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Effects of Ionizing Radiation on Synchronous Cultures of *Escherichia coli* B/r

D. JOSEPH CLARK[1]

Virus Laboratory, University of California, Berkeley, California 94720

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The sensitivity of *Escherichia coli* B/r to X-irradiation is correlated with the replication cycle of deoxyribonucleic acid (DNA). The sensitivity to X-irradiation in the wild type can be attributed to the presence of nuclear targets plus DNA repair mechanisms. The effects of nuclear targets are observed in the recombination-deficient (*rec*–) mutant B/r, but the sensitivity reflected by changes in the slope of killing curves is absent. A study of different growth conditions indicates that maximal resistance to X rays occurs toward the middle of the division cycle. Evidence is offered that branched chromosomes respond as one-hit targets to X-irradiation. The killing effects of heavy-ion bombardment on *E. coli* are due primarily to ionizing radiation.

Overwhelming evidence implicates deoxyribonucleic acid (DNA) as the primary target for ultraviolet and X-ray damage to biological systems (8). Agents which are incorporated uniquely into DNA affect the radiosensitivity of the cell. The incorporation of ^{3}H-thymidine proves more lethal to the cell than the incorporation of ^{3}H-uridine or ^{3}H-histidine (30). Incorporation of 5-bromodeoxyuridine induces greater sensitivity to X rays, while incorporation of 5-fluorodeoxyuridine has no effect (2, 22, 32). Szybalski and Lorkiewicz (8) demonstrated that the increased sensitivity of *Bacillus subtilis* to X-irradiation as a result of the incorporation of bromodeoxyuridine in whole cells was the same as the inactivation of the transforming DNA as a result of X-irradiation (10, 12, 13). Haynes (15) showed synergistic effects between ultraviolet and X-ray damage, implying that they have similar targets. Since the mechanism of ultraviolet action is known to be the production of thymine dimers in DNA (37), one concludes that DNA is the primary target for both ionizing and nonionizing irradiation.

We have shown, using synchronous cultures grown on glucose, that the end of the round of DNA replication occurs in the middle of the cell cycle (6, 7); these results have been confirmed by others (16). It became of interest to investigate the period of radiosensitivity of the cell and to relate it to the DNA replication cycle. Deering (9) found that the sensitivity of *Escherichia coli* B to ultraviolet and X-ray irradiation correlated with the number of nuclei in filaments. Owen and Mortimer (29) have shown that diploid yeast is twice as sensitive to X-ray damage as haploid yeast. Others have shown that each nuclear body in strain B/r was a radiosensitive unit (3, 28, 33). One might expect, therefore, that the time of nuclear division (which corresponds to the end of a round of DNA replication in our system) would be associated with increased resistance to ultraviolet and X-irradiation. Earlier studies (19, 36) indicated that this might be the case. However, these data were not extremely convincing and they were open to question due to the method of cell synchronization. In addition, there was no way to determine whether the increase in resistance observed during the cell growth cycle was due to DNA repair phenomena, to the stage of DNA replication, or to nuclear division. In our system, the end of the round, nuclear division is well defined. We have also included studies using *rec*$^-$ mutants of *E. coli* B/r in an effort to define the contribution to changes in radiosensitivity as a result of fluctuations in the repair activity throughout the growth cycle.

MATERIALS AND METHODS

Bacterial strains and culture methods. Strains of bacteria used for these studies include *E. coli* B/r (American Type Culture Collection 12407), and *E. coli* B/r *rec*$^-$, a "reckless" strain derived from *rec-56* which was isolated by A. J. Clark (5). Cells were grown in a mineral salts medium, referred to as 007, that has been described previously (7). A synthetic medium that supported rapid growth was prepared by supplementing 007 with 20 of the most

[1] Present address: Department of Microbiology, University of British Columbia, Vancouver 8, B.C., Canada.

common amino acids: 5 nucleosides (adenine, thymine, guanosine, uracil, and cytosine), and the following vitamins; nicotinamide, B_{12}, pyridoxal phosphate, ascorbic acid, and biotin. Amino acids were added to a final concentration of 20 μg per ml, nucleosides at 10 μg per ml, and vitamins at 1 μg per ml. The doubling time for *E. coli* B/r in this medium is approximately 25 min at 37 C.

Synchronous cultures of bacteria were obtained by a method developed by Helmstetter and Cummings (18), which employs a Millipore membrane as the binding agent for parent cells. Medium passing over the surface of the membrane allows bound cells to grow and release progeny with the effluent as a result of fission. Samples collected during a short interval of time contain cells which presumably are "newborn." Subsequent growth of such cultures exhibits the stepwise growth expected of synchronous cultures.

Irradiation of samples. Synchronous cultures were incubated in a shaking water bath at 37 C. At intervals, samples were removed and diluted, and 0.1 ml of the final dilution was spread on a slightly dehydrated nutrient agar plate. After the surface of the plate was dry (generally less than 1 min), the plate was exposed to ultraviolet, X-ray, or heavy-ion irradiation. The plates were incubated overnight at 37 C and the survivors were recorded the following day. Dose curves were obtained by chilling cells at predetermined ages and plating them on nutrient agar plates just prior to irradiation.

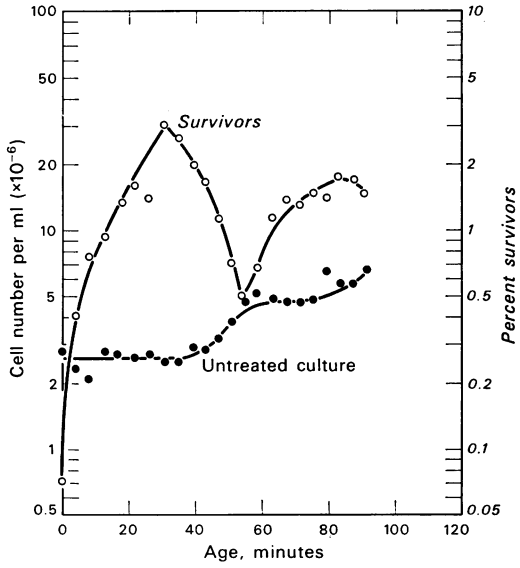

FIG. 1. *Sensitivity of E. coli B/r to heavy-ion (Argon) bombardment. A synchronous culture of E. coli B/r is treated with a constant dose of heavy-ion (Argon) irradiation at different times during the division cycle. Symbols: (●), growth of the untreated culture; (○), percentage of survivors after 80 krad of heavy-ion bombardment, showing survivors as a function of age of the synchronous culture.*

RESULTS

Sensitivity to ionizing irradiation as a function of age. The effect of heavy-ion (Argon) bombardment on synchronous cultures of *E. coli* B/r is illustrated in Fig. 1. A growth curve of the synchronous culture is given for reference, indicating that the division time of *E. coli* B/r grown in minimal medium and glucose is approximately 45 min. Treatment with 80 krad of Argon yields about 0.1% survivors at age 0 min. There is an immediate increase in survivors to a final value of approximately 3% at age 30 min, and then a decline in survivors.

The sensitivity of synchronously growing *E. coli* to ultraviolet and X-irradiation was examined. Survivors as a function of age are shown in Fig. 2. The increase in cell number during synchronous growth is given for reference. Survivors after X-ray treatment increase from approximately 0.1% at age 0 min to about 1% at 30 min. The survivors after ultraviolet irradiation increase from 0.4% to about 2% at 20 min. This general pattern of increased resistance to irradiation towards the middle of the cycle, followed by increased sensitivity to irradiation at or shortly before cell division, is observed for ionizing as well as nonionizing irradiation. The increased resistance to irradiation near the middle of the division cycle is correlated with the time at which a round of DNA replication is terminated which, under these special conditions, also corresponds to the time at which a new round of replication is started (6, 7, 16, and Fig. 3). If the increased resistance is due to the number of targets, for example the number of nuclei, then the kinetics of X-ray killing should change as a function of age.

Kinetics of X-ray killing as a function of age. Cultures at various stages in the division cycle were tested for the kinetics of survival to X-ray treatment. Figure 4 shows the dose-response of *E. coli* B/r to X ray at different ages. In Fig. 4A, killing curves for ages 0, 10, and 20 min are shown. Linear dose-response curves are obtained for 0 and 10 min which extrapolate to 1, while a curvilinear function is obtained for cells treated at age 20 min.

However, the slopes of the curves are not the same. The slope of the killing curve at 20 min is intermediate between that of 0 and 10 min. An analysis of the slopes and hitness of the curves in Fig. 4A and 4D is summarized in Table 1. A plot of the extrapolated values obtained from such dose curves is given in Fig. 5. The hitness is 1 for young cells and starts to increase at 15 min. It becomes 2 at 20 min and remains at this level until about 40 min, where it starts to return to a hitness of 1.

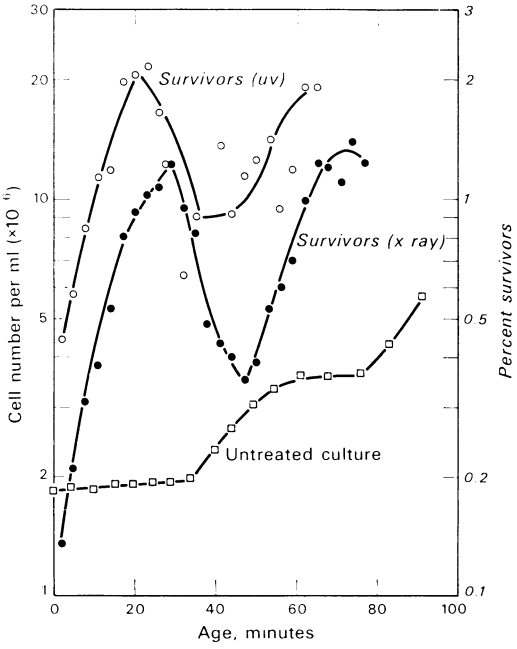

FIG. 2. *Sensitivity of E. coli B/r to irradiation. A synchronous culture of E. coli B/r is treated with a constant dose of X rays or ultraviolet irradiation at different times during the division cycle. Symbols:* (●), *growth of the untreated cultures;* (○), *percentage of survivors as a function of time after 20 krad of X-irradiation;* (■), *survivors as a function of time after exposure to 12 sec of an ultraviolet lamp (2,537 C) at 1.03 μw/cm².*

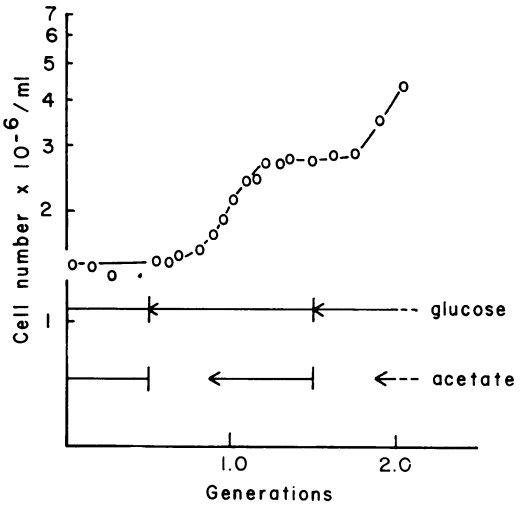

FIG. 3. *Diagram of DNA replication relative to the division cycle of E. coli B/r in glucose and in acetate cultures. Symbols:* (○), *a typical growth curve of synchronous B/r/1 for glucose and acetate cultures;* (←), *the start of a round of replication;* (|), *the end of the round of replication.*

An analysis of the killing curves (Table 1) indicates that in addition to an increase in hitness there is an increase in the slope. Since nuclear division occurs near the middle of the growth cycle, one expects increased resistance on the basis of increased targets. The increase in slope must indicate a change in resistance other than number of targets. R. H. Haynes suggested that the change in slope might be due to increased DNA repair mechanisms available to the cell at different times during the growth cycle. Therefore, we examined a reckless mutant of *E. coli* B/r for its response to X rays.

Effect of X rays on E. coli B/r rec⁻. Survivors as a function of age for *E. coli* B/r *rec⁻* are presented in Fig. 6. An increase in survivors is observed from 12 to 15% at 0 min to nearly 50% at 30 min. The survivors decline as the cells divide and they increase again during the second generation. Dose curves of *E. coli* B/r *rec⁻* are given in Fig. 7. A summary of these curves is included in Table 1.

Cultures of age 0 min exhibit one-hit killing kinetics, while cultures of 25 min exhibit two-hit kinetics. The slopes of the killing curves are similar, having a specific killing constant of 0.2 krad^{-1}.

Effect of X-irradiation on rapidly dividing cells. Bacterial cultures that are grown in rich medium are thought to have more than one chromosome (17, 24, 31). We examined the sensitivity to X rays of cells that were grown in a glucose medium supplemented with amino acids. Survivors as a function of age for synchronous *E. coli* after exposure to a constant dose of X rays are indicated in Fig. 8. An increase in survivors from approximately 0.5 to 4% is observed between 0 and 15 min. The division period is about 25 min, as indicated by the growth curve included in Fig. 8. Dose-response curves for ages 0, 5, 10, 15, 20, and 25 min are shown in Fig. 9. Cells at 0 age exhibit curvilinear killing with an extrapolated hitness value of approximately 1.5. The hitness as a function of age is plotted in Fig. 10 and is included in Table 1. Figure 10 shows hitness to change from 1.5 at 0 age to 3.5 at age 10, and then decline at about 15 min. As before, after division there is an increase in the hitness required to kill the cell.

Effects of X-irradiation on slowly dividing cells. Several investigators have demonstrated that the DNA replication period in slow-growing cells does not occupy the full division time (17, 25, 26). In particular, *E. coli* B/r that is grown in minimal salts plus acetate as the sole carbon and energy

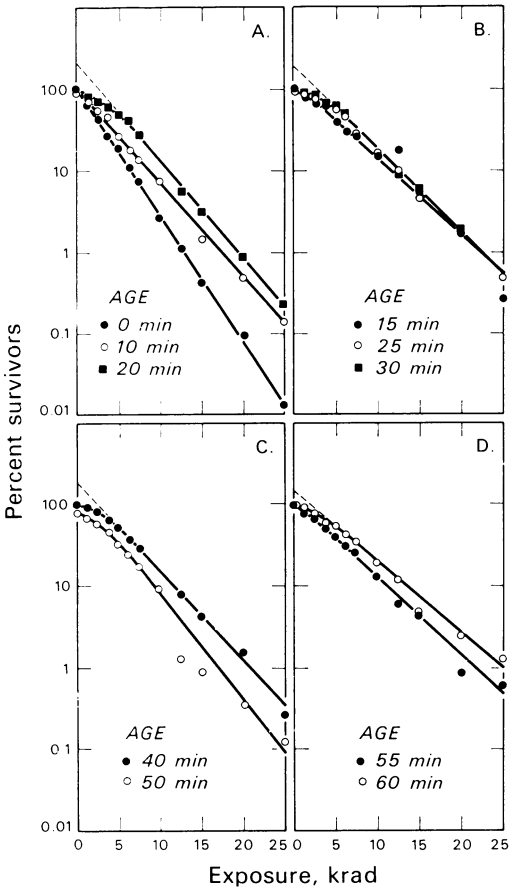

FIG. 4. *Kinetics of X-ray killing at different ages of synchronous glucose cultures of E. coli B/r grown in glucose medium. In Fig. 4A, percentages of survivors as a function of dose for cultures 0, 10, and 20 min old are indicated by* (●), (○), *and* (■), *respectively. In Fig. 4B, percentages of survivors of 15-, 25- and 30-min cultures are indicated by* (●), (○), *and* (■), *respectively. In Fig. 4C, percentages of survivors of 40- and 50-min cultures are indicated by* (●), *and* (○), *respectively. In Fig. 4D, percentages of survivors are indicated for 55- and 60-min cultures by* (●) *and* (○), *respectively.*

source appears to have a gap in DNA synthesis (17; Clark, *unpublished data*). If X-irradiation has its primary effect on DNA, then irradiation of slow-growing cells should respond in a manner that would indicate the time at which chromosome replication is completed. Figure 11 shows the response at sequential ages of *E. coli* B/r that was grown on acetate to a constant dose of X rays. An immediate increase in survivors (from 0.02 to 0.3%) is observed between the ages of 0 and 50 min. The growth curve for these cells that were grown in a minimal salts medium plus acetate is shown in Fig. 11 as a reference curve. The division time is approximately 140 min under the conditions of these experiments.

DISCUSSION

The similarity between the effects of X-ray and heavy-ion irradiation (Fig. 1 and 2) indicate that the lethal effect is due to a common agent, ionizing radiation. Although the survival curve after ultraviolet treatment is qualitatively similar to those obtained with X ray and with heavy-ion bombardment, there is some difference in the exact ages at which cells are the most sensitive and the times at which they are most resistant to ultraviolet. Maximal resistance to ultraviolet occurs at about 22 min, while it occurs at about 30 min for X rays. Maximal sensitivity to ultraviolet occurs at 0 and 35 min compared to 0 and 45 min for X-ray treatment. An explanation for this discrepancy is not apparent, although post-irradiation effects might effect DNA repair in a differential manner, which could shift the minimum and maximum of the survival curve (35).

An analysis of the kinetics of X-ray killing of *E. coli* B/r (Fig. 4 and Table 1) indicates that cultures irradiated at age 0 min always respond to one-hit kinetics, implying that a single target is involved as the sensitive unit. A plot of the hitness as a function of cell age is given in Fig. 5. There is an increase in hitness from 1.0 to 2.0 between the ages of 10 and 20 min. This curve is almost identical to that obtained when the rate of thymidine incorporation is determined as a function of age (Fig. 3). We concluded earlier that the doubling in the rate of thymidine incorporation reflected the age at which a new round of replication is started (7). It was noted that glucose cultures do not have a gap in DNA synthesis; therefore, the age at which a new round of replication begins is equivalent to the time at which a previous round of replication is completed. Our interpretation of the curves using X ray and heavy-ion bombardment is that increased resistance reflects the completion of a round of replication, or nuclear division, or both. Since DNA synthesis is continuous in glucose cultures, young cells (age 0 min) have chromosomes that are half replicated. One might expect that the only type of cell that would give precisely one-hit killing would be one in which there is a single nucleus that contains a single genome. The fact that cells containing half completed chromosome exhibit one-hit kinetics of killing is surprising. Stent (34) suggested that only double-strand breaks in DNA are lethal in *E. coli* and in phage. Other experiments support this view (1, 21). It is clear that double-strand breaks

TABLE 1. *Comparison of the sensitivity of E. coli B/r grown in minimal medium with cells grown in rich medium and with E. coli B/r rec⁻ grown in glucose medium*

Growth conditions	Age (min)										
	0	5	10	15	20	25	30	40	50	55	60
B/r, glucose											
Slope[a]	.36	.26	.21	.39	.23	.23	.25	.35	.35	.20	.22
Hitness[b]	1		1	1.3	2	1.9	2	2	1.5	1.1	1.5
B/r, glucose plus aa											
Slope	.22	.20	.20	.20	.21	.27					
Hitness	1.5	2	3.4	1.9	2	3					
B/r, *rec*⁻ glucose											
Slope	.88			.84		.77	.84				
Hitness	1			1.4		2	1.4				

[a] Expressed as the specific killing constant (k) in the equation: $2.3 \log (X_1 - X_0) = -k(D_1 - D_0)$ where X = number of survivors and D = dose in krad.

[b] Expressed as the extrapolated value from dose-response curves (Fig. 3A-D).

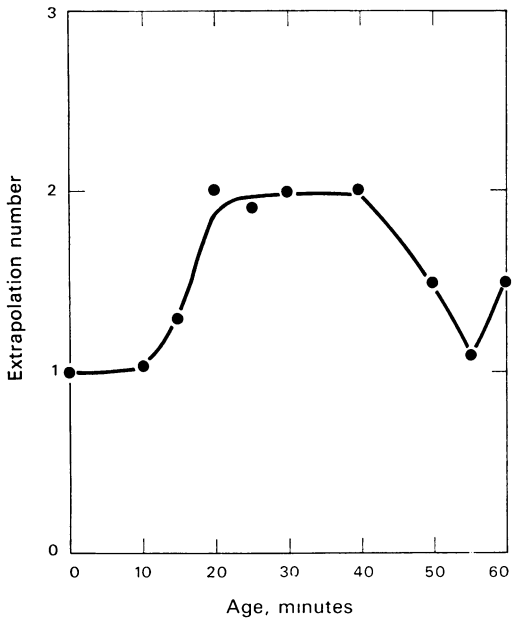

FIG. 5. *Hitness of X-ray killing as a function of age of E. coli B/r grown in glucose medium. Extrapolated values from dose-response curves in Fig. 3A–D are plotted as a function of age of the culture.*

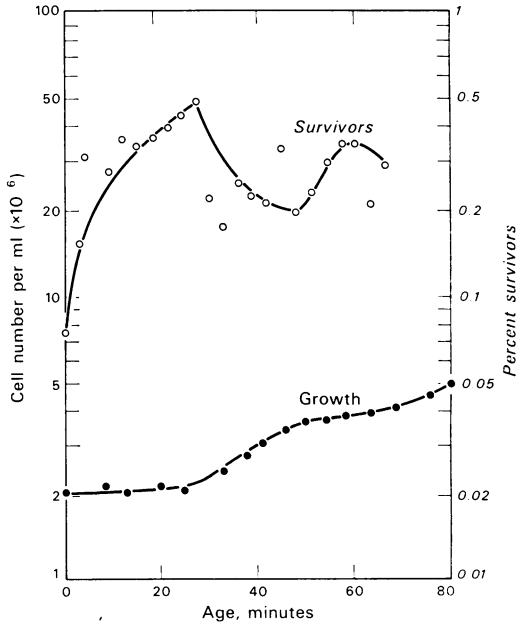

FIG. 6. *Sensitivity of E. coli B/r rec⁻ to X-irradiation. A synchronous culture of E. coli B/r is treated with a constant dose of X rays at different times during the division cycle. Symbols: (●), growth of the untreated culture; (○) percentage of survivors as a function of time after 3 krad of X-irradiation.*

can be repaired (20), but we assume that in this system the major cause of lethality is double-strand breaks. Such lesions, which cannot be repaired, represent dominant lethals in the sense that integrity of the opposite strand is not sufficient to save the cell from death. In other words, *a double-strand break in one branch cannot be masked by an allele or by the integrity of the intact branch.*

Figure 12 is constructed from the curves in Fig. 4. It indicates the relationship between the sensitivity of the cells (reflected by the slope of the killing curves and defined by the specific killing rate constant (k) and the age of the cells). Within a single generation, there is a variation of

approximately 50% in the sensitivity of *E. coli* B/r grown in glucose which is not associated with the hitness. The maximal resistance is observed during the period of 10 to 20 min (k = 0.22), while the most sensitive period corresponds to the time of division (k = 0.37).

The B/r *rec*⁻ mutant shows only about 10% difference in sensitivity to X rays. The slopes of the killing curves at different ages are almost identical (k = 0.84 ± 0.05), and the variation in killing at a constant dose (Fig. 6) can be attributed

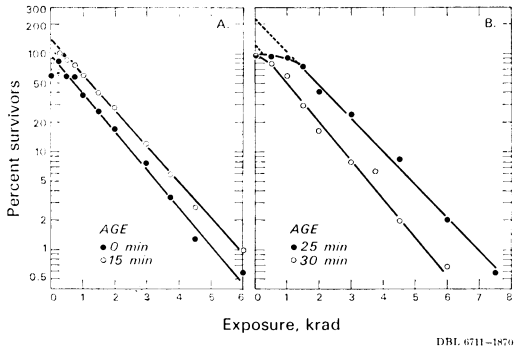

FIG. 7. *Kinetics of X-ray killing of E. coli B/r rec⁻ at different times of synchronous growth in minimal medium plus glucose. In Fig. 7A, percentages of survivors as a function of dose are indicated for 0 and 15 min by (●) and (○), respectively. In Fig. 7B, percentages of survivors are plotted as a function of dose for 25 and 30 min by (●) and (○), respectively.*

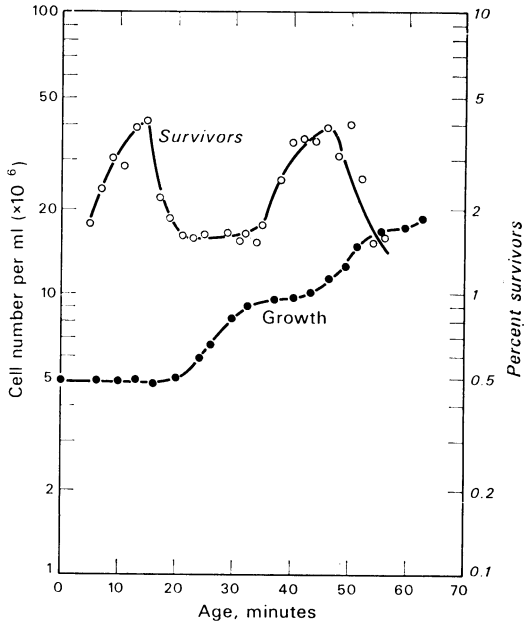

FIG. 8. *Sensitivity of E. coli B/r to X-irradiation when grown in rich medium. A synchronous culture of E. coli B/r grown in minimal medium plus supplements is treated with a constant dose of X rays at different times during the division cycle. Symbols: (●), showing cell numbers per ml as a function of time, reflects growth of the untreated culture; (○), percentage of survivors as a function of age.*

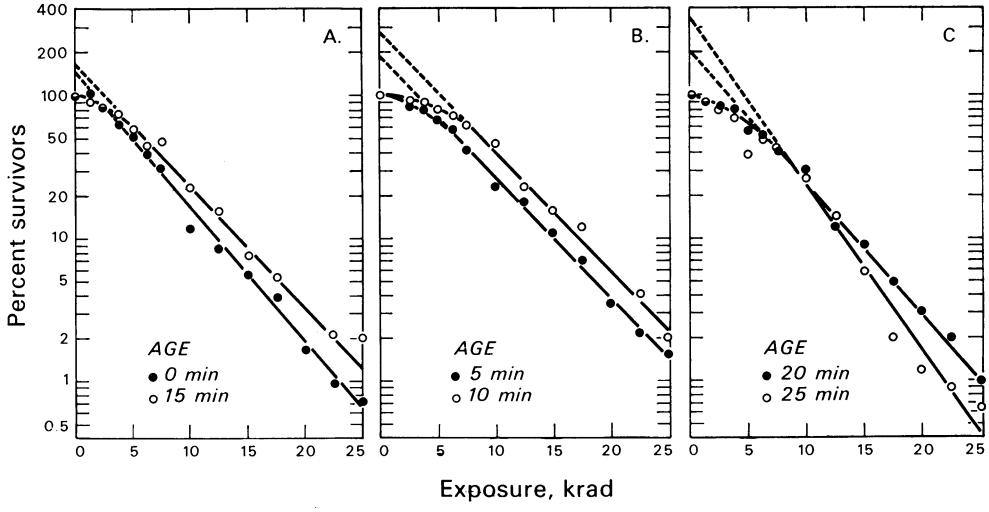

FIG. 9. *Kinetics of X-ray killing of E. coli B/r grown in rich medium. In Fig. 9A, percentages of survivors as a function of age are indicated by (●) and (○) for ages 0 and 15 min, respectively. In Fig. 9B, percentages of survivors as a function of age are indicated by (●) and (○) for 5 and 10 min, respectively. In Fig. 9C, percentages of survivors as a function of age are indicated by (●) and (○) for ages 20 and 25 min, respectively.*

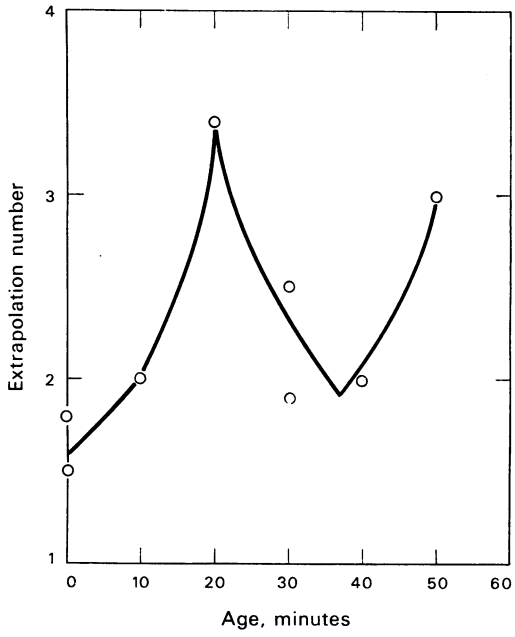

FIG. 10. *Hitness of X-ray killing as a function of age of E. coli B/r grown in rich medium. Extrapolated values from dose-response curves in Fig. 8A–C as a function of age of the culture.*

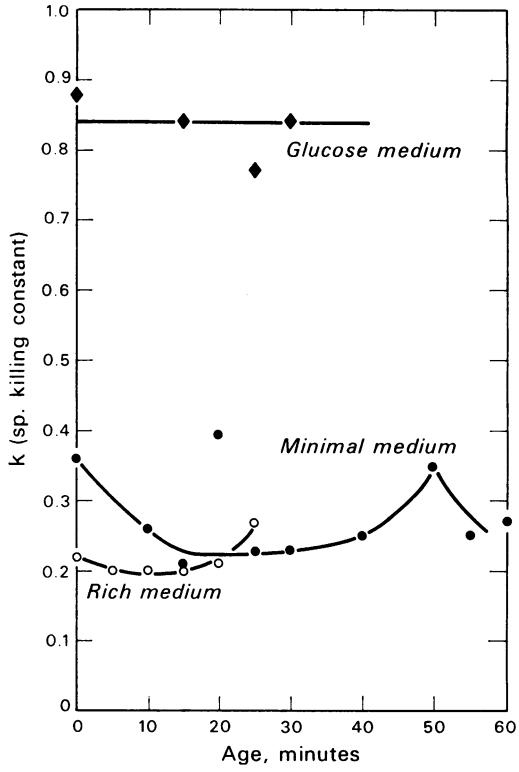

FIG. 12. *Sensitivity of E. coli B/r grown in acetate medium to X rays. Symbols:* (●), *synchronous growth, showing cell numbers per ml as a function of time;* (○), *percentage of survivors as a function of time after 18 krads of X rays.*

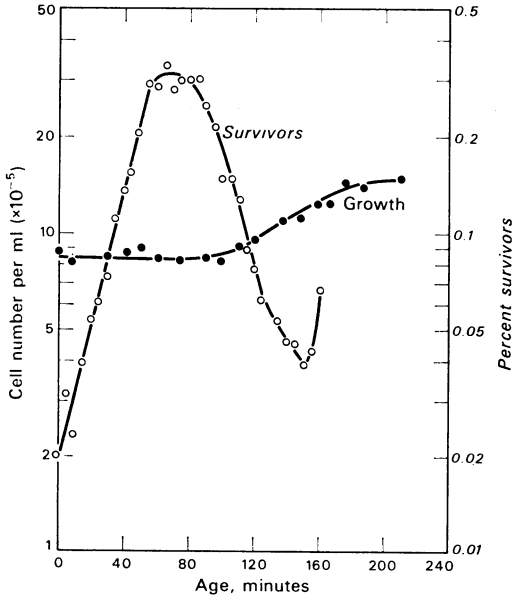

FIG. 11. *Comparison of E. coli B/r grown in minimal medium* (●), *with cells grown in rich medium* (○), *and with E. coli B/r rec⁻ grown in glucose medium* (■).

almost entirely to a difference in hitness. The particular activity associated with *E. coli rec⁻* mutation is unknown. The strain is a reckless mutant derived from JC 2761, containing a lesion in the *rec⁻-56* allele (5). According to A. J. Clark (*personal communication*), the most likely action of the *rec⁻-56* allele is the repair of gaps opposite thymine dimers. On this basis, we conclude that the disappearance of the change in slope as a function of age associated with *E. coli* B/r *rec⁻* is due to a lack in some repair functions. The difference in sensitivity between the *rec⁺* and the *rec⁻* may be a gene-dose effect in which the *rec* allele is replicated during the period of increased resistance in the wild type. The increased activity provided by two copies of the gene may lead to more DNA repair at the time of increased resistance. The validity of the gene-dose hypothesis is well documented (11, 14, 23, 27; M. L. Pato, M. S. Thesis, Univ. of Calif., Berkeley, 1968), and it is reasonable to suppose that it operates as well for the recombination-repair enzymes.

There is good evidence that the polymerization of DNA occurs at a constant rate per growing point in *E. coli* (4, 7, 16). One would assume a priori that the polymerase for DNA replication would proceed at a constant speed along the chromosome, providing that the polymerase is not substrate-limited and the temperature is constant, since there is only one site of DNA replication. There is strong support for the idea that the time required for the growing point to traverse the chromosome is approximately 40 to 45 min (17). This presents an apparent paradox. How do cells with a generation period shorter than 40 min accommodate DNA replication so that genomes are duplicated within the generation period? The answer appears to be that the cells start new growing points on the chromosome prior to the completion of a full round of replication. Thus, although the time required for a single growing point to traverse the entire chromosome is 40 min, the distance between sequential growing points is equal to or less than the generation period of the cell. In this manner a genome is finished at intervals of the generation period and each cell receives its due share of the genetic information. Experiments by Helmstetter (17) indicate that the end of the round for replication occurs very near the division period for cells growing in a rich medium with a doubling time of 20 to 25 min. The results from our experiments (Fig. 9), indicating that young cells exhibit 1.5-hit kinetics when exposed to X rays, can now be explained. We imagine that there is a statistical probability that a cell has finished a round of replication at the time of division. Some of the newborn cells would receive a single chromosome that has three growing points, because the cell has divided early compared to DNA replication, while others would have finished replication of the chromosome with three growing points, yielding two chromosomes with a single growing point. Thus, intermediate hitness is due to a heterogeneity in the population that is composed of cells with one nucleus and cells with two nuclei. This further agrees with the notion that the chromosome acts as a single unit even though it is branched.

A slow-growing culture of *E. coli* B/r was irradiated with X rays and the survivors were recorded (Fig. 11). There was an increase in survivors toward the middle of the division cycle. The sensitivity to X rays increases as the cells divide, presumably because they become one-hit targets. If X-ray sensitivity reflects completion of the chromosome, we can use the technique to determine the time at which chromosomes are completed. Results of this experiment suggest that the completion of the chromosome division, or the nuclear division, or both, occurs at about 30 to 50 min in cells that have a generation time of approximately 140 min.

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