Effect of Ultraviolet Light on Division and Deoxyribonucleic Acid Synthesis in Haemophilus influenzae

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The effects of ultraviolet (UV) light on cell morphology, deoxyribonucleic acid (DNA) synthesis, and protein synthesis in UV-sensitive and UV-resistant strains of Haemophilus influenzae were examined. Relatively low doses of UV induce lyses in the sensitive strains but not in the resistant mutant; however, UV temporarily blocks cell division of the resistant mutant, and elongated cells are formed after a period of incubation. Low doses of UV do not stop DNA synthesis in any of the strains examined; however, they do slow the rate of DNA synthesis in a manner consistent with the model correlating the kinetics of postirradiation DNA synthesis with the cell's ability to repair UV-induced DNA lesions. The data are not consistent with a model in which UV causes all DNA synthesis to stop for a time linearly dependent on dose.

Barnhart and Cox (4) recently reported the isolation of mutants of Haemophilus influenzae that were sensitive or resistant to ultraviolet (UV) light as defined by colony-forming ability after exposure to UV light. A comparison of the effects of UV on specific parameters in the wild type and mutants should lead to a better understanding of the response of this organism to UV irradiation. The effects of UV light on cell morphology, deoxyribonucleic acid (DNA) synthesis, DNA degradation, and protein synthesis were studied, and the results are presented here.

Postirradiation DNA synthesis was examined by measuring incorporation of a radioactive DNA precursor by H. influenzae cultures previously labeled with the same radioactive precursor. Results obtained for the wild type differ from the interpretation of data previously presented (14). However, a comparison of the results obtained for the wild type and mutants supports the correlation between the ability of cells to repair radiation-induced DNA lesions and to synthesize DNA after irradiation (14, 23).

MATERIALS AND METHODS

Bacterial cells. H. influenzae strain Rd [isolated by Alexander and Leidy (3)] and UV-resistant strain BC200 and UV-sensitive strain BC100 (both isolated by Barnhart and Cox (4)) were used in all experiments. Cells grown in 3% Difco Brain Heart Infusion Medium supplemented with 10 μg of hemin per ml (see reference 5) and 2 μg of nicotinamide adenine dinucleotide (NAD) per ml at 37°C to about 10^8 cells/ml were made 15% (v/v) in glycerol, frozen, and stored at −85°C. The Brain Heart Infusion Medium supplemented with hemin and NAD is referred to as BH medium.

Bacterial growth conditions. BH medium was inoculated with a thawed sample of frozen cells and incubated overnight at 37°C. A sample of the overnight culture was diluted into fresh BH medium in a 500-ml nepheloscope flask and incubated with shaking in a water bath at 37°C. Cell concentrations were routinely determined by observing optical density (OD) with a colorimeter (Bausch and Lomb, Spectronic-20) at 650 nm calibrated against the number of viable centers. Log-phase cells harvested at a titer of about 10^9/ml were used in all experiments. For growth on solid medium, 4% Difco Brain Heart Infusion Agar supplemented with hemin and NAD (BH agar) was used.

UV-irradiation techniques. Cells collected by centrifugation were resuspended in an ice-cold phosphate buffer described by Setlow et al. (18) at a concentration of 5 × 10^7 to 10^9 cells/ml. A 5-ml amount of the suspension was placed in a 9-cm diameter glass petri dish and irradiated with continuous agitation. This volume gave a liquid depth and light path of about 1 mm. The UV radiation source was a General Electric 15-w germicidal lamp. Incident intensities were determined using a phototube and voltmeter which were calibrated against a thermopile that had been previously calibrated against a standard lamp. Corrections calculated by Morowitiz (15) to obtain an average effective intensity throughout the
sample were made. Survival of colony-forming ability (CFA) was determined by diluting irradiated cells in phosphate buffer and plating on BH agar medium by using the pour-plate technique.

Measurement of protein and DNA synthesis and DNA degradation. Cells were collected by centrifugation (4,000 × g for 10 min) and assayed for protein by using the method of Lowry et al. (13) and for DNA by measuring diphenylamine-reacting material as described by Burton (10). Synthesis of DNA was also followed by measuring uptake of radioactive thymidine into cold 5% trichloroacetic acid-precipitable material. Cells were grown at 37°C in BH medium containing 5 μc of ³H-thymidine (thymidine-methyl-³H, 16 c/m mole; New England Nuclear Corp., Boston, Mass.) per ml or 0.5 μc of ¹⁴C-thymidine (thymidine-2-¹⁴C, 59.2 mc/m mole, New England Nuclear Corp.) per ml for at least four generation times before UV irradiation. After UV irradiation, cells in buffer were either collected by centrifugation and resuspended in BH medium or were diluted 1:5 into BH medium. Radioactive thymidine was added to a concentration equal to that used before UV irradiation. All transfer and irradiation steps were done at 4°C. The effect of UV light on DNA degradation was determined by treating cells in an identical manner, except that radioactive thymidine was not added to the BH medium after irradiation. Samples (0.1 ml) were taken periodically and assayed for radioactivity by using the filter-paper technique of Bollum (8). Samples were placed in vials with 10 ml of Bray’s scintillation fluid (9) and counted in a Packard Tri-Carb scintillation spectrometer.

Microscopic observations. UV-irradiated cells were spread on BH agar, incubated, and observed at 37°C by using a Zeiss phase-contrast microscope equipped with 16X eyepieces and a 40X objective. The technique was similar to that previously described (2). A map marking the location of cells in a particular field of view was drawn, and this view was continuously observed over a 150-min period.

RESULTS

Protein synthesis. UV-irradiated *H. influenzae* strains Rd and BC100, a strain more UV-sensitive than Rd, lyse after a short period of incubation in liquid nutrient medium at 37°C, but similarly treated BC200, a strain more UV-resistant than Rd, does not (5, 22). UV doses great enough to cause lysis of the majority of cells have little initial effect on mass increase as measured by increase in optical density (5). Figure 1 shows that similar UV doses also have little initial effect on protein synthesis. A dose of 150 ergs/mm² causes the rate of protein synthesis to decrease only slightly in both Rd and BC200. However, after 60 min, total protein present in the Rd culture in the form of cells that can be pelleted by centrifugation (4,000 × g for 10 min) stops increasing and decreases to a value at 120 min nearly equal to the original value. Protein synthesis in BC200 continues unaffected. A dose of 25 ergs/mm² affects protein synthesis in BC100 in a manner similar to that observed for Rd except that synthesis appears to stop at about 80 min after UV irradiation. The decrease in amount of protein is attributed to lysis of the cells. Microscopic observations of these cultures showed that both Rd and BC100 cells lysed at the time total protein stopped increasing. Similar observations of the irradiated BC200 culture

![Figure 1](http://jb.asm.org/jb INTERVAL/d18745a7a2e04f01a0d606d373e9a56b.jpg)

**FIG. 1.** Effect of UV light on protein synthesis in *H. influenzae*. Control nonirradiated and UV-irradiated cells were collected by centrifugation, resuspended in BH medium, and incubated at 37°C. Samples (5 ml) were taken at the times indicated and assayed for protein. Results are expressed in micrograms of protein per milliliter of culture. Open symbols, control; closed symbols, UV-irradiated: (●) BC200, 150 ergs/mm²; (▲) Rd, 150 ergs/mm²; and (■) BC100, 25 ergs/mm².
indicated that, although mass increase and protein synthesis continued, cell division was inhibited and that elongated cells were formed.

**Morphological changes.** UV-irradiated cells incubating at 37 C on nutrient agar surfaces were observed under the microscope to determine whether their responses were similar to those in liquid medium and to observe and quantitate changes in cell morphology (Table 1). At the doses used, the primary cellular response for Rd was cell elongation followed by lysis; for BC200, cell elongation. UV-induced cell elongation and lysis have previously been observed and reported for strain Rd by Stuy (22). Rd cells began to lyse after the first 30 min of incubation, although the majority of cells lysed between 60 and 100 min. Most cells elongated to two or four times normal length before lysing. In a few cases, cells divided but both daughters subsequently lysed. Most BC200 cells initially failed to divide (92%) but continued to increase in length, some becoming 10 to 20 times normal length. These elongated cells were capable of division, and some gave rise to two to three microcolonies along their length. At lower UV doses, the responses were similar, but less lysis of Rd and less division inhibition of BC200 were observed. Unirradiated control cells incubated under similar conditions proceeded to divide normally. Lysis of BC200 has never been observed, regardless of the UV dose or post-UV-irradiation growth conditions. Since the results obtained

**TABLE 1. Summary of observations of morphological changes in ultraviolet (UV)-irradiated Haemophilus influenzae**

<table>
<thead>
<tr>
<th>Strain</th>
<th>UV dose (ergs/mm²)</th>
<th>Survival (CFA)</th>
<th>No. of cells observed</th>
<th>Cells that lysed</th>
<th>Cells that elongated to at least 4 X N</th>
<th>Cells that divided at least once</th>
<th>Cells that neither lysed, divided, or elongated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rd</td>
<td>100</td>
<td>%</td>
<td>10</td>
<td>108</td>
<td>85</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>BC200</td>
<td>250</td>
<td>%</td>
<td>10</td>
<td>115</td>
<td>0</td>
<td>92</td>
<td>42</td>
</tr>
</tbody>
</table>

*a Cells were UV-irradiated with doses that give approximately equal survivals as measured by colony-forming ability, placed on agar blocks at 37 C, and observed under the microscope for 150 min.

*b Most Rd cells elongated without dividing but lysed before reaching a length of 4 X N (normal size).

*c For Rd, 7% divided at least once, in good agreement with CFA results. For BC200, 42% divided at least once, indicating that one division does not mean the cell will give rise to a colony.

for Rd and BC200 were similar to those obtained in liquid culture, similar experiments on agar surfaces were not done with BC100.

The percentage of Rd cells that divided at least once (7%, Table 1) is in good agreement with the per cent survival of CFA (10%). The majority of cells (85%) lysed, indicating that at this UV dose the major cause of lethality is cell lysis. For BC200, 42% divided at least once, whereas the per cent survival of CFA was only 10%. Evidently, one division does not mean that daughters will continue to divide and give rise to colonies.

**DNA synthesis.** DNA synthesis in control and irradiated cultures was measured by a direct chemical method (10) and by observing incorporation of radioactive thymidine by previously labeled cells into cold trichloroacetic acid-precipitable material. Results obtained for Rd control and irradiated (150 ergs/mm²) cells are shown in Fig. 2. Mass increase, as measured by increase in OD, and viable centers are shown in Fig. 3. Log-phase cells grown in BH medium containing ¹⁴C-thymidine were UV-irradiated

![Fig. 2. DNA synthesis in H. influenzae strain Rd after exposure to UV light. Control nonirradiated cells and UV-irradiated (150 ergs/mm²) cells previously labeled with ¹⁴C-thymidine were suspended in BH medium containing ¹⁴C-thymidine and incubated at 37 C. Samples taken periodically were assayed for (a) DNA by chemical means and (b) trichloroacetic acid-precipitable ¹⁴C: (+) control nonirradiated cells and (○) UV-irradiated cells.](http://jb.asm.org/)

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and incubated in fresh BH medium plus \(^{14}\)C-thymidine (see Materials and Methods). At the desired times, samples were taken for measurements of DNA synthesis and viable centers. Results show that, at lower cell titers, uptake of labeled thymidine into trichloroacetic acid-precipitable material parallels DNA synthesis measured by direct chemical means for both control and irradiated cultures. A modified Schmidt-Thannhauser extraction procedure (17) using both control and irradiated cultures indicated that approximately 95% of the \(^{14}\)C was in the DNA fraction. Uptake of labeled thymidine stops abruptly in control cells at a viable cell titer of about \(2 \times 10^{9}/\text{ml}\), although DNA synthesis as measured by chemical means continues. It is necessary, therefore, to use cell titers considerably lower than \(2 \times 10^{9}/\text{ml}\) to observe incorporation of labeled thymidine. Possibly the presence of adenosine in the growth medium would permit continued uptake of exogenous thymidine (14) at the higher cell titers. DNA synthesis in the UV-irradiated cells appears to stop for approximately 15 min and then proceeds at a rate considerably reduced from that of the control. At around 60 min, when cells are observed to lyse (Fig. 3b), a drop in the amount of DNA per sample is observed. The results are similar regardless of whether acid-precipitable \(^{14}\)C or DNA by chemical means is measured. A greater drop in amount of DNA is observed when diphenylamine-reacting material is measured than when \(^{14}\)C label is measured. This is because only whole cells capable of being pelleted by mild centrifugation (4,000 \(\times\) g for 10 min) are used for chemical assay, whereas 0.1-ml samples taken directly from a growing culture are trichloroacetic acid-precipitated and used for \(^{14}\)C measurements. Evidently, DNA released from cells upon lysis is not completely degraded so as to be trichloroacetic acid-soluble. Similar experiments comparing DNA synthesis by these two methods were done for both BC200 and BC100. Results showed that incorporation of labeled thymidine was a valid measurement of DNA synthesis in these strains also.

Approximately 2% of viable centers in the experiment of Fig. 2 survived the UV exposure and were able to give rise to visible colonies (Fig. 3). As the time of incubation in liquid medium increased, the number of viable centers at first dropped and then, after about 60 min, began to increase at a rate similar to that of the control. The drop is attributed to a decrease in plating efficiency of some cells, which apparently become fragile upon incubation and can no longer survive the plating procedure they are capable of surviving immediately after UV and before any incubation. At a later time, they can recover their ability to survive the plating technique. Similar experiments with BC200 showed that no similar decrease in viable centers occurred, although a definite lag in viable center increase did occur.

**UV dose effect on DNA synthesis.** The effect of increasing doses of UV on DNA synthesis in all three strains was examined by observing incorporation of tritiated thymidine into acid-precipitable material (Fig. 4). In all experiments, prelabeled cells were resuspended after UV radiation at the same cell titer in fresh medium containing labeled thymidine. Results for BC200 show that increasing doses of UV irradiation decrease the rate of DNA synthesis until, at 150 ergs/mm\(^2\), an actual lag of about 15 min is observed. At later growth times, synthesis approaches the rate of the control. Similar results during the first 60 min of postirradiation incubation are observed for Rd. Results obtained for Rd at times later than 60 min are confused by lysis of the cells; the number of cells that lyse increases with UV dose. As for BC200, a dose of 150 ergs/mm\(^2\) is needed to completely stop DNA synthesis in Rd cells. DNA synthesis in BC100 is affected more drastically by lower doses. A dose of 20 ergs/mm\(^2\) has an effect similar to that of about 100 ergs/mm\(^2\) in both BC200 and Rd. Again, as with Rd, results for BC100 are
confused at later times by lysis of the cells. In all experiments, lysis was confirmed by observing the OD of the cultures and by microscopic observations.

To quantitate the effect of UV radiation on rate of DNA synthesis, slopes of the lines presented in Fig. 4 and from other similar data were calculated and plotted as a function of dose in Fig. 5. Slopes were calculated in all cases only for the first 40 min of postirradiation incubation. All data were normalized to the control rate of DNA synthesis in a given experiment. Data shown in Fig. 5 indicate that rates of DNA synthesis in Rd and BC200 are affected similarly, decreasing in a linear manner with increasing dose. Rate of synthesis in BC100 also decreases in a linear manner but is about seven times more sensitive than that of Rd and BC200.

**Effect of UV on DNA degradation.** The observed rates of DNA synthesis after UV irradiation could be a net result of DNA synthesis plus DNA breakdown. The effect of UV irradiation on DNA breakdown was examined, therefore, as part of the same experiments in which DNA synthesis was measured. After exposure to UV radiation, prelabeled cells were resuspended in BH medium containing no labeled thymidine and incubated at 37°C. Samples were assayed for trichloroacetic acid-precipitable material at various times (Fig. 6). No UV-induced solubilization of DNA could be detected for any of the strains during the first 60 min after UV irradiation. A drop in acid-insoluble label occurred for both Rd and BC100 after 60 min—at a time when a major fraction of cells of both of these strains start to lyse. The drop in acid-insoluble label is interpreted as meaning that some of the DNA is degraded upon lysis of the cells. BC200 does not lyse after UV irradiation, and no drop in acid-insoluble material is observed. The data in Fig. 6 show results obtained for only one dose; however, each strain was examined for DNA degradation at the several doses used in the experiments of Fig. 4. No DNA degradation was observed during the first 60 min after UV irradiation for any of the strains, regardless of UV dose. It appears that, since UV causes no DNA degradation during the first 60 min after exposure to UV, the observed rates of thymidine incorporation during this time (Fig. 4) are a true measure of DNA synthesis rates.

![Fig. 4. UV dose effect on DNA synthesis in H. influenzae.](https://example.com/f4.jpg)

**Fig. 4.** UV dose effect on DNA synthesis in *H. influenzae*. 3H-thymidine-labeled cells were UV-irradiated in phosphate buffer, diluted 1:5 into BH medium containing 3H-thymidine, and incubated at 37°C. Samples (0.1 ml) were taken at the times indicated and assayed for cold trichloroacetic acid-precipitable 3H. Cultures were irradiated with the doses indicated in the figure: (a) BC200, (b) Rd, and (c) BC100.

![Fig. 5.](https://example.com/f5.jpg)

**Fig. 5. Effect of UV light on rate of DNA synthesis in *H. influenzae*.** Rates of DNA synthesis for the first 40 min after UV irradiation were calculated from slopes of lines in Fig. 4, and other similar data and were plotted as a function of dose. All data were normalized to the rate of DNA synthesis in the non-irradiated control. Open symbols, BC200; closed symbols, Rd; and (+ and ×) BC100. Different symbols refer to different experiments.
**DISCUSSION**

Exposure of *H. influenzae* strains Rd and BC200 to relatively low doses of UV temporarily blocks division without inhibiting mass increase. After a period of incubation in growth medium, cells four to eight times normal length can readily be observed in these cultures; however, Rd cells start lysing at a time when long cells are easily distinguishable from normal-sized cells in the culture, and it is difficult, therefore, to quantitate division inhibition in this strain. BC200 long cells are capable of division and appear to divide at random along the length of the cell. Cells split off by division are capable of further division as evidenced by the appearance of several microcolonies in the position of the original long cell. When observed under the microscope, the long cells reported here appeared similar to UV-induced filamentous cells of *Escherichia coli fil* + and *lon* − mutants. However, UV doses needed to induce filament formation in the majority of cells in *E. coli fil* + or *lon* − cultures have little or no effect on DNA synthesis or mass increase (1, 12), whereas UV doses needed to inhibit division in the majority of *H. influenzae* Rd and BC200 cultures have initially a considerable effect on the rate of DNA synthesis without affecting mass increase. Therefore, the DNA-to-mass ratio is considerably reduced from that of a normal-sized cell and differs from the situation observed in *E. coli*. Log-phase *H. influenzae* cells have two to four chromosomes per cell (7). These chromosomes are probably dispersed along the length of an UV-induced long cell, accounting for the observation that division occurs at random along the cell length.

Postirradiation DNA synthesis in all strains was observed by following the uptake of radioactive thymidine using cells labeled with radioactive thymidine before UV irradiation, as suggested by Smith and O'Leary (20). Since no breakdown of DNA was observed during the first 60 min after exposure to UV, uptake of radioactive thymidine is an accurate measure of DNA synthesis during this time. Relatively low doses of UV do not stop DNA synthesis in *H. influenzae* cultures, as was observed for *E. coli* (20, 23) or as was suggested for *H. influenzae* (14), but only slow the rate of DNA synthesis for a short time. These data can be interpreted as either (i) all cells synthesizing DNA at a reduced rate or (ii) a fraction of cells making no DNA and a fraction of cells continuing DNA synthesis. Randomly growing log-phase cells used in the experiments were irradiated by using conditions in which all cells, on the average, received the same number of hits. It seems likely that DNA synthesis would be affected in a similar manner in all cells and that postirradiation DNA synthesis curves are due to a reduced rate of synthesis in all cells.

Our data do not appear to be consistent with the model in which UV irradiation causes all DNA synthesis to stop for a time linearly dependent on dose (23). There are several possible modes of DNA synthesis that could occur and might explain our data:

(i) If more than one replicating point per chromosome is present in *H. influenzae*, photoproducts could stop DNA synthesis at one replicating point without immediately affecting DNA synthesis at the other replicating points, causing a "reduced" rate of DNA synthesis to be observed.

(ii) It is possible that randomly spaced photoproducts are repaired not within a given time but within a given region of the chromosome in proximity to the replicating point so as to affect the rate of semiconservative replication.

(iii) UV irradiation may induce new sites of semiconservative replication (11), preventing observation of a DNA synthesis lag.
(iv) Rd and BC100 both lye after UV irradiation, suggesting that a defective prophage may be induced (21). Replication of this defective prophage could account for the observed post-irradiation DNA synthesis.

(v) Another possibility is that photoproducts do not act as irreversible blocks to DNA synthesis but only serve to slow replication. DNA synthesis may proceed by skipping over photoproducts as reported for an E. coli excision-defective mutant (16). If one assumes that dimers act as irreversible blocks to DNA synthesis, that 1 erg/mm² induces two dimers per H. influenzae chromosome (19), that the molecular weight of the H. influenzae chromosome is \( 8 \times 10^8 \) daltons (7), and that there is one replicating point per chromosome, then the amount of DNA synthesis observed in the first 30 min after UV irradiation in a BC100 culture (20 ergs/mm²) is 10 times greater than expected. The data on postirradiation DNA synthesis in BC100 further suggest that nonsurviving cells, as defined by lack of ability to form a colony after UV, are capable of synthesizing DNA. Only 0.01% of BC100 cells are capable of surviving a dose of 20 ergs/mm². If one assumes that only surviving cells are making DNA at the control rate, we should not be able to detect DNA synthesis in this culture—at least not at short incubation times. A considerable amount of DNA synthesis was detected, indicating that nonsurviving BC100 cells are making DNA. We feel that “repair replication” can be ruled out as accounting for the initial post-UV-irradiation DNA synthesis, since measurement of DNA by using direct chemical means gave results identical to radioactive thymidine incorporation experiments. Direct chemical measurement of DNA should not be affected by repair, non-semiconservative replication.

The reduced rate of DNA synthesis after UV irradiation can be observed to increase eventually to the control rate in cultures of all three strains if a low enough UV dose is used. BC200 is the most capable of resuming the control rate of DNA synthesis and does so for all doses used. Rd and BC100 both show some capability for resuming the control rate of DNA synthesis, although this capability is greatly reduced from that of BC200. Rd cultures are capable of recovering during the 150-min postirradiation observation time from a dose of 20 ergs/mm², whereas BC100 cultures are capable of recovering from a dose of only 2 ergs/mm². Both Rd and BC100 cells lye after UV irradiation. This lysis destroys cell integrity and prevents one from observing the full extent of “repair” these mutants are capable of performing. Since the initial rate of synthesis after UV irradiation is affected similarly for both BC200 and Rd, we conclude that these two strains have similar DNA repair capabilities. This conclusion is supported by experiments measuring DNA repair capabilities indirectly (5) and by experiments measuring excision of thymine dimers (6). The extrasensitivity of Rd is due to some factor other than DNA repair ability and may be due to induction of a defective prophage that leads to cell lysis. BC100 is defective in the excision step of the repair process (6), making DNA replication in this strain more sensitive to UV irradiation than it is in Rd or BC200. Since BC100 lyes after UV irradiation, we conclude that it also harbors the same factor responsible for the extrasensitivity of Rd.

Our data and interpretation of data on effects of UV on DNA synthesis in H. influenzae differ from those previously presented (14). Part of this difference can be attributed to the use, in our case, of prelabeled cells. Arguments advocating the use of prelabeled cells for this kind of experiment have been presented (20) and will not be discussed further. Although UV doses used here are in some instances higher, this was necessary to observe any effect. Preliminary experiments indicate that the wild-type strain used here is the same as the wild-type strain used by previous authors (14), but there is no indication of lysis occurring in their work (14, 18). Possibly, differences may be accounted for in the laboratory handling of the cells.

The kinetics of postirradiation DNA synthesis in E. coli are affected by the ability of a cell to repair radiation-induced lesions in DNA (23). DNA synthesis in UV-sensitive BC100 is much more sensitive to UV irradiation than it is in UV-resistant BC200; it appears, therefore, that the same is true for H. influenzae.

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LITERATURE CITED


