heavy peaks coincide with the hybrid density absorbance peak. Most of the $^3H$-TdR incorporation into hybrid DNA molecules after irradiation consequently occurs in molecules that are distinct from those made by semiconservative replication without irradiation.

The intermediate density $^3H$ in controls and in the light region of irradiation profiles must be due to the density of the parental DNA strands since $^3H$-TdR was the only label employed. The label and inhibitors were supplied between 20 and 23 hr, approximately one cell cycle after BUdR had been first added to the cultures,

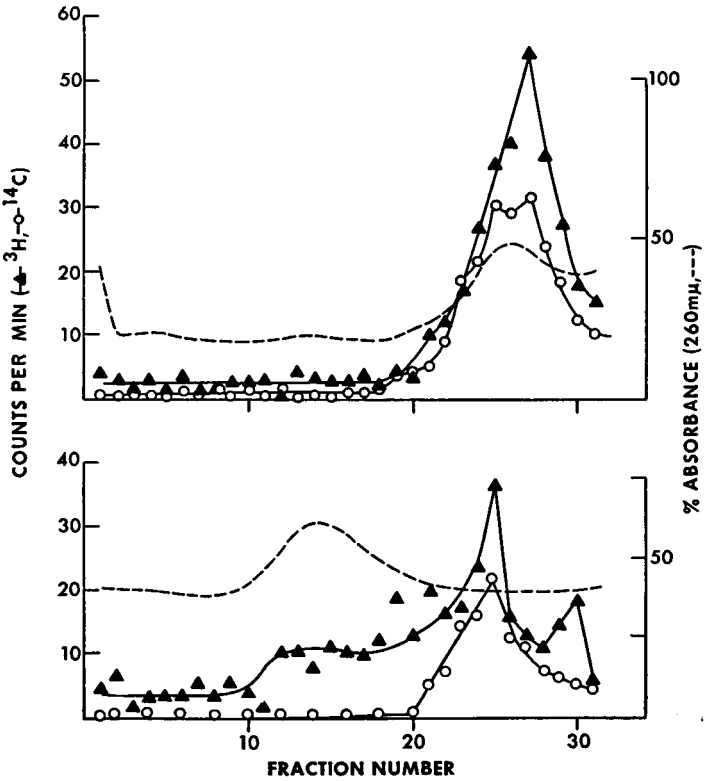


FIGURE 5 Density gradient profiles pH 7.0 in SW 40 rotor of selected regions from hydroxyurea control profiles in Fig. 4, after shearing. —▲— $^3H$, —○— $^{14}C$-TdR-labeled DNA added as marker, ----- absorbance at 260 mμ. Top, normal density DNA. Bottom, hybrid density DNA.

though in the presence of the inhibitors only a small amount of DNA is replicated. The parental strands of the DNA replicated with $^3$H-TdR consequently contained regions in which BUdR had first been incorporated one cell cycle earlier. Intermediate density $^3$H-labeled DNA should thus contain parental strands which are of normal density at one end and BU density at the other. These two regions can be separated by shearing, and Fig. 5 shows the neutral pH rebands of sheared material from both $^3$H peaks in the hydroxyurea control gradient. The peaks of $^3$H activity in the sheared gradients now coincide with the absorbance and $^{14}$C marker peaks. Similar results were obtained with each of the intermediate density $^3$H peaks of Fig. 4. The intermediate densities consequently arose in the way described by the preceding argument. The profiles were thus due solely to the time sequence of the experiment, not to any specific effect characteristic of hydroxyurea and arabinosyl cytosine.

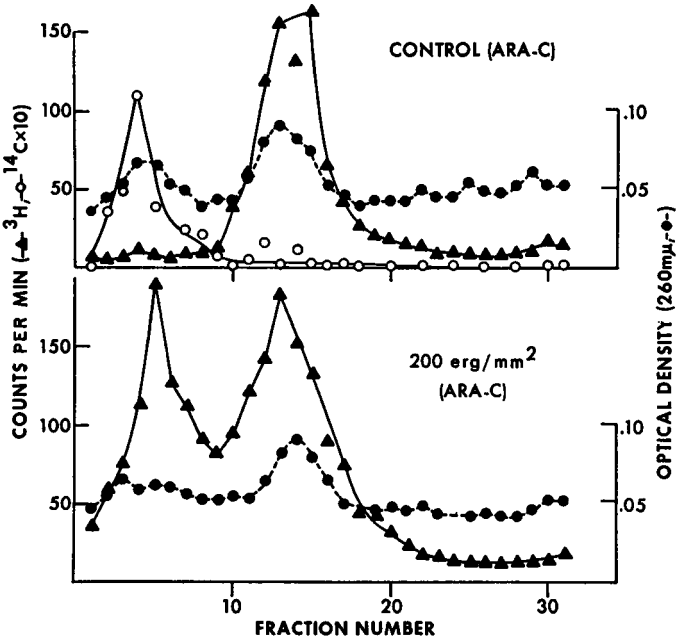


FIGURE 6 Density gradient profiles pH 12.5 in SW 39 rotor of hybrid density regions from arabinosyl cytosine profiles in Fig. 4. —▲— $^3$H, —○— $^{14}$C-BUdR-labeled DNA added as marker, ----- absorbance at 260 mμ.

The hybrid density material from each gradient was also rebanded in an alkaline gradient to determine the $^3$H distribution between the strands. The rebands from the arabinosyl cytosine gradients are shown in Fig. 6, and similar results were obtained from the hydroxyurea gradients. In the control reband all of the $^3$H is at normal density, further confirming the conclusions drawn from the sheared rebands of Fig. 5: that the intermediate densities in Fig. 4 were due to the parental BU-

containing strands. In the irradiated reband, the $^3H$ profile shows two peaks, at the positions of heavy BU strands and light normal strands. With the SW 39 rotor, the BU strands band several fractions from the bottom of the tube whereas in the SW 40 rotor (Figs. 1 and 3), the BU strands band on the bottom of the tube. This profile is very similar to the 200 erg/mm$^2$ alkaline reband shown in Fig. 3, which was obtained without the use of inhibitors. A quantitative comparison of the amount of $^3H$-TdR incorporated during repair replication of normal and BU-DNA with a number of inhibitors will be reported later.[1] From preliminary quantitative results, as suggested by the gradient profiles of Figs. 3 and 6, hydroxyurea and arabinosyl cytosine have no influence on the amount of repair replication. In the density gradient profiles from irradiated cultures (Fig. 4) the $^3H$ activity peak thus coincides with the peak absorbance of the hybrid DNA of the gradient because the $^3H$ is distributed throughout the hybrid DNA molecules. In the profiles from the control cultures (Fig. 4), however, the $^3H$-TdR is incorporated into only the small fraction of the DNA that contains intermediate density parental strands.

*DNA Degradation after Irradiation*

The previous experiments have shown that much of the incorporation of $^3H$-TdR into BU-DNA after irradiation occurs in the BU strand. In accordance with the "cut and patch" model for repair of UV damage (9), much of the excision of material from DNA should occur from the BU strand. To test this prediction, cultures were grown in $^3H$-TdR or $^{14}C$-TdR to prelabel the normal density DNA and then hybridized with $^{14}C$-BUdR or $^3H$-BUdR, respectively. Experiments with hybrid BU-DNA were done with $^3H$ on the BU strand and $^{14}C$ on the opposite normal strand, and vice versa. This was to ensure that results could be ascribed to the presence of BU rather than to differential radiation damage on the two strands from the extremely short-ranged $^3H$ $\beta$ particles. The cultures were irradiated, the medium changed, and the release of material from DNA into the medium was determined by comparing the $^3H/^{14}C$ ratios in DNA with the $^3H/^{14}C$ ratios for radioactivity found in the medium. (Radioactivity in the acid soluble fraction was indistinguishable from background.) Table I shows the results that were obtained for BU-hybridized cultures (Expts. 1 and 2) and for an experiment (Expt. 3) in which cells were labeled with $^{14}C$-TdR in one DNA strand and $^3H$-TdR in the other. In each experiment the medium from control and irradiated cultures contained a significant amount of radioactivity. At 1000 erg/mm$^2$ the radioactivity, both $^3H$ and $^{14}C$, in the medium 4 hr after irradiation in Expts. 2 and 3 is significantly greater than that found in unirradiated cultures. At shorter times and lower doses, the activity in the medium is not significantly different from that in unirradiated cultures. The $^3H/^{14}C$ ratios in the medium for the cultures labeled with TdR in both strands, 3.98–7.97 (Expt. 3), are similar to the $^3H/^{14}C$ ratio in DNA,

[1] J. E. Cleaver. Paper in preparation.

TABLE I

## RADIOACTIVITY IN DNA, AND RELEASED TO THE MEDIUM, FOR HeLa CELLS HYBRIDIZED WITH BROMOURACIL AND IRRADIATED WITH ULTRAVIOLET LIGHT

*Experiment 1*

Protocol: $^{3}$H-TdR, 1 μc/ml 0.36 c/mmole, 24 hr/*/$^{14}$C-BUdR 0.4 μc/ml 20 mc/mmole, 20 hr/*

| | | | |
|---|---|---|---|
| DNA specific activity‡ | | | |
| $^{3}$H | | 74,500 ± 970 | |
| $^{14}$C | | 2,140 ± 30; without $^{3}$H prelabel 6,000 | |
| $^{3}$H/$^{14}$C ratio | | 34.8 ± 6.4 | |
| Medium 2 hr postirradiation§ | | | |
| | Control | *200 erg/mm²* | *1000 erg/mm²* |
| $^{3}$H | 710 ± 51 | 825 ± 102 | 787 ± 127 |
| $^{14}$C | 214 ± 15 | 229 ± 31 | 297 ± 15 |
| $^{3}$H/$^{14}$C ratio | 3.32 | 3.60 | 2.65 |

*Experiment 2*

Protocol: $^{14}$C-TdR 0.1 μc/ml 50 mc/mmole, 90 hr/*/$^{3}$H-BUdR, 20 μc/ml 5 μg/ml, 20 hr/*

| | | | |
|---|---|---|---|
| DNA specific activity‡ | | | |
| $^{3}$H | | 75,800 ± 5,800; without $^{14}$C prelabel 85,800 | |
| $^{14}$C | | 16,000 ± 2,020 | |
| $^{3}$H/$^{14}$C ratio | | 4.73 ± 0.32 | |
| Medium 2 hr postirradiation§ | | | |
| | Control | *200 erg/mm²* | *1000 erg/mm²* |
| $^{3}$H | 376 ± 27 | 186 ± 9 | 358 ± 37 |
| $^{14}$C | 9.2 ± 1.8 | 7.0 ± 1.5 | 6.1 ± 0.9 |
| $^{3}$H/$^{14}$C ratio | 42.0 | 26.6 | 58.7 |
| Medium 4 hr postirradiation§ | | | |
| $^{3}$H | 294 ± 9 | 214 ± 39 | 417 ± 25 |
| $^{14}$C | 6.9 ± 3.1 | 6.9 ± 3.1 | 15.3 ± 1.2 |
| $^{3}$H/$^{14}$C ratio | 43.3 | 31.0 | 26.3 |

*Experiment 3*

Protocol: $^{14}$C-TdR 0.1 μc/ml 50 mc/mmole, 90 hr/*/$^{3}$H-TdR 0.1 μc/ml 0.36 c/mmole, 20 hr/*

| | | | |
|---|---|---|---|
| DNA specific activity‡ | | | |
| $^{3}$H | | 95,900 ± 7,660; without $^{14}$C prelabel 98,200 | |
| $^{14}$C | | 20,020 ± 2,350 | |
| $^{3}$H/$^{14}$C ratio | | 4.79 ± 0.09 | |
| Medium 2 hr postirradiation§ | | | |
| | Control | *200 erg/mm²* | *1000 erg/mm²* |
| $^{3}$H | 63.5 ± 6.4 | 78.8 ± 21.6 | 55.9 ± 5.1 |
| $^{14}$C | 8.6 ± 0.6 | 11.3 ± 4.6 | 8.9 ± 2.1 |
| $^{3}$H/$^{14}$C ratio | 7.38 | 6.98 | 6.28 |
| Medium 4 hr postirradiation§ | | | |
| $^{3}$H | 68.6 ± 1.3 | 58.5 ± 3.8 | 122 ± 12 |
| $^{14}$C | 8.6 ± 0.3 | 14.7 ± 1.8 | 27.2 ± 1.2 |
| $^{3}$H/$^{14}$C ratio | 7.97 | 3.98 | 4.48 |

*A 2 hr interval in nonradioactive medium was used between each change of label and between the end of labeling and irradiation.

‡ Mean dpm/μg DNA ± standard error. Control and irradiated values at 2 and 4 hr post-irradiation have been combined since there was no trend in values with time or dose.

§ Mean dpm/0.1 ml of protein-free medium ± standard error.

4.79. For the cultures hybridized with BU (Expts. 1 and 2) there is a 5-10–fold difference in the ratios from medium and DNA. The difference in the $^{3}H/^{14}C$ ratios for both experiments is in the direction of greater release of label from the BU strand, irrespective of which strands contained the $^{3}H$ and $^{14}C$. In Expt. 1, for example, in which the DNA was labeled with $^{14}C$-BUdR the $^{3}H/^{14}C$ ratio in DNA is 34.8, whereas the ratio in the medium was between 2.65 and 3.60. This indicates that $^{14}C$-BU was released from the DNA to a greater extent than $^{3}H$ from the opposite strand. Unfortunately, this preferential release of BU from DNA appears to be partly due to causes other than irradiation, since the release occurs from both UV-irradiated and unirradiated cultures. Possible causes may be radiation damage from $^{3}H$ $\beta$ particles and the abnormal metabolism of BU itself. In Expt. 1 the incorporation of $^{14}C$-BUdR into DNA was reduced by prelabeling with $^{3}H$-TdR as compared to cultures labeled with $^{14}C$-BUdR without $^{3}H$ prelabeling. This is presumably due to reduction of the rate of DNA replication by $^{3}H$ $\beta$-particle irradiation, with the result that less DNA was hybridized than in the cultures which were not prelabeled (26). Such reduction in the rate of DNA replication was not observed with $^{14}C$-TdR prelabeling (Expts. 2 and 3).

Since the results of Table I suggest that there is a small increase in the release of BU into the medium after large doses, a further experiment was performed to determine approximately the fraction of DNA released as a consequence of irradiation. Cultures were grown in $^{14}C$-BUdR or $^{14}C$-TdR to obtain three categories of labeled DNA. One category was $^{14}C$-TdR, only, in the DNA; another was $^{14}C$-TdR in one strand and nonradioactive BUdR in the other strand; the third category was $^{14}C$-BUdR in one strand and no label in the other. The detailed time schedules are shown in Table II, and in each category the cultures were grown in nonradioactive medium for at least 15 hr (i.e. overnight) immediately prior to irradiation. The medium was then changed and the cultures irradiated with 1000 erg/mm$^{2}$ and harvested 6 hr after irradiation. At this time there was no visible necrosis although a small amount cannot be completely excluded. Any radioactivity in the medium should be due to degradation of DNA within the cells, rather than death and lysis of a small part of the population. At 6 hr after irradiation the cell number, and the total radioactivity in the medium, in the acid soluble fraction, and in DNA was determined. From these values the radioactivity per cell in DNA and the activity released into the acid soluble fraction and the medium were calculated. These are shown in Table II. In cultures labeled with $^{14}C$-TdR only, 1.7% of the DNA was released into the medium as a result of irradiation. From hybrid BU-DNA, 1.9% of the normal strand was released into the medium but 17.5% of the BU strand. The amount of degradation of the normal strand is thus independent of the presence of BU in the complementary strand, but about 10 times as much of the BU strand is degraded as compared to the normal. These results thus confirm the conclusions drawn from the double labeling experiments summarized in Table I. Also,

in all of the experiments the amount of activity in the acid soluble fraction is much less than that found in the medium.

TABLE II

LOSS OF RADIOACTIVITY FROM PRELABELED CELLS AT 6 HR AFTER 1000 ERG/MM² OF UV LIGHT*

| | Control | Irradiated | Percentage loss due to irradiation |
|---|---|---|---|
| **Prelabeled with $^{14}$C-TdR (100 hr followed by 15 hr in unlabeled medium)** | | | |
| DNA | 6.01 ± 0.14 | 5.14 ± 0.19 | |
| DNA (corrected)‡ | 7.33 ± 0.17 | — | |
| Acid soluble | $1.85 \times 10^{-4}$ | $4.13 \times 10^{-4}$ | |
| Percentage of DNA | 0.0025 | 0.0081 | 0.0056 |
| Medium | $(2.28 \pm 0.06) \times 10^{-2}$ | $(10.40 \pm 0.34) \times 10^{-2}$ | |
| Percentage of DNA | (0.3 ± 0.1) | (2.0 ± 0.1) | 1.7 ± 0.2 |
| **Prelabeled with $^{14}$C-TdR (36 hr followed by 31 hr in unlabeled BUdR)** | | | |
| DNA | 0.469 ± 0.034 | 0.925 ± 0.164 | |
| DNA (corrected)‡ | 0.572 ± 0.042 | — | |
| Acid soluble | $(0.10 \pm 0.1) \times 10^{-2}$ | $(0.35 \pm 0.08) \times 10^{-2}$ | |
| Percentage of DNA | (0.17 ± 0.03) | (0.38 ± 0.11) | 0.2 ± 0.1 |
| Medium | $(0.34 \pm 0.03) \times 10^{-2}$ | $(2.28 \pm 0.40) \times 10^{-2}$ | |
| Percentage of DNA | (0.6 ± 0.1) | (2.5 ± 0.6) | 1.9 ± 0.6 |
| **Prelabeled with $^{14}$C-BUdR (30 hr followed by 15 hr in unlabeled medium)** | | | |
| DNA | 0.144 ± 0.005 | 0.138 ± 0.007 | |
| DNA (corrected)‡ | 0.176 ± 0.006 | — | |
| Acid soluble | $3.62 \times 10^{-5}$ | $13.8 \times 10^{-5}$ | |
| Percentage of DNA | 0.02 | 0.10 | 0.08 |
| Medium | $(0.124 \pm 0.003) \times 10^{-2}$ | $(2.51 \pm 0.09) \times 10^{-2}$ | |
| Percentage of DNA | (0.7 ± 0.3) | (17.9 ± 1.1) | 17.2 ± 1.1 |

* All values given as mean ± standard error of the mean in dpm/cell.

‡ All samples were harvested 6 hr after the time of irradiation. The control DNA dpm/cell are corrected for 6 hr exponential growth in unlabeled medium in order to express the loss of radioactivity as a percentage of the activity in DNA at the time of irradiation and activity in medium. Such correction is not applied to the irradiated DNA dpm/cell because 1000 erg/mm² stops cell division and DNA synthesis within a few minutes (21, 22).

## DISCUSSION

BU, itself, produces a wide variety of photoproducts on irradiation with UV light (6) and in BU-hybridized DNA, more radiation damage occurs in the BU strand

than in the complementary normal strand. Single strand breaks, for example, are not produced in normal DNA by UV light but in BU-hybridized DNA, UV light produces breaks in the BU strand (27). The alkaline density gradients of Figs. 3 and 6 show that after irradiation with UV light, $^3$H-TdR was incorporated into the BU strands without affecting their density. Such incorporation must have been in the form of small numbers of nucleotides inserted at infrequent intervals throughout the DNA fragments, otherwise the density of $^3$H-labeled BU strands would have been lighter than unirradiated BU strands. Insertion of small numbers of bases into DNA after irradiation is characteristic of the nonconservative mode of repair replication which is observed in irradiated *E. coli* (9), *Tetrahymena* (28), and mammalian cells (7, 23, 37). A reduced ability to perform repair replication is correlated with increased radiation sensitivity in both mammalian cells (36) and bacteria (37), and this process is therefore important in the recovery of irradiated cells. The results of this paper show that upon irradiation of BU-substituted cells, much of the damage and much of the repair replication occurs in the BU strand.

Excision of damaged regions of DNA strands is a prerequisite for repair, according to current theories (9, 29), and the results given in Tables I and II show that there is more excision of material from the BU strand than from the normal strand. Excision, however, involves only a small proportion of the DNA of the cell, which is in contrast to the large amount of excision that occurs from UV-irradiated bacteria (1, 2, 9). In unsubstituted *E. coli* approximately 50% of the DNA is degraded, and some of this replaced by repair replication, within 3 hr of irradiation with 500 erg/mm$^2$ (9). In BU-substituted *E. coli*, the BU interferes with repair replication, and degradation of up to 70% of the DNA occurs after 1000 erg/mm$^2$ (2). In mammalian cells, as the results of Tables I and II show, DNA degradation is a small part of the UV response, even at large doses. In mammalian cells containing unhybridized DNA, 1000 erg/mm$^2$ results in the conversion of approximately 0.5% of the thymines to thymine dimers (30). The amount of material released from such cells (1.7%, Table II) is only slightly more than required for excision of the dimers, though all are not necessarily excised (31). The increased excision from BU-hybridized cells is probably due to the damage associated with BU photoproducts, but quantitative data on numbers and types of biologically important BU photoproducts are unavailable. Also, the incorporation of $^3$H-TdR into BU strands during repair replication is at such infrequent intervals throughout the DNA that there is no detectable alteration of the density of the strands (Figs. 3 and 6), whereas the extensive replacement of material that occurs in bacteria can result in the formation of a large proportion of intermediate density DNA (9).

Repair replication in the BU strand appears to be unaffected by the presence of hydroxyurea or arabinosyl cytosine. Hydroxyurea has also been shown to have no effect on repair replication in unsubstituted mammalian cells after irradiation with UV light (8) or large doses of X-rays (24). The basis for enhanced killing of irradiated cells by these agents after irradiation with X-rays (25) thus may not be due to

interference with repair replication of damaged DNA. The most likely reason that the inhibitors do not affect repair replication in mammalian cells is that they inhibit DNA replication by merely reducing the level of nucleotide triphosphates. Hydroxyurea may act mainly at the nucleotide diphosphate reductase step (32, 33) and arabinosyl cytosine on the deoxycytidine synthetic pathway (34). The small numbers of nucleotides required for repair replication may still be available even in the presence of these inhibitors.

The presumed sequence of events upon irradiation of mammalian cells containing DNA hybridized with BU is as follows. The DNA suffers more damage to the BU strand than the normal strand. Damaged regions are then excised, more from the BU strand than the normal strand, and repair replication follows to replace the excised regions. Only a small amount of excision occurs, presumably enough to remove the photoproducts without also removing large numbers of undamaged bases. Sensitization of mammalian cells to UV light by BU substitution may consequently be due to the excessive damage from BU photoproducts which overload repair mechanisms.

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