X-RAY AND ULTRAVIOLET SENSITIVITY OF SYNCHRONOUSLY DIVIDING *ESCHERICHIA COLI*

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ABSTRACT A paper pile filtration technique was used to obtain synchronously dividing populations of *E. coli* strains B and B/r from cultures in the exponential growth phase. Three generations of highly phased cell division were obtained by rapid pressure filtration which selected approximately 1 per cent of the exponentially growing culture. The sensitivity of *E. coli* strain B to x-ray and UV inactivation as a function of the cell division cycle was determined on synchronous populations. *E. coli* strain B showed a sharp decrease in sensitivity to inactivation by both radiations in the middle of the division cycle, and a further decrease near the end of the cycle. The sensitivity of *E. coli* strain B/r to x-irradiation was also investigated. Only the mid-cycle decrease in sensitivity was found during the division cycle of this strain. It was concluded that the repetition of the observed sensitivity patterns in both strains through the first three cycles after synchronization indicates that the same basic sensitivity patterns are probably also present in the individual cells of an exponential phase culture.

INTRODUCTION

During the past few years a number of techniques for the induction of synchronous cell division in *Escherichia coli* have been described. Phasing of cell division has been obtained by temperature shock (1, 2), nutritional deprivation (3), and size selection (4-6). These methods, and a variety of others, have already been reviewed and discussed (7-10).

Various previously reported procedures for obtaining synchronously dividing populations were utilized by us to investigate the radiation sensitivity of *E. coli* as a function of the cell division cycle. The synchronization procedures used (first, temperature and glucose regulation, Scott and Chu (1); second, size selection by paper pile filtration, Maruyama and Yanagita (4), as modified by Abbo and Pardee (5); and third, size selection by membrane filtration, Anderson and Petti-

john (6)) all produced phasing of cell division for at least one generation. Of these, the size selection methods are generally considered to have the least effect on cellular metabolism. However, in our hands all of these techniques yielded bacteria which did not meet the criterion of showing repetitive properties extending over a few division cycles.[1] Therefore, a procedure was sought in which the radiation sensitivity patterns, in general, would repeat in at least two or three cycles of phased cell division immediately following synchronization. A procedure which appears to satisfy this requirement is the filtration method of Maruyama and Yanagita (4), as modified by Abbo and Pardee (5), with additional necessary modifications. This paper describes the synchronization technique and the synchronous growth obtained as evidenced by two observations. These observations are first, the phasing of cell fission, and second, the phasing of x-ray and UV sensitivity during the division cycles of the first three generations of growth.

MATERIALS AND METHODS

Bacteria and Media. The organisms used were *Escherichia coli* strain B (ATCC 11303) and strain B/r (ATCC 12407). The minimal medium was essentially the C medium of Roberts *et al.* (11), and contained NH_4Cl, 2 gm; Na_2HPO_4, 6 gm; KH_2PO_4, 3 gm; NaCl, 3 gm; $MgSO_4$, 0.013 gm; Na_2SO_4, 0.011 gm; and glucose, 1 gm in 1 liter of distilled water. The buffer consisted of Na_2HPO_4, 6 gm; and KH_2PO_4, 3 gm; per liter of distilled water. The broth medium contained nutrient broth, 8 gm; and NaCl, 5 gm per liter, and 15 gm agar was added for the plating medium.

Synchronization. The synchronization procedure was a modification of the paper pile fractionation technique of Maruyama and Yanagita (4), incorporating the modifications of Abbo and Pardee (5), for selecting the small cells of an exponentially growing population of *E. coli.* In their technique a culture of the bacteria was filtered through a stack of filter papers under suction. The stack was constructed so that only small cells would get through into the filtrate. The small cells collected in this manner constituted a population which divided in synchrony. The procedure used in the experiments described here was based on the same principle, but employed pressure rather than vacuum filtration. The filtration apparatus is shown in Fig. 1. The filter paper pile was that of Abbo and Pardee (5) and consisted of, from bottom to top, one No. 42, sixteen to eighteen No. 1, one No. 42, and two No. 1 Whatman 15 cm diameter filter papers. The paper pile was firmly bolted in the stainless steel holder with four wing nuts which were wrench-tightened. A stainless steel screen (5 × 5 mesh per linear inch, 0.041 inch diameter wire) was recessed into the top section of the holder, flush with the outer rim. This screen acted as a spacer between the top of the paper pile and the entrance port for the culture. The spacer permitted flow of the culture across the entire surface

[1] To insure that studies on such populations reflect the properties of single cells in balanced growth, it is at least necessary that some properties of the culture, other than fission synchrony, repeat over several division cycles. The most sensitive and demanding criterion for evaluation of the synchrony procedures tested was the sensitivity of *E. coli* to visible light inactivation in the presence of the basic dye acridine orange. All of the techniques attempted, other than the procedure described, yielded bacteria which were unusually resistant to photodynamic inactivation as compared to *E. coli* in the exponential growth phase. This work will be reported elsewhere.

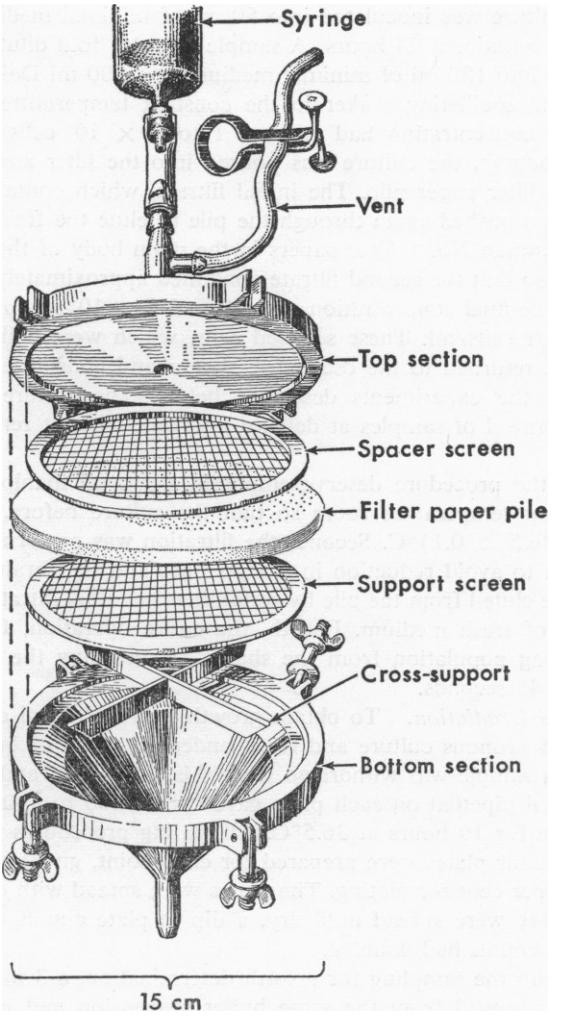

FIGURE 1 Pressure filtration apparatus.

of the filter papers while keeping the dead space between the papers and the entrance port at a minimum. The paper pile was held in position above the funnel-shaped lower section by a second inset screen and a stainless steel cross-support. The top section of the holder was connected to a 100 ml Pyrex syringe.[2] Sterilization of the apparatus or the filter papers was not necessary.

In preparation for each experiment, an inoculum of the bacteria from a refrigerated agar slant was grown in 5 ml of broth for 24 hours with shaking at 36.5°C. A 0.1 ml

[2] In more recent work, the 100 ml Pyrex syringe has been replaced by a lever-actuated pump attached directly to the top of the paper pile holder. This arrangement provides, especially in inexperienced hands, higher flow rates during filtration and more reproducible yields and results.

aliquot from this culture was inoculated into 50 ml of minimal medium and incubated with shaking for an additional 24 hours. A sample of a 10^4 fold dilution of this culture was then inoculated into 150 ml of minimal medium in a 500 ml DeLong culture flask[3] and incubated on an oscillating shaker in the constant temperature room at 36.5°C. When the bacterial concentration had reached 1 to 2 $\times$ 10^8 cells/ml in exponential growth (18 to 20 hours), the culture was poured into the filter assembly syringe and pushed through the filter paper pile. The initial filtrate, which contained 1 to 5 $\times$ 10^4 bacteria/ml, was then pushed again through the pile to elute the fraction of small cells. The number of Whatman No. 1 filter papers in the main body of the pile was adjusted between 16 and 18 so that the second filtrate contained approximately 1 per cent of the initial population. The final concentrations were 2 to 2.5 $\times$ 10^6 strain B cells/ml or 1 to 2.5 $\times$ 10^6 strain B/r cells/ml. These selected cells, which were collected in a 500 ml DeLong flask, were returned to the oscillating shaker and constituted the synchronous population used in the experiments described below. The culture flask was briefly uncapped for withdrawal of samples at definite intervals, without removing it from the shaker.

Four aspects of the procedure deserve emphasis. First, all manipulations were performed in a constant temperature room so that the culture before, during, and after treatment was at (36.5 $\pm$ 0.1)°C. Second, the filtration was performed under pressure rather than vacuum to avoid reduction in gas pressure and temperature in the filtrates. Third, the cells were eluted from the pile by rerunning the initial filtrate in order to avoid the possible shock of fresh medium. Fourth, the entire operation, from removing the exponentially growing population from the shaker to returning the selected cells, was performed in 30 to 45 seconds.

Plating and Irradiation. To obtain growth curves, 0.1 ml aliquots were withdrawn from the synchronous culture and resuspended in 10 ml of buffer at room temperature (24°C). A sample was withdrawn from this suspension and further diluted in buffer, so that 0.1 ml pipetted on each plate gave rise to 200 to 400 colonies per plate following incubation for 19 hours at 36.5°C. All plating procedures were performed at room temperature. Four plates were prepared for each point, giving standard deviations of approximately 4 per cent for plating. The plates were spread with one revolution of a glass spreader. If they were spread until dry, a dip in plate counts was frequently observed just after the counts had doubled.

Simultaneously with the sampling for growth determination, a 3 ml aliquot for radiation exposure was removed from the same buffer suspension and put into a silicone-coated glass vial 3 cm in diameter and 1 cm high. The experimental procedures for irradiation were similar to those of Uretz (12). For ultraviolet irradiation the vial was placed in a holder which rotated about its own axis near the periphery of a turntable. The sample was located 44 cm below a GE 15-watt germicidal lamp operated through a Sola regulator. The incident flux, of which approximately 90 per cent is at 2537 A, was measured with an Epply thermopile as 6 ergs/mm^2/sec., and was routinely monitored with a 935 phototube. To avoid photoreactivation, all work involving UV was performed in red light, and the resulting plates were incubated in the dark. For x-irradiation the vial was placed in a Lucite holder, and mixed with a magnetic stirrer during irradiation. The Norelco MG 150 x-ray unit was operated at 100 kv and 12 ma with external filtration of 0.5 mm Al. The dose rate, 5.5 kilorads/min., was determined with a ferrous sulfate dosimeter and converted to energy absorption in cells.

[3] Obtained from Bellco Glass, Inc., Vineland, N. J.

The x-ray and ultraviolet irradiation experiments were performed on separate synchronized cultures. The plating procedures following the radiation exposure were performed in the same manner (with suitably adjusted dilutions) as described for the growth curves. All manipulations for each point of the growth curve and the radiation sensitivity curve were performed well within the 2.5 minute interval between successive points. The ultraviolet and x-ray irradiation times were 8 seconds and 30 seconds respectively for strain B, and the x-irradiation time for strain B/r was 50 seconds. Each sample was diluted and plated within 1 minute, and the plates were placed in the incubator when the subsequent sample was withdrawn. Rapid treatment was used to minimize changes in the samples following withdrawal from the growing culture. However, once the bacteria had been diluted 1:100 in buffer, several minutes of delay prior to plating controls or irradiating samples had no effect on the results. Post-irradiation conditions for *E. coli* have been shown to have striking effects on subsequent colony-forming ability (13-15). The experiments reported here were performed under standardized post-irradiation times and temperatures. Relaxation of these parameters resulted in marked changes in survival levels which have not yet been fully explored.

The extended curves for survival as a function of dose at a given time after synchronization were obtained by suspending 0.3 ml of the synchronously dividing culture in 30 ml of buffer and irradiating separate 3 ml aliquots of this suspension. Following irradiation each sample was immediately plated and placed in the incubator. In all cases the criterion for survival was the ability to form a visible colony after 19 hours incubation at 36.5°C.

RESULTS

The paper pile filtration technique generally yielded highly synchronous division of *E. coli* for at least three generations. This synchrony is clear from the sustained stepwise nature of the growth curves (Figs. 2A, 3A, 6A). The time after filtration when the first approximate doubling of cell number took place was usually at least 2.5 to 5.0 minutes (one to two sampling intervals) shorter than that obtained in subsequent division cycles. We shall define a division cycle to extend from the mid-point of one doubling to the mid-point of the next. The second and third doublings normally occurred at about 43 minute intervals, although the second doubling occasionally took place approximately 47.5 minutes after the first.

Radiation Sensitivity of Strain B. For the experiments on radiation sensitivity, each sample from a synchronous culture was exposed to a single constant dose of radiation and plated. Fig. 2 shows the first three cycles of synchronous fission and the corresponding x-ray sensitivity of a culture of *E. coli* strain B. Fig. 2B gives the survivors/ml at constant x-ray dose (2.8 kilorads). In the first generation the number of survivors/ml was constant until mid-cycle when it increased abruptly; it continued at this new level until just prior to cell fission, when it increased again to twice the initial value. This doubling of survivors anticipated the cell doubling. The survivors/ml then remained constant through fission and repeated with the same pattern in the succeeding cycles. Fig. 2C gives the surviving fraction (curve B divided by curve A) at the constant x-ray dose, *i.e.*, the repeating

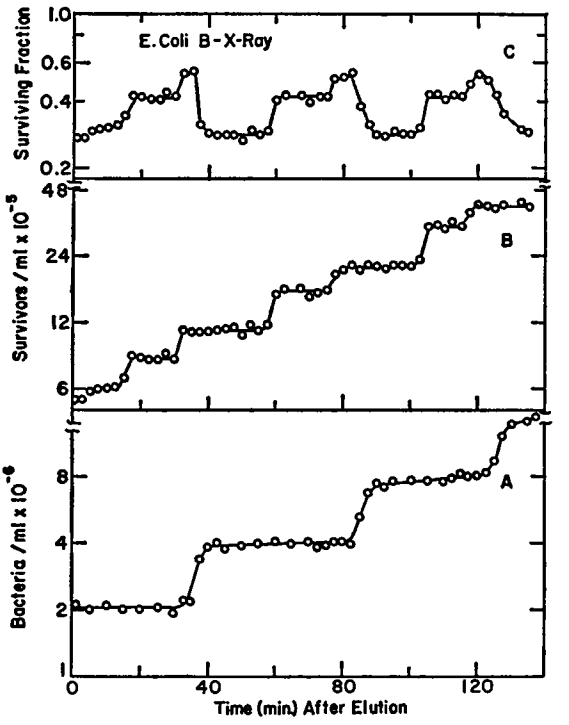

FIGURE 2 X-ray sensitivity during synchronous growth of *E. coli* strain B. A. Growth curve showing synchronous cell fission as determined by colony counts. B. Total number of survivors/ml of a 2.8 kilorads x-ray dose. C. Fraction of bacteria which survive the 2.8 kilorads x-ray dose.

pattern of sensitivity. Again the sharp decreases in sensitivity are seen in mid-cycle and just prior to cell fission. Within the resolution of this system the surviving fraction decreased by a factor of two during fission, and the same pattern repeated in each cycle. The pattern of UV sensitivity in relation to the cell division cycle is shown in Fig. 3 for a separate synchronous population of *E. coli* strain B. The pattern of survivors/ml, Fig. 3B, and surviving fraction, Fig. 3C, at constant dose (48 ergs/mm^2) was basically the same as in the case of x-irradiation. Although the curves shown were chosen for their high quality, the infrequency of points intermediate between plateaus was not unusual, particularly in the first generation, and indicates that these shifts occur within the 2.5 minute intervals.

A partial uncoupling of the changes in radiation sensitivity from the fission period was often observed. That is, the time the population remained in each sensitivity stage, or the relative time of appearance of each stage, varied in each cycle. The changes in sensitivity tended to occur earlier in relation to the fission period in subsequent cycles. This observation is particularly noticeable in Fig. 3.

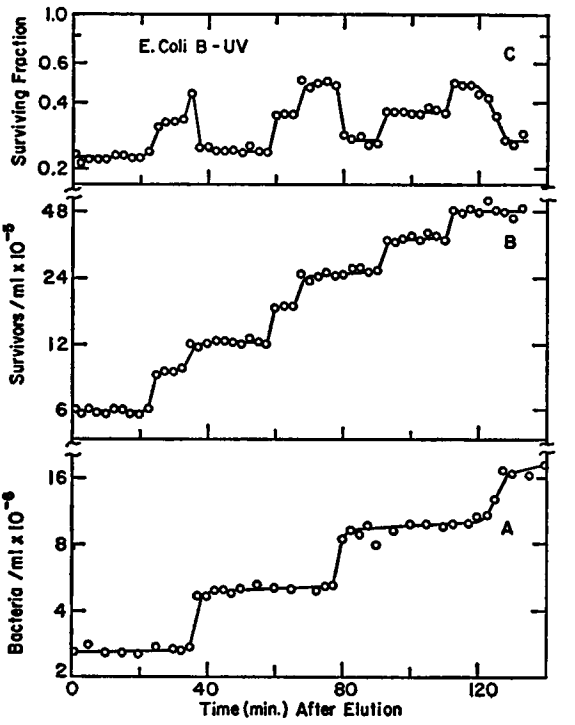

FIGURE 3 UV sensitivity during synchronous growth of *E. coli* strain B. A. Growth curve showing synchronous cell fission as determined by colony counts. B. Total number of survivors/ml of a 48 ergs/mm² UV exposure. C. Fraction of bacteria which survive the 48 ergs/mm² UV exposure.

Survival Curve Analysis. Survival curves from samples in the three distinct sensitivity plateaus are shown in Fig. 4 for x-ray inactivation of strain B. Survival curves of this nature have been obtained throughout the first and second cycles after synchronization, but it was difficult to obtain curves for all sensitivity stages in an individual experiment due to the unpredictable degree of uncoupling of the sensitivity period from the fission period. In the initial portion of the interdivisional period (10 minute curve) the x-ray survival curve was exponential. Following the decrease in sensitivity at mid-cycle, the survival curve became sigmoid in shape (30 minute curve). Just prior to cell fission (80 minute curve), the survival curve was sigmoid but with an increased shoulder. The constant dose x-ray experiments were performed at 2.8 kilorads, and, therefore, at a survival level which was below the shoulder of the sigmoid survival curves. The shape of the 80 minute survival curve explains the continuity in the number of survivors/ml through the cell fission interval. Following fission of the bacteria the survival curve became exponential, but then there were twice as many cells, as indicated by the

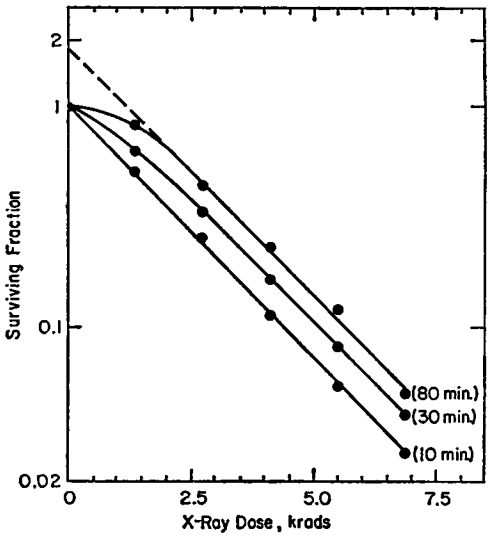

FIGURE 4 X-ray survival curves for *E. coli* strain B at 10, 30, and 80 minutes after synchronization.

dashed line in Fig. 4. Thus, the survivors/ml just before fission corresponded to the number attained in the initial portion of the next cycle, as long as the x-ray dose was large enough to reduce the surviving fraction to a level below the shoulder.

Ultraviolet survival studies for the three sensitivity regions of strain B were also performed. As shown in Fig. 5, these survival curves were more complex than those for x-ray, but the changes in sensitivity during the division cycle were again due to the appearance of a shoulder on the survival curve following the mid-cycle

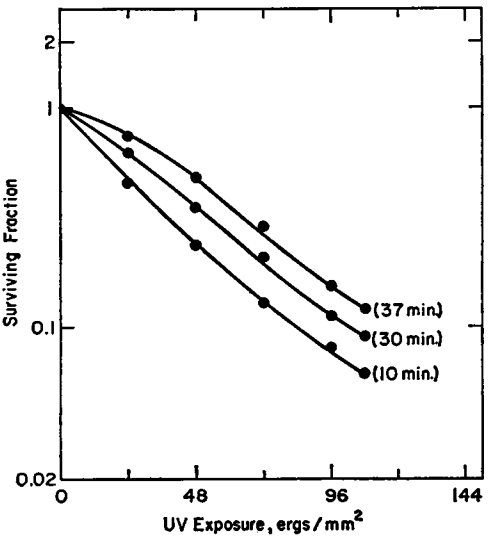

FIGURE 5 UV survival curves for *E. coli* strain B at 10, 30, and 37 minutes after synchronization.

jump and an increased shoulder at anticipation of fission. The concave form of these curves at high doses is difficult to interpret in terms of the homogeneity of the synchronous population. It may be that during the filtration process we have selected or induced a small fraction of highly resistant cells. The presence of such an unusual fraction would go undetected in the synchronous growth curves or in the constant dose irradiation experiments. It may also be that the population was homogeneous, and the results reflect increasing resistance to ultraviolet inactivation with increasing dose.

X-Ray Sensitivity of Strain B/r. Nearly complete doubling of bacteria during the fission burst, as is shown in Figs. 2A and 3A, was not always obtained in *E. coli* strain B. Occasionally, as few as 50 per cent of the cells may fission during this period. Excluding the small fraction which formed filaments, the remaining cells fissioned randomly during the subsequent generation time. However, the variations in radiation sensitivity and the doubling of the number of survivors/ml of x-ray and ultraviolet irradiation in each cycle were consistently obtained, independent of the doubling of the bacterial concentration. Since we felt that this fission delay (disregarding the 10 per cent which were completely out of phase) might be related to the filament-forming tendency of strain B, we studied strain B/r, which does not have this tendency, primarily for verification of the technique. Fig. 6 shows the synchronous growth curve and the corresponding sensitivity to x-ray inactivation for strain B/r. In this case, in repeated experiments, nearly complete doubling was consistently obtained during the burst of fissions in each generation. Fig. 6B shows that the number of survivors/ml at constant x-ray dose (4.5 kilorads) again increased in mid-cycle, but there was no second increase in survivors/ml prior to fission in each cycle. The second increase in survivors/ml in each cycle occurred at the same time as, rather than prior to, the sudden doubling of the bacterial concentration. As is seen from Fig. 6C, following the mid-cycle change the sensitivity returned to the initial value at fission without any further changes. There may, of course, be other sharp changes in sensitivity in both strains which have not been detected here because of imperfect synchrony.

DISCUSSION

The experiments presented demonstrate the sharp phasing of cell fission and the equally sharp changes in sensitivity to x-ray and UV inactivation that can be obtained during three generations of synchronous growth of *E. coli.* Microscopic examination of the synchronous culture showed that small cells were selected by the filtration process used. However, we do not believe that size selection alone was sufficiently fine to account for the narrow time intervals within which drastic shifts in the properties of the vast majority of the cells occured. The surprisingly sharp steps which appeared in both the growth and the sensitivity curves suggest that additional explanations may be necessary to account fully for the synchrony. One

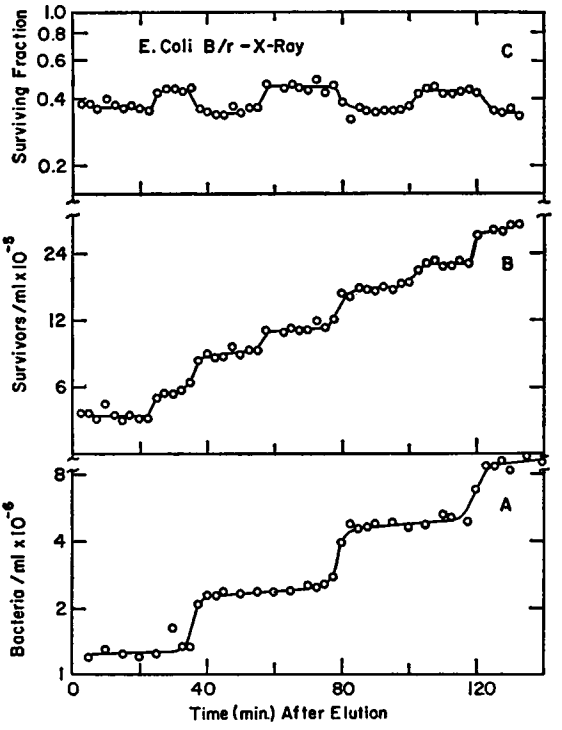

FIGURE 6 X-ray sensitivity during synchronous growth of *E. coli* strain B/r. A. Growth curve showing synchronous cell fission as determined by colony counts. B. Total number of survivors/ml of a 4.5 kilorads x-ray dose. C. Fraction of bacteria which survive the 4.5 kilorads x-ray dose.

possibility could be associated with concentration effects in strain B. The results presented were obtained with cultures which contained 2 to 2.5 $\times$ 10^6 bacteria/ml after filtration, and which were diluted to give 200 to 400 colonies per plate. If the concentrations of cells were dropped below these values, the fission synchrony deteriorated. The effect of concentration in the synchronous culture of strain B/r was not as pronounced, but may be just as important. Excellent and reproducible fission synchrony was obtained with strain B/r in the range 1 to 2.5 $\times$ 10^6 bacteria/ml.

All of the results were obtained with the use of viable plate counts as the sole scoring criterion. The mechanical agitation of the cells in the sampling, diluting, and plating procedures may have caused doubling of the colony counts during a sharper interval than the actual doubling of the bacteria in the growth flask. This possibility is indicated by the observation that the cells immediately after fission appear to be particularly sensitive to plating method, as was described in *Materials and Methods*. In addition, the slightly shortened first generation following elution also indicates that selection and subsequent synchrony may be functions of factors other than simply selecting and observing the smallest cells of the initial population.

Nevertheless, it is unlikely that the extent of synchrony observed can be accounted for on the basis of periodic induction of fission by scoring procedure.

E. coli strain B showed a sharp decrease in sensitivity to inactivation by both x-ray and UV near the middle of the division cycle and again near the end of the cycle. The degree of fission synchrony in strain B was very sensitive to the detailed experimental parameters which have been discussed, and, even under the optimal conditions described, nearly complete doubling of cells during the fission burst was not always obtained. However, the patterns of sensitivity to x-ray and UV radiation were consistently sharp and reproducible even when the bacterial concentration was dropped slightly below the optimum for sharp fission synchrony. Occasionally growth curves were obtained which showed poor fission synchrony while giving radiation sensitivity patterns as sharp as those obtained in conjunction with good fission synchrony. The radiation-sensitive cell functions do not appear to be coupled to cell fission. Similar disassociations of certain cell processes (*e.g.*, growth in mass) from cell fission have been discussed by Swann (16). These disassociations are particularly pronounced in the filament-forming strain B. In addition, the number of radiation survivors (*i.e.*, colony-forming units) doubled in each cycle regardless of the fraction of cells which fissioned. The results indicate that in the synchronous population the division of *E. coli* strain B into radiobiologically independent units is highly phased, well regulated, and independent of the less precise fission of this strain into spatially detached units. In strain B/r the doubling of cell number at the fission period was consistently obtained. In contrast to the results with strain B, strain B/r appears to show a highly regular fission period which is closely related to the division of the cells into radiobiologically independent units. The consistently sharp radiation sensitivity curves in both strains is clear evidence of the synchrony. It is apparent that in studies on synchronized cells the phasing of cell fission or of radiation sensitivity may in itself be insufficient for characterizing the over-all phasing of the cells in the culture.

The repetition of the sensitivity patterns in both strains through the first three cycles after synchronization suggests that the same basic pattern is probably also present in the cells of the pre-filtration exponential phase culture. However, the uncoupling of the sensitivity period from the fission period in strain B may imply that some stresses have been imposed in the procedure. Therefore, the fraction of cells in a randomly dividing population which are in each of these sensitivity stages cannot be calculated with confidence from the timing of these stages in the synchronous population. This conclusion is strengthened by the fact that even larger disparities in periodicities have been observed in bacteria synchronized by chilling. Thus, Hotchkiss (17) found the period for transformability of synchronized *Pneumococcus* to be longer than the cell division period, and Sinai and Yudkin (2) found the period of resistance to proflavin in synchronized *E. coli* to be shorter than that of cell division.

Yanagita *et al.* (18) investigated UV sensitivity as a function of the division cycle of *E. coli* strain B synchronized by paper pile filtration. This report confirms their observation that the bacteria became progressively more resistant to UV inactivation during the interdivisional period. They also found that survival curves from samples taken at intervals during growth were sigmoid with parallel limiting slopes. The extrapolation number of these curves were found to vary from 1.6 just after synchronization to 4.4 before the subsequent synchronous division. These numbers were correlated with the number of nuclei, which was found to be 2 after division and 4 from the middle to the end of the interdivisional period. Although the absolute differences between our survival curves and theirs may simply reflect different post-irradiation treatment, the relative changes in shape during the division cycle are consistent. However, interpretation of even our x-ray results in terms of hit numbers or extrapolation numbers is invalid since the limiting slopes of the sigmoid survival curves cannot be determined without extending the curves to considerably higher dose levels.

Romig *et al.* (19), using bacteria synchronized by temperature cycling, also found that *E. coli* was most sensitive to inactivation by UV following division and most resistant prior thereto. Stapleton and Sicard (20) studied the x-ray sensitivity of the thymine-requiring mutant of *E. coli* strain 15 which was synchronized by withholding and then resupplying the required pyrimidine (Barner and Cohen (3)). They observed large changes in radiosensitivity during the first cycle of synchronized growth, but found it difficult to relate these changes to the division process. Weatherwax (21) and Weatherwax and Landman (22) investigated the sensitivity of *E. coli* strain 15_{T^-} to ultraviolet irradiation in cultures also synchronized by starvation for thymine and then resupplementation of the pyrimidine. During the period of starvation the cells became increasingly sensitive to UV inactivation. After addition of thymine, the sensitivity returned to the initial level and remained constant during synchronous division. Bruce and Maaløe (23) observed the ultraviolet sensitivity of *Salmonella typhimurium* which was synchronized by temperature cycling (Lark and Maaløe (24)). In contrast to our results, they found that these cells were most sensitive prior to division and most resistant after division. The relation of these observations to the results reported here is difficult to determine because of the basically different treatments used to induce synchrony.

In this investigation no attempt has been made to relate the observed changes in radiation sensitivity to physiological state during growth in the synchronous culture. However, the abrupt decrease in radiation sensitivity in mid-cycle may correspond to nuclear division since Schaechter *et al.* (25) have observed cytologically that nuclear division took place shortly after the middle of the cell division cycle in synchronized bacteria. We had originally thought that the second decrease in sensitivity in strain B was due (by virtue of the continuity of survivors through fission) to the division of the cell into two radiobiologically independent units just

before fission. In this case both units would have to be inactivated to be scored as a cell death. This hypothesis fits the data exactly. However, the occasional strong uncoupling of the sensitivity period from the fission period, and the tendency for this sensitivity shift to occur earlier in successive cycles, suggest that the uncoupling phenomenon must be carefully investigated before interpretation of the results is attempted.

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