DIVISION PATTERNS FROM SINGLE *ESCHERICHIA COLI* CELLS

WARREN G. YEISLEY *and* ERNEST C. POLLARD

From the Department of Biophysics, The Pennsylvania State University, University Park, Pennsylvania 16802. Dr. Yeisley's present address is Department of Molecular Biophysics, Yale University, New Haven, Connecticut 06520

ABSTRACT A method has been worked out for studying the division of single bacterial cells (*Escherichia coli* B/r) in a uniform environment. Under optimal conditions the daughters of one single cell are found to divide at different times, a fact which indicates that they are not identical. The spread in generation times can be estimated quantitatively. When cells are irradiated with gamma rays in nutrient broth there is an increase in the spread in generation times, and the number of three-cell progeny (which require considerable difference between the daughters) rises. The results are consistent with the idea that damage to a segment of DNA has taken place and that there are three growing points on the DNA at any one time. In nutrient broth there is some evidence for repair of damage. For cells irradiated in minimal medium the pattern is different. The increase in generation times is not so marked, and only slight increase in three-cell progeny is seen. The results suggest that there is the same class of damage to the DNA, with only one growing point present and less repair. Using the criterion that no increase in generation time at all is permitted, we can estimate the total escape probability after radiation. The resulting calculation of critical target size is much closer to the whole bacterial chromosome than is found from normal plating experiments. The behavior of cells that have been exposed to irradiated medium is quite different, involving very long lags and cell death.

INTRODUCTION

A bacterial cell has a characteristic behavior that is beginning to be understood in terms of molecular biology. Several aspects of the cell operate in terms of small numbers of units; for example, the growing points on the chromosome at which the DNA is synthesized may be no more than three. It can be expected, therefore, that at a division, unequal numbers of many components will be shared among the daughters. The natural question arises, whether the control and regulation systems in the cell can act to keep the behavior of such cells essentially identical. Furthermore, exposure to hostile environments (e.g., irradiation) that affect cells in ways that are not yet understood may act to produce a diversity in progeny where normally no such diversity is found.

In the experiments to be described we studied the multiplication of single cells of *Escherichia coli* under conditions in which the environment could not have any selective effect on the growth of cells. On a surface, cells may have a varied relation to sources of oxygen or of metabolites; in liquid medium this can hardly be true. Accordingly, we followed the growth and division of single cells in liquid medium. The daughter cells were found to be nearly equal in division time. At the same time, the effects of irradiating cells and of subjecting them to irradiated medium were studied. Both these agents can produce diversity in progeny that is different under different conditions. Quantitative estimates of division delay can be made from our data, and it is clear that there can be a loss of daughter cells. This loss can be estimated.

Many past studies have determined the composition of a whole population, where the contributions of individuals are considered to be representative. This has yielded a general description of cellular components and their activities. Studies with cell populations in the exponential phase of growth have shown that the cell mass (mostly protein) and the DNA and RNA contents of cells occur in quantities characteristic of the growth rate supported by the medium in which the cells were grown (Maaløe and Kjeldgaard, 1966). For a population, at least, the average composition per cell growing normally in a particular medium is a constant. It has been shown that DNA synthesis is essentially continuous for cells growing in glucose minimal medium (Pachler et al., 1965; McFall and Stent, 1959). Yoshikawa et al. (1964) postulated that bacterial cells growing at the fastest rates, e.g. in rich medium, have three points of synthesis on the chromosome. More recent evidence indicates that this is true for rapidly growing *Escherichia coli* (Helmstetter and Cooper, 1968).

Studies of a sequence of events during the cell cycle or of cell generation time have usually been done with synchronized cells. Synchronized cells permit correlation of the contributions from a large number of individual cells. However, as pointed out by Engelberg (1964), the very feature of difference that we wish to study is made apparent in the loss of synchrony. Our experimental results underscore the inevitability of the loss of synchrony and, in fact, show up the limitations on the use of synchronized cultures.

Differences in individuals have been shown most directly by microscope observations of single cells. Interesting early photomicrographic studies by Adolph and Bayne-Jones (1932) with *Bacillus megaterium* and by Bayne-Jones and Adolph (1933) with *Bacterium coli* cells growing on agar showed that the conditions and stage of growth were important factors influencing the size of the individuals and their time of cell division. Other studies also showed the variability of individual bacterial cells (Kelly and Rahn, 1932; Powell, 1955; Kubitschek, 1962; and Schaechter et al., 1962). All the above studies of individual cells were done with bacteria growing on an agar surface, however, rather than in liquid medium.

In an important theoretical study Powell (1956) derived an expression for the age (time after division) distribution of bacterial cells in an exponentially growing

population. This study shows that despite individual variation, the relative number of cells in any age group in such a culture is constant and can be used in setting up a theoretical age distribution for a culture.

To examine the fate of cells subjected to a hostile environment, we chose to use ionizing radiation. The colony-forming ability of a cell culture is highly sensitive to ionizing radiation. Extremely small energy releases have drastic effects. Furthermore, irradiation of the medium can have an effect. Our principal study is on ionization within the cells; radiation action on the medium, we show, acts in a different way.

MATERIALS AND METHODS

Cells

The basic technique employed was to inoculate many tubes containing liquid medium with very dilute inocula of bacteria such that the average initial number of cells per tube was less than one. Growth was then permitted for varying times, and the liquid medium phase was stopped by adding molten agar that set and prevented the migration of any cells. The cells were then grown out into colonies and observed.

The organism used for this study was *Escherichia coli* B/r, obtained from Dr. S. Person of our laboratories. Before a culture was used for an experiment, two criteria were applied: 1) Cells were always grown for at least four generations in exponential phase to eliminate lag at the beginning of an experiment. The conditions for Campbell's definition of balanced growth were presumably met (Campbell, 1957). 2) The cell titer used was always less than 4×10^7 cells/ml, to insure that the cells were at least two generations away from the onset of stationary phase.

Media

Nutrient broth solution (8 grams Difco per liter) was used in one series of experiments. Since nutrient broth is not a chemically defined medium, certain precautions were observed to maintain uniformity of growth conditions. The same jar of Difco nutrient broth was used for all experiments in liquid culture. The same prepared solution was used for culture flasks, dilution blanks, and growth medium for the isolated cells that were used. Nutrient agar for ending the growth phase in liquid culture was prepared by adding Difco agar (8g liter) to nutrient broth.

For another series of studies, Roberts's C-minimal medium was used (NH_4Cl, 2 g; Na_2HPO_4, 6 g; KH_2PO_4, 3 g; $MgCl_2 \cdot 6H_2O$, 62 mg; Na_2SO_4, 80 mg; NaCl, 3 g; glucose, 5 g per liter). after sterilization, the culture flasks, dilution blanks, and growth medium for isolated cells were placed in the water bath for at least 1 hr before use, to equilibrate to the water bath temperature of 30°C. All experiments were carried out at 30°C (± 0.1°C) in a shaking water bath. The agar was held at 46–48°C before being used for filling test tubes.

Isolation of Cells in Nutrient Broth or Glucose Minimal Medium

Cells were inoculated into 100 ml broth or minimal medium in a culture flask at a titer of about 10^5 cells/ml. The experiment was begun when cells grown in either culture medium had reached a titer of about 10^7 cells/ml as determined with a Coulter counter (model *A* or *B*,

Coulter Electronics, Inc., Hialeah, Fla.). The cells were diluted by using growth medium in the dilution tubes so as to give a cell concentration of 0.5 cell/ml in a culture flask containing 200 ml of the growth medium. By means of a pipetting syringe, 1 ml samples of cells and medium were delivered to 90 bacteriological test tubes (13 × 100 mm) for each experiment.

To end the liquid culture growth interval, 7 ml of 46–48°C agar solution of the growth medium type was added to each of the test tubes by means of a pipetting syringe. The test tubes were incubated until visible colonies were formed, which were then counted. In the case of colonies growing in glucose minimal agar, in which gas bubbles formed by large colonies tended to break up the agar, colonies were counted before this occurrence.

Cleaning the test tubes proved to be important. Those used for glucose minimal medium experiments were cleaned, after scrubbing, by heating to about 90°C in 50% sulfuric acid. They were subsequently thoroughly rinsed with distilled water.

Irradiation of the Cells

Cells grown in either medium to about 10^7 cells/ml were diluted to give a cell concentration of about 10^5 cells/ml. For the cells grown in glucose minimal medium, catalase was added to the 10 ml of culture in the glass irradiation vial to give a final catalase concentration of 2 μg/ml. The cells were irradiated in a Gammacell 100 cobalt 60 gamma source (Atomic Energy of Canada, Ltd., Pinawa, Manitoba, Canada). Doses of 1350 and 9350 roentgens were given to the cells in nutrient broth in two sets of experiments. The cells in glucose minimal medium were given a dose of 5670 R. The final dilution was made with allowance for killing to give a surviving cell concentration of 0.5 cell/ml in the culture flask before distribution.

Irradiation of Glucose Minimal Medium

200 ml of glucose minimal medium were placed in each of three irradiation vials (for three experiments). The medium in each vial was bubbled with oxygen at a rate of 55 cc/min for 1 min. The vials with medium were then irradiated with 2230 R in one series or 5900 R in another series of experiments. 0.5 ml aliquots of irradiated medium were delivered to each of 270 test tubes that had previously been replaced in the water bath.

Cells that had been grown to about 10^7 cells/ml were placed in the culture flask at 1 cell/ml, and 0.5 ml aliquots of cells and medium were delivered to each of the 90 tubes involved in each experiment.

RESULTS AND DISCUSSION

Normal Growth of Isolated Cells in Nutrient Broth or Glucose Minimal Medium

The results of an experiment with cells growing in nutrient broth are shown in Fig. 1. The time interval (Δt) for this experiment was 1 hr from the time the medium and the cells were placed in the test tubes until agar was added. During this interval the cells were permitted to divide, separate, and disperse throughout the growth medium. Upon the addition of agar, any cell is restricted to the space in which its colony was formed. For the interval $\Delta t = 1$ hr the number of tubes that contained a particular

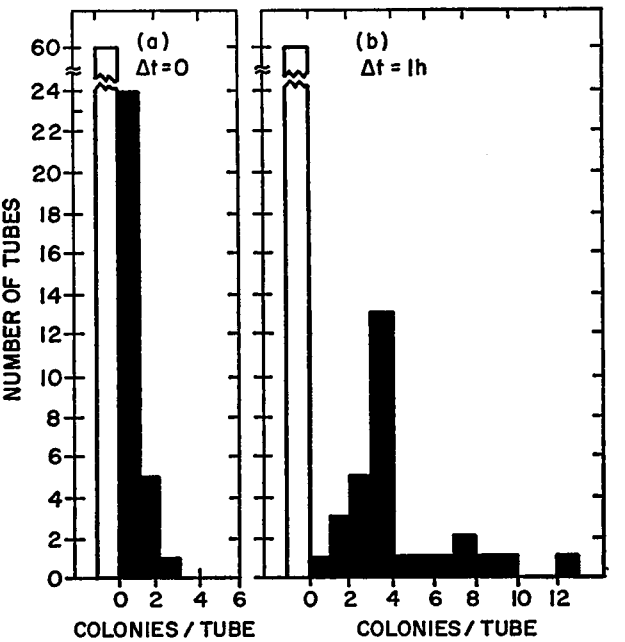


FIGURE 1 Histograms showing data obtained from an experiment. (*a*) Initial distribution calculated on the basis of the experimentally observed number of tubes having zero colonies and an assumed Poisson distribution. (*b*) Experimental data for cells in nutrient broth. In this instance, for 1 hr growth the most frequent number of daughters is four. Quite clearly, other numbers have also developed.

number of colonies was recorded against the number of colonies per tube. In this sample, the number of colonies that occurred most frequently was four.

The number of tubes that contained no colonies is of considerable importance because it allows computation of the distribution of one, two, three, and so on colony-forming centers that had been placed randomly in the tubes at the beginning of the experiment, assuming that Poisson statistics describe the situation. The computed values are shown in Fig. 1 *a*.

The ratio of the total number of colonies, N, to the calculated initial number of colony-forming centers, N_0, plotted against the growth time of the experiment yields a growth curve. Exponential growth was found in nutrient broth and glucose minimal medium (Fig. 2). The average doubling times were 36 min in nutrient broth and 88 min in minimal medium. Little, if any, lag in growth was introduced into the experiments by diluting from a cell concentration of 10^7 cells/ml.

The lines in Fig. 2 can be used to estimate the correctness of the use of Poisson statistics. The exponential growth beginning at unity on the ordinate, which should be expected even for small numbers of cells (Kubitschek, 1962), lends support to our method of treatment of the data.

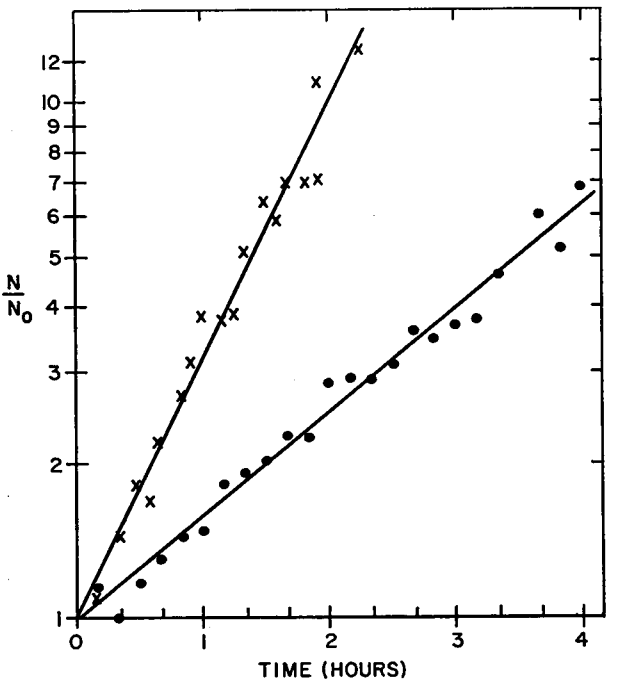


FIGURE 2 Normal growth of cells in nutrient broth (crosses) and glucose minimal medium (solid circles). Ratio of total colonies counted in all the separate tubes, N, to calculated initial colony-forming centers, N_0. Exponential growth is observed, as expected.

Analysis of Numbers of Progeny as a Function of Time

If the two daughters in every division are truly identical, then a very simple pattern of numbers of progeny developed from a single cell should be observed. As the number of single cells goes down, the number of two-cells should rise until this number reaches the original number of singles. The same would then be true for four-cells and eight-cells and so on. This simple behavior was not observed; so, in order to present a means for seeing the deviation graphically and numerically, we have devised a method for presenting the ratio of a given number of cells, n, to the original number of single cells. This ratio we designate as $R_n(t)$.

Our experiments very nearly permit a simple measurement of this ratio. To a first approximation it is possible to divide the observed number of tubes carrying the first number, n, of colonies by the calculated number of tubes with one cell. However, it is clearly only an approximation, for cells do develop from the initial two-cell and three-cell inocula that Fig. 1 *a* shows are actually present. We have corrected for this by estimating the probabilities for starting from two cells and deducting these. The estimate of probabilities was made from the first approximation. When we make these allowances the corrections prove to be small and, in fact, hardly relevant. In the Appendix we give the details of the method of calculation.

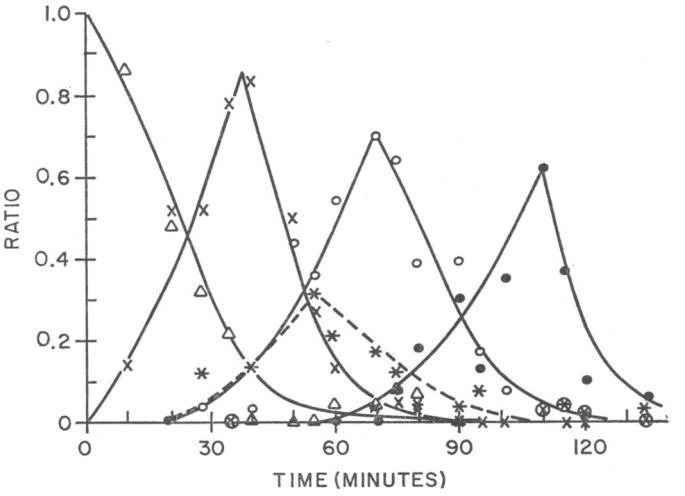


FIGURE 3 Normal growth pattern for cells in nutrient broth growing exponentially. The ratio $R_n(t)$ is calculated as the number of tubes containing n colonies to the calculated number containing one colony. Ratio curves $R_1(t)$ (triangles), $R_2(t)$ (crosses), $R_3(t)$ (asterisks), $R_4(t)$ (open circles), $R_8(t)$ (closed circles) are shown for 1, 2, 3, 4, and 8 cells. The peaks do not reach unity and there is an abnormal number of 3-cell tubes.

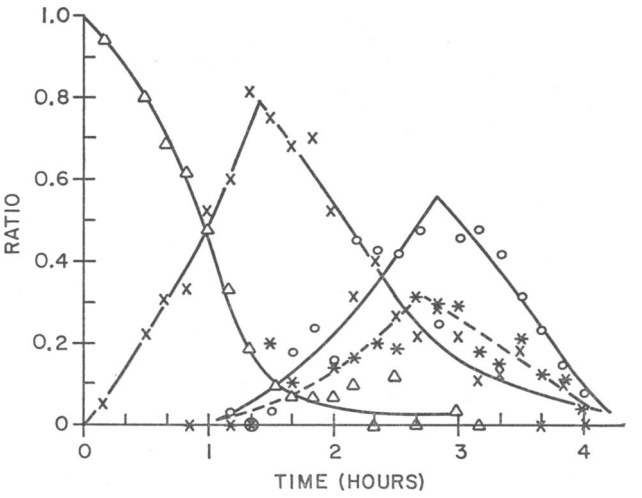


FIGURE 4 Normal growth pattern for cells in glucose minimal medium growing exponentially. Ratio curves $R_1(t)$, triangles; $R_2(t)$, crosses; $R_3(t)$, asterisks; $R_4(t)$, open circles. The drop away from a unity peak is more marked and the number of three-cell tubes is more apparent than in nutrient broth.

In Fig. 3, $R_n(t)$ was plotted versus time for cells grown in nutrient broth. Fig. 4 shows the same experimental function for cells in glucose minimal medium. Both these figures show that the fraction of original single cells that remained as one cell decreased, from unity at the origin, with time. After one culture doubling time, only

a small fraction were left. The fraction of original single cells that divided once to form two-progeny cells increased from none at zero time until a maximum was reached, after which they decreased toward zero.

The maxima in the plots for two- and four-progeny cells occurred at approximately one and two doubling times, respectively, for both media. Each curve approaches zero at a time equal to a culture doubling time later. In the case of nutrient broth, the progeny which reached eight cells are shown in Fig. 3; the maximum was reached three doubling times after zero time.

It can be observed from the figures that the fraction of the original cells that became three-progeny was a significant portion of the population. The maximum for this number of cells occurred between the maxima for two cells and four cells.

It can be seen from examining Figs. 3 and 4 that at the time the maximum occurred on the curve for two cells, a small fraction of single cells remained to divide, while some of the two-progeny cells had begun to divide to give three and four cells. The overlap was due to variation in cell generation times among the cells. The leading edge of the curve gained from dividing cells, and the trailing edge lost through cell division.

Comparison of Fig. 3 with Fig. 4 shows that cells growing in nutrient broth had higher maxima for the two- and four-cell progeny curves than cells growing in minimal medium. Correspondingly, the leading edges of curves for four cells and three cells in minimal medium occurred sooner with respect to the two-cell peak than they occurred in nutrient broth. The peak for three cells shifted toward the four-cell peak in minimal medium.

Theoretical Consideration of an Ideal Population

The ideal population of interest is one in which all cells have an equal cell division time although growing exponentially at random. The number of cells in a culture with ages from the last division between a and $a + da$ can be shown to be $\phi(a)da$, where $\phi[a] = 2\nu_m e^{-\tau m^a}\, da$ for ages less than the division time τ (Powell, 1956). This is plotted in the inset of Fig. 5. If this distribution is applied to calculate the ratio $R_2(t)$ theoretically, it is readily shown to be $e^{\tau m^t} - 1$. This yields a nearly straight line rising to the value 1 at $t = \tau$. The corresponding ratio $R_4(t)$ for an ideal culture will be exactly the same line rising from the ratio zero at $t = \tau$ to unity at $t = 2\tau$, and the difference between this and unity measures the falling values of $R_2(t)$ at times beyond the division time. Thus the theoretical lines of Fig. 5 represent the behavior of $R_n(t)$ for an ideal population of identically dividing cells.

The result of unequal cell division times is to lower the peak as in Figs. 3 and 4. The consequence of inequality appears when one of the two daughter cells from a single cell divides before the other. For a time, three cells will be found for isolated cells. If such three-cell tubes are found, they clearly indicate that individuals have a variation in generation time.

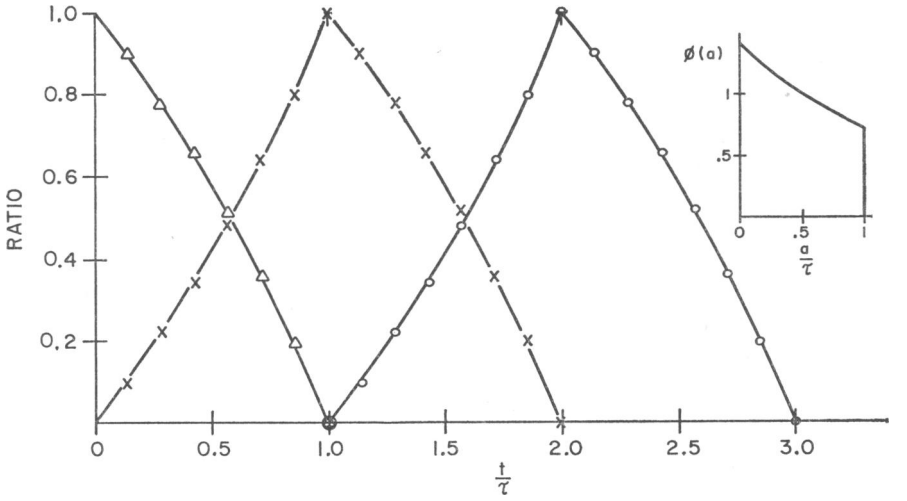


FIGURE 5 Theoretical pattern of growth for an ideal population of cells. Inset shows age distribution for ideal population (from Powell, 1956). Ratio curves $R_1(t)$, triangles; $R_2(t)$, crosses; $R_4(t)$, open circles.

Observation of Figs. 3 and 4 shows that the two features of diminution of peak height and presence of three-cell tubes are found. Therefore in both cases there is a variation in generation time among daughters even under these optimal conditions. The variation is somewhat greater in the glucose minimal medium case of Fig. 4; nutrient broth apparently shows more nearly ideal behavior.

An alternative hypothesis to explain the finding of three cells in a test tube experiment is provided by the possibility that some cells never divide. This is a special case of unequal generation times in which one cell has an infinite generation time whereas the sister cell, if the population is otherwise ideal, has a generation time equal to that of its cousins. This hypothesis assumes a constant probability of cell death per generation. The effect of this hypothesis may be seen by drawing progeny "family trees" and striking out every *n*th cell for each generation. The approximate results for a probability of one-cell-in-ten death per generation are illustrated in Fig. 6, using the same distribution of ages as in Fig. 5.

The significant findings of this hypothesis are the delays produced, which are always an integral number of generation times. This gives the discontinuities in the ratios that occur at integral numbers of generation times from the origin. An interesting feature of this pattern is that between the second and third generation time intervals from the origin, for this example, a test tube experiment would have a large number of tubes containing two, three, and four colonies per tube. However, a comparison of Fig. 6 with Figs. 3 and 4 shows that this hypothesis alone does not explain the experimental results for normal growth. Although Fig. 4 gives some indication that this pattern may have been occurring in glucose minimal medium, this hypothesis cannot explain the early appearance of three- and four-cell progeny.

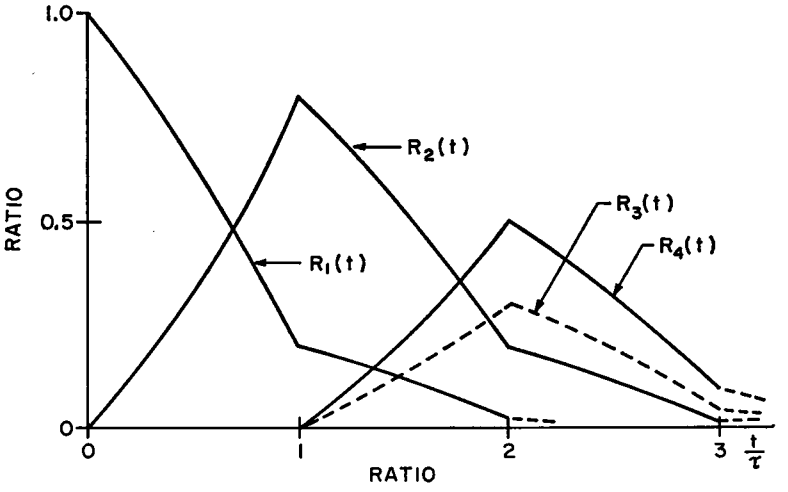


FIGURE 6 Ratio curves illustrating growth pattern with a constant probability of cell death per generation of 0.1.

Previous investigations (Powell, 1955; Kubitschek, 1962) have in fact directly demonstrated variations in the growth of individuals. The results obtained in Figs. 3 and 4 are in agreement with those observations.

Growth of Irradiated Cells in Nutrient Broth

The organism used (*E. coli* B/r) was determined to have a multihit survival curve with an extrapolation number of 2 when grown in log phase in nutrient broth. The response of single cells was observed for two doses: a low dose (1350 R; 95% survival) corresponding to the shoulder, and a higher dose (9350 R; 50% survival) corresponding to the exponential part of the survival curve.

The total growth curves obtained for the two doses are shown in Fig. 7. The curves describe growth of all cells in the tubes which ultimately formed visible colonies. By calculating the survival level, we arranged the dilution that gave the same average number of colony-forming cells per tube as the control. The survivors were assumed to be distributed according to Poisson statistics in the same manner as the unirradiated cells had been. In so arranging, we distributed among the tubes extra cell bodies which were unable to form colonies.

The irradiation treatment occurred an average of 15 min before distribution of cells to the tubes. This time was necessary for transporting the sample from the source, diluting, and distributing the cells to the tubes. The design of the experiment is such that for normally growing cultures this increased time will result in no change, since all that is done is to sample at a slightly increased cell number with no change in population distribution (Powell, 1956). However, the irradiated cultures, plotted without allowance for the manipulation time, still show a deviation that appears as

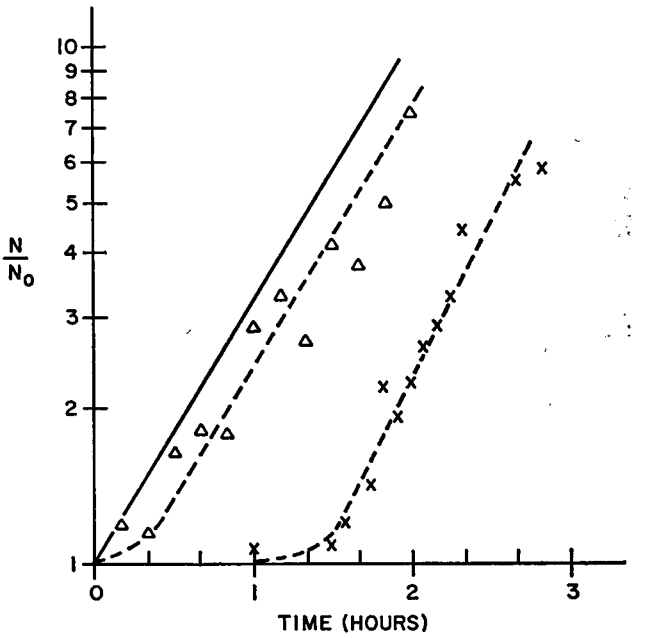


FIGURE 7 Growth of irradiated cells in nutrient broth. Solid lines, control; dashed lines, irradiated cells. Cells irradiated with 1350 R, triangles; with 9350 R, crosses.

a lag in time. This lag is certainly longer than plotted, but it cannot be simply increased by the manipulation time, since some cells will divide in that time while others will not, because of the effect of radiation. Thus, all we can say is that the observed lag is *less* than the total, but the precise lag is hard to estimate. The population lags can be obtained by extrapolating growth in Fig. 7 to the abscissa.

The progeny growth patterns for cells irradiated with 1350 R are compared separately with the respective curves for unirradiated cells in Fig. 8. It is emphasized that these were progeny of irradiated cells capable of forming colonies. Some features of the treatment are readily apparent. There was a slight delay of up to 25 min, since the start was delayed by 15 min as explained above. This can be seen in the first division as shown in Fig. 8 *a* and also by the leading edge on the two-progeny curve in Fig. 8 *b*. The trailing edge on the two-progeny curve is extended, showing a long delay before the second division took place. The shift in the irradiation-produced curve in Fig. 8 *c* (the three-cell curve) means that there was a considerable lag before either of the cells which reached the two-cell stage was ready to divide. Unlike the two- and four-cell cases, the three-cell case does not show a diminution in the peak. Figure 8 *d* shows that, as compared with the control, a smaller part of the population had divided to the four-cell stage.

The ratio of (surviving) irradiated cells reaching the four-cell stage at 68 min as compared with the control peak at that time is 0.25. The chance of total escape is

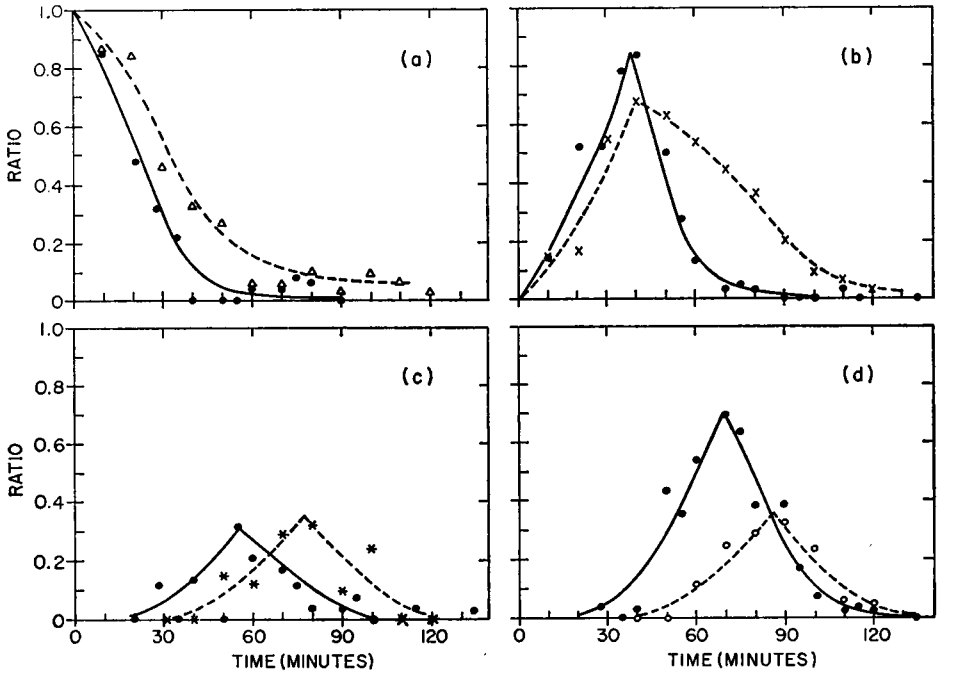


FIGURE 8 Comparison of irradiated cells with control in nutrient broth. Irradiation dose 1350 R. Solid lines and circles, controls; dashed lines, irradiated cells. (*a*) Ratio $R_1(t)$ for remaining as one cell (triangles). (*b*) Ratio $R_2(t)$ for two cells (crosses). (*c*) Ratio $R_3(t)$ for three cells (asterisks). (*d*) Ratio $R_4(t)$ for four cells (open circles). The fall of the peak from unity is marked; there is a lag in division, and the three-cell tubes are still prominent.

not greater than 25%; it is probably very much less, since the cells which could have totally escaped belonged to a particular age group in the population in that the first 30% of the control population older cells had reached the four-cell stage of growth in 50 min, whereas essentially none of the cells in the irradiated population had. This total escape group is of interest if target theory is applied. If it is claimed that for 1350 R no more than 10% have totally escaped, then we can apply the probability relation

$$\ln \frac{N}{N_0} = - M \cdot I$$

where N/N_0 is the total escape ratio, M the mass of the sensitive target, and I the number of primary ionizations per gram. We find $-M \cdot I = -2.3$, and since for 60 ev per primary ionization each rad produces 10^{12} primary ionizations per gram, $I = 1.35 \times 10^{15}$, so $M = 2.3/1.35 \times 10^{15} = 1.7 \times 10^{-15}$ gram. Using Avogadro's number, this gives a sensitive target of $6.03 \times 10^{23} \times 1.7 \times 10^{-15} = 1.03 \times 10^{9}$ daltons. One of the paradoxes of radiation studies on microorganisms is the lack of correlation between any reasonable known sensitive element in the cell and the sensitive

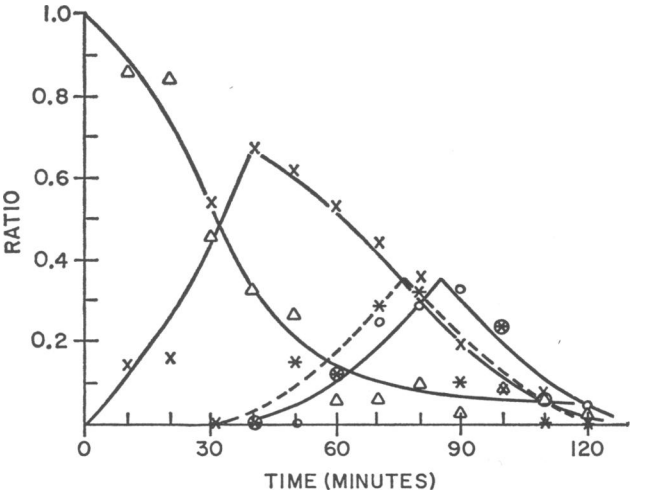


FIGURE 9 Growth pattern for irradiated cells in nutrient broth. Irradiation dose 1350 R. Ratio curves $R_1(t)$, triangles; $R_2(t)$, crosses; $R_3(t)$, asterisks; $R_4(t)$, open circles.

target. These studies suggest that if the criterion that the cell actually has totally escaped is applied, then something like the whole DNA is actually the target. There is still a discrepancy because in a broth-grown cell the whole DNA content is in the vicinity of 5×10^9 daltons.

The one-, two-, three-, and four-cell cases are plotted together for comparison in Fig. 9. The considerable overlap of the three- and four-cell curves can be clearly seen. The trailing edges of both curves appear to be paralleled by the final part of the trailing edge for the two-cell case. This pattern of progeny development seems quite significant, especially when compared with Fig. 6. It suggests that daughter cells were lost as nonviable cells after the second division.

The patterns of progeny growth after the high-dose irradiation treatment as compared with those of unirradiated cells are shown in Fig. 10. It is clear from Fig. 10 *a* that essentially all original cells have had a division delay, as evidenced by the long lag. About 95 min after irradiation (80 min in the figure) the cells began division in a manner similar to that of unirradiated cells. A residual plateau was reached of 20% of undivided cells, which must eventually divide, since after addition of agar colonies are formed.

Figure 10 *b* shows that the two-cell progeny curve has a lower peak than the control and in addition a long trailing edge. Although the three-cell curve (Fig. 10 *c*) is not unlike the control, the four-cell curve (Fig. 10 *d*) is certainly different. The irradiated cells after the irradiation-produced lag had a different progeny pattern from that found for unirradiated cells. Such results would not have been anticipated from the data shown in Fig. 7. Growth remained abnormal after the lag period.

The statistics for total escape show the same character as discussed previously. If

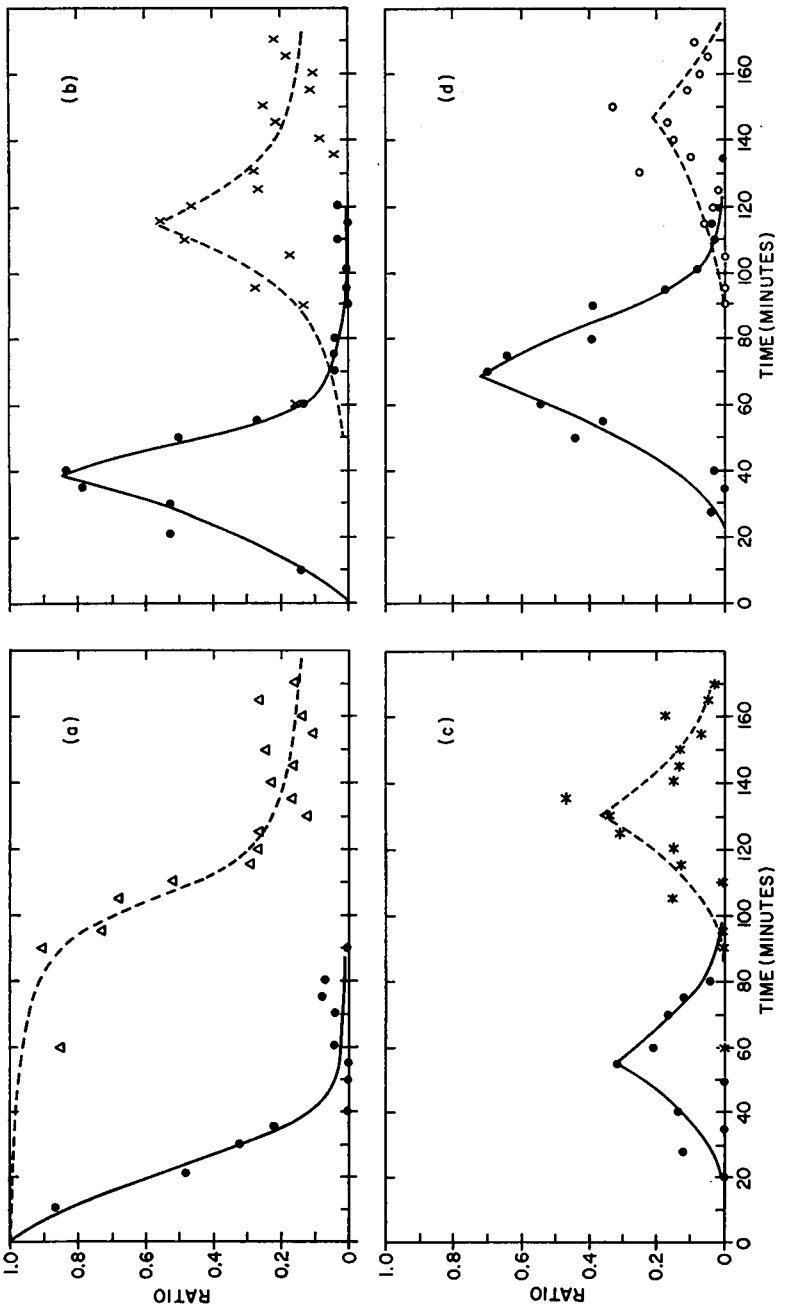


FIGURE 10 Comparison of irradiated cells with control in nutrient broth. Irradiation dose 9350 *R*. Solid lines and circles, controls; dashed lines, irradiated cells. (*a*) Ratio $R_1(t)$ for remaining as one cell (triangles). (*b*) Ratio $R_2(t)$ for two cells (crosses). (*c*) Ratio $R_3(t)$ for three cells (asterisks). (*d*) Ratio $R_4(t)$ for four cells (open circles).

Fig. 10 *d* is consulted, it can be seen that no cells can be said to behave like the control group. Thus for 9350 R the "total escape probability" must be less than 1 %. This also suggests that there is a large sensitive target and that the normal plating experiments reflect a considerable ability in the cells to repair damage.

Growth of Irradiated Cells in Glucose Minimal Medium

Considerably different results were obtained when cells grown in glucose minimal medium were exposed to ionizing radiation. Fig. 11 shows the population growth of irradiated cells given a dose of 5670 R compared with the exponential growth of the unirradiated cells.

The one- through four-cell progeny curves obtained after irradiation are compared with the growth of unirradiated cells in Fig. 12. The main feature was a delay in division of single cells, as shown in Fig. 12 *a*. The corresponding delay for increase to two cells appears in Fig. 12 *b*. The irradiated two-cell peak is lower and lags behind the control curve peak. Division of irradiated cell progeny proceeded more slowly than for unirradiated cells, as shown by the slow rate of descent of the former curve's trailing edge. The trends seen for the two-cell curve reappear in the four-cell curve. The three-cell curve for irradiated cells, however, is nearly the same as the control curve (Fig. 12 *c*). The general effect of irradiating cells grown in minimal medium was the large initial delay in division for nearly all cells. The pattern of progeny development for those irradiated cells had some features similar to the

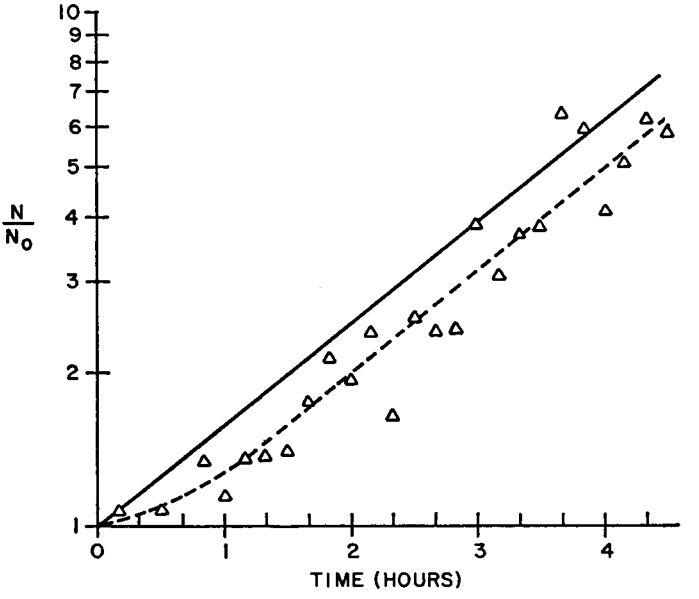


FIGURE 11 Growth of irradiated cells in glucose minimal medium. Irradiation dose 5670 R. Control, solid line; irradiated cells, dashed line with triangles.

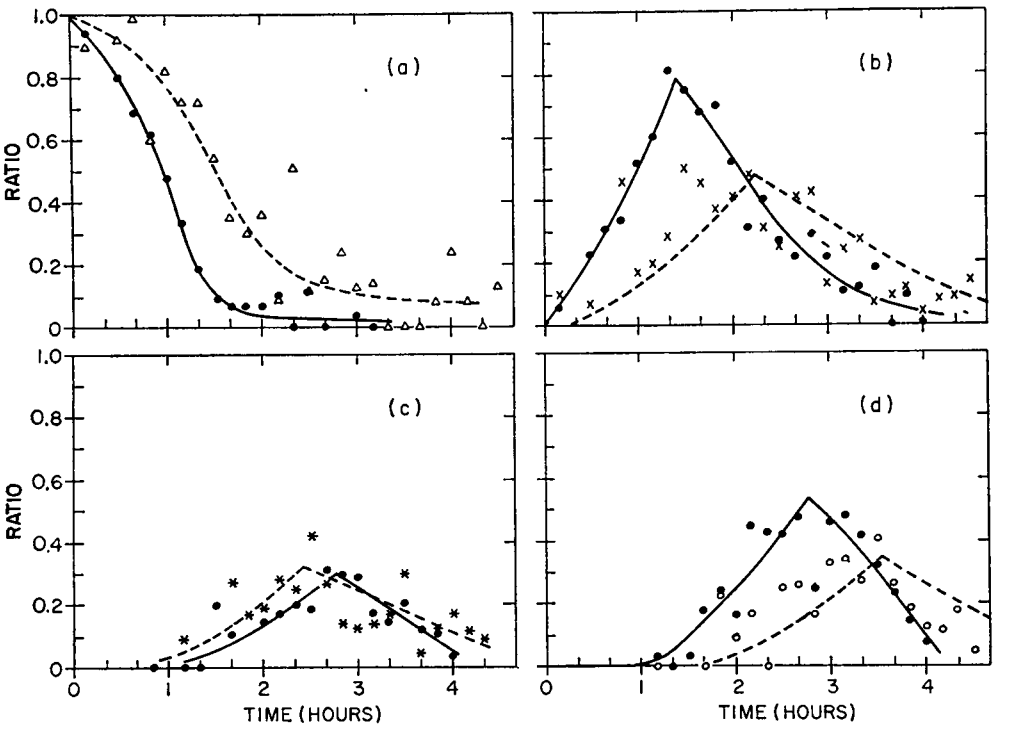


FIGURE 12 Comparison of irradiated cells with control in glucose minimal medium. Irradiation dose 5670 R. Solid lines and circles, controls; dashed lines, irradiated cells. (*a*) Ratio $R_1(t)$ for remaining as one cell (triangles). (*b*) Ratio $R_2(t)$ for two cells (crosses). (*c*) Ratio $R_3(t)$ for three cells (asterisks). (*d*) Ratio $R_4(t)$ for four cells (open circles).

pattern for the higher dose that produces exponential killing of nutrient broth-grown cells.

The total escape can again be estimated for the four-cell case. Taking the leading edge of Fig. 12 *d* as a basis and allowing for survivors which have hits, then 0.04 of the cells could be said to have escaped completely and to have acted precisely as the unirradiated cells did. This is a much higher proportion than for nutrient broth–cultured cells and suggests a very much more all-or-nothing type of damage. Since only 33% of the original culture would plate, the 25% figure must be multiplied by this, yielding 8% for true total escape. Using the same statistical reasoning as formerly, we find for the sensitive target size for 25% total escape at 5670 R a figure of 2.74×10^8, which is less than that for nutrient broth–grown cells. This suggests that cells in minimal medium are on the one hand inherently less sensitive and on the other hand less able to repair their damage.

Hypothesis for Radiation Action

Ionizing radiation is known to produce drastic action on the chromosome; for example, considerable degradation (Pollard and Achey, 1966). If it is supposed that the

radiation damage is to the chromosomes and that an intact chromosome is ultimately necessary for survival, then an explanation of the results can be given. We need to assume the relation of cell age to the number of chromosomes per cell and the number of branches per chromosome as illustrated by Cooper and Helmstetter (1968). Table I shows chromosome figures with possible damaged regions. For a chromosome with only one growing point as expected for glucose minimal medium, three possibilities are given: total escape at (*a*), damage to a postreplication branch at (*b*), and damage to a prereplication branch at (*c*). The consequence of (*a*) is a fully normal cell in the usual way, of (*b*) is two daughter cells in double the usual time, and of (*c*) is no cells at all.

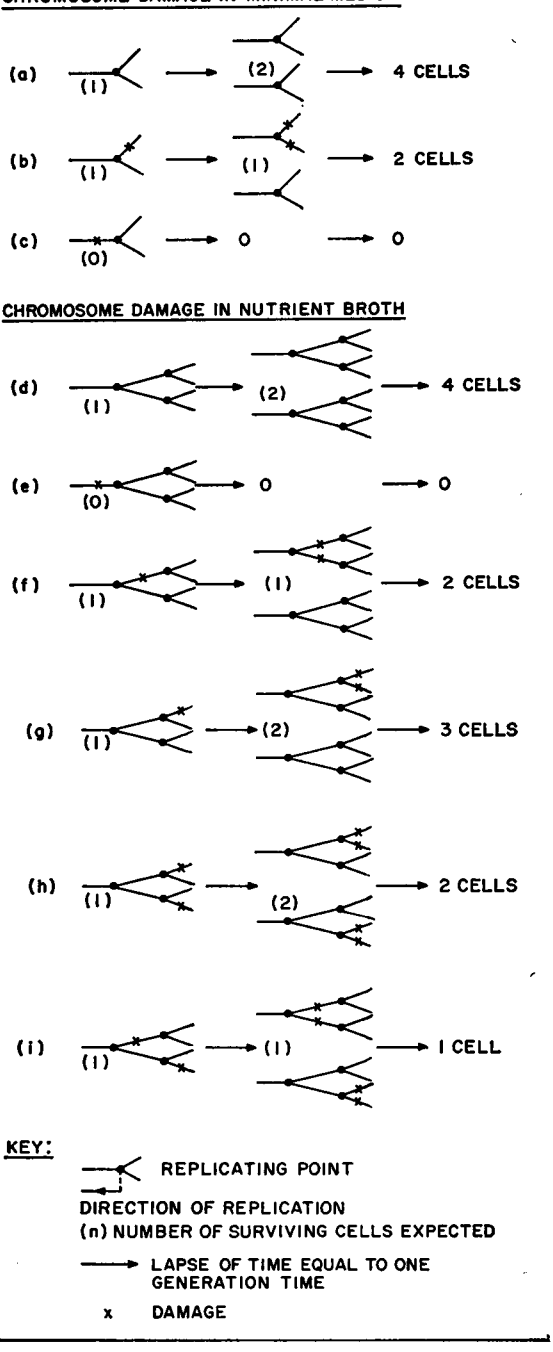
During the procedure time of about one-sixth generation between irradiation and isolation of cells into tubes, the oldest cells would divide into young cells having a single chromosome with damage as illustrated in (*a*), (*b*), or (*c*) of Table I. Young cells of type (*a*) can be expected to divide as normal cells between 75 and 90 min after isolation. Of the remaining cells which have not divided during the procedure time, the oldest cells with two chromosomes having any combination of (*a*) and (*b*) would divide to two cells soon after isolation. These cells account for 30% of the population dividing to two cells during the first generation time after isolation. The remaining cells would first divide to two cells after one generation time. An increase in production of three cells would be expected among those which divided during the first generation time, because a significant number of cells should have one chromosome with no damage (*a*) and one with damage (*b*).

In nutrient broth the number of replication forks that are simultaneously in action is greater than one. Three have been illustrated for a single chromosome. It is quite clear that under these circumstances quite a different pattern of progeny is to be expected. Again we assume that the older cells have two chromosomes, each with three replication points. The procedure time for the low-dose experiments averaged about one-third of the doubling time, permitting most of the older cells to divide once after irradiation and before isolation. This means that cells having two chromosomes at the time of irradiation divided to cells having one chromosome at the time of isolation. The effect of this circumstance is that for any cell age the chromosome damage can be pictured as in the lower part of Table I. At (*d*) the total-escape case is shown: four normal cells should be seen. We have already commented that this was not readily observed experimentally, a fact which suggests that total escape for any chromosome is unlikely. Again, if the prereplication branch receives damage as at (*e*) no cells will ever result. Now, if the other branches receive damage, there can be found two cells for case (*f*) at double the normal time and three cells for case (*g*) at the normal time for four cells. Additional damage as at (*h*) and (*i*) is possible, causing longer delays in progeny formation. By referring to Fig. 9 we show that this was the case and that the timing was also in accord with our hypothesis.

The higher-dose case fits this hypothesis also with the exception of the 20% plateau on the trailing edge. We have to suppose that some secondary recovery process acts

TABLE I

HYPOTHESIS FOR RADIATION DAMAGE TO CHROMOSOME



to reclaim these otherwise hopelessly damaged cells. We have no clear suggestion as to what it might be. Cells of *E. coli* B/r are thought to have a defective prophage, and Grady and Pollard (1968) have found that in such cells there is less DNA degradation and more cell survival. Possibly the plateau is in some way related to this finding.

Since these experiments, Clark (1968) has studied X-ray effects on synchronized cultures of *E. coli* B/r using Helmstetter's technique (Helmstetter and Cummings, 1965). He finds that as the age of culture after synchrony increases, the sensitivity measured by the slope becomes less. This fits our hypothesis very well, for the total loss of colony-forming ability must be due to a hit in the prereplication fork as indicated in Table I (*c*) or (*e*). As the culture progresses, the prereplication fork will become shorter and the cell less sensitive.

Growth in Irradiated Minimal Medium

The *E. coli* B/r cells were inoculated into previously irradiated glucose minimal medium. The total growth of isolated cells is shown in Fig. 13 for medium given a dose of 2230 R. Initially the cells grew as though their medium had not been altered. However, they reached a stage of growth where they no longer divided in the normal way. Comparison shows that the cell growth rate was arrested sooner when the medium had been given a higher dose (Fig. 13). The higher dose to the medium

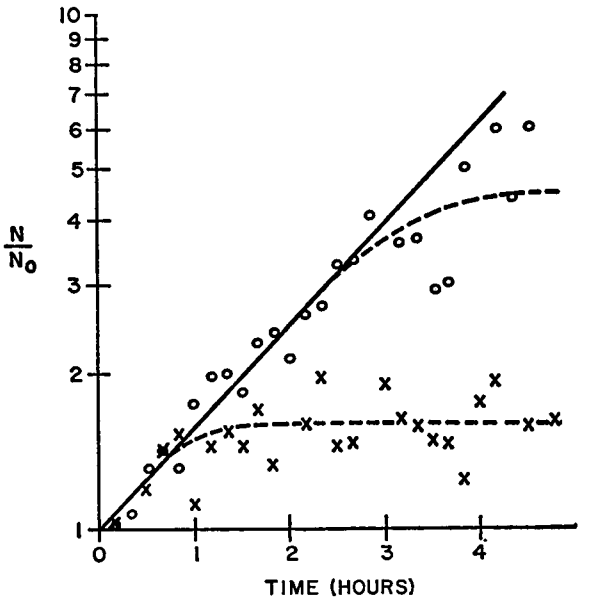


FIGURE 13 Growth of cells in irradiated glucose minimal medium. Solid line, control; dashed lines, cells in irradiated medium. Irradiation dose 2230 R, open circles; irradiation dose 5900 R, crosses.

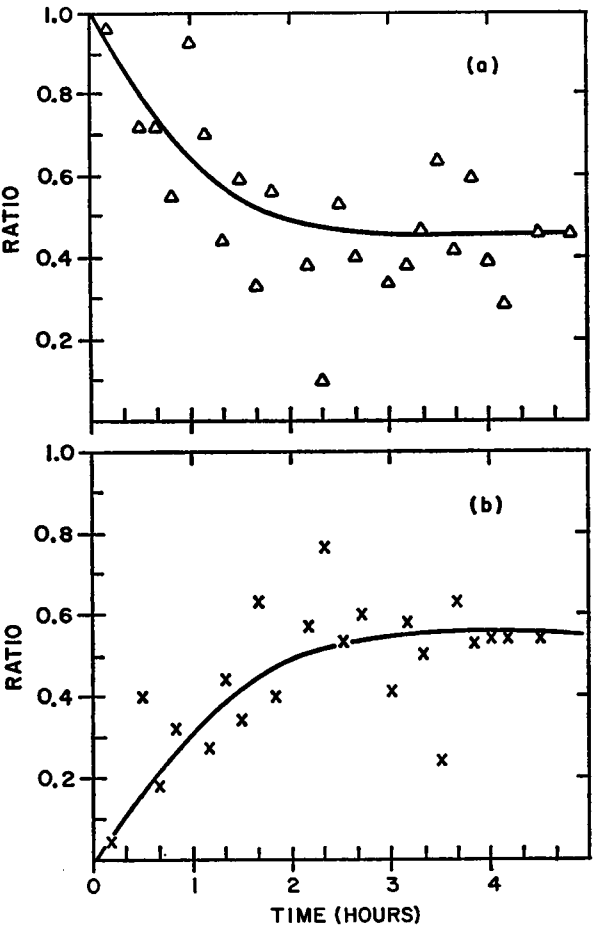


FIGURE 14 Growth of cells in glucose minimal medium irradiated with a dose of 5900 R. (*a*) Ratio $R_1(t)$ for remaining as one cell (triangles). (*b*) Ratio $R_2(t)$ for two cells (crosses).

caused a distinct plateau after 1 hr of cell growth. Growth in the other case appeared normal for nearly 3 hr, although subsequent growth was not.

The detailed patterns for growth of individual cells in medium irradiated with a dose of 5900 R are presented in Fig. 14. The scatter is apparently due to the nonuniform way in which the cells react to irradiated medium. There appears to be a statistical scattering about the mean mode of action as indicated by the line. Nevertheless, a basic trend is clearly apparent.

In Fig. 14 *a* the fraction remaining as one cell is shown. The curve shows that some single cells in the population divided leaving the remainder to form the plateau. Fig. 14 *b* shows that those cells which had divided once did not divide again during the course of the experimental intervals of time. It was the subsequent eightfold dilution with glucose minimal agar which permitted these cells to begin dividing again to

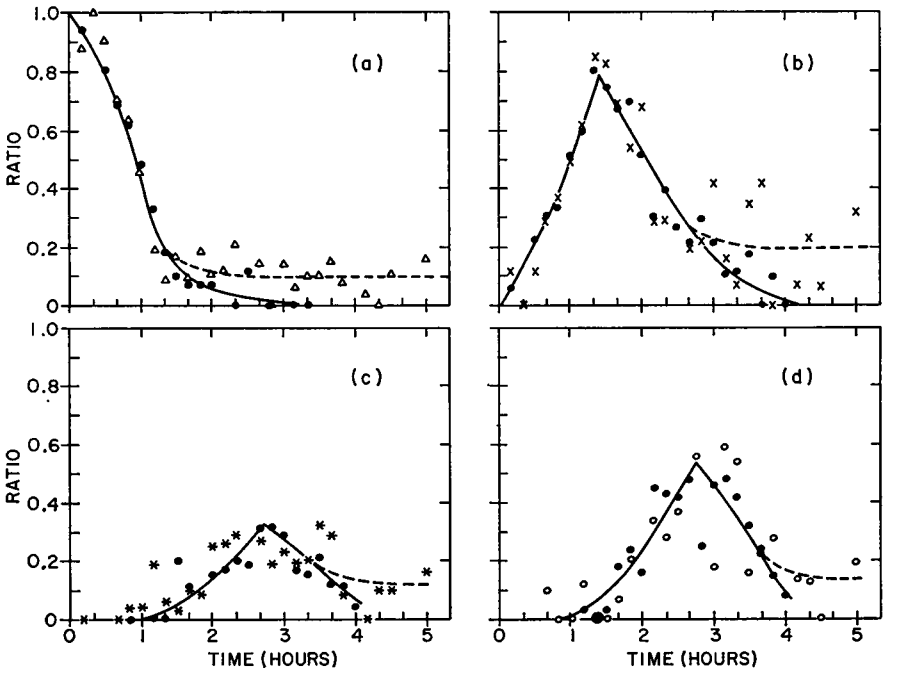


FIGURE 15 Growth of cells in glucose minimal medium irradiated with a dose of 2230 R, compared with the control. Solid lines and circles, controls; dashed lines, cells in irradiated medium. (*a*) Ratio $R_1(t)$ for remaining as one cell (triangles). (*b*) Ratio $R_2(t)$ for two cells (crosses). (*c*) Ratio $R_3(t)$ for three cells (asterisks). (*d*) Ratio $R_4(t)$ for four cells (open circles).

form colonies. Thus it seems that with this dose the cells, whether or not they divide once, soon reach a static condition.

With a lower irradiation dose, the medium permits a course of development that proceeded in a nearly normal manner, as shown by the comparison with the control populations in Fig. 15. However, a residual portion of the population remained undivided from each of the first four curves of progeny number. For example, in Fig. 15 *c* for three progeny, the residual portion is interpreted to mean that after the first division one of the two daughter cells divides once more while the other daughter cell remains undivided. Hence, these static stages of growth must cause the total growth in Fig. 13 to depart from normal exponential growth.

The initial growth of these cells is in contrast to the immediate cessation of growth reported by Frey and Pollard (1966). The growth situation for the cells, however, was different in two important respects. The medium used by Frey and Pollard was given a much higher dose of irradiation; also they had a much higher cell concentration (5×10^7 cells/ml). Since their cells were found to remove the main irradiation product, hydrogen peroxide, from the medium, those cells could have subsequent growth. In fact they did after a long delay.

By contrast, however, the present experiment had a cell concentration in the test tube of 1–4 cells/ml. During the duration of the experimental interval, this cell concentration could not remove a quantity of hydrogen peroxide sufficient to permit growth. Instead, presumably, the cells continually accumulated enough irradiation products to stop their growth, because the initial amount of irradiation product obtained here must have been low as compared with that reported by Frey and Pollard (1966).

The results obtained here and those reported by Frey and Pollard suggest that the irradiation product must exceed a certain threshold to become effective. In their case, growth ensued after the irradiation product was reduced below the threshold, whereas in our case the irradiation product was accumulated, whereupon growth ceased after the threshold was reached.

It is possible that the threshold level varies from cell to cell. For instance, the cells may accumulate hydrogen peroxide at different rates. It is also possible that the synthetic processes reported by Pollard et al. (1965) to be affected by irradiated medium may vary in sensitivity. The residual portions of the population which remain undivided might be due to sensitivity of synthetic processes during a part of the cell division cycle. If a cell manages to escape the block, it proceeds with otherwise normal growth until it again reaches the sensitive state.

The above considerations could account for the scatter observed, because they could thrust a nonuniform effect upon the cells.

CONCLUSIONS

Exponential growth was found for isolated cells growing in liquid culture. Dilution of the cells from 10^7 cells/ml to 1 cell/ml produced no lag in growth. Cells grown in nutrient broth more nearly correspond to an ideal population than those grown in glucose minimal medium. In either medium, most of the cells are capable of dividing. However, the results of this study suggest that a real population of cells could not maintain synchrony. The reasons for variation of the individual cells need further investigation.

The irradiation of cells with small doses of gamma rays produced a significant effect on the pattern of progeny development from single cells. Since only surviving cells are observed, the pattern of growth represents the recovery of the cells from the effects of radiation damage. The results clearly show a difference in recovery from irradiation between cells growing in nutrient broth and those growing in glucose minimal medium. The results suggest that, during recovery, the cells discard nonviable progeny cells, and indicate that the effect produced by irradiation is damage to elements in the cell which correspond to chromosomal segments. The pattern of growing points and replication suggested by Cooper and Helmstetter (1968) can be correlated reasonably well with the development of different numbers of progeny after irradiation.

The results obtained for irradiated glucose minimal medium were quite different from those for irradiated cells. Cells grown in medium irradiated with a dose of 5900 R reach a static state after a short time. The cells recovered after a dilution of the irradiated medium. In medium irradiated with a dose of 2230 R, the cells had a nearly normal pattern until their cell division was blocked. A residual number of progeny were blocked at each stage. Cells were affected in a nonuniform way, which indicated a threshold effect.

APPENDIX

The details of the method of analysis are illustrated by example using the experimental data from Fig. 1 *b* and calculations from the Poisson distribution represented by Fig 1 *a*. From the experimental data of Fig. 1 *b*, the test tube which was found at $\Delta t = 1$ hr to contain one colony must have started from one cell (as opposed to two or more). After an interval of 1 hr, the proportion of original single cells which remain as one cell to form one colony per tube is calculated as a ratio, $R_1(1\text{ hr})$.

$$R_1(1\text{ hr}) = \frac{(\text{no. tubes with 1 colony at } \Delta t = 1\text{ hr})}{(\text{calc. no. tubes with 1 cell at } \Delta t = 0)} = \frac{1}{24} = 0.04$$

where the number 24 is obtained by the Poisson distribution as in Fig. 1 *a*. Owing to the magnitude of the denominator (determined, in effect, by a large number of zeros), a variation of one or two tubes in the numerator has little effect on the result, because it is the relative magnitude of the ratio which is important for the method of analysis. Also, assuming continuity to neighboring intervals, less weight is attached to a particular calculation.

The chance that two cells initially in one tube at zero time will produce two colonies is the product of the probability for each, $P_1(t)$, to remain as a single cell. If the fraction of single cells remaining as one cell at $\Delta t = 1$ hr, which is $R_1(1\text{ hr}) = 0.04$, may be assumed to approximate the actual probability, $P_1(1\text{ hr})$, then

$$[R_1(1\text{ hr})]^2 = (0.04)^2 = (0.0016).$$

The number of tubes calculated, from the Poisson distribution in Fig. 1 *a*, to start with two cells is 5. To the extent that $R_1(1\text{ hr})$ approximates its corresponding probability $P_1(1\text{ hr})$, the number of tubes giving two colonies which started with two cells is given by $[R_1(1\text{ hr})]^2$, the calculated number of test tubes which started with two cells at $\Delta t = 0$ is

$$(0.0016)5 = 0.008 \Rightarrow 0.$$

The number 0.008 is assumed to be negligible; rounding off to the nearest test tube, this case would be 0. A more accurate computation might be made by rounding off to the nearest one-tenth of a test tube as the ratios are expressed by two significant figures (for purposes of plotting). The accuracy of the ratios obtained does not warrant such fine detail.

Continuing with the method, the number of test tubes with two colonies at $\Delta t = 1$ hr that began with one cell is determined by subtracting the number of tubes calculated to have begun with two cells,[1] 0, from the experimentally counted number, 3, of tubes which have two

[1] Note that for other intervals of time the numbers of test tubes to be subtracted are not 0 but are rounded off to the nearest integral number.

colonies (but which could have originally contained either one or two cells). The ratio obtained for progeny of one cell giving two colonies at $\Delta t = 1$ hr to the total number of single cells at $\Delta t = 0$ is

$$R_2(1\text{ hr}) = \frac{\left(\begin{array}{c}\text{no. tubes with 2}\\ \text{colonies at } \Delta t = 1\text{ hr}\end{array}\right) - \left(\begin{array}{c}\text{no. tubes with 2 colonies calc.}\\ \text{to have begun with 2 cells at } \Delta t = 0\end{array}\right)}{(\text{calc. no. tubes with 1 cell at } \Delta t = 0)}$$

$$= \frac{3 - 0}{24} = 0.13.$$

Considering next the tubes that contained three colonies, the chance that one cell in a tube initially containing two cells forms one colony while the other forms two colonies is given by the product $P_1(t) \cdot P_2(t)$. However, since it was just as likely that the reverse happened, the occurrence is given by twice the product. Therefore the chance that three colonies resulted from two cells originally in the same test tube is $2P_1(t) \cdot P_2(t)$. The corresponding calculation for the ratios at $\Delta t = 1$ hr is

$$2R_1(1\text{ hr}) \cdot R_2(1\text{ hr}) = 2(0.04)(0.13) = 0.01.$$

Since for this example five tubes began with two cells, the number of tubes having three colonies which started with two cells is $(0.01)5 = 0.05 \Rightarrow 0$. Then the ratio for a tube starting with one cell which for $\Delta t = 1$ hr contains three colonies is

$$R_3(1\text{ hr}) = \frac{5 - 0}{24} = 0.21.$$

The ratio for a tube that contains four colonies descending from one cell is calculated in the same way. At this stage, however, four colonies from one cell could be confused with two sets of possibilities starting from two cells. The two cells could collectively produce four colonies by one and three, or the reverse, or by two each. Hence,

$$[R_2(1\text{ hr}) \cdot R_2(1\text{ hr}) + 2R_1(1\text{ hr}) \cdot R_3(1\text{ hr})](\text{calc. no. tubes with 2 cells at } \Delta t = 0)$$

$$= [(0.13)^2 + 2(0.04)(0.21)]5 = 0.15$$

which is rounded off to 0. Then

$$R_4(1\text{ hr}) = \frac{13 - 0}{24} = 0.54.$$

Owing to the larger number of combinations by which two cells can produce any number of colonies greater than four, the method of calculating ratios becomes cumbersome and uncertain. So far, however, for this example only 22 of the 24 tubes which started with single cells have been accounted for. For the purpose of tabulating, the five-colony and six-colony tubes are next most likely. The data in Fig. 1 *b* for higher numbers of colonies per tube are presumably the consequence of two (or three) starting cells per tube.

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