STUDIES ON THE RADIATION INACTIVATION OF MICROORGANISMS

VI. X-RAY-INDUCED BREAKDOWN OF DEOXYRIBONUCLEIC ACID IN HAEMOPHILUS INFLUENZAE AND IN OTHER BACTERIA

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The formation of deoxyribonucleic acid (DNA) in ultraviolet-inactivated microorganisms is receiving considerable attention. The general picture is that ultraviolet light specifically inhibits DNA synthesis in actively dividing cells, but, in time, the synthesis may be resumed. In a previous study (Stuy, 1959) with a multimarker strain of Haemophilus influenzae, two additional striking phenomena were observed: (a) ultraviolet-induced damage of cell DNA (measured as a reduction in transforming principle activity) was quickly repaired by the noncolony-forming, killed cells, (b) after a certain period in all (or at least most of) the treated cells, DNA was rendered nonfunctional without being depolymerized. This period was followed by cell lysis and simultaneous DNA breakdown into small fragments. The conclusion was that killing of the vigorously growing cells was attributable mainly to the extent of the radiation damage to their DNA and to the length of the period necessary for repair. Inhibition of DNA formation was thought to be a secondary effect.

In contrast, very little is known about DNA formation in X-irradiated logarithmic growth phase cells. Doudney (1956) reported that DNA formation was blocked in X-irradiated Escherichia coli pretreated with cysteamine. It is not certain, however, whether his cultures were in logarithmic growth. Addition of a mixture of certain amino acids together with guanine and uracil eliminated this block. In his discussion, Doudney introduced the "unbalanced growth" hypothesis to explain recovery, the term "unbalanced" being used in the broadest sense. The ultraviolet study on H. influenzae (Stuy, 1959) yielded more specific examples of such unbalanced growth, only there it was called "radiation-induced disarrangement." The study was repeated with X rays to find out whether ionizing radiation produces similar effects in growing bacteria.

Although, in the experiments reported here, extraction and assay of transforming principle was basically carried out as described elsewhere (Stuy, 1959), the methods were improved with respect to yields of transforming principle. In every experiment, three different markers were scored.

METHODS

Bacteria. The following bacteria were treated with X rays: H. influenzae strain Rd4(3M), which was resistant to streptomycin (1 mg per ml; Abbott Laboratories), cathomycin (2.5 µg per ml; Merck, Sharp and Dohme), and erythromycin (6.25 µg per ml; Abbott Laboratories); E. coli strains B and B/r (provided by Dr. M. Demerec); E. coli strain H (a porphyrinless mutant; Latarjet and Beljanski, 1956); Bacillus cereus strain p2/SM (a streptomycin-resistant mutant of strain p2; Stuy, 1956); and Diplococcus pneumoniae strain Rx ery2, str, which was resistant to streptomycin and erythromycin (obtained from D. M. Green of this laboratory). In transformation reactions, H. influenzae strain Rd served as the recipient strain.

Stock transforming principle was prepared from H. influenzae strain Rd(MM) (Stuy, 1959). Activity of the streptomycin- and erythromycin-resistance markers derived from this strain was about 50 and 75 per cent with respect to that of the cathomycin-resistance marker. Strain Rd4-(3M) possessed equivalent streptomycin- and cathomycin-resistance markers, whereas its erythromycin marker was the same as the one just described. It is not known why such markers are found with a lower activity, but the phenomenon may be similar to the depressor effect for pneumococci (Green, 1958). The low yields of


2 Operated by Union Carbide Corporation for the U. S. Atomic Energy Commission.
streptomycin marker mentioned in the previous study (Stuy, 1959) are thus actually more satisfactory than would seem.

Media. All media for \textit{H. influenzae} were prepared as described previously (Stuy, 1959). Horse (burro, \textit{Equus asinus asinus}) blood was obtained from the University of Tennessee, Atomic Energy Commission Agricultural Research Laboratory. The author is very much indebted to Larry Jones, who performed the frequent bleedings.

The peptone medium contained per L: peptone (Difco), 10 g; glucose, 5 g; Na$_2$HPO$_4$, 4.74 g; and KH$_2$PO$_4$, 4.53 g.

The chemically defined medium was Anderson’s M9 (Anderson, 1946) supplemented with 2 mg of ferric sulfate per L.

Growth conditions. All liquid cultures were grown by shaking (180 strokes per min) in a water bath at 38 C in Levinthal broth (Stuy, 1959) except where otherwise stated. \textit{E. coli} strain H$_7$ behaves like normal \textit{E. coli} if grown in this way because it takes up all the porphyrins it needs from the medium. The latter strain was also grown anaerobically in the porphyrin-deficient peptone medium and therefore it lacked all porphyrin-containing enzymes (catalase and cytochromes (Latarjet and Beljanski, 1956)). This presumably kills it even after X-ray treatment has been stopped (Adler, 1958). \textit{E. coli} strain B/r was also grown in M9. \textit{D. pneumoniae} cultures were shaken at 90 strokes per min for optimal growth. Cultures were diluted with Eugon broth (BBL) where necessary.

After treatment, suitably diluted samples were plated in Levinthal agar, which was cooled to 44 C. Transformed cells were plated as described (Stuy, 1959). Plates were incubated at 38 C for at least 24 hr before colonies were counted.

Preparation of stock transforming principle. This was carried out as described by Rupert and co-workers (1958) from \textit{H. influenzae} strain Rd(MM). The preparation was stored frozen and where necessary it was diluted with buffered saline. In the preparation only the cathomycin marker was underpressed.

The DNA concentration was 32 \mu g per ml. Its activity for cathomycin-resistance transformation was about $4 \times 10^{14}$ transformations per g of DNA. Since one cell contains approximately $2 \times 10^{-16}$ g of DNA and since this is presumably one set of markers (genes), the activity of the preparation was about the expected value, $5 \times 10^4$. This shows further that preparations reported to be extremely active should give the above number of transformations per g of DNA. This is also true for \textit{D. pneumoniae} since its DNA content per cell is about $2 \times 10^{-16}$ g.

Routine transformation system. This system consisted of 1.6 ml of buffered saline (9 g of NaCl and a few mg of K$_2$HPO$_4$ per L, pH of 7.0) and 0.2 ml of a pretreated recipient culture in an 18-mm tube. After 75 min at room temperature, 0.2 ml of the transforming principle solution to be tested was added and the tube was gently shaken at room temperature. Shaking was necessary for a rapid and quantitative uptake by the cells of small quantities of transforming principle. Exposure to DNA was always terminated after 15 min by simply diluting the system.

Preparation of competent (transformable) cultures. About 13 ml of recipient culture were shaken in a 50-ml flask until turbidity had reached an optical density value of ~0.280. The culture was then poured into a 25-mm tube and

\textbf{Figure 1.} Preparation of highly transformable cultures of \textit{Haemophilus influenzae} strain Rd. Total cell count remained constant at $2 \times 10^9$ per ml of culture for period shown. Marker scored was the cathomycin marker.
incubated without shaking for 90 min. Such a pretreated culture contains few or no competent cells; this is in contradiction to an earlier statement (Stuy, 1959). All cells become competent, however, on dilution of the culture with 9 volumes of buffered saline and incubation at room temperature. This is demonstrated by the following experiment. To a series of tubes, containing 1.8 ml of diluted, pretreated culture, 0.2 ml of (excess) stock transforming principle was added at time zero and after 15-min intervals. From the first tube (zero time), samples were removed at 15-min intervals. From the other tubes samples were removed after 15 min of exposure to transforming principle. In all samples, the number of transformed cells was scored, and, in some samples, the total number of cells was determined. Total viable cell count remained constant at about $2 \times 10^9$ per ml for at least 2 hr. Both series of samples showed identical numbers of transformants. Figure 1, giving the numbers of cathomycin-resistant cells, shows that all cells quickly became competent and remained so for at least 75 min.

The transformation frequency for a single underpressed marker obtained in this way was very reproducible. In all experiments carried out with the cathomycin markers, it fluctuated only between 3 and $5 \times 10^{-2}$.

Pretreated cultures, after addition of 15 per cent (final concentration) glycerol, could conveniently be frozen and stored at $-80\,^\circ\mathrm{C}$ for at least 6 months without appreciable loss in subsequent activity. To make cultures highly competent by incubating them in saline, $\sim 10$ per cent of the original medium had to be present. If cells were free of Levinthal broth, subsequent saline incubation gave no competent cells. However, once cells had become competent, they could be spun down and resuspended in buffered saline without any loss in activity.

**Routine extraction and assay of transforming principle.** Extraction of transforming principle from donor cells (Stuy, 1959) was carried out at 37 $\,^\circ\mathrm{C}$. This was considered unsatisfactory since some repair of radiation damage could have occurred before cell lysis and an improved method was used. Samples of donor cell cultures (0.5 ml) were added to 1 ml of chilled citrate solution (9 g of NaCl and 0.05 mole of sodium citrate per L). The bacteria were spun down and stored frozen when necessary. They were lysed in the cold by resuspending them in chilled citrate solution of which the pH had been adjusted to 9.5 to 10.0 by addition of NaOH solution. After 10 min in an ice bath, 0.5 ml of citrate-sodium lauryl sulfate solution (final concentration, 0.4 per cent) was added, and the suspensions were gently shaken at room temperature for another 10 min. They were then deproteinized by shaking briefly with 0.25 ml of a chloroform-isooctanol mixture (3:1, v/v). After centrifugation, 0.2 ml of the 1000-fold diluted supernatant was added to the transformation system. In this dilution range, the number of transformants was proportional to the dilution. The ratio of the number of markers scored to the number of cells extracted was always from 0.7 to 1.0. If it is assumed that a cell contains a single set of markers (genes), the above procedure is quantitative.

X-ray-damaged transforming principle could be processed as described without further loss in activity. This was established by X-ray inactivation of transforming principle dissolved in 1 per cent yeast extract (Difco). The 10 per cent activity remaining immediately after irradiation was recovered completely after the solution had gone through extraction procedure.

In every assay of transforming principle, the transformation frequency of the system was determined.

**Viable cell counts.** The cells of the *H. influenzae* strains used had a tendency to stick together while in logarithmic growth phase, thus precluding accurate viable cell counts. From microscopic observations and from comparisons with stationary phase cells (which are predominantly single), it was concluded that the true number of viable cells was about twice that found by the plating technique.

The same was more or less true for all other strains studied. Survival thus was measured as the reduction in number of colony-forming units. It is, however, given in the legends as reduction in number of single cells, the initial number of cells being estimated from colony counts and microscopic observations of the cell aggregates. As such, these data are approximate.

**Irradiation.** The X-ray source was a General Electric Maxitron 250 operating at 30 ma. No filter was used. About 100 ml of culture in a 500-ml beaker placed in an ice bath were irradiated while being vigorously stirred by a magnetic
stirrer. The distance from window to bottom of beaker was 16 cm. The dose rate in the center of the suspension was about 4.8 kr per min.

The irradiation temperature chosen was between 0 and 10 C because, at higher temperatures, the cells lost much of their DNA during the treatment, whereas, at the temperatures used, losses did not exceed 15 per cent. At room temperature, a culture of H. influenzae showed a 50 per cent loss after 10 min of irradiation.

Assays. DNA contents were determined as described by Burton (1956); and RNA was assayed as described elsewhere by Stuy (1958). In both assays very pure and highly polymerized preparations were used to evaluate the commercial standard preparations.

Optical density of cultures was measured in 12-mm tubes at about 600 μM with a Beckman model C photometer.

RESULTS

About 120 ml of medium were inoculated with 10⁶ to 10⁷ cells. In each of seven 50-ml flasks, 17 ml of this culture were shaken at 38 C. Growth (turbidity) was observed, and it was determined that cultures grew logarithmically at the time they had reached an optical density value of about 0.200. The seven cultures were poured into a 500-ml beaker placed in an ice bath. When the temperature of the magnetically stirred culture had dropped below 10 C, samples for viable cell count, DNA, and transforming principle assay (0.1, 5, and 0.5 ml, respectively) were removed. Another 13 ml were removed and kept in ice to serve as a nonirradiated control culture. The remaining culture was irradiated and then the necessary samples were taken. Thirteen ml of treated culture were pipetted into each of six 50-ml flasks, and all flasks, including the control, were again shaken at 38 C. At stated intervals a flask was taken out of the water bath for turbidity measurements and removal of samples. Only turbidity measurements were made on the control culture.

Preirradiation DNA samples after processing had an optical density of about 0.050. Values from 0.010 to 0.005 were considered highly inaccurate but were noted, nevertheless, and lower values were ignored. Transforming principle values were scored more accurately in that region; but at the 1 per cent level, accuracy had greatly disapp-
Figure 3. X-ray-induced breakdown of DNA in growing *Haemophilus influenzae* strain Rd4(3M). Dose, 19.2 kr; survival, 5.2 × 10^{-2}. Growth (turbidity) of nonirradiated control; •, growth treated culture; □, DNA contents; ---, activity of markers scored. Markers, streptomycin Δ, cathomycin ○, and erythromycin ▲. Preirradiation values: growth, 0.220; viable cells, estimated at 7.7 × 10^8 per ml of culture; DNA content, 1.7 μg per ml; Δ, 6.5 × 10^8; ○, 7.2 × 10^8; and ▲, 6.0 × 10^8 transformations per ml.

Figure 4. X-ray-induced breakdown of DNA in growing *Haemophilus influenzae* strain Rd4(3M). X-ray dose, 26.4 kr; survival, 1.5 × 10^{-2}. Symbols: see figure 3. Preirradiation values: growth, 0.220; viable cells, estimated at 7.7 × 10^8 per ml of culture; DNA content, 1.8 μg per ml; Δ, 6.4 × 10^8; ○, 7.2 × 10^8; and ▲, 6.1 × 10^8 transformations per ml.

Figure 5. X-ray-induced breakdown of DNA in growing *Haemophilus influenzae* strain Rd4(3M). X-ray dose, 33.6 kr; survival, 2.5 × 10^{-3}. For symbols see figure 3. Preirradiation values: growth, 0.260; viable cells, estimated at 9.1 × 10^8 per ml of culture; DNA content, 2.0 μg per ml; Δ, 8.7 × 10^8; ○, 8.7 × 10^8; and ▲, 6.7 × 10^8 transformations per ml.

Figure 6. X-ray-induced breakdown of DNA in growing *Haemophilus influenzae* strain Rd4(3M). X-ray dose, 48 kr; survival, 5.0 × 10^{-4}. For symbols see figure 3. Preirradiation values: growth, 0.210; viable cells, estimated at 7.4 × 10^8 per ml of culture; DNA content, 1.6 μg per ml; Δ, 7.0 × 10^8; ○, 7.5 × 10^8; and ▲, 5.8 × 10^8 transformations per ml.
after irradiation. No transforming principle activity could be detected in the medium, which would indicate a breakdown of DNA and not a mere release of DNA into the medium.

Since no reports on DNA breakdown in X-irradiated bacteria have been published, experiments were extended to a variety of other bacteria. All strains but one were exposed to two X-ray doses, 48 and 96 kr. In every case a picture similar to that of *H. influenzae* was found although the DNA breakdown rate was much lower. These results are summarized by figures 7 and 8, which show the postirradiation effects for *E. coli* strains B and B/r grown in Levinthal broth.

RNA data have been omitted from the figures for simplicity. The curves had shapes similar to corresponding growth curves, but synthesis was more inhibited and the final plateau was considerably lower.

Viable cell counts in *H. influenzae* experiments indicated an apparent lag in cell division by the survivors. If, however, aggregate formation was taken into account, it followed that surviving cells showed no inhibition of cell division. In contrast to this was the microscopic observation of cell elongation of most of the (killed) cells after small to medium X-ray doses. The picture was, however, much more complicated than that observed after ultraviolet irradiation (Stuy, 1959).

**DISCUSSION**

As pointed out earlier (Stuy, 1959) the fraction of cell DNA assayed for by the described transforming principle method is very small. From a practical point of view it is impossible to include all cell DNA in the determination. Because of identical behavior of the three markers studied, the author is convinced that such behavior reflects that of the total DNA. Thus an activity drop of the markers means a drop in biological activity of total cell DNA.

The experiments performed revealed three interesting facts. First, X-irradiation damaged cell DNA as had ultraviolet light (Stuy, 1959). Figure 9, in which averages of all data have been plotted, shows the X-ray sensitivity in *vivo* of cell DNA. There were no signs of repair but the rapid breakdown of DNA may have obscured such a repair.

Second, X-ray treatment of actively dividing cells induces a breakdown of their DNA. This study shows that this is a general phenomenon independent of external factors such as media. Damage of cell DNA could be the reason for this degradation but it is more likely that a DNase is activated by the irradiation.

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**Figure 7.** X-ray-induced breakdown of DNA in growing *Escherichia coli* strain B. X-ray doses, (1) 48 kr and (2) 96 kr; survival, (1) $6.0 \times 10^{-6}$ and (2) less than $10^{-7}$. For symbols see legend of figure 3.

**Figure 8.** X-ray-induced breakdown of DNA in growing *Escherichia coli* strain B/r. X-ray doses, (1) 48 kr and (2) 96 kr; survival, (1) $2.6 \times 10^{-3}$ and (2) $7.5 \times 10^{-7}$. For symbols see figure 3.
Figure 9. Inactivation in vivo of cell DNA of Haemophilus influenzae strain Rd4(3M). All data on marker inactivation have been averaged. Differences in X-ray sensitivity shown by the three markers have been ignored. Plotted are averages (from 3 to 9 points per average) and ranges (lowest and highest values). For comparison the killing curve of cells is given.

Last, after 20 to 60 min, depending on dose and strain, there was an increase in DNA content. Because of the breakdown it is impossible to state that immediately after irradiation DNA formation was inhibited. The “resumed” synthesis of DNA shows that, if the DNA synthetic mechanism had been injured by the X rays, some recovery of this damage could take place. Figure 10 shows that the effect is not the result of survivor growth. Curves in this figure were derived from those of figure 3 by subtracting from all values the contributions by the survivors. Survival was 5.2 per cent, and surviving cells grew at a normal rate. It was assumed that these cells were normal in all respects. Thus DNA formation occurred in inactivated cells, which causes some speculation. It is unlikely that all DNA was completely broken down in 70 per cent of the population and not at all in the remaining 30 per cent. The latter cells certainly could not have resumed synthesis of DNA at the observed rate. Rather, DNA was probably degraded in every cell, except the survivors, and to varying levels, giving an average decrease to 30 per cent. All three markers behaved identically, which indicates that the entire culture showed no preference as to which part of the DNA would be broken down. If the DNA template-DNA replication theory is valid, two possibilities exist. The first is that the DNA strands in the cells, at the time of resumption of DNA formation, were broken but kept in their place and thus still served as a template (Stent and Fuerst, 1955). These non-precipitable fragments (which are too small to have transforming principle activity) come apart only when treated with cold perchloric acid and thus escape the diphenylamine reaction. The other, and more likely, possibility is that only the remaining 30 per cent of DNA can serve as a template and be more or less completely replicated. The approximate doubling of DNA contents observed in most cases after a mild X-ray treatment suggests the latter rather than the former possibility. If this is the case, it is clear.

Figure 10. Behavior of DNA in X-ray-treated inactivated cells of Haemophilus influenzae strain Rd4(3M). Experiment was that of figure 3, but data have been corrected for contributions by survivors. Except for growth of nonirradiated control, all data are for cells which on plating failed to form visible colonies. For symbols see figure 3.
that cells do not need an intact and complete genetic structure to start replicating.

Although in X-ray-treated *H. influenzae* DNA breakdown is a very injurious process, experiments with other bacteria have thrown doubt on the possible conclusion that this breakdown is the radiation-induced process leading to cell inactivation. This doubt is best illustrated by experiments with *E. coli* strains B and B/r. Whereas strain B is much more sensitive to X-rays than strain B/r as far as killing is concerned, the reverse is slightly true for the observed postirradiation growth phenomena. Results with other strains also fail to show a relation between killing and DNA breakdown. This leads the author to believe that X-ray-induced DNA breakdown is a secondary effect that may aggravate the condition of the treated cells but is not directly responsible for cell death.

Then what is the cause of radiation-induced killing? The target theory in its simplest form probably does not hold, but, if it is assumed for the moment that killing is effected only through destruction of vital genes, then data in this paper allow for calculation of the number of these genes per *H. influenzae* cell. An X-ray dose of 48 kr killed a growing culture to about $5 \times 10^{-5}$ and reduced the biological activity of the cell DNA to about 75 per cent (figure 9). Marmur and Fluke (1955) reported for *D. pneumoniae* transforming principle that four different markers had equal sensitivity to ionizing radiation. Experiments with *H. influenzae* transforming principle have shown probable variations but they are very small (Stuy, unpublished data). It is therefore not unreasonable to assume that all genes have equal X-ray sensitivity. From the data given, it can then be calculated by the equation

$$0.75 p = 5 \times 10^{-5}$$

that one *H. influenzae* cell contains about $p = 35$ vital genes.

This number may seem small but does not exclude the possibility that radiation-induced death occurs through DNA. As pointed out earlier (Stuy, 1959), the vigorously growing cells probably depend very much on the functional state of their DNA. X-ray treatment impaired part of the cell DNA while growth, although partially inhibited, continued. Thus the treated cells synthesized large amounts of substances during a period in which their DNA could not supply all information needed for harmonious cell development. As a consequence, the whole cell structure was disorganized, which led ultimately to the cessation of all cell functions. As in the case of ultraviolet irradiation, it is obvious that the physiological state of the bacteria both during and after X-irradiation can affect survival. The postirradiation treatments may accelerate repair of DNA damage. They may also help the cells counterbalance the structural disorganization, thus giving them more time in which to carry out the necessary repair processes.

This hypothesis is still speculative. Repair of X-ray-damaged DNA has not yet been demonstrated in bacteria. Establishment of this process would support the views presented. Furthermore, support may also be obtained by finding a positive correlation between protection against killing and DNA injury.

The decrease in activity of intracellular transforming principle by X rays was found to be dose dependant (figure 9). Furthermore, the X-ray sensitivity in vivo of *H. influenzae* DNA was comparable to the sensitivity in vitro of the same DNA frozen in 1 per cent yeast extract (unpublished data). This confirms a recent communication by De Filipes and Guild (1959) and indicates that X-ray damage in vivo of DNA is a predominantly direct-hit affair. The data on the small but significant decrease of transforming principle activity caused by X-irradiation of living *H. influenzae* cells are in contradiction to an earlier report by Drew (1955), who reported that 100,000 rep of γ-rays, administered in vivo did not noticeably reduce the activity of pneumococcus DNA. This fact is probably due to the much greater inaccuracy of the procedures used in the pneumococcus work.

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SUMMARY

Treatment of actively dividing bacteria with X rays was followed by a breakdown of cell DNA. After a certain period this process stopped and an increase in DNA occurred.
These phenomena have been studied in several bacteria. In *Haemophilus influenzae*, the biological activity of cell DNA was also determined. It appeared that all remaining DNA was functionally normal. This was true also for DNA formed after irradiation.

No relation was found between killing and severeness of DNA breakdown as far as the various strains were concerned. From this it is concluded that the observed DNA breakdown is not the immediate radiation-induced process leading directly to cell death.

REFERENCES


