GROWTH AND MUTATION OF BACTERIA DURING CONTINUOUS IRRADIATION

B. A. RUBIN

Brookhaven National Laboratory, Upton, New York

Received for publication September 24, 1953

For a long time now, microorganisms have been employed as a tool in the interpretation of the biological activities of radiations, usually in terms of lethal and genetic effect. In order to maintain standard conditions, recent investigations have been careful to prevent growth during irradiation. Whereupon, the lethal and genetic effects usually have been affected proportionately over a wide dosage range and indeed have been considered causally related (Lea, 1946).

In studies of the genetic effects of absorbed radioactive isotopes, it is not possible to avoid the growth or metabolism of the organisms during irradiation. Radioactive material must be present in the ambient medium in order to have the isotope taken up. Indeed, many workers have grown bacteria successfully in radioactive culture media, where equal or smaller radiation levels have been shown to be rapidly lethal to the resting organisms (Schmidt, 1948; Hershey et al., 1951; Deynse and Bonet-Maury, 1951; Harper and Morton, 1952).

This study is concerned with the characteristics of bacterial growth during continuous irradiation and with a genetic change that occurs under such conditions. Since a widely prevalent concept attributes mutagenesis to errors in the synthesis of new genetic material (Mueller, 1952), it has been of interest to examine mutagenic effect as a function of the amount of growth taking place during an irradiation period. Conversely, such a study also could indicate the contribution of genetic damage to the total lethal effects of radiation.

MATERIALS AND METHODS

The organisms used in these studies were strains B and B/r of Escherichia coli, obtained from the Carnegie Institution at Cold Spring Harbor. The culture medium used in the X-ray irradiation experiments was usually brain heart infusion broth (Difco). With less complete media, the observed effects were qualitatively the same, but since the brain heart infusion broth gave the largest crop of bacteria (and mutants), it was employed in the interests of improved statistics. When P⁴⁰ in solution was used as an irradiation source, the medium consisted of glutamic acid (neutralized with NaOH), 2 per cent; nutrient broth (Difco), 0.5 per cent; KCl, 0.2 per cent; glucose, 0.2 per cent; MgSO₄ (anhydrous), 0.012 per cent; and a mixture of phosphate salts (pH 7.0) calculated to give 2 mg of total P per ml of medium (including the P content of the other ingredients).

In the X-ray experiments, turbidimetric measurement of culture growth was made with the Coleman spectrophotometer whose wavelength was adjusted to minimize the effect of the medium. In the equipment used for irradiation, the treated cultures were contained in matched tubes, especially fabricated to fit the Coleman tube holder, in order to permit periodic observations of the turbidity with a minimum of manipulation. A control tube containing only the medium was always run as a treatment and optical blank. The turbidity of cultures containing P⁴⁰ was measured in an automatic recording turbidimeter whose readings corresponded to those obtained with the Coleman. These instruments have been described in detail (Rubin, 1950).

Counts of the viable organisms were made by dilution plating. Three separate dilution series and three plates at each dilution level were always prepared. The triple agar layer technique was used consistently. In this technique the middle layer containing the organisms is sandwiched between two layers of the same agar, in this case nutrient agar. Plates were incubated at 37°C for at least three days before counting.

Mutation frequency was measured by a method that has been described in detail elsewhere (Rubin, 1954). In essence it consisted in finding the number of organisms resistant to 100 μg per
ml of streptomycin. Irradiated cells were plated in an alkaline triple layer agar plate containing no streptomycin. After a period of growth to allow for phenotypic expression, a fourth layer containing streptomycin and brain heart infusion broth was added and allowed to diffuse. After a period of four days of incubation at 37 C, the surviving colonies were counted as streptomycin resistant mutants.

In order to relate the genetic changes to growth during irradiation, it was necessary to vary the number of generations occurring during an irradiation period. This was done by changing the proportion of inoculum to fresh medium in a culture. If one volume of a sterile medium is added to an equal volume of a freshly grown culture, only one generation of growth can occur before the medium is saturated. If the medium is three times the volume of the inoculum, there is a fourfold increase in cell count, corresponding to an average of two generations of growth. If n generations of growth is desired, the ratio would be 1 part of inoculum to 2 - n parts of fresh medium.

Nucleic acid determinations were done in the manner described by Morse and Carter (1949) but with the careful preparation of reagents recommended by Ogur and Rosen (1950).

Irradiation techniques used in this work have been described in detail (Rubin, 1950). Most of the X-ray experiments were done with the aid of the "external irradiation chamber" discussed in that paper. In all cases the equipment was set up in the same position using the same X-ray machine settings. Under these conditions, the cultures received 5000 Roentgens (R) per hour for the entire period of irradiation. Seven tubes could be irradiated simultaneously in the machine described, and in a later model of the same general construction as many as 21 tubes could be treated simultaneously and equally. Since the cultures in the tubes were stirred constantly and vigorously, each of the organisms was presumed to receive an equal dose. In experiments where it was necessary to deal with a large, uniformly irradiated culture, especially prepared two liter, round bottom flasks containing up to one liter of culture were irradiated at the same dose rate (5000 R per hour). A flat surface was made in these flasks to correspond with the position opposite the point of entrance of the X-rays. Below this flat surface was a magnetic stirrer, and each flask contained an appropriate stirring magnet to provide continuous agitation. The incubation temperature (37 C) was maintained by thermostating and humidifying the entire X-ray room.

Irradiation with Pm usually was carried out in the matched tubes of the recording turbidimeter. The calculation of radiation dosage has been described (Richards and Rubin, 1950). In these tubes one millicurie of Pm provided about 1500 R per hour, and, here too, constant agitation was provided. The preparation, handling, and sterilization of the radioactive media have been described (Rubin, 1950). At the activity levels used in these experiments, special handling precautions were essential.

RESULTS

From our own observations (Rubin and Steinglass, 1950) as well as from those of other workers (mentioned above), it was evident that the growth of bacteria has occurred in media containing radioactive phosphorus (Pm) in solution. The development of an accurate recording turbidimeter permitted a detailed study of the characteristics of bacterial growth in highly radioactive culture media where frequent observations could be made during any desired time period. Although it was possible to measure the turbidity in each of 24 tubes as often as once in 1.2 minutes, for the maximum clarity of the recording it was preferable to read no more often than once each 7.2 minutes. Since the design of experiments containing large amounts of Pm required careful preparation consuming most of the working day, it was essential to be able to collect data all through at least one night, and some of the recordings were run continuously for as long as four days.

In all cases, each run of the machine contained all of the variables that were being directly compared, always at least in duplicate. The machine also held at least two culture medium blanks. Since the radioactive solutions were held in thick walled glass tubes and were continuously immersed in the water bath, there was very little cross irradiation between the tubes.

The recording turbidimeter could supply accurate information about the length of the lag period, the characteristics and rate of the logarithmic growth (generation time), and the total level of growth attained. A comparison between growth in radioactive and nonradioactive media is presented in figures 1 and 2. The only obvious
BACTERIAL GROWTH DURING CONTINUOUS IRRADIATION

Figure 1. The growth of Escherichia coli, strains B and B/r, in media containing various levels of P32—as determined with a recording turbidimeter. Alternate points provided by the machine are plotted here. Each treatment was repeated at least four times without significant variation. In all cases one percent of an 18 hour culture was used for inoculum. Lag time for each level of P32 was calculated from the point of intersection of the curves with the “X” axis—subtracting the lag time of the control, containing no P32.

The difference observed was in the length of the lag period. Since the presence of some effect on the logarithmic growth would be significant, very careful comparisons were made to see what differences were detectable. Figure 1 shows some of the curves taken from one experiment in which increasing levels of P32 in the media were compared carefully with controls. From this and similar experiments, it was concluded that no measurable differences in the logarithmic growth rate could be found by the present method. Similarly, there were no important differences in the total growth obtained in the various radioactivity levels.

The effect of increasing amounts of P32 in the medium on the lag period is shown in figure 3. In the experiment represented there, five levels of P32 were compared in both the B and B/r cultures. An analysis of these curves indicated that they were of the same family of hyperbolic functions differing only in their constants. In both cases 8 millicuries of P32 per ml increased the lag time to a period exceeding 4 days. Changes in the amount of inoculum or using older inoculum did not alter this picture significantly (nonirradiated controls were always subtracted from each value). Repetition of this experiment with the radiation grown cultures for inoculum did not give significantly different results. Microscopic examination of the bacteria after complete growth in radioactive media failed to reveal any striking morphological differences from controls.

Figure 2 illustrates the effect of using actively growing cultures for inoculum. A short period of
Figure 2. The growth of *Escherichia coli*, strain B, in different levels of \( P^{22} \)—as determined with a recording turbidimeter. Alternate points given by the machine are plotted. The inoculum in these cases was one per cent of a 6 hour culture. When the linear portion of the curves are extrapolated to the “X” axis, the effect of \( P^{22} \) on the lag time is equal to that obtained with older inocula (see figure 1).

Continued growth is followed by a period of inhibition proportional to the amount of \( P^{22} \) in the medium. Then the growth is resumed to provide a final picture equivalent to that described above, namely, equal generation times and equal total growth.

The use of a radioactive substance in solution permitted an accurate study of bacterial growth during continuous radiation with turbidity as a criterion. Irradiation continued even during the measurements, and it was not necessary to remove the cultures from their thermostated water bath during any part of the growth periods. But studies requiring estimations of viable cells were more difficult. It was not easy to stop the radiation (by washing) when desired without affecting the plate count. Also, the procedures involved in the usual type of plate counting were complicated somewhat by the radioactivity. For the aspects of these experiments in which determination of viable counts was required, the use of X-ray machine radiation proved more simple as well as more accurate.

When the development of turbidity in B/r cultures irradiated at 5000 R per hour was compared with nonirradiated controls, the increase in lag was equivalent to that obtained with about 3.5

Figure 3. The delay in the inception of growth (lag) as a function of \( P^{22} \) concentration in the culture medium (see figure 1). The points are averages of three experiments in which one per cent of an 18 hour culture was used for inoculum. Both curves fit the hyperbolic expression:

\[
A = y(\log x + B)^n
\]

where \( y \) is the lag time and \( x \) the \( P^{22} \) concentration. \( B \) is a constant which is equal for both cultures, while \( A \) and \( n \) are constants that differ in the two curves.
BACTERIAL GROWTH DURING CONTINUOUS IRRADIATION

Figure 4. The effect of continuous X-ray irradiation (5000 Roentgens per hour) on Escherichia coli, strain B/r. As described in the text, from 0 to 14 generations of growth were permitted during irradiation by varying the inoculum concentration. All levels from 0 to 14 generation cultures were assayed for viable count during 12 hour irradiation periods. Representative curves are shown here in which the measured points are indicated by the number of divisions which could occur in the culture. The curve following the zeros shows the effect of irradiating an aliquot of the whole inoculum (0 generations of growth). The dotted zeros (O) show the effect on the same inoculum aged for two days at 37 C. All points are the averages of three experiments.

The rate of logarithmic growth and the total crop both appeared to be equal to controls, as in the P³² experiments. Since there have been reports (Lea, 1946; Billen et al., 1953) of the growth of irradiated cells, without corresponding increase in viable (plate) counts, it was of interest to look into the viability of radiation grown cultures. A comparison of the progression of viable counts was made in cultures of different size inocula, allowing from 0 to 14 generations of growth during irradiation (figure 4). Even in the last case, when the inoculum was only 0.006 per cent, the growth which was observed differed from nonirradiated controls only in a somewhat increased lag period. Whenever the whole growth cycle could be ob-
served, there was an increase in viable count, followed by a period of relative stability of count, and finally a logarithmic decrease. The rate of killing of the radiation grown cultures by further irradiation was not affected by the previous period of growth in the radiation field.

In the experiment illustrated by figure 4 an aliquot of the inoculum used for the rest of the experiment was irradiated and called zero generations of growth. It may be noted that the lethal effect of the radiation does not commence immediately but follows what may be called a "multi-hit" curve. When this (18 hour) culture was allowed to age for two more days and then irradiated under the same conditions, the usual type of logarithmic (one hit) killing curve was obtained.

When the nucleic acid content of radiation grown cells was compared with that of resting controls, certain differences were first noted.

Figure 5. The frequency of streptomycin resistant mutants in cultures grown during continuous X-ray irradiation of 5000 Roentgens per hour. The assayed points in the various cultures are represented by numbers equal to the length of time (in hours) of irradiation and growth. Since a horizontal line could be fitted for each time period, the factor of growth during irradiation does not affect the genetic response to radiation dose. The inserted curve shows the relation of the radiation to mutation frequency as obtained by extrapolating each line to the "Y" axis. Each point is an average value from 4 to 8 experiments.
BACTERIAL GROWTH DURING CONTINUOUS IRRADIATION

1954

In this way one might explain the two phase curves which are seen when actively growing inoculum is used to seed the radiation grown cultures.

Even after complete growth in radiation has been attained, the antipoison effect is still visible in the relatively long plateau period before radiation killing commences. To a smaller extent this effect may also be the cause of the "multi-hit" killing curves of fresh cultures without any previous irradiation history.

The rapid growth rate occurring at the various radiation levels, equal to the logarithmic growth rate of controls, possess several very interesting implications. Evidently there is no race between killing and growth during actual growth. If there were, a detectable and increasing effect on the slope of the logarithmic growth period should be detectable as the radiation dose increases. The equal growth rate at all radiation levels also indicates that, in order for growth to start, some effect of the radiation must be reversed completely. For that reason it takes even more time for growth to start; with higher dose rates, a greater antiradiation effect must be achieved.

This effect of irradiation, which is somehow metabolically reversible, appears to be the major cause of radiation death since there is no detectable lethal effect during the actual growth. Since genetic damage goes on at the same rate, regardless of whether or not the cells are growing, one must conclude that lethal mutations contribute relatively little to the total lethal effect. As a corollary one might deduce also that the so-called "direct" effect of radiation constitutes an insignificant portion of the total lethality, at least under the experimental conditions described.

The growth of bacteria (and nucleic acid synthesis) during irradiation implies a basic difference from higher organisms where it is generally considered that the growing cell is most sensitive. Perhaps we have an indication here that the process of cell division in bacteria is somehow different from the mitotic processes which have been shown to be disturbed so easily by radiation.

The possibility that radiation resistant cells are being selected during the growth process seems rather unlikely. When radiation grown cells were used for inoculum, there was no shortening of the increased lag period. When cultures grown for different times in radiation were then irradiated during their resting phase, the rate of killing was
the same for all and equal to the killing rate of a control (not radiation grown) culture.

The lack of effect of growth on the mutation rate also has several important implications. Radiation mutations seem to be caused by a different mechanism than is responsible for most of the lethal effect in bacteria. This concept is supported by the observation (Demerec and Latarjet, 1946) that strains B and B/r respond to radiation equally as far as genetic effect but quite differently with regard to the lethal effect. Resting cultures of strain B/r of different ages, that are differently sensitive to the lethal effect of radiation, also show the same dose response with regard to mutations.

If the fraction of a population which is found to have mutated remains constant, regardless of whether or not growth is going on, then as the population increases, the changed and unchanged cells must be increasing at about the same rate. After only a few generations of radiation growth, this proportionality holds very well, probably because most of the mutants are not yet expressed phenotypically. With a greater amount of growth, there is a somewhat greater spread in the results indicating the differences in the growth rate of the mutants. The repeated reduplication of genetic material during irradiation, without increasing genetic effect, seems to imply that mutation is not the result of mistakes in gene synthesis. From the data of these experiments, it seems more likely that radiation affects the already formed gene since repeated opportunities to interfere with accurate synthesis are without effect on the mutation rate.

SUMMARY

Escherichia coli, strains B and B/r, has been grown during continuous irradiation of up to 10,000 Roentgens per hour. Growth (viable count as well as turbidity) was observed to occur at a rate equal to nonirradiated controls, and the maximum crop of cellular material was also equal. Only the period before the inception of growth (lag) increased as a function of a dose. Under these conditions the mutation frequency increased as a function of total radiation dose, regardless of how much growth (if any) had occurred during irradiation. Nucleic acid content of radiation grown cultures was indistinguishable from controls.

REFERENCES


Richards, P. I., and Rubun, B. A. 1950 Irradiation of small volumes by contained radioisotopes. Nucleonics, 6(6), 42–49.


