VARIATIONS IN THE SENSITIVITY OF *ESCHERICHIA COLI* TO IONIZING RADIATIONS DURING THE GROWTH CYCLE[1]

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The literature is replete with information concerning the variability in susceptibility of bacterial cells to inactivation by a variety of chemical and physical agents during the growth cycle. The majority of investigators obtained data which supported the premise that "young" bacteria are more sensitive to deleterious agents than are older or more "mature" cells. Winslow and Walker (1939) summarized most of the pertinent information available and proposed an explanation based on a "physiological youth" of cells in a growing bacterial culture.

It is important to consider the difficulties in attempting to correlate the findings of the various investigators, whose test organisms and growth conditions were almost as numerous as the investigations involved. It is highly improbable that "young" and "old" were used to describe cells in a similar physiological state in the various investigations. A knowledge of the role of physiological and metabolic states in determining the radiation sensitivity of the test organism is of utmost importance in radiobiological research. This knowledge should aid in explaining variability so often encountered in such research as well as permitting an understanding of the mechanism of action of radiation on microorganisms. From this point of view an investigation was undertaken to attempt a correlation of variations in X-ray sensitivity of *Escherichia coli* with the various phases of the growth cycle. Experiments were so designed that the growth and radiation sensitivity could be assayed simultaneously and at frequent intervals during the positive portion of the culture cycle (i.e., through the maximum stationary phase), thereby assuring measurements of radiation sensitivity characteristic of the several phases of the growth cycle. It was found that (1) the lag phase is characterized by an increased resistance to X-rays, and (2) the phase of logarithmic growth coincides with a slow but steady decay in resistance, resulting in extreme sensitivity of the cells. The maximum stationary phase is distinguished by a gradual return to the sensitivity displayed by cells after 24-hr incubation at 37 C.

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MATERIALS AND METHODS

Escherichia coli strain B/r (Witkin, 1946) was carried in stock at refrigerator temperature on nutrient agar slants. Twenty-four hours prior to the beginning of a particular experiment, a small loopful of surface growth was removed from a slant and inoculated into 20 ml of sterile 1 per cent nutrient broth (Difco). After 24-hr incubation with constant aeration in a water bath at 37 C, 0.1 ml of this culture was inoculated into 100 ml of sterile nutrient broth at 37 C. The culture was contained in a 250-ml Erlenmeyer flask fitted with a cotton plug through which was inserted an aeration tube. Aeration was continuous from the time of inoculation. The cell concentration in this freshly inoculated culture was approximately 3×10^6 cells/ml. For determination of cell count, 0.1-ml aliquots of this culture were removed from the flask at 30-min intervals, the first being taken immediately after inoculation. Each sample was diluted in sterile M/15 phosphate buffer (pH 6.8) and surface plated on nutrient agar plates which had been previously dried for 15 min at 37 C.

For irradiation, 10-ml aliquots were removed at 1-hr intervals from the same flasks used in determination of cell counts. Cells were harvested by centrifugation, washed, and resuspended by vigorous shaking in M/15 phosphate buffer. (The total time which elapsed between removal of cells from the culture medium and resuspension in non-nutrient medium was approximately 30 min.) This final buffer suspension was irradiated

in equilibrium with air at ice-bath temperature. Once active cell division was under way in the culture, dilution of the irradiated suspensions was necessary for maintenance of constant cell concentration.

All estimates of relative sensitivities of the bacteria were based on ability of the cells to form visible colonies on nutrient agar with incubation at 37 C for 24 hr. In one part of the experiments, incubation at various temperatures was employed as described in the text. Surviving fractions of irradiated cells were determined by comparison of the number of colony-forming organisms in irradiated suspensions with similarly prepared but unirradiated control suspensions. Quadruplicate platings were made in all cases, and usually several dilutions of irradiated suspensions were plated. Surface platings were made by pipetting 0.05 ml of the diluted suspensions onto solid agar and then spreading this aliquot over the entire agar surface with a thin glass spreader.

The X-rays used in this work were delivered by a General Electric Maxitron 250 unit operated at 250 kvp and 30 ma with 3 mm of added Al filter (HVL, 0.55 mm of Cu). All bacterial exposures were made in a uniform field at 20 cm from the target. The dose measured in air at this distance with a Victoreen thimble chamber was 2000 r per min. The chamber had been recently calibrated by the manufacturer and checked against a Co^{60} standard in this laboratory.

The suspensions were irradiated in thin-walled pyrex tubes in a ring-type lucite holder which permitted the simultaneous exposure of 8 tubes immersed in ice water mixture within the uniform radiation field.

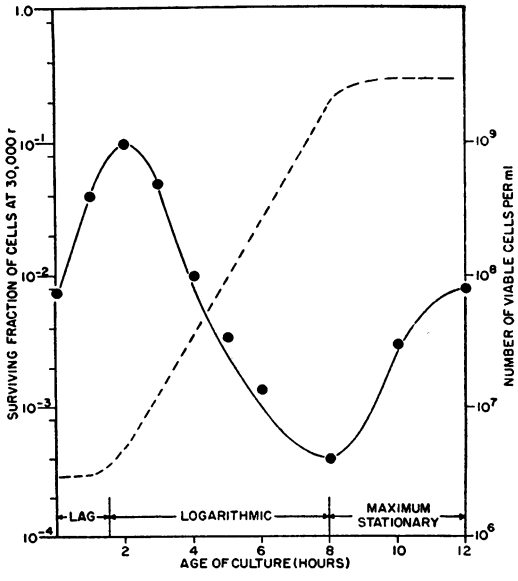

Figure 1. Survival at a constant dose of X-rays as a function of the age of the culture. Dose rate in all experiments was 2000 r per min. - - - - , growth curve; ——, surviving fraction at 30,000 r for cells at various ages indicated on the abscissae.

RESULTS

X-ray studies. When cells are removed from a growing culture of *E. coli* and suspended and irradiated as described previously, the surviving fraction at a constant X-ray dose varies with the age of the culture as shown in figure 1. The data plotted in this manner show the phasic changes in radiation sensitivity which occur during the growth cycle. The growth curve for this culture is shown also for convenience in comparing the variations in sensitivity with the various phases of the growth cycle. The individual points on the curves are averages of several experiments carried out under similar experimental conditions. It is clear from these data that *E. coli* is more susceptible to inactivation by X-rays during the logarithmic phase than elsewhere in the growth cycle. Equally evident is the finding that cells in the lag phase are more resistant than cells from any other phase of the growth cycle. These findings compare favorably with those of Elliker and Frazier (1938) and White (1951) for heat inactivation of *E. coli* and *Streptococcus faecalis*, respectively. The data shown in figure 1 indicate a rapid decay of resistance during the period when cells are in a stage of rapid cell multiplication, the most sensitive cells being obtained from cultures at the end of the logarithmic phase. This period of extreme sensitivity is followed by a gradual return to initial sensitivity during the maximum stationary phase.

One approach which could be used to obtain information concerning the nature of resistance or sensitivity which appears characteristic of cells of various ages is to determine the dose-effect relation for these organisms. Since the intensity of the radiation in these experiments was constant, the dose received was proportional to the duration of exposure. The survival curves

obtained for cells of various ages are shown in figure 2. The logarithm of surviving fraction of cells is plotted as a function of X-ray dose. Individual curves are shown for cells of various ages and are indicated by T_0, T_1, T_2, etc., representing cells at time zero, 1 hr, 2 hr, etc., from the time of inoculation. It should be noted that cells at T_0 show first-order inactivation kinetics, indicated by the exponential survival curve. The cells found to show greater resistance, 1, 2, and 3 hr old, however, yield survival curves of sigmoidal shape.

The nature of these sigmoid curves suggests that cells during the lag phase may be multinucleate or multicellular in nature. It is obvious that the inactivation kinetics would be similar regardless of which condition exists. Atwood and Norman (1949) and Norman (1951) have interpreted such curves on the basis of first order kinetics with respect to nuclei rather than the cell. Lea (1947) has applied similar kinetics to inactivation of clumped bacteria where first order inactivation kinetics hold for the individuals within the clump. Since determination of the multiplicity of the curves presented is dependent on the limiting slopes of the survival curves no accurate extrapolation can be made. Microscopic observations have been made of smears of cells from the very young culture, utilizing the technique of Robinow (1947). Although the picture is somewhat confused as regards size and stainability of cells, there appeared to be a great preponderance of large cells, most of which contained a multiplicity of "nuclear staining bodies." There are good data in the literature in support of the premise that the nuclei of cells are the sensitive sites, and that the extent of nuclear or chromosomal replication determines the sensitivity of cells to ionizing radiation (Latarjet and Ephrussi, 1949; Zirkle, 1932, 1952; Atwood and Norman, 1949).

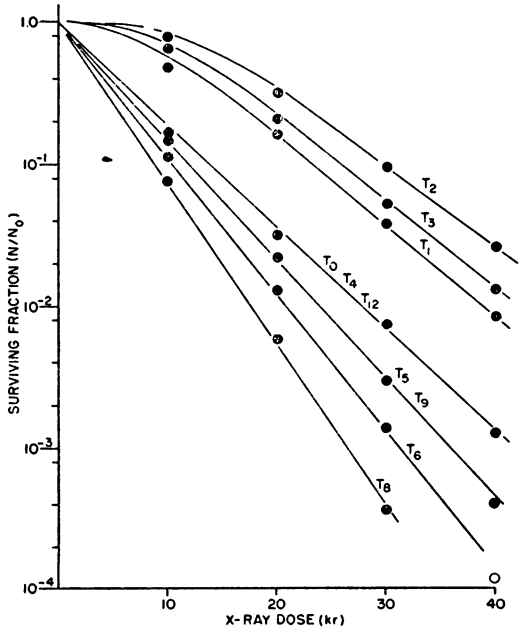

Figure 2. Surviving fraction as a function of X-ray dose for cells of various ages. T_0, T_1, T_2 etc. represent cells taken from growing culture at time zero, 1 hr, 2 hr, etc. from the time of inoculation.

Since it had been reported in the literature and reviewed by Winslow and Walker (1939) that, in general, enzyme activity of growing cells is greatest during the early phases of growth, we were interested in determining if the oxygen content of the cells at the various stages might in some way influence their sensitivity. Hollaender *et al.* (1951) had shown that a lowering of the oxygen concentration in bacterial suspensions before irradiation brought about a systematic reduction in the number of cells inactivated per unit dose. Duplicate suspensions were prepared of cells of various ages from the growing culture. One of each duplicate set was bubbled with oxygen for 30 min prior to irradiation, the other was bubbled with nitrogen. The effect of oxygen removal from suspensions was similar for cells from any stage of the growth cycle as shown in table 1. Within experimental error, the efficiency of the radiation is reduced by a factor of 3. This

TABLE 1

Comparison of dose-efficiency ratios in oxygen and nitrogen saturated irradiated E. coli suspensions*

Age of Culture	Dose-Efficiency Ratios
hr	
0	3.0
2	3.1
4	3.0
12	3.1
24	3.2

* Dose-efficiency ratio = D_{N_2}/D_{O_2} = $\frac{\text{dose in absence of oxygen}}{\text{dose in presence of oxygen}}$ to inactivate a given fraction of cells. See Burnett *et al.* (1951).

indicates that possible variations in the oxygen content play no important role in determining the differential sensitivity of cells from different phases of the growth cycle.

Gamma-ray studies. Essentially similar results were obtained when γ radiation was substituted for X-rays in the aforementioned experiments. A high-intensity Co^{60} source of the type described by Ghormley and Hochanadel (1951) served for these exposures. The source was calibrated with a special thimble chamber dosimeter described by Darden and Sheppard (1951). Similar thin-walled pyrex tubes were used as previously described for X-ray exposures. Four such tubes, filled with suspension and immersed in ice water, could be irradiated simultaneously in approximately the geometric center of this source. The dose rate in the position occupied by the samples of bacteria was about 2000 r per min. As a result of some dosimetry experiments, in which bacterial suspensions were exposed in all possible positions in the center of the long axis of this source, in thin-walled plastic tubes and in glass tubes, inside the cylindrical holder and outside the holder, and with and without surrounding ice water, it was found that absorption in the exposure system was negligible, i.e., less than 5 per cent.

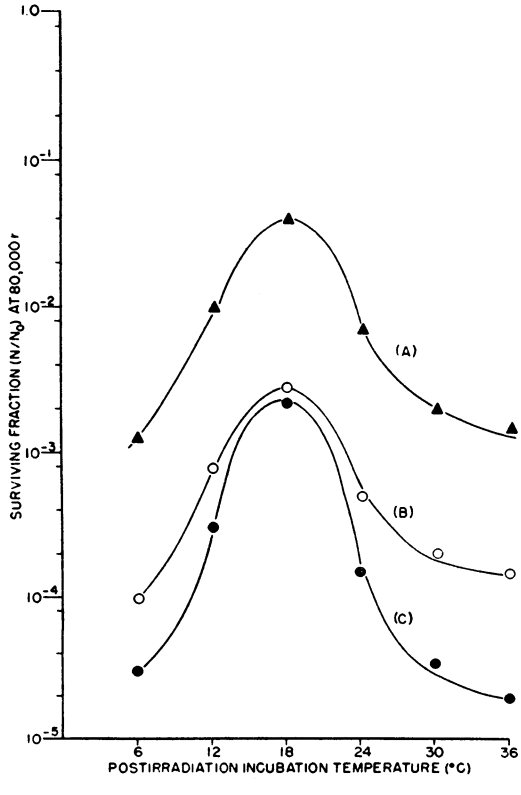

Figure 3. Survival at a constant γ-ray dose for cells of various ages as a function of the postirradiation incubation temperature. Curve A shows the surviving fraction at 80,000 r for T_2 cells; curve B, for T_0 cells (at the time of inoculation); curve C for T_6 cells. The γ-ray dose rate was 2000 r per min.

Relation of recovery to variations in sensitivity. In a few experiments the effect of postirradiation incubation temperature on survival of cells of various ages was studied. Stapleton *et al.* (1953) demonstrated that *E. coli* grown on nutrient broth and irradiated with X or γ rays show increased viability, based on ability to form colonies, if they are incubated, following irradiation, at a temperature suboptimal for growth. This effect was apparently related to repair processes in the irradiated cells. A plot of surviving fractions of bacteria as a function of the temperature at which the cells are incubated after irradiation shows a maximum at 18 C. Washed cells from a growing culture at the stages of high (T_2), intermediate (T_0 or T_{12}), and low resistance (T_6) were irradiated at a constant γ-ray dose and plated and incubated at several temperatures of 6 to 37 C. The results of this experiment are shown in figure 3. Surviving fractions of cells, after 80 kr of γ rays, are plotted as a function of the postirradiation incubation temperature. The data obtained here are essentially similar to those reported previously by Stapleton *et al.* (1953). Survival at all temperatures for T_2 cells is ten times as great as that for T_0 cells, or cells in the maximum stationary phase, indicating that the T_2 cells are intrinsically more resistant and that this resistance is probably not related to recovery. However, the most sensitive cells (T_6) show a greater sensitivity to incubation temperature than the more resistant cells, indicating that their relatively greater sensitivity may be intimately related to their inability to recover after irradiation. It is interesting to note that greater thermolability of logarithmic-phase cells has been shown by both Elliker and Frazier (1938) and White (1951). The reason for increased thermolability of normal bacteria in the stage of rapid multiplication, and also for increased thermolability of X- or

γ-irradiated bacteria, as evidenced in this work by a markedly reduced optimal survival temperature after exposure, is not answered by the data accumulated here.

DISCUSSION

The dynamic nature of sensitivity changes which occur during the growth cycle is demonstrated by the experiments described in this paper. The necessity for caution in describing cells in the culture cycle as "young" and "old" without specification as to the stage of development is obvious. As was shown, "young" cells from the lag phase are more resistant to radiation than cells in the logarithmic phase, described by many previous investigators as "young" bacteria. It appears at present that, in describing the age of cells, reference to the particular phase of growth is more meaningful than to the time from inoculation. For example, it is clear that the duration of the logarithmic phase is dependent on the size of inoculum, the temperature, and the cultural conditions. Investigations of Elliker and Frazier (1938), White (1951), and the present work indicate consistencies, independent of species and chemical or physical agent in that non-dividing lag phase cells are more resistant and cells in rapid multiplication are less resistant than are cells in the maximum stationary phase. The abnormal resistance of the large cells from the lag phase may be explicable either on the basis that these cells are multinucleate or that these cells are in reality multicellular forms which do not separate, and for all practical purposes are chains of cells. In the latter case, an artifact is induced in experiments where viable cell counts are used to measure the total number of cells, in that each of these chains of cells yields one colony on a plate. Direct counting of the cells is of no help since the decision must be made as to the nature of these cells. Development of better cytological methods may reveal the nature of abnormal resistance.

The abnormal sensitivity of cells in rapid division (logarithmic phase) is the most consistent finding in all reported investigations. Indeed, rapidly dividing cells in tissues of mammalian forms are the most sensitive cells to radiations as well as to other agents. The explanation has been offered by Lea (1947) and others that the damaging effects of radiations are chiefly genetic and are not displayed until the affected cells divide. Our data suggest that in a rapidly dividing cell there is not sufficient time for normal repair or recovery processes to take place. As seen in the data presented, some slowing down of division rate by reduction of the incubation temperature after irradiation may be conducive to better survival of these cells.

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SUMMARY

Variations in radiation sensitivity of *Escherichia coli* strain B/r during the positive portion of the growth cycle can be correlated with the recognized phases of the cycle. The phasic nature of these changes, as represented in this work and in that of Elliker and Frazier (1938) and White (1951), give a somewhat more dynamic picture than heretofore presented. A description of cells as "young" and "old," without reference to the particular phase of development, should be made with caution since these two often-used words do not describe the same cells under various cultural conditions. The nature of the variations in radiosensitivities which occur during the growth cycle can be simply summarized as follows:

(1) From the moment of inoculation into a fresh culture, cells of *E. coli* increase in their radioresistance for the duration of the lag phase, coincident with increase in cell volume and number of nuclear staining bodies.

(2) The logarithmic phase is marked by a rapid and steady decay in radioresistance. The rate of decay parallels the rate of cell division in the population. This decay in resistance progresses until the end of this phase, at which time the cells are approximately twice as sensitive as they are initially.

(3) The passing of the culture into the maximum stationary phase coincides with a return to initial sensitivity. This return to initial sensitivity is to be expected since the cells with which the culture was originally inoculated were obtained from this phase of a previous culture.

REFERENCES

ATWOOD, K. C., AND NORMAN, A. 1949 On the interpretation of multi-hit survival curves. Proc. Natl. Acad. Sci. U. S., **35**, 696–709.

BURNETT, W. T., STAPLETON, G. E., MORSE, M. L., AND HOLLAENDER, A. 1951 Reduction of X-ray sensitivity of *Escherichia coli* B/r by sulfhydryl compounds, alcohols, glycols, and sodium hydrosulfite. Proc. Soc. Exptl. Biol. Med., **77**, 636–638.

DARDEN, E. B., JR., AND SHEPPARD, C. W. 1951 A thimble type gamma-ray dosimeter for measurement of the radiation from lumped and distributed type sources. Atomic Energy Commission Unclassified Rept., ORNL-1002.

ELLIKER, P. R., AND FRAZIER, W. C. 1938 Influence of time and temperature of incubation on heat resistance of *Escherichia coli*. J. Bacteriol., **36**, 83–98.

GHORMLEY, J. A., AND HOCHANADEL, C. J. 1951 A cobalt gamma-ray source for studies in radiation chemistry. Rev. Sci. Instr., **22**, 473–483.

HOLLAENDER, A., STAPLETON, G. E., AND MARTIN, F. L. 1951 X-ray sensitivity of *Escherichia coli* as modified by oxygen tension. Nature, **167**, 103–105.

LATARJET, R., AND EPHRUSSI, B. 1949 Courbes de survie de levures haploïdes et diploides soumise aux rayons X. Compt. rend., **229**, 306–308.

LEA, D. E. 1947 *Actions of radiations on living cells*. The MacMillan Company, New York.

NORMAN, A. 1951 Inactivation of *Neurospora conidia* by ultraviolet radiation. Exptl. Cell Research, **2**, 454–473.

ROBINOW, C. F. 1947 Nuclear apparatus and cell structure of rod-shaped bacteria. Pp. 353–377 in *The bacterial cell*, edited by R. J. Dubos. Harvard University Press, Cambridge, Mass.

STAPLETON, G. E., BILLEN, D., AND HOLLAENDER, A. 1953 Recovery of X-irradiated bacteria at suboptimal incubation temperatures. J. Cellular Comp. Physiol., **41**, 345–358.

WHITE, H. R. 1951 Variation with age in the resistance of bacterial cells. Nature, **168**, 828–829.

WINSLOW, C.-E. A., AND WALKER, H. H. 1939 The earlier phases of the bacterial culture cycle. Bacteriol. Revs., **3**, 147–186.

WITKIN, E. M. 1946 Inherited differences in sensitivity to radiation in *Escherichia coli*. Proc. Natl. Acad. Sci. U. S., **32**, 59–68.

ZIRKLE, R. E. 1932 Some effects of alpha radiation on plant cells. J. Cellular Comp. Physiol., **2**, 251–274.

ZIRKLE, R. E. 1952 Speculations on cellular actions of radiations. In *Symp. on radiobiology*, Ed. J. J. Nickson. John Wiley and Sons, New York.