Effects of Ionizing Radiation on the Capacity of *Escherichia coli* to Support Bacteriophage T4 Growth

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Received for publication 24 November 1971

The loss in capacity of irradiated bacteria to support the growth of T4 phage has been studied for two strains, *Escherichia coli* B and *E. coli* B_{s-1}. Following a dose of 25 krad, the capacity is quite rapidly lost during postirradiation incubation so that after two hr of such incubation at 37 C only 12% remains in strain B and 3% in strain B_{s-1}. Evidence that capacity is lost in an all-or-none fashion was provided by two types of experiments: (i) a single-burst analysis of those cells which survived to give a burst, and (ii) an analysis of the regulation of an early phage enzyme. Several processes were examined to try to determine the cause of the loss in capacity. These were the ability of the irradiated and postirradiation incubated cells to respire, to allow phage adsorption and injection of phage deoxyribonucleic acid, to support the transcription and translation of an early gene, and to support replication of phage deoxyribonucleic acid. Of these processes, transcription and translation appeared to be the most closely associated with the capacity loss, although respiration was reduced to 50% after 2 hr.

Since the pioneer experiments of Anderson (3) in which he showed that heavily ultraviolet (UV)-irradiated *Escherichia coli* would sustain the growth of T2 phage, several studies have been made on the differential response of cells to various agents as tested by the remaining capacity to grow viruses (7, 19, 23, 27). The general picture that has emerged is that the more functions for which the phage can code (i.e., the less the phage requires of the cell), the less sensitive is an irradiated cell in terms of capacity loss for that phage. Thus, *E. coli* can withstand doses of several hundred krad before the capacity for T-even phage is significantly reduced. However, a very different picture of the capacity for T-even phage appeared if the irradiated bacteria were incubated under growth conditions *prior* to infecting them with phage. Labaw, Mosley, and Wyckoff (19) showed that after a dose of 160 krad the capacity for T6r+ fell to, and leveled off at, just less than 1% when 2.5 hr of postirradiation incubation intervened between the irradiation and the measurement of capacity.

In studies of *E. coli* and its response to ionizing radiation there are several advantages to observing the interaction between the cell and a virus. Two are used in this investigation. The first advantage is that it permits an analysis of the various cellular processes following irradi-

ation without the complication of damaged bacterial genetic material. In other words, we give the irradiated cell good viral deoxyribonucleic acid (DNA) and ask what the cell can do with that DNA. The second advantage is that we can investigate the response of *single* cells to the viral infection, and this permits us to examine the interesting problem of the character of the cellular response to ionizing radiation.

The physical action of ionizing radiation is local and drastic. A cell that receives a dose of 25 krad (the dose used in this study) has received an average of 70,000 energy releases of 60 ev each. This is a large number, though not out of line with normal numbers in cells [5×10^6 adenosine triphosphate molecules, 2×10^4 transfer ribonucleic acids (tRNA)], but much more significant is the small volume occupied by these events. One can obtain an estimate of this volume either by noting that the energy released from a primary ionization is dissipated within a very small volume (17) or by calculating the fraction of chemical bonds in the cell which could possibly be disrupted by such an energy deposition. Either method indicates that only one part in 10^6 of the cell volume is affected. Because the cell is not an inert mechanism but a responsive living system, it would be expected that, for the most part, this much damage would not matter at all. Only if the radiation event

occurs in some essential unit should there be any response of consequence. Accordingly, ionizing radiation should have the characteristic of all-or-nothing action. That is, a vital unit in a cell should completely escape or be utterly destroyed. The observation of the capacity of cells after irradiation offers a potent means to test this suggestion. The observations made are not purely of a statistical nature and so are not dependent on interpretation. The response of single cells can be studied and the development of phage from such unitary systems observed. If the effects are all-or-nothing, then a cell should support either a normal phage development or no development at all. We have used this approach in this study.

The findings, reported below, show that the great insensitivity to ionizing radiation of the ability of cells to grow T4 phage is only present if the cells are not incubated after irradiation. But as first discovered by Labaw et al. (19), postirradiation incubation causes drastic reduction in that capacity. Our preliminary studies of the phenomenon observed by Labaw suggested that attachment and injection processes are not involved and that phage bursts were affected in all-or-none fashion (Marsden et al., Biophys. Soc. Annu. Meet. Abstr., 236a, 1970). Here we report an extension of those studies which, in addition, show that the ability of the phage DNA to be transcribed and translated is involved. Whatever the critical process, it is apparent from our studies that the action of ionizing radiation does partake of the all-or-nothing character.

Boyle and Swenson (7) recently used a similar approach by studying the capacity of *E. coli* B/r cells for T4D in response to UV irradiation.

MATERIALS AND METHODS

Growth media. Minimal A (Min A) medium contained the following per liter of distilled water: 2.0 g of NH_4Cl , 5.0 g of NaCl , 6.0 g of Na_2HPO_4 , 3.0 g of KH_2PO_4 , 5.0 g of glucose, 0.34 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, and 0.115 g of Na_2SO_4 . Cells were grown at 37°C in Min A medium supplemented with 50 μg of L-tryptophan and 1.0 mg of Difco Casamino Acids (vitamin-free) per ml. This supplemented medium is referred to as Min A'.

Strains. *E. coli* strains used were B (local laboratory stock), B_{s-1} (from Ruth Hill), and CR63 (from S. E. Luria). The phage strains used were T4D (from A. Kozinski), T4amN55 (a mutant defective in gene 42), and T4amB22 (a mutant in gene 43). Both amber strains were obtained from R. S. Edgar. Their reversion frequencies are less than 10^{-6} .

Assays. The standard double-layer technique of Adams (1) was used to obtain phage titer and concentration of infective centers. Dilutions of cells and phage were made in a solution containing 1.0 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and 9.0 g of NaCl per liter of distilled

water. This medium is referred to as MgSal. Radioactive measurements were made in Bray's (8) scintillation fluid and samples were counted in a Packard Tri-Carb scintillation counter except for experiments involving the assay of deoxycytidine monophosphate (dCMP) hydroxymethylase activity (see Fig. 8-10) where 'Aquasol' (a scintillation fluid purchased from New England Nuclear Corp.) and a Beckman LS230 scintillation counter were used.

Irradiation of cells. Cell growth was followed on a Coulter counter (model B). Two min before the cell cultures reached the desired concentration they were brought to ice-bath temperature and vigorously aerated for several min. They were then transferred to a precooled flask packed in ice and irradiated in a Gammacell 200 (Atomic Energy of Canada, Ltd.) with a dose rate of about 10 krad/min. This gave D_{37} with the same values as for oxygenated bacteria (*unpublished results*). Where several hr of irradiation were required, the cultures were aerated throughout the irradiation, and the ice-bath temperature was also maintained. Irradiated cultures were held in an ice bath for not longer than 1 hr before use. Different holding times up to 1 hr had no effect on the capacity-loss kinetics.

Postirradiation incubation. Following irradiation the cells were kept at 0°C until they were incubated at 37°C with aeration. When necessary, the cell concentration during postirradiation incubation was followed on a Coulter counter, and the optical density was measured at 620 nm by using a 1-cm light path (Hitachi Perkin-Elmer model 139, UV-VIS spectrophotometer). In separate experiments the size distribution was measured at various times during the postirradiation incubation by using a series of window settings on the Coulter counter.

Measurements of cell DNA degradation. Cells were grown to 5×10^7 /ml in Min A'. The bacterial genome was labeled with thymidine-*methyl*- ^3H (^3H -thymidine; Tracerlab, 6.7 Ci/mmol). ^3H -thymidine was added at a specific activity of 750 mCi/mmol to give a final concentration of 3.6 μg /ml. Deoxyadenosine was added to give a final concentration of 250 μg /ml. Deoxyadenosine increases incorporation of the ^3H -thymidine (6). Growth was continued to 3.0×10^8 cells/ml when the cells were removed from the radioactive medium by centrifuging the culture at $7,000 \times g$ for 3 min and resuspending the pellet in the same volume of Min A' supplemented with 50 μg of unlabeled thymidine per ml. This washing was repeated. The cells were then aerated at 37°C until they reached 5.0×10^8 cells/ml to deplete the pools of labeled precursors. They were chilled to 0°C in an ice bath, aerated, and then irradiated.

Following irradiation the cells were incubated at 37°C with aeration. Trichloroacetic acid-precipitable radioactivity was measured by the method of Bollum (4). Samples of 0.1 ml were removed from the irradiated culture at various times and spread over 2 by 1-inch rectangular strips of Whatman no. 1 filter paper. These were immediately placed into a beaker containing 750 ml of iced 5% trichloroacetic acid. No sooner than 1 hr after the last sample had been taken the filter papers were stirred gently and the acid

poured off. About 750 ml of fresh trichloroacetic acid at about 4°C was added to the beaker. After a few minutes this washing procedure was repeated. A total of five such washings was used. The filter papers were then dried on paper towels and the radioactivity counted.

Control pieces of filter paper with no culture on them were placed in the beaker of trichloroacetic acid along with the sample pieces and were treated identically. They showed less than 0.1% of the counts which appeared on the filter paper with the highest number of incorporated counts.

Capacity assay. Cells were grown to 5×10^8 /ml in Min A'. These were chilled and irradiated as described above. Bacteria which received no post-irradiation incubation were kept in ice until assayed for capacity. The assay for capacity is the same for both unirradiated bacteria and bacteria which have been irradiated and subsequently incubated. The assay consisted of adding 0.5 ml of strain T4D in Min A' to 0.5 ml of the bacterial suspension to give a MOI of less than 0.1. Five minutes at 37°C were allowed for phage adsorption, then 0.5 ml of the adsorption mixture was added to 0.5 ml of strain T4-specific antiserum at 37°C and allowed to incubate for 5 min. This is sufficient to reduce unadsorbed phage to 5×10^{-3} of the unadsorbed titer. The tube was then plated for infectious centers. Immediately after removing 0.5 ml for the antiserum treatment, the remaining 0.5 ml in the adsorption tube was titered for unadsorbed phage following chloroform treatment.

Single-burst analysis. Cells were grown to 5×10^8 /ml. One sample was used immediately as a control and the remainder was irradiated and incubated as described. To 5.0 ml of cells T4D phage were added at an MOI of 0.1. After 5 min was allowed for adsorption, 0.5 ml was transferred to a tube containing 0.5 ml of T4 antiserum. After an additional 3 min the antiserum tube was diluted with Min A' at 37°C to give about 0.5 infectious center per ml in a total volume of 150 ml. This was quickly distributed, 1.0 ml at a time, into each of 100 tubes which were then incubated, with shaking, at 37°C. Forty minutes after infection, one drop of chloroform was added to each tube, the tube was vortexed, and the entire contents were plated after standing at room temperature between 10 and 15 min. A control experiment showed that the plating efficiency was 75% of the unchloroformed phage samples after 10 min of standing and 85% after 15 min. All sets of experimental samples (unirradiated, irradiated, and postincubated cells) were treated in the same manner except that a smaller dilution was necessary for the irradiated cells in order to produce 0.5 infectious center per tube. The required dilution was estimated from previous assays of the capacity.

Controls were run in all experiments to measure the extent of adsorption and the removal of unadsorbed phage with antiserum.

Adsorption kinetics. Cells were grown to about 1×10^8 /ml in Min A'. One sample served as a control and the other was irradiated. Adsorption kinetics were determined by mixing a 3.0-ml sample of the bacterial

suspension with T4D phage to give a MOI of less than 0.1. At suitable times after mixing, a 10^2 dilution was made into MgSal saturated with chloroform at 0°C. This was thoroughly stirred and left in ice for 1 min. This procedure will lyse infected cells (1). The contents of this tube were then diluted and plated for free unadsorbed phage.

Injection of phage DNA. Instead of using the traditional Hershey-Chase method (15), injection was measured by using the phenomenon first observed by Lesley et al. (20), which is that during the normal course of infection, a fraction of the parental genome is degraded to acid-soluble fragments. Controls show that contact between the cell and phage is necessary for this degradation to occur. The test consists of labeling the phage genome and measuring the amount of DNA that is rendered acid-soluble following infection. The rationale is that if there is no injection, then the parental genome will not be degraded. There is one slight complication in the method. We have shown that the proportion of the phage genome that is degraded depends upon the MOI (*unpublished results*). It is therefore important to determine the cell concentration accurately before infection so that the number of phage may be adjusted to keep the multiplicity constant.

One serious criticism of both this method and the Hershey-Chase method (15) is that they do not show where the phage DNA is localized within the cell after injection. A discussion of the implications of this appears later.

To label the phage a culture of *E. coli* B_{s-1} was grown to 5×10^8 cells/ml in Min A'. ³H-thymidine was added to give a final concentration of 3.6 µg/ml at 6.7 Ci/mole and deoxyadenosine to give a final concentration of 250 µg/ml. T4D phage were added to give a MOI of 3. After 1 hr of incubation the culture was lysed with chloroform.

The labeled phages were purified as described below. Bacterial debris was removed by centrifuging at $7,000 \times g$ for 5 min. The supernatant fluid was treated with about 50 µg of deoxyribonuclease and ribonuclease at 37°C for 10 min. Deoxyribonuclease was obtained from Calbiochem (pancreatic, B grade); ribonuclease was obtained from Worthington Biochemical Corp. An equal mixture of chromatographically prepared ribonuclease A and B was used. The treated supernatant fluid was transferred to a dialysis sac, which was then suspended in a current of warm air to concentrate the preparation. It took about 2 hr to reduce this volume from 15 to 3 ml, and every 15 min the contents were dialyzed for 5 min against MgSal.

One ml of this concentrated lysate was layered on a 30-ml 5 to 20% sucrose gradient containing 0.1 M NaCl, 10^{-3} M MgCl₂, and 10^{-2} M tris(hydroxymethyl)-aminomethane, pH 7.0, and centrifuged at 24,000 rev/min for 36 min at 4°C in an SW25.1 Beckman rotor. Tubes were punctured and about 30 fractions per gradient were collected; 0.05 ml of each fraction was assayed for radioactivity and the peak two or three fractions were combined and dialyzed against three changes of Min A and two changes of Min A', each of 750 ml and at 4°C.

To reduce the number of phage required in the

experiment, while maintaining a fast adsorption rate, the infection was initiated with cells at a concentration of 5×10^8 /ml and later diluted to give the volume necessary for the assay of trichloroacetic acid-soluble material. The detailed procedure is described below.

A 25-ml culture was grown to 5×10^8 cells/ml. A sample was removed for the irradiated control, and the remainder was chilled and irradiated with 25 krad. At $t = 0$, 1.0 ml of the unirradiated cells was inoculated with labeled phage at a multiplicity of infection (MOI) of 7.6. At $t = 3$ min, 7.0 ml of Min A' supplemented with 50 μ g of unlabeled thymidine per ml at 37 C was added. At $t = 4$ min and at subsequent regular 4-min intervals 0.9 ml of the infected culture was transferred to 0.1 ml of 50% trichloroacetic acid in a centrifuge tube in an ice bath. The final trichloroacetic acid concentration in the sample is thus 5%. These were set aside for 1 hr or so to await further processing. The procedure to determine the amount of phage genome degraded in cells that had been incubated for various amounts of time following irradiation was identical to that for the unirradiated control. However, the number of cells in the culture was followed closely with a Coulter counter to insure that the phage multiplicity was kept constant. Again, samples were taken into centrifuge tubes.

To each centrifuge tube was added 0.1 ml of 10% bovine serum albumin. The mixture was shaken, then left for 1 hr in the ice bath. It was centrifuged at $8,000 \times g$ to pellet the trichloroacetic acid-precipitable material, and 0.5 ml of the supernatant fluid was added to 20 ml of scintillation fluid.

To show that the degradation was not brought about by some extracellular bacterial enzyme, the following experiment was performed. A culture at 5×10^8 cells/ml was filtered through a 0.22- μ m membrane filter (Millipore Corp.) to remove the cells. Seven ml of the filtrate was incubated with the same number of labeled phage per ml as was used in the experiment described above. The experiment was also performed with the filtrate of cells which had been incubated for 90 min following irradiation. The sampling procedure for trichloroacetic acid-soluble material was identical to that described above.

Respiration. Respiration was measured with an oxygen electrode (Yellow Springs Instrument Co., Inc., biological oxygen monitor, model 53). The monitor was connected to a recorder (Sargent, model SRL) to give a continuous reading of the percentage of saturation of oxygen in the suspending medium. A 10-ml culture of *E. coli* B₈₋₁ was grown to 5.0×10^8 cells per ml in Min A', then chilled in an ice bath with aeration. A 1-ml amount of the culture was saved for the control and the remainder was irradiated and incubated as usual. Three ml of suspending medium in the reaction cell was maintained at 37 C and saturated with air. The background oxygen consumption was measured for about 10 min when 200 μ liters of the sample was injected. The percentage of saturation of oxygen was measured for an additional 12 to 15 min and thus the volume of oxygen consumed in 10 min could be calculated.

Two problems were encountered. The first concerns the hydrogen peroxide that is produced when

growth media are irradiated (13). Control experiments showed that irradiated media alone produce an apparent oxygen consumption. This is probably because the hydrogen peroxide, a postulated intermediary in the reactions at the electrodes, diffuses across the Teflon probe membrane to produce a current. This problem was overcome by adding 10^{-7} M catalase (Calbiochem, C grade) to the suspending medium (13). The second problem was that the suspending medium gave a background consumption of about 9 μ liters of O₂ per hr as opposed to 2 μ liters/hr by using distilled water. This was between 15 and 20% of the rate with bacteria present and had to be subtracted out.

dCMP hydroxymethylase assay. Hydroxymethylase, a phage-specific "early" enzyme of T-even phages (11, 12) was assayed by the method of Yeh and Greenberg (28). They showed that the enzyme specifically catalyzes an exchange between the hydrogen atom on carbon-5 of dCMP and water in the solution. Thus, ³H-dCMP labeled on carbon-5 will exchange its radioactive label with H₂O to give tritiated water, which is separated from the unreacted ³H-dCMP by an active carbon column and assayed by a scintillation counter.

Samples of phage-infected cells were assayed for dCMP hydroxymethylase in the following manner. Three ml of the sample in a 15-ml Corex centrifuge tube (Sorvall, Inc.) was sonically treated in an ice bath (Branson sonifier, model W140D, at a power setting of 2½ for 3 min with a micro tip). The tube containing the crude enzyme was placed in a 30 C water bath and 0.6 ml of phosphate buffer (1.0 M, pH 8.9) containing 0.1 M mercaptoethanol was added. Controls showed that this brought the pH of all samples to 7.5 which is close to the optimum for the enzyme (28). After 4 min, tetrahydrofolate (Nutritional Biochemicals Corp.) supplied in 1 M mercaptoethanol at 167 mg/ml was added. The volume of tetrahydrofolate added was constant within an experiment and is given in the figure legends. It was chosen on the basis of separate, more detailed experiments which showed that, in experiments where up to 6% of the substrate was used, a plot of tritiated water formed against reaction time was found to be linear up to 60 min. Thus, the concentration of tetrahydrofolate was chosen so that no more than 6% of the substrate would be used during the reaction. After an additional 4 min, 0.4 ml of ³H-dCMP (Amersham/Searle) (30 μ moles/15 μ Ci/ml) was added (0 time) to the tube. [The ³H-dCMP as supplied from the manufacturer was found to contain 1% tritiated water, which we removed by lyophilization. The lyophilized ³H-dCMP was redissolved in water containing 10% ethanol and stored at 4 C. It was adjusted to the specific activity indicated above with cold dCMP (Calbiochem, A grade).] At 5, 20, and 40 min, 0.6-ml samples were taken, heated to 75 C for 2.5 min to inactivate the enzyme (11), and chilled to 0 C until they were assayed for tritiated water formed in the reaction mixtures. Identical results were obtained when the enzyme was inactivated by adding 1 M NaOH to the reacted 0.6-ml sample to bring the pH to 12.

The amount of tritiated water formed was determined by passing the reacted mixtures through activated charcoal columns. The columns were made by placing a small cotton plug in the end of a serum transfer disposable pipette (7 mm by 6 inches with a 2-inch tip; Rochester Scientific Co., no. 3800). A water suspension of activated charcoal (Eastman Organic Chemicals, practical grade) was poured into the column and packed down under slight air pressure to give a height of 1 to 1.5 inches. The tube which had contained the reacted mixture was washed first with 1.8 ml and then 3.2 ml of water. Each wash was applied to the column and allowed to run through to the level of the charcoal bed before adding the next. The eluate and washes (5.6-ml total) were collected in a scintillation vial to which 10 ml of scintillation fluid was added and the vial was counted.

In the experiment to test the production of hydroxymethylase in irradiated and postirradiation incubated cells, a 300-ml culture of *B*_{s-1} was grown at 37°C in Min A'. When the culture reached about 3×10^8 cells/ml a 30-ml sample was removed to a separate bubbler. This control culture was infected at 4.7×10^8 cells/ml with T4D at a MOI of 7. At various intervals after infection, 3.0-ml samples were transferred to a Corex centrifuge tube in a dry ice-acetone bath which rapidly froze the samples. These samples were stored at -60°C until they were to be sonicated after thawing slowly in an ice bath at 0°C. A few minutes prior to the infection of the control culture the 270 ml remaining in the main culture was rapidly chilled by pouring it into a 4-liter flask in an ice bath. The flask was rocked for several min to insure adequate aeration. The cells were then irradiated at ice temperature with aeration as described above. These cells were at a concentration of 4.2×10^8 /ml. At a given duration of postirradiation incubation at 37°C a 30-ml sample of the cells was withdrawn to a separate bubbler tube and infected with T4D at a MOI of 7 (except for the 120-min sample which was mistakenly infected with a MOI of 10.5). Samples were taken and frozen as for the culture control.

In an additional control experiment a 90-ml culture of unirradiated *E. coli* *B*_{s-1} was split into three 30-ml cultures, and each culture was infected at 5×10^8 cells/ml with either T4D, T4amN55 (hydroxymethylase mutant) or T4amB22 (T4 DNA polymerase mutant) at a MOI of 7 (the final volume of the cultures was adjusted to be the same in all cases).

Phage DNA synthesis. DNA synthesis was measured by the uptake of ³H-thymidine into trichloroacetic acid-precipitable material following phage infection. Acid-precipitable material was measured as described above.

A culture of *E. coli* *B*_{s-1} was grown to 5.0×10^8 cells/ml. One sample was used immediately as a control, and the remainder was irradiated and incubated as described. A 2.4-ml sample of the cells was added to 0.6 ml of a mixture consisting of the following: 50 µg of L-tryptophan per ml, 1,250 µg of deoxyadenosine per ml, 18 µg of ³H-thymidine (specific activity < 0.5 Ci/mmol) per ml, and phage T4D. The concentration of the phage was such that with zero postirradiation incubation the MOI

was 10 to 11, which means that after 2 hr of incubation the MOI was still > 6, thus assuring that > 99% of the cells were infected.

RESULTS

The interpretation of several of our experiments demands a knowledge of the number of cells in the culture during postirradiation incubation. Accordingly, the cell growth was monitored in those experiments. The results, not presented, can be summarized as follows. The total cell mass in cultures of *E. coli* *B*_{s-1} and *B* increases after 25 krad of irradiation by 40 to 50% (measured by optical density at 620 nm). For *B*_{s-1} this reflects an increase in the total number of cells in the culture, whereas the cell size remains constant as measured by (i) Coulter counter size distribution and (ii) an increase in optical density per cell number. For *B*, it reflects the formation of filaments as the cell size increases while the cell number remains constant.

The effect of irradiation on the ability of cells of *E. coli* *B* and *B*_{s-1} to support phage growth is shown in Fig. 1. It is obvious that very large doses are necessary to produce any reduction of the capacity. It is also clear that there is a large extrapolation number, not easy to estimate, but in the general region of hundreds to thousands. It is tempting to associate the loss of capacity with the destruction of ribosomes and RNA polymerase. The same idea based on similar results has previously been suggested by Wallace (Ph.D. thesis, Cornell Univ., Ithaca, N.Y., 1965). She obtained additional evidence for it by studying the average burst size in cells after various X-ray doses. She found that the average burst size began to fall off without any shoulder and that at a dose which left 90% capacity the burst size was only 1%. For our purposes, we observe that the capacity for unincubated cells is indeed very insensitive.

If the same experiment is carried out on cells which have received 25 krad (a dose that has no measurable effect on the capacity immediately after irradiation) and which are incubated for the times shown, it is clear that the effect of incubation is drastic. The results are shown in Fig. 2. The left-hand side refers to *B*_{s-1}, and for comparison the per cent of capacity remaining and the per cent of acid-insoluble DNA left in the cells after this dose are both shown. The right-hand side of Fig. 2 shows the same comparison for *E. coli* *B*.

Whatever the reason for the loss of capacity on incubation, it is very interesting to see whether the ability to grow phage is lost uniformly with all cells or whether it is totally lost in some and not at all in others. This can be examined be-

FIG. 1. Effect of radiation on the capacity of *Escherichia coli* B and B_{s-1} to support the growth of T4D. (○) *E. coli* B; (●) *E. coli* B_{s-1} ; (-----) colony-forming ability of *E. coli* B_{s-1} .

FIG. 2. DNA degradation and loss of capacity at 37°C after 25 krads. (●) ^3H in trichloroacetic acid precipitate; (○) infective centers.

cause the yield of phage from a single cell can be studied. Figure 3 shows the results of such a study on B_{s-1} which have received a 25-krad dose as compared to cells that were unirradiated. The value of the residual capacity is also given. It can be seen that the unirradiated cells yield a distribution of individual bursts from single cells at about a mean of 72. With an average of 0.6 cell per tube, as determined from the number of control plates with no plaques, one would expect 10 tubes out of 100 to contain two cells, two tubes to contain three, etc. This has been allowed for in calculating the various mean

FIG. 3. Burst-size distribution at 37°C from T4D-infected *Escherichia coli* B_{s-1} . N_0 is the number of plates out of 100 which have no plaques. The arrow indicates the mean of the size distribution.

burst sizes and explains the number with high burst sizes.

In the irradiated case, at 45 min of postirradiation incubation the capacity is only 15% of normal. Therefore, to obtain the same number of zero-plaque plates the number of cells per tube was increased proportionally. The number of plaques per plate now affords a sharp test of the all-or-nothing hypothesis. In the 45-min case there are roughly seven times as many cells per tube, a situation in which each cell develops one-seventh the normal burst, which means that the probability of a zero-plaque tube is about e^{-70} , an extremely small number. Therefore, in the average response hypothesis there should be no zero-plaque tubes at all. In fact, the expected number was found, and this only can mean that the great majority of cells gave *no burst at all*. At the same time, many cells gave the normal burst size (Fig. 3). Thus, there are present at least two major populations: cells which have no capacity at all and cells with about normal capacity.

When the postirradiation incubation time is 120 min, the same finding is seen. There are nearly 30 times as many cells per tube and 29 out of the 30 give no burst. Those that do give bursts produce widely distributed burst sizes,

including sizes in the upper edge of the distribution. Therefore, even under conditions that remove the capacity from almost all the cells, there are cells that give normal yields. There are also more of these cells that produce reduced yields. It is premature to offer suggestions because the nature of the process that is damaged by radiation has to be understood.

In Fig. 4 we show the same experiment with *E. coli* B as the host cell, which differs from *B*_{s-1} in having a considerable nondegradable DNA fraction, lesser radiation sensitivity, and ability to respond to radiation by the formation of filaments. The same conclusion as before is reached regarding the number of zero-plaque plates. With 45-min postirradiation the number of cells per tube was increased by a factor of three and, if the average burst size of 20 were uniformly distributed among all cells, the probability of zero-plaque tubes would be e^{-60} . This was not found. The actual burst distribution reflects the formation of filaments as mentioned earlier. Deering and Christian (9) have shown that the burst size of T2 in *E. coli* B is linearly related to the length of the filament. Accordingly, the burst size can go up and the distribution become more spread out. Similar conclusions can be drawn from the 120-min postirradiation incubation data.

These experiments thus lead us to the important conclusion that whatever in the cell is essential to the development of phage, the damage produced in it by radiation and incubation is of the all-or-nothing character. Additional evidence for an all-or-none character of capacity loss was obtained when the processes of transcription and translation were studied. The remainder of this study is concerned with narrowing down the scope of explanation for the loss of capacity.

The first step in phage infection is adsorption and is not responsible for the loss of capacity as is shown in Fig. 5 where the fraction of phage

FIG. 4. Burst-size distribution at 37°C from T4-infected *Escherichia coli* B. N_0 is the number of plates out of 100 which have no plaques. The arrow indicates the mean of the size distribution.

adsorbed at various times is plotted for various treatments and incubations of the cells for both B and *B*_{s-1}. The latter shows very little difference. *E. coli* B shows, in fact, an increase in adsorption, but this increase is correlated with the increase in cell mass due to the formation of filaments.

The next significant step is injection. To test injection we measured the amount of parental phage DNA degradation present after infection. This is done by observing the amount of ³H-labeled phage DNA that appears in acid-soluble form after infection. As mentioned earlier, neither this method nor the Hershey-Chase method show that the phage DNA is injected into the cytoplasm of the cell. If the DNA is only injected into the periplasmic space, then the phage would probably not be able to form a plaque. Our method has an additional disadvantage in that either the nuclease levels or the accessibility of the phage DNA to the nucleases may change in postirradiation-incubated cells and thus confuse the results.

Bearing in mind these limitations, we can look at the results of the experiment summarized in Fig. 6, which shows the amount of ³H-labeled phage DNA appearing in acid-soluble form after infection. The data has been normalized to the number of cells in the culture. There are three aspects to the figure. First, the hatched area represents the spread of data from three separate control experiments using unirradiated cells; the variability probably reflects slightly different MOI(s). Second, the figure shows that there is no breakdown if the phages are incubated with the filtrate of cells which have received 90 min of postirradiation incubation. An identical result (data not shown) is obtained with the filtrate of unirradiated cells. These controls show that the degradation is not caused by some extra-cellular

FIG. 5. Adsorption of phage to irradiated (25 krad) and incubated cells at 37°C. (○) Control, no irradiation. Time of postirradiation incubation: (●) 5 min, (□) 10 min, (■) 30 min, (△) 60 min, (▲) 90 min, (×) 120 min. Original cell concentrations: *Escherichia coli* B = 1.1×10^8 cells/ml; *E. coli* B_{s-1} = 1.3×10^8 cells/ml.

FIG. 6. Degradation of the parental phage genome in irradiated (25 krad) and postirradiation-incubated *Escherichia coli* B_{s-1} (multiplicity of infection = 7.6; temp = 37 C). The hatched area represents the variability of the control as deduced from three separate experiments using unirradiated cells. The data are normalized to the number of cells in the culture. (●) Control, supernatant fraction of 90-min cells. Time of postirradiation incubation: (○) 15 min, (□) 45 min, (Δ) 90 min, (×) 120 min.

enzyme and that the phage must come into contact with the cell for breakdown to occur. Finally, Fig. 6 shows the behavior for four different postirradiation times. In all cases there is appreciable degradation (about 10% of the parental label), meaning that the phage DNA was injected. The drop below the control value does not correspond to the much greater loss of capacity. Moreover, the data, as presented in the graph, have been normalized to the number of cells in the culture which, as mentioned, increases during postirradiation incubation. Our unpublished experiments using chloramphenicol indicate that in phage in which several ³H decays have taken place, a part of this degradation is brought about by host enzymes and it could very well be that the amount of enzyme responsible for the degradation does not increase in proportion to the cell number, resulting in a slight decrease per cell. We therefore conclude within the limitations discussed above that the important factor in loss of capacity is not the loss of injection.

A factor that certainly could be involved is the respiration of the irradiated cells. Data by Pollard and Schuster (quoted in reference 22) suggest that B_{s-1} is insensitive to radiation in this respect. However, they did not measure respiration for longer than 40 min of postirradiation incubation. Accordingly, the measurements on respiration ability were extended to 130 min of postirradiation incubation with the results shown in Fig. 7. The oxygen consumption relative to the initial rate of the unirradiated cells actually rises for the first 40 min or so and then falls. Some of the rise probably can be attributed to the increase in numbers of cells after irradiation. If correction is made for this, there is a steady fall in the respiration rate per cell (Fig. 7). However, this loss of respiration does not match the loss of capacity, although it is more of a factor than the injection findings. This result is supported by the recent work of Swenson et al. (26) who have observed that the richer the incubation medium, the more resistant B/r is to respiration loss as a result of gamma irradiation. Like Pollard and Schuster they find B_{s-1} is more resistant than is B/r. Our medium is richer than any they used, and we find our cells correspondingly more resistant.

Observations on two related processes showed much closer agreement with the loss of capacity. These are the processes of transcription-transla-

FIG. 7. Rate of oxygen consumption at 37 C in irradiated (25 krad) and postirradiation-incubated *Escherichia coli* B_{s-1}. (○) Raw data; (●) data normalized to the number of cells in the culture.

tion and of DNA synthesis. The ability of the cells to transcribe the phage genome and then to translate the message into protein was measured by observing the amount of deoxycytidylate hydroxymethylase activity resulting from phage infection of postirradiated incubated cells. Figure 8 shows the findings for unirradiated cells and irradiated cells which have been incubated for various times prior to infection. By 120 min of postirradiation incubation very little enzyme is seen. The observations are made for various times after infection, and it should be noted that in all cases (except possibly for the 140-min case) the enzyme production reaches a maximum about 20 min after infection. In Fig. 9 we show a composite graph for the amount of enzyme produced relative to the control as determined by the length of postirradiation incubation. The data have been normalized to the number of cells per ml in the unirradiated control culture. Also shown for comparison are (i) data from Fig. 2 showing the amount of capacity remaining and (ii) data from Fig. 11(b) showing the rate of DNA synthesis relative to the unirradiated con-

FIG. 8. Appearance of dCMP hydroxymethylase in phage-infected *Escherichia coli* B_{s-1} . The cells were irradiated (25 krad) and postirradiation incubated at 37 C. (○) Control, no irradiation. Times of postirradiation incubation: (△) 10 min, (●) 25 min, (▲) 45 min, (□) 80 min, (■) 121 min, (◇) 140 min. The multiplicity of infection was 7 in each experiment, except for the 121-min case where it was 10.5. The reaction mixture contained per ml: 9.0 μ moles of tetrahydrofolate, 2.9 μ moles and 1.45 μ Ci of 3 H-deoxycytidine monophosphate, 38 μ moles of 2-mercaptoethanol, 190 μ moles of sodium phosphate buffer (pH 7.5), and 3.5×10^8 infected cells (this increased for the postirradiation incubated cells by about 50%). The infected cells were sonicated prior to the reaction to obtain the crude enzyme extract. Each data point represents a separate reaction (at 30 C) with enzyme extracted at the time indicated on the abscissa.

FIG. 9. Relative amount of deoxycytidine monophosphate hydroxymethylase activity in phage-infected cells expressed as a percentage of the unirradiated control. The cells were *Escherichia coli* B_{s-1} irradiated with 25 krad and incubated at 37 C for the times shown. The data has been normalized to the number of cells in the culture. Also shown in the figure are the data from Fig. 2 (----) showing the capacity loss in irradiated and incubated B_{s-1} and also the data from Fig. 11 (-.-) showing the rate of synthesis relative to the unirradiated control in such cells.

trol in postirradiation-incubated cells. The drop in amount of enzyme produced is considerable and certainly bears a relationship to the loss of capacity shown in Fig. 2.

A second line of evidence referred to earlier indicates that the capacity loss which occurs during postirradiation incubation has an all-or-none character. It has been shown (21) that mutants of T4 which are incapable of synthesizing phage DNA produce abnormally high levels of dCMP hydroxymethylase and other early enzymes required for DNA synthesis. This is demonstrated by the use of T4amb22, a polymerase-negative mutant. As shown in Fig. 10 the enzyme activity in cells infected with this mutant increases sharply after 7 min, about the time when DNA synthesis would normally begin, in contrast to cells infected with the wild-type phage, and attains by 28 min after infection a level about four times higher than the control. This implies that DNA synthesis is required in order that some temporal control mechanism can shut off the production of this early enzyme. The exact nature of this control mechanism is not known. However Bolund and Sköld (5) have shown that dCMP hydroxymethylase (among other early phage enzymes) is regulated at the transcriptional level and appears to be operative even in cells that have had various amounts of postirradiation incubation (Fig. 8). Thus, the

FIG. 10. Appearance of deoxycytidine monophosphate (dCMP) hydroxymethylase in *Escherichia coli* B₈₋₁ infected at 37°C with control T4D (curve B), T4amB22 (curve A), and T4amN55 (curve C). The reaction mixture contained per ml: 4.5 μ moles of tetrahydrofolate, 2.9 μ moles and 1.45 μ Ci of 3 H-dCMP, 38 μ moles of 2-mercaptoethanol, 190 μ moles of sodium phosphate buffer (pH 7.5), and 3.6×10^8 infected cells. The infected cells were sonicated prior to the reaction to obtain the crude enzyme extract. Each data point represents a separate reaction (30°C) with enzyme extracted at the time indicated on the abscissa.

data indicate that if such a cell is capable of supporting the production of the early enzyme then it is also capable of producing phage DNA and a functional control mechanism. Figure 10 (curve C) also shows that in cells infected with T4amN55, a hydroxymethylase-negative mutant, there is, as expected, no production of the enzyme.

Fig. 11a shows DNA synthesis in infected unirradiated *E. coli* B₈₋₁ cells and cells which have been irradiated and incubated for 20 and 75 min before infection. Synthesis between 10 and 30 min after infection is linear. The radioactivity incorporated between 15 and 25 min has been taken as a measure of the rate of DNA synthesis. The uptake probably reflects phage DNA synthesis rather than residual cellular synthesis since (i) even ghosts are known to shut off host DNA synthesis (10) and (ii) the activity extrapolates back to 7 min, the time when synthesis normally commences in the control.

The data from three such experiments is presented in Fig. 11b where the rate of phage DNA synthesis in postirradiation-incubated and infected cells has been expressed as a percentage

FIG. 11. DNA synthesis in phage-infected, irradiated (25 krad), and postirradiation-incubated *Escherichia coli* B₈₋₁. (a) Single experiment showing the uptake of 3 H-thymidine into trichloroacetic acid-precipitable material. Control ($\circ$), no irradiation. Time of postirradiation incubation: (Δ) 20 min, ($\square$) 75 min. (b) Summary of data from three experiments expressed as a rate of synthesis relative to the unirradiated control. The data have been normalized to the number of cells in the culture. The different symbols refer to different experiments.

of the unirradiated control after normalization of the rate to the number of cells in the culture. The yield of phage DNA closely followed the yield of the early enzyme, and because the enzymes for the synthesis of DNA have to be transcribed and translated this is not surprising.

DISCUSSION

This work confirms Anderson's (3) early experiments which showed that the capacity of *E. coli* cells to support T4 phage growth is very resistant to ionizing radiation. A strikingly different picture is obtained, in agreement with the earlier work of Labaw et al. (19), if the same cells are irradiated with relatively low doses and incubated under growth conditions prior to infection with phage. Following a dose of 25 krad the capacity is quite rapidly lost during post-irradiation incubation so that after 2 hr of such incubation only 12% remains in strain B and 3% in strain B₈₋₁.

The objectives of this study were twofold. The first was to investigate the character of the capacity loss. Evidence that capacity is lost in an all-or-none fashion was provided by two types of experiments: (i) a single-burst analysis of those cells which survived to give a burst and (ii) an analysis of the regulation of an early phage enzyme. The all-or-nothing character of the loss of capacity after irradiation and incubation is interesting both for the reason that it fits with the widely distributed but highly energetic character of ionization events and because

it also supports the idea that for this type of radiation action there could be no threshold. It is often claimed that threshold processes are unlikely in radiation action, but it is difficult to conduct experiments at extremely low doses to prove the point. Here the basic nature of the action, the all-or-nothing character, requires that there be no threshold. Generalization to all other actions of ionizing radiation is, of course, not justified.

As we pointed out, we feel that all-or-nothing responses to ionizing radiation are likely. Such responses have been demonstrated for several processes, namely DNA degradation (16, 22, 24) and the two in this paper. Boyle and Swenson (7) found that in *E. coli* B/r capacity is lost quite rapidly during incubation following low doses of UV which have no effect on capacity measured immediately after irradiation (results analogous to ours with gamma irradiation). They decided to look at the character of those cells which retained their capacity. They found that the cells produced an essentially normal burst. It would be interesting to look at the response of some other processes to UV to see whether the all-or-nothing phenomena could be generalized to UV damage.

It is worth mentioning two differences between the results of Boyle and Swenson with UV and ours with gamma irradiation. The first is that they observed a correlation between the number of cells surviving to support phage growth and to form colonies and concluded that after 120 min of postirradiation incubation following 520 ergs/mm², "phage growth appeared to occur only in those cells that would eventually form colonies." In our experiments 25 krad reduced survival of B_{s-1} and B to 10⁻⁶ and 10⁻⁴, respectively, so that even if the survivors started to grow without any lag the ratio of surviving infective centers to colony formers was after 120 min, 1,500 to 1 and 60 to 1 for the two cells. Therefore, we must conclude that some cellular function which is essential for cell division but not for phage production was inactivated by ionizing radiation. The second difference is that with UV there is a lag of some 30 min before any capacity is lost during postirradiation incubation, whereas with gamma irradiation there is no lag.

The second objective of this study was to determine the cause of the capacity loss during postirradiation incubation, because it was felt that this would give information on the cause of radiation-induced cell lethality. We had thought at one time that there may be a relationship between the extent of cellular DNA degradation and the extent of capacity loss. However we

found that the ratio between these two quantities could be altered by using different postirradiation incubation media and doses smaller than those necessary to produce maximal degradation of the cellular DNA.

It was not possible to ascribe the cause of the loss in capacity to any one of the processes investigated, although the following conclusions can be made. (i) The capacity loss is not due to the loss of the ability of the cell to adsorb phage. (ii) Capacity loss is probably not due to injection failure. To the extent that phage DNA must be ejected somewhere into the cell (either the periplasmic space or the cytoplasm) in order to be degraded, this conclusion is valid. But if by injection we mean normal progression of the phage DNA into the cytoplasm, the conclusion is not valid. Additional work, such as the high-resolution autoradiographic study done by Anderson et al. (2), would be necessary to settle this question rigorously for the loss of the ability of irradiated cells to allow *normal* injection would be a clearcut explanation of the all-or-none effect observed in this work. (iii) Respiration is affected, although not to an extent that would completely account for the loss in capacity. After 120 min of postirradiation incubation only 3% capacity remains, whereas 50% respiration remains. (iv) There is a large effect on transcription and translation. The end product of the two processes was measured and therefore it is not possible to distinguish directly between them. DNA synthesis also falls off rapidly and quite closely parallels the loss in transcription and translation. This result is to be expected because transcription and translation are necessary to produce the early enzymes for DNA synthesis.

Subject to the conditional conclusion mentioned above in connection with injection failure, postirradiation incubation damage to the processes of transcription and translation most clearly parallels capacity loss. Any explanation of the capacity loss must be able to account for the all-or-none nature of this loss. Kućan and Kućan have shown evidence for ribosomal damage in several strains of *E. coli* (18). They incubated cells which had received a dose of 23.5 krad. After a lag of 30 min there was a linear breakdown of rRNA to trichloroacetic acid-soluble material so that by 120 min 40% had been broken down. This certainly suggests some major change within the ribosome, possibly inactivation. On the other hand, Solosko (*unpublished data*) has shown that the latent period of T4 in postirradiation-incubated cells is not increased. This implies that the ribosomes are operating at their normal rate and therefore are

not damaged. Regardless of whether the ribosomes are damaged or not, it is difficult to envisage, with such a large multiplicity of ribosomes, how one could produce the observed all-or-none effect. Such an effect is more likely to result from damage to some macromolecular species of which there are only a few copies in the cell. A large transcription complex on the membrane or the membrane itself are suitable candidates.

The following postulated scheme is compatible with the experimental results. As a consequence of some initial lesion there may occur any or all of a number of phenomena. These include DNA degradation, induction of a prophage, membrane damage, and impairment of DNA synthesis and transcription. The data indicate that transcription is particularly sensitive and it is therefore hypothesized that the decrease in transcription leads to a general "running down" of the cell, the reduced respiration being one manifestation of this. If the phage genome is injected when the ability of the cell to transcribe the phage genome has been destroyed, there will be no burst. If, on the other hand, the phage genome enters the cell before transcription has completely failed, then the phage-induced enzymes may be able to repair the cellular structures necessary to produce a normal burst. In support of this conjecture is the work of Furrow and Pizer (14) who have shown a redirection of the phospholipid synthesis of the cell during a normal T4 infection.

One additional aspect of this scheme would occur if transcription failed during phage development. Evidence that this may happen is indicated by a comparison of Fig. 2 and 9 which shows that the capacity curve and the dCMP hydroxymethylase curves, although similar in shape, are displaced by 15 to 20 min. This can be rationalized as follows: there are about 15 to 20 min between the transcription of the early enzymes and lysis of the infected cell, and, therefore, transcription must continue 15 to 20 min after expression of dCMP hydroxymethylase in order to get a burst. Thus, any decrease in capacity should occur 15 to 20 min earlier than the decrease in the enzyme curve.

ACKNOWLEDGMENTS

We wish to acknowledge the excellent technical help of Joyce Cox and Zivile Khoury with some of the experiments. The help of Friederica Spencer in preparation of the manuscript was also appreciated.

This work was supported by a grant from the National Science Foundation to W. Ginoza and grant AT(30-1)-2804 from the Atomic Energy Commission to E. C. Pollard.

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