VARIATIONS IN SEVERAL RESPONSES OF HeLa CELLS TO X-IRRADIATION DURING THE DIVISION CYCLE

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ABSTRACT Several responses of synchronized populations of HeLa S3 cells were measured after irradiation with 220 kev x-rays at selected times during the division cycle. (1) Survival (colony-forming ability) is maximal when cells are irradiated in the early post-mitotic (G_1) and the pre-mitotic (G_2) phases of the cycle, and minimal in the mitotic (M) and late G_1 or early DNA synthetic (S) phases. (2) Markedly different growth patterns result from irradiation in different phases: (*a*) Prolongation of interphase (division delay) is minimal when cells are irradiated early in G_1 and rises progressively through the remainder of the cycle. (*b*) Cells irradiated while in mitosis are not delayed in that division, but the succeeding division is delayed. (*c*) Persistence of cells as metabolizing entities does not depend on the phase of the division cycle in which they are irradiated. (3) Characteristic perturbations of the normal DNA synthetic cycle occur: (*a*) Cells irradiated in M suffer a small delay in the onset of S, a slight prolongation of S, and a slight depression in the rate of DNA synthesis; the major delay occurs in G_2. (*b*) Cells irradiated in G_1 show no delay in the onset of S, and essentially no alteration in the duration or rate of DNA synthesis; G_2 delay is minimal. (*c*) Cells irradiated in S suffer an appreciable S prolongation and a decreased rate of DNA synthesis; G_2 delay is shorter than S delay.

INTRODUCTION

Study of the nature and origin of the several distinct responses to ionizing radiation that can be observed in cultured animal cells (Puck and Marcus, 1956; Tolmach, 1961) may lead not only to an increased understanding of the radiation syndrome, but to greater insight into normal cellular processes as well. For example, examination of the effects of radiation on DNA synthesis in a variety of cell types, both *in vivo* and *in vitro*, has suggested that certain differences with respect to the synthesis of nuclear material may exist between cells of normal tissues and of tumors

(Seed, 1961). In an effort to more fully characterize the radiation syndrome in one particular cell strain, we have made a detailed analysis of several of the responses of HeLa S3 cells to x-irradiation at different stages of the cell division cycle.

A number of studies directed toward the description of changes in certain of the radiation responses of cells during the division cycle have been carried out with randomly dividing cultures. The stage of a cell in the division cycle may be determined by measurement of any one of a variety of non-constant growth parameters, *e.g.*, nuclear volume (Woodard *et al.*, 1961), cell length (Swann, 1962), or, in particular, DNA synthesis, usually by an autoradiographic method (Howard and Pelc, 1953). Although such procedures have yielded important information (*e.g.*, Painter and Robertson, 1959; Yamada and Puck, 1961; Whitmore *et al.*, 1961; Harrington, 1961; Dewey and Humphrey, 1962), the use of non-synchronous populations has severe limitations which arise from the need to determine simultaneously both the stage of the cells in the division cycle, and their response to the radiation insult.

Accordingly, in the experiments reported here, we have employed synchronously dividing cultures of HeLa S3 cells. The cultures were prepared by a purely selective method which does not distort the normal pattern of DNA synthesis (Terasima and Tolmach, 1962). Three types of responses to 220 kev x-rays have been studied at selected stages throughout the division cycle: (A) loss of reproductive integrity (*i.e.*, ability to proliferate indefinitely), on which we have previously reported briefly (Terasima and Tolmach, 1961); (B) alterations in growth characteristics, including both the loss of the cells' ability to persist as metabolizing entities and the induction of division delay; and (C) disturbances in the pattern of DNA synthesis.

METHODS

The cell strain employed in these studies was a derivative of S3-9IV (Puck *et al.*, 1956; Marcus, 1959) kindly provided by Dr. T. T. Puck and by Dr. P. I. Marcus. Cell culture procedures (Ham and Puck, 1962) and the technique of synchronization, in which mitotic cells are selectively harvested by taking advantage of their tenuous attachment to the growth surface, are described in detail elsewhere (Terasima and Tolmach, 1962), as are the procedures employed for labeling with H^3-thymidine (for autoradiographic studies) or C^{14}-thymidine (for Geiger counting). The autoradiographic procedures, criteria for scoring labeled cells, and method of counting grains employed here are also described in that paper; in general, they follow the techniques described by Doniach and Pelc (1950) for the use of stripping film, Messier and Leblond (1957) for the use of liquid emulsions, and Stanners and Till (1960).

Irradiations, which were all performed in single exposures at dose rates of 25 to 70 rads per min., depending on the experiment, were carried out with a constant potential unit operated at 220 kv and 15 ma, with 1 mm Al + 0.5 mm Cu added filtration (HVL 1.9 mm Cu). Doses were measured with a suitably located ionization chamber. During irradiation, culture dishes were placed on a rotating plastic platform housed in a chamber

maintained at about 30°C and fed with a stream of (3.5 per cent carbon dioxide + 96.5 per cent air).

Cell growth measurements for assessment of the degree of synchronization and determination of division delay were made by repeated observation of selected microscopic fields (Marcus and Puck, 1958), and always involved more than 100 cells. Measurement of cell division followed a number of arbitrary rules: (1) A cell first appearing in a field subsequent to the initial observation was not scored. (2) Even if a cell's morphology suggested that it had arisen by fusion of 2 sister cells, it was counted as one cell, unless it had actually been observed in the 2-cell condition. (3) The slopes of the cell number *versus* time curves tended to decrease with increasing interdivisional time, and division delay was measured as the difference between the median rise times for the irradiated and control cultures, respectively. (4) On the other hand, normalization of the time axis for purposes of collation of data from replicate experiments was based on the minimum interdivisional time, taken as the intersection of the steepest segment of the growth curve with the abscissa.

A reproductively surviving cell was scored as one which gave rise to a colony containing 50 or more cells after 12 days of incubation following irradiation (Puck and Marcus, 1956).

RESULTS

A. *Cell Killing.* We reported previously (Terasima and Tolmach, 1961) that survival of the reproductive integrity of HeLa S3 cells showed cyclical alterations during the division cycle. The data available at that time suggested (1) that the survival curves were of the multihit type, (2) that the fluctuations in survival arose mainly from changes in the slopes of the survival curves, and (3) that the greatest interphase sensitivity occurred during the DNA synthetic (S) phase of the division cycle. In further study of this phenomenon, we have attempted (*a*) to obtain sufficiently precise data to establish the true shapes of the survival curves, and to determine the survival curve parameters, and (*b*) to locate precisely the period of maximum sensitivity with respect to the intermitotic pattern of DNA synthesis. We have been more successful in endeavor (*b*) than (*a*).

Figs. 1 and 2 show the results of two survival experiments, typical of the 10 that have been carried out. Certain features of the curves are noteworthy:

(1) The curves appear to be of the usual multihit type for 1-2 decades of inactivation. However, further irradiation often reveals an increased slope of the survival curve for the remainder of the population. The restricted numbers of synchronous cells that can be harvested conveniently (about 10^5) preclude detailed study of the survival kinetics at high doses.

(2) The initial exponential slopes form a consistent pattern, cyclically varying between maximal values for mitotic (M) cells (mean lethal dose, D_0, about 70 rads) and mid-interphase cells (D_0 about 100 rads), and minimal values for post-mitotic (G_1) cells (D_0 about 180 rads) and pre-mitotic (G_2) cells (D_0 about 160 rads). The slopes differ maximally by about a factor 2.5.

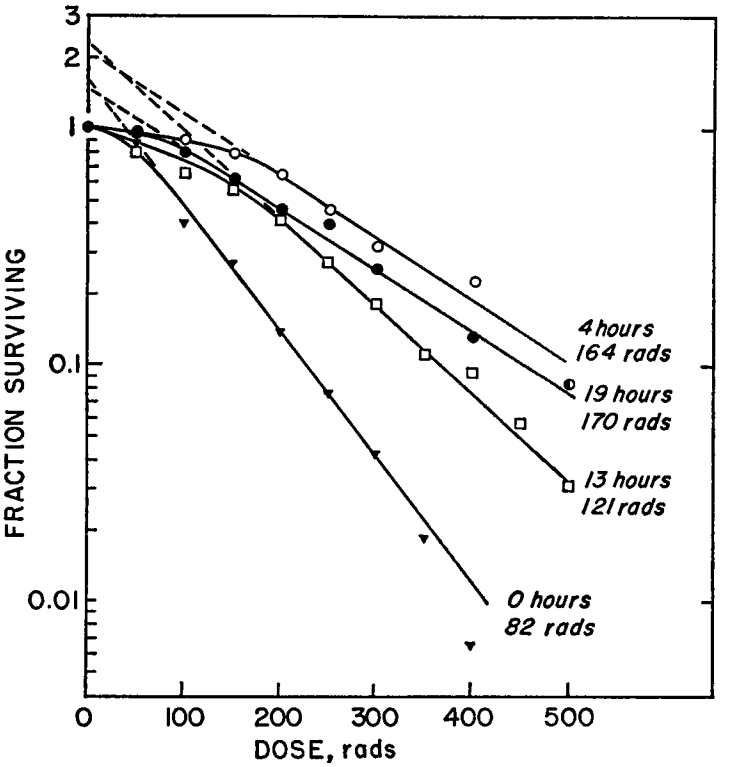

FIGURE 1 Survival of reproductive capacity in aliquots of a synchronous culture after x-irradiation at 4 different times during the cell division cycle, measured from mitosis (collected cells). The lines have been drawn by eye.

(3) No pattern is discernible in the variation of extrapolation numbers of the initial curves for interphase cells, which generally fall between 1 and 3, but the curves for mitotic cells tend to exhibit extrapolation numbers slightly smaller than those for interphase cells. This finding is expected (for independently inactivated cells) as the mitotic population consists mainly of single cells, while nearly every interphase cell is a member of a pair of sister cells (however, see below).

The finding that more mitotic cells than interphase cells are inactivated by a given dose of x-rays is not surprising in view of the special state of the reproductive elements during mitosis. However, the markedly steeper slopes of the survival curves for mitotic cells may be partly artifactual. In this system, cells in mitosis, in contrast to all other phases of the division cycle, are not attached to the growth surface when irradiated, and in spite of the equivalence of scattered radiation received by cells free in suspension or attached to and spread on the plastic dishes used (Hood and Norris, 1961), the different geometrical configurations of the two might lead to slightly different effects. Accordingly, comparison was made of the survival of a

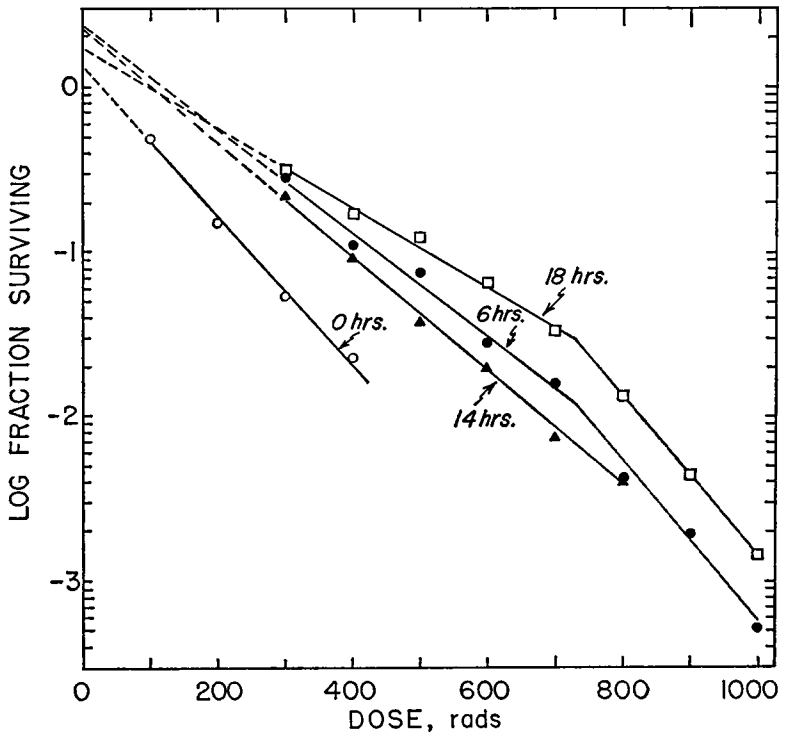

FIGURE 2 Similar to Fig. 1, except that inactivations were carried out to larger doses, permitting detection of the apparent change in slope of the survival curves. The data are representative of those found in 10 experiments.

freshly trypsinized, randomly dividing culture and of an aliquot of that population 3 hours later when the cells had attached and spread. Fig. 3 shows that the trypsinized cells are considerably more sensitive. Although the validity of such a test may be doubtful, as trypsinized cells are under great physiologic stress, having lost many intracellular components (Puck *et al.*, 1956), and thus may not be equivalent to collected mitotic cells, pre-irradiation treatment of mitotic cells with trypsin for 12 minutes had no effect on their survival. This finding suggests that the condition of a cell with respect to attachment to a growth surface may indeed affect its response to x-rays.

Interpretation of the survival curves, at best a hazardous undertaking, is particularly difficult in this system because of both experimental limitations and the complex nature of the curves. The observed shapes of the survival curves could in principle arise from any of a number of factors, some of which may be examined.

(*a*) The occurrence of cell clumping is unlikely, as periodic microscopic observation of the collected synchronous population is made routinely, and reveals individual cells.

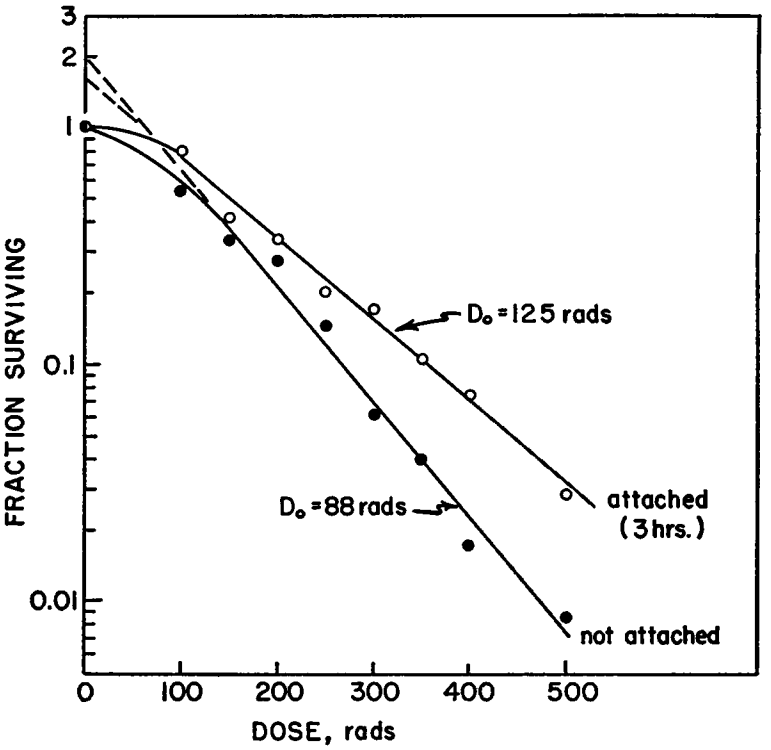

FIGURE 3 Survival curves for 2 aliquots of a randomly dividing culture x-irradiated immediately after trypsinization, when the cells were not attached to the plastic culture dish (solid circles), and 3 hours later, when the cells were attached and spread (open circles). The curves have been drawn by eye.

(*b*) If a second type of lesion, requiring the accumulation of many hits for expression, became important at high doses, survival curves with the same shape should be observed with random populations. Although curves of this general form have been reported (Barendsen, 1962), they are not the rule, and we have not observed them in random populations.

(*c*) Distortions induced by the scoring criterion for surviving cells, *viz.*, an underestimate of survivors at high doses where colony formation is slow and many small colonies are found after 12 days of incubation, should occur equally in survival experiments with random populations.

(*d*) Cell interactions (non-independence of cell killing) could give rise to such curves (Person *et al.*, 1961). Although, under the cultural conditions obtaining, the two sister cells constituting each micro-colony (except for mitotic cells) at the time of irradiation do not in general appear to remain in contact, a significant fraction of them may do so, for when irradiations were carried out at 30 to 35 hours after harvesting, by which time all of the cells had again doubled, no increase in extrapo-

lation number was noted in 2 experiments. Hence it is possible that interactions are in fact occurring in this system.

In view of the ambiguities involved in the interpretation of the survival curves, it seemed useful to represent the fluctuations that occur during the division cycle in terms of cell survival after irradiation with a constant dose of 300 rads. The upper curve of Fig. 4 shows the survival values measured in a large number of experiments, each denoted by a different symbol. The time scales have been normalized with respect to the minimal interdivisional time for the control (unirradiated) cells in each experiment, which was set equal to 18 hours, the most usual value in the later experiments. Essentially the same pattern is obtained if we plot the mean lethal doses, estimated from the initial exponential portions of each inactivation curve. Although there are no points in Fig. 4 between 6 and 10 hours, other experiments show that the location of the minimum at about 10 hours is correct. At the 300 rad dose chosen, survival levels differ at least 4-fold between the maximum and minimum values of interphase sensitivity. This difference could be enhanced, of course, by comparing survivals at a larger dose.

The lower portion of Fig. 4 shows the pattern of DNA synthesis as determined in 3 experiments from the fraction of cells incorporating H^3-thymidine in 20 minute periods. The time scales have been normalized as in the upper curve. It is clear that the greatest number (about 95 per cent) of cells are synthesizing DNA at about 13 hours, at least 3 hours later than the time when cells show greatest inactivation by x-rays. Because of the asynchrony which has developed by this time in the cycle (Terasima and Tolmach, 1962), it is not possible from these data to determine whether the latter part of G_1 or the early part of S is the period of maximal response.

In this connection, Dewey and Humphrey (1962) note that mouse L and ascites tumor cells are more sensitive to x-rays when irradiated during S than during G_1. However, since those workers used random cultures, and x-ray sensitivity changes throughout the division cycle, it is not possible to evaluate that report in terms of our findings.

B. *Effects on Cell Growth and the Induction of Division Delay.* Until recently, investigations of radiation effects on the course of cell division have involved for the most part either determinations of alterations in the mitotic frequency (see Lea, 1956) or study of population dynamics (Kohn and Fogh, 1959; Tolmach, 1961). Although such studies showed that induction of a delay in cell division was a widespread consequence of exposure to radiation, only those investigations in which sea urchin eggs or grasshopper neuroblasts were used as the experimental material yielded information from which the relation between division delay and the location of the cell in the cycle at time of irradiation could be established (see Carlson, 1954). The latter information led to the generalization that the closer cells are to a critical period (located in prophase) when irradiated, the greater the

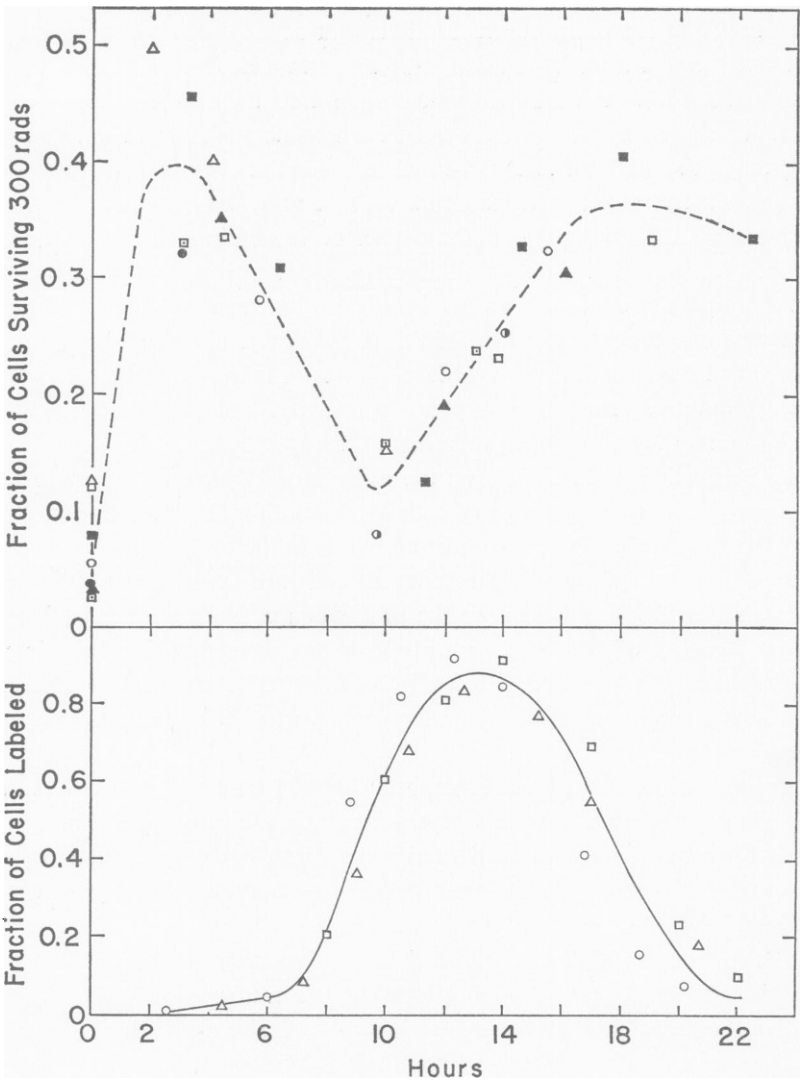

FIGURE 4 The upper curve shows the fraction of cells surviving reproductively after receiving 300 rads of x-rays administered at different times in the division cycle, in which mitosis is taken as zero hours. Each symbol represents a separate experiment, the time scales of which have all been normalized to a minimum interdivisional time of 18 hours. The lower curve shows the pattern of DNA synthesis found in 3 separate experiments with synchronous cells. The fraction of cells showing incorporation of H^3-thymidine after 20 minute sojourns in medium containing that tracer is plotted against the time after mitosis, normalized as in the upper curve.

delay produced; and a mathematical treatment of division delay was developed by Lea (1956) based on the assumption that repair processes, whose completion constitutes the delay, begin to occur immediately after irradiation.

More recent experiments, utilizing tracers for detection of DNA synthesis, have confirmed the dependence of division delay on the state of the cell at the time of irradiation, and have also indicated that the increased interdivisional time which results from irradiation often comes about at least in part from a delay in G_2 (Whitmore, *et al.*, 1961; Yamada and Puck, 1961), or in S (Painter and Robertson, 1959; Painter, 1962). Because blocking of cells in the G_1, S, or G_2 periods might reflect fundamentally different radiation-produced lesions, a complete resolution of the over-all mitotic delay would be highly desirable. Our attempt to effect such a resolution is described in section C.

In the experiments described in this section, we examined in detail the relation between the pattern of cell proliferation and the location of cells in the division cycle at the time of irradiation. Growth was monitored in large numbers of cells after a single 300 rad dose, by repeatedly examining selected microscope fields; the procedure adopted for measuring delay is described under Methods. Fig. 5 shows

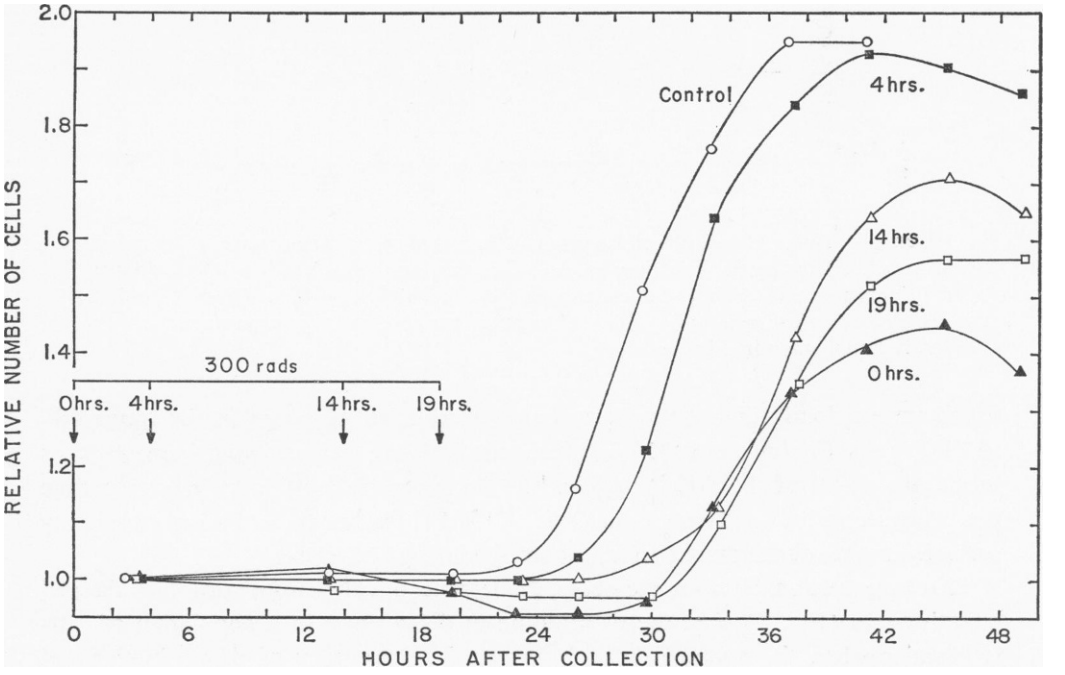

FIGURE 5 Growth curves of aliquots of a synchronous culture x-irradiated with 300 rads at the times after mitosis indicated above the arrows. The control curve refers to an unirradiated aliquot of cells.

typical growth curves for cells irradiated at four different times in the division cycle. Fig. 6 shows the delays observed in several experiments, each denoted by a different symbol; delay is expressed on a per rad basis, and the time scale has been normalized so that the control cells in each experiment have a minimum interdivisional time of 18 hours. The following observations may be noted:

(1) The extent to which cells are delayed in interphase depends strongly on the phase of the cycle at which they are irradiated (Fig. 6). Mitotic cells (zero time) show a delay of about 1 min. per rad, while cells in early G_1 show minimal delay, about 0.3 min. per rad. Thereafter delay increases progressively to about 1.4 min. per rad, just before the next mitosis. The delay of about 1 min. per rad previously

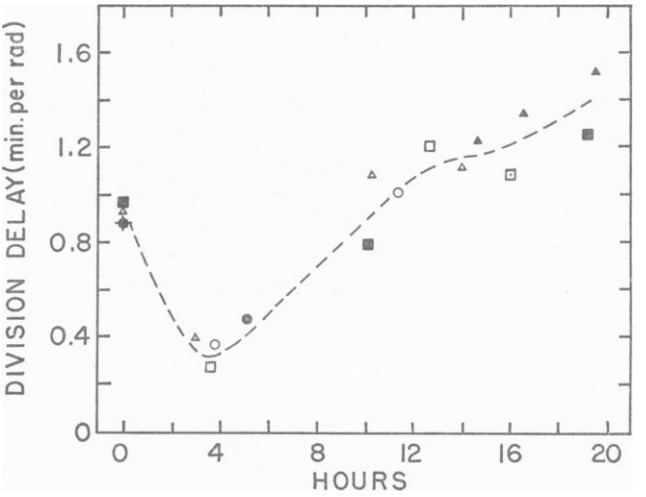

FIGURE 6 Delay in cell division measured in populations x-irradiated at the times after mitosis indicated. The time scales have been normalized to a minimum interdivisional time of 18 hours for each experiment, each denoted by a different symbol. Although delay is reported in units of minutes per rad, in most experiments only a 300 rad dose was administered.

found for randomly dividing cultures exposed to doses of x-rays in the range 400 to 2000 rads (Tolmach, 1961) is consistent with the values found here for cells in various stages of the division cycle, but the latter are considerably smaller than the delays reported by Yamada and Puck (1961). Part of these discrepancies may arise from the development of asynchrony during the cycle.

(2) In general, the longer the delay, the less steep is the rising portion of the cell growth curve (Fig. 5); *i.e.*, the distribution in delay times in a population of cells is broader when the average delay is longer. The accuracy of determinations of division delay is accordingly reduced.

(3) The fraction of cells that undergoes even one post-irradiation division may be markedly reduced when cells are irradiated in certain phases of the cycle. For

example, the growth curve for cells irradiated at zero hours, shown in Fig. 5, exhibits a significantly lower maximum than was found previously for randomly growing populations (Tolmach, 1961), even after the 300 rad dose is adjusted, on the basis of the level of reproductive survival, to a larger effective dose of radiation.

Although the foregoing data show that irradiation of mitotic cells results in a relatively long delay in the inception of the succeeding division, they do not give information about possible protraction of the mitosis in which the cells are irradiated. We have examined the effect of irradiation on the completion of this division, and find no evidence for delay (Fig. 7). That is, once mitosis is initiated, radiation does not hinder its completion in this system, as is true also in many types of animal cells (see Carlson, 1954).

Fig. 8, which extends the observations shown in Fig. 5 to longer times, illustrates another feature of growth curves of irradiated cell populations. When the observational period is sufficiently prolonged, the loss of cells through detachment and degeneration may begin to be detected. The growth curves then exhibit shapes that depend on the severity of the radiation insult. Thus, as has been pointed out previously (Tolmach, 1961), this type of measurement may magnify the extent of radiation damage. However, as the parameter scored is a complex function of reproductive inactivation, cellular degeneration, and division delay, it is not readily susceptible to interpretation. Nevertheless, the sensitivity of this measure of radiation damage may render it useful.

The decrease in the number of cells remaining attached to the surface of the culture dish, which is responsible for the falling curves of Fig. 8, has been studied in greater detail by making repeated observations of selected microscope fields during several days following irradiation. It was found that the persistence of cells irradiated with 1700 or 1830 rads did not vary significantly with the stage in the division cycle at which irradiation was carried out. This result is not unexpected in view of the relative dose-independence of persistence previously observed in random cultures (Tolmach, 1961). In this connection, the finding of Painter *et al.* (1961) that the probability of giant-cell formation is essentially independent of the stage in the division cycle at which HeLa S3 cells are irradiated is pertinent. Both giant-cell formation and persistence may be taken as indicators of the general metabolic activity of irradiated cells. Although these functions may be indirectly related to damage to a cell's reproductive apparatus (Tolmach and Marcus, 1960), their relative insensitivity to both dose and stage of the division cycle is consistent with the interpretation that they are physiologically remote from the primary radiation lesion which causes reproductive death, and that metabolic processes in general are only transiently (*e.g.* DNA synthesis) or indirectly affected by radiation insult.

C. *Effects on DNA Synthesis.* Irradiation of HeLa S3 cells in three distinct phases of the reproductive cycle, mitosis, G_1, and S, has been carried out, and

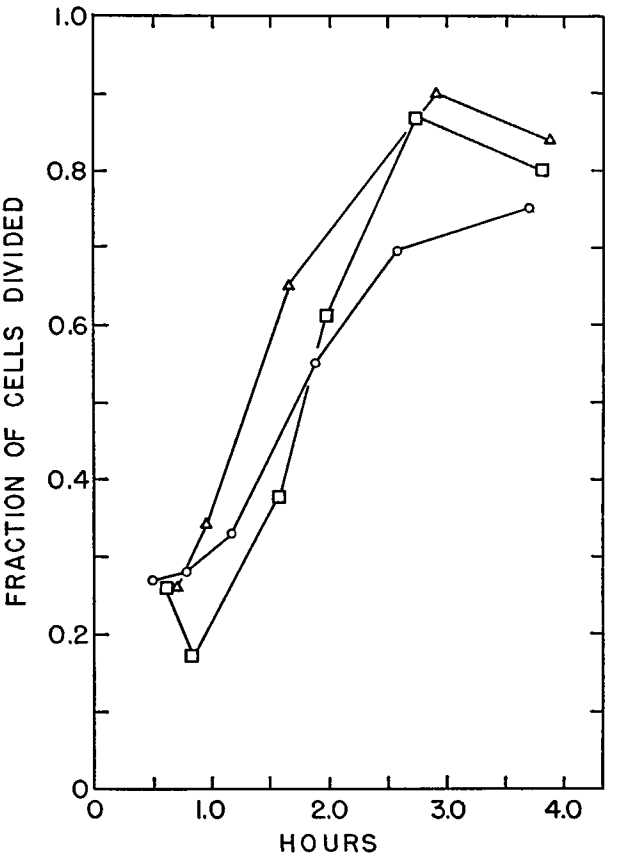

FIGURE 7 Division of cells immediately after irradiation during mitosis. Synchronized mitotic cells were harvested at room temperature (which slowed their growth), and distributed to three dishes. One was irradiated with 300 rads (squares), another with 1000 rads (triangles), and the third served as an unirradiated control (circles). At 0.5 hour, all three cultures were placed at 37°, and the proportion of cells which divided was measured periodically. After most of the cells had attached (1 hour), scoring was straightforward; prior to that time a divided cell could be detected by its dumbbell-like configuration, and an undivided cell by its large size. Failure to obtain division of 100 per cent of the cells may be attributed to the presence of a few nonsynchronous and dead cells, and to incorrect scoring of a few divided cells.

effects on the pattern of DNA synthesis determined. In most experiments, replicate cultures of synchronized cells, irradiated with a single dose of 300 rads at the desired times, were sequentially exposed for 20 minutes to growth medium containing H^3-thymidine, immediately after which they were fixed and prepared for autoradiography.

Three different functions were scored: (1) inception and (2) duration of DNA synthesis, measured by the fraction of labeled cells (upper curves of Figs. 9A

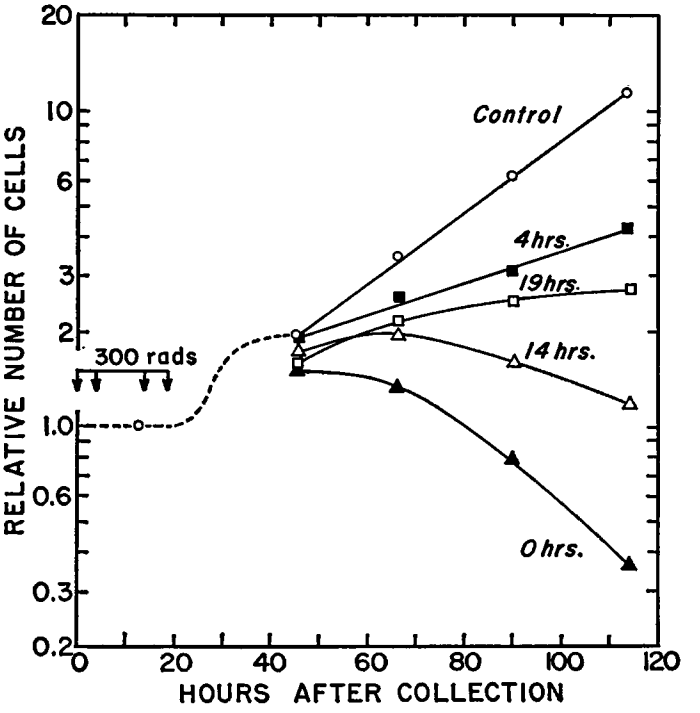

FIGURE 8 Extension of the growth curves of Fig. 5 to longer times.

and 9B), and (3) rate of DNA synthesis, measured by the relative grain count over labeled cells (midde curves). In addition, cell division (see section B) was determined in each experiment (lower curves). It has been possible, therefore, to determine which of the various phases of the division cycle are prolonged by irradiation.

The results, of which Figs. 9A and 9B are typical, reveal a complicated pattern of responses:

(1) Fig. 9A shows that cells irradiated with 300 rads while in mitosis suffer a very short delay in the inception of DNA synthesis (less than 0.2 min. per rad) and a small prolongation of S for the majority of cells. However, a small fraction of cells appears to be held in S for as long as 0.7 min. per rad. The average rate of synthesis is depressed, as shown by the grain counts between 12 and 18 hours (although not always so markedly) and it may be reasonable to suppose that the rates in those cells which are held in S are the most depressed. The delay in cell division induced by irradiation of mitotic cells is considerably longer than the delay in the inception of S; hence an appreciable G_2 delay is suffered, of the order of 0.4 min. per rad. The dependence of delay on dose has not yet been studied.

(2) Irradiation of cells in G_1 with 300 rads (Fig. 9B, triangles) causes no delay in the inception of DNA synthesis; indeed, a slightly accelerated entry of

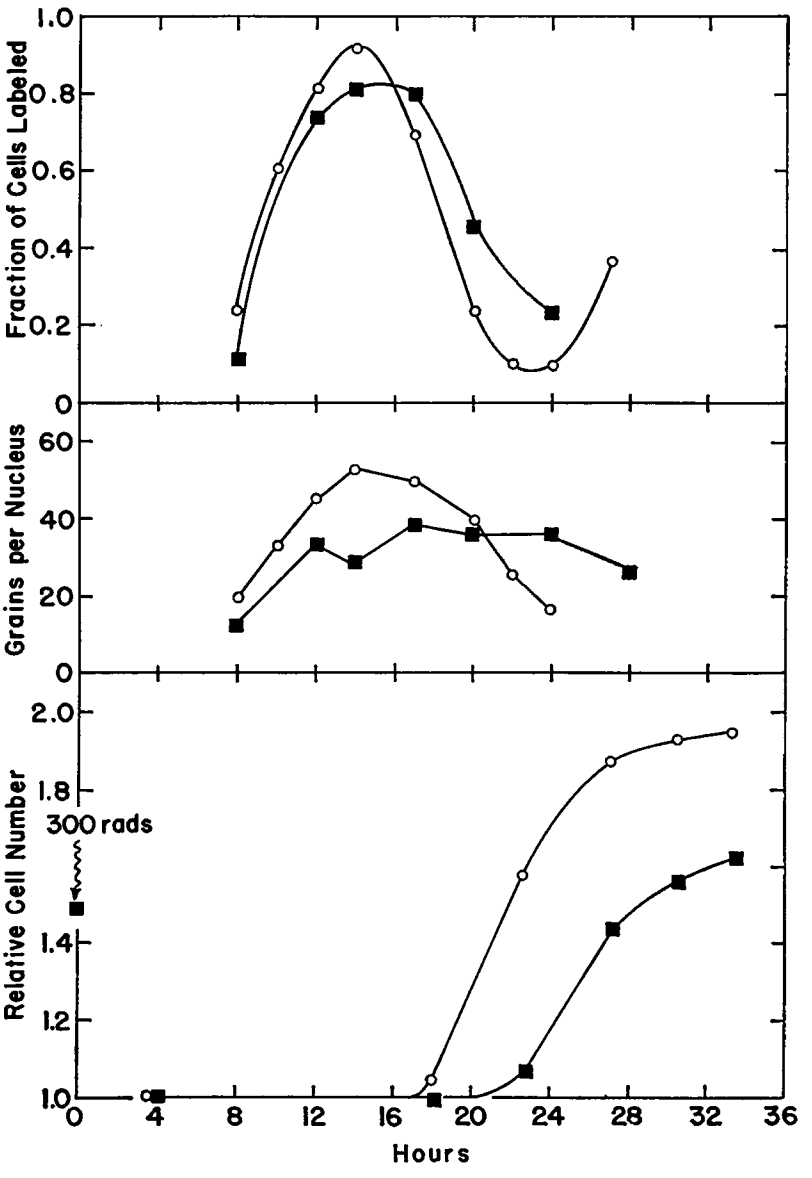

FIGURE 9A Solid squares show three effects of a 300 rad dose of x-rays administered to synchronized cells during mitosis (zero hours). The open circles refer to an unirradiated control culture. The upper curves represent autoradiographic determinations of the fraction of cells synthesizing DNA at the times shown. The middle curves show the average rate of DNA synthesis in those cells which are in the S phase. The lower curves show cell division, and reveal the delay in division discussed in section B (Effects on Cell Growth and the Induction of Division Delay).

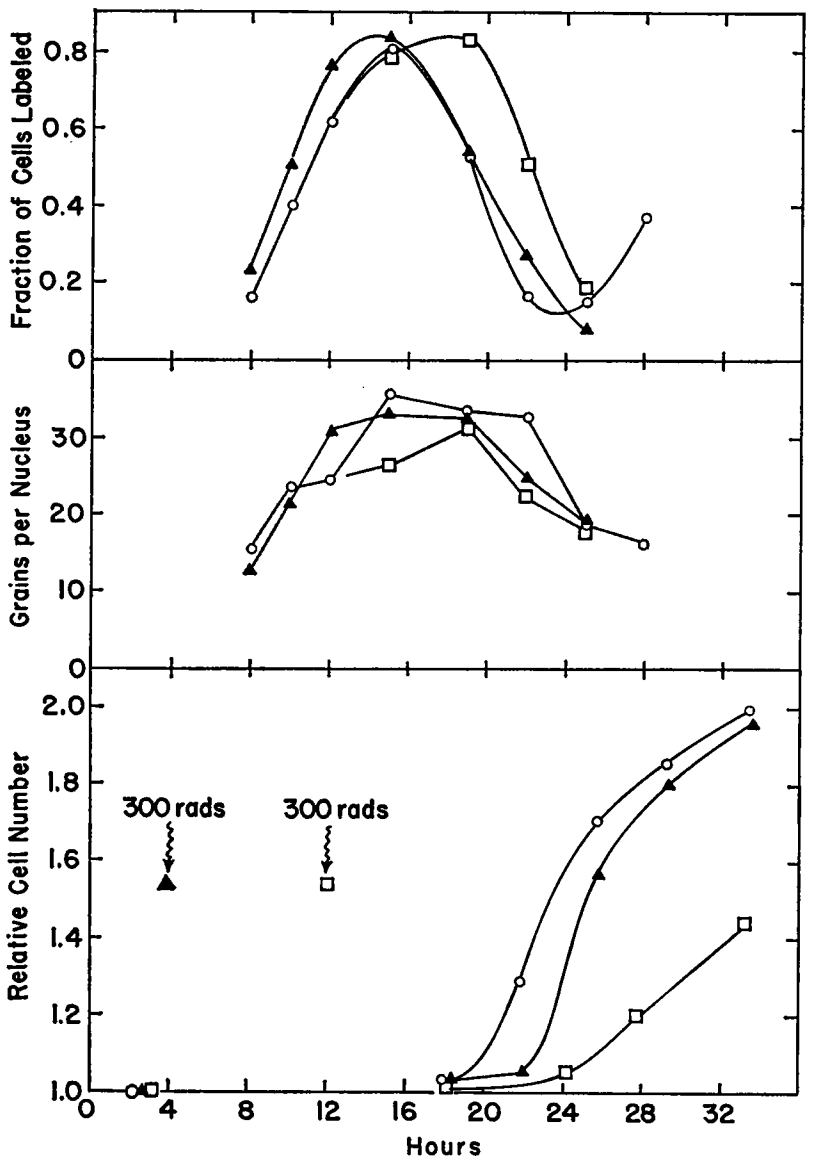

FIGURE 9B Similar to Fig. 9A, except that cells were irradiated during the G_1 phase (4 hours; triangles) or the S phase (12 hours; open squares). The open circles again refer to an unirradiated control culture.

cells into S (0.2 min. per rad) has been noted. As cells enter G_2 at the normal time, the duration of S is very slightly prolonged. The rate of DNA synthesis is very slightly depressed.

The failure of irradiation during G_1 to affect the pattern of over-all DNA synthesis was confirmed in experiments in which synthesis was measured after the administration of graded doses of x-rays between 100 and 1000 rads. In these experiments DNA synthesis was followed by measuring C^{14}-thymidine incorporation, as detected with a low background Geiger counter. It may be noted, however, that such measurements give the product of the fraction of cells labeled, times the average amount of label incorporated per cell, and would fail to show deviations from the normal pattern if the two functions were equally and oppositely modified.

The short delay in division experienced by cells irradiated in G_1 (about 0.3 min. per rad; Fig. 6) is assignable to a G_2 block entirely.

(3) Cells already synthesizing DNA at the time of irradiation with 300 rads are retained in S for an appreciable period, about 0.6 min. per rad (Fig. 9B, squares). The rate of DNA synthesis is correspondingly depressed. Furthermore, because the maximum fraction of labeled cells does not decrease, and the grain count distribution curve does not undergo any alteration in shape, but merely shifts towards lower values, it may be assumed that the radiation does not selectively affect certain of the synthesizing cells.

The possibility that this radiation-induced decrease in thymidine incorporation results from a breakdown of nucleic acid, with consequent dilution of labeled precursor, and does not indicate a reduced rate of DNA synthesis, was investigated. When cells that had been grown in the presence of C^{14}-thymidine for one generation were irradiated with 300 rads, they failed to show any loss of radioactivity. This finding suggests that we are indeed observing a reduction in the rate of DNA synthesis in irradiated cells, although other interpretations cannot be ruled out.

Again, delay of cell division in cells irradiated during S is greater than the prolongation of S, so that a G_2 delay must occur also. It is estimated to be of the order of 0.3 min. per rad, although a detailed study of its dose dependence has not yet been carried out.

From the foregoing findings, it is apparent that x-irradiation of cells in any phase of the division cycle causes a block in the G_2 period, and that a plot of prolongation of G_2 *versus* phase in the cycle, while generally falling below that for total division delay, shown in Fig. 6, would never go to zero. Although the exact shape of the curve cannot be determined from the available data, it may be that G_2 delay, in contrast to total delay, does not rise continuously from the minimum value at 3 to 4 hours to the end of G_2, but instead remains relatively small and constant throughout much of G_1 and S. However, it is not clear that any greater significance can be attached to G_2 delay than to total delay.

Although the data concerning the duration and rate of DNA synthesis are not

sufficiently precise for reliable calculation of the total amount of DNA synthesized in irradiated cells during interphase, the finding that a prolongation of S is accompanied by a decreased average rate of synthesis suggests that the total amount of DNA formed may tend to be constant.

DISCUSSION

A. *Reproductive Cell Killing.* The fluctuations in reproductive survival observed when cells are irradiated at successive times throughout the division cycle form a complex pattern, and may reflect the interplay of several kinds of radiation lesions, fluctuations of intrinsic sensitivity, a number of different repair processes, and variable periods of time available for repair of the lesions before they become irreversible. In addition, the development of asynchrony during the division cycle adds complexity to the interpretation of the experiments reported here. However, the findings will be discussed in terms of only a few of the many alternative models that can be proposed for the events that occur at each stage of the cycle.

Because cell killing is likely to be strongly correlated with damage to the cell's reproductive apparatus (Puck and Marcus, 1956), the maximal response of mitotic (zero time) cells might reasonably be attributed to the particular state of the genetic elements at that time; *e.g.*, the condensed condition of the chromosomes might lead to a high frequency of chromosomal disorganization. However, alternative explanations for increased sensitivity of mitotic cells should not be ignored; for example, the absence of a nuclear membrane could be of importance (Bozeman and Metz, 1949). The abrupt decrease in sensitivity that develops as soon as mitosis is completed is consistent with this interpretation that the mitotic state *per se* is responsible for the high sensitivity of collected cells.

Soon after cells enter interphase, sensitivity with respect to reproductive survival again begins to increase in an approximately linear fashion until the cells reach a critical time (10 hours after mitosis in the population as a whole) at which about 60 per cent of them are involved in DNA synthesis. A similar increase in recessive lethal mutation frequency has been observed in paramecia exposed to x-rays at intervals during G_1 (Kimball, 1961). A possible explanation of the increased sensitivity was suggested by Kimball, and can be adopted here for killing of HeLa S3 cells: The DNA of a cell irradiated in G_1 might suffer damage of a type that is repaired with time. This damage does not retard the cell's entry into S, as is evidenced by the absence of a G_1 delay (see section C (Effects on DNA Synthesis) and Fig. 9B). Hence, the later the cell is irradiated in G_1 the shorter will be the period available for repair before it reaches the critical time which occurs at the time of, or subsequent to, the inception of DNA synthesis. According to this hypothesis, completeness of repair would be inversely related to the number of abnormalities transferred to the newly formed DNA, and, therefore, to the probability that the cell or its progeny will not be able to reproduce.

Subsequent to the maximal sensitivity observed at 10 hours, resistance increases in parallel with the accumulation of DNA within the cells, reaching a maximum near the end of interphase at 18 hours. Recent experiments of Erikson and Szybalski (1962) with human D98/AG cells that were forced into synchronous division by treatment with fluorodeoxyuridine have also shown an increase in x-ray resistance accompanying the synthesis of DNA. This increase in resistance, which ultimately amounts to a factor of about 1.6, as measured from the change in mean lethal doses (Fig. 1), could be related to the increasing amount of DNA being replicated. Thus, at the time of maximal sensitivity 60 per cent of the population is already engaged in DNA synthesis, and rough calculation shows that on the average the cells have already synthesized about 30 per cent of the total amount of DNA that they will ultimately make. That is, the critical time appears not to coincide with the inception of DNA synthesis, but rather to occur some time later. Perhaps a minimum amount of DNA replication is needed before resistance begins to increase. Alternatively, the process which begins to be manifested at the critical time might be some sort of structural stabilization of the newly formed DNA, or a capacity of the DNA to undergo repair of radiation damage. It must be emphasized in connection with these considerations, that the asynchrony present in the culture by this time in the cycle makes difficult the precise determination of the critical time, and may distort the shape of the rising portion of curve of Fig. 4. Accordingly, the apparent correlation of resistance with DNA synthesis might be fortuitous. The possibility that a change in some cellular constituent other than DNA is responsible for the increasing resistance in this phase of the cycle cannot be excluded on the basis of currently available information.

It was of interest to determine whether an artificial cell population with a composition consistent with the data of the upper curve of Fig. 4, *i.e.*, composed of suitable numbers of 14 types of cells, each obeying 2-hit kinetics and having survival parameters estimated from data of the kind shown in Fig. 1, would in fact exhibit a survival curve of the same general shape as is shown by cultures of randomly dividing cells. Accordingly, a theoretical survival curve was constructed (Fig. 10), assuming a population with the characteristics given by Equation (1) and Table I, in which C_i is the fraction of cells with sensitivity given by the mean lethal dose, D_i, S is the surviving fraction, and D is the dose in rads.

$$S = 1 - \sum C_i(1 - e^{-D/D_i})^2 \tag{1}$$

The points in Fig. 10 could not conceivably be distinguished experimentally from a straight line over the entire 5 decades of inactivation for which survival was calculated, except for the initial shoulder. (It may be noted also that the extrapolation number of the least squares straight line fit to the points between 150 and 2000 rads is 1.5, though each component of the population was assumed to obey 2-hit kinetics.) The behavior of mixtures of cells with differing survival parameters has

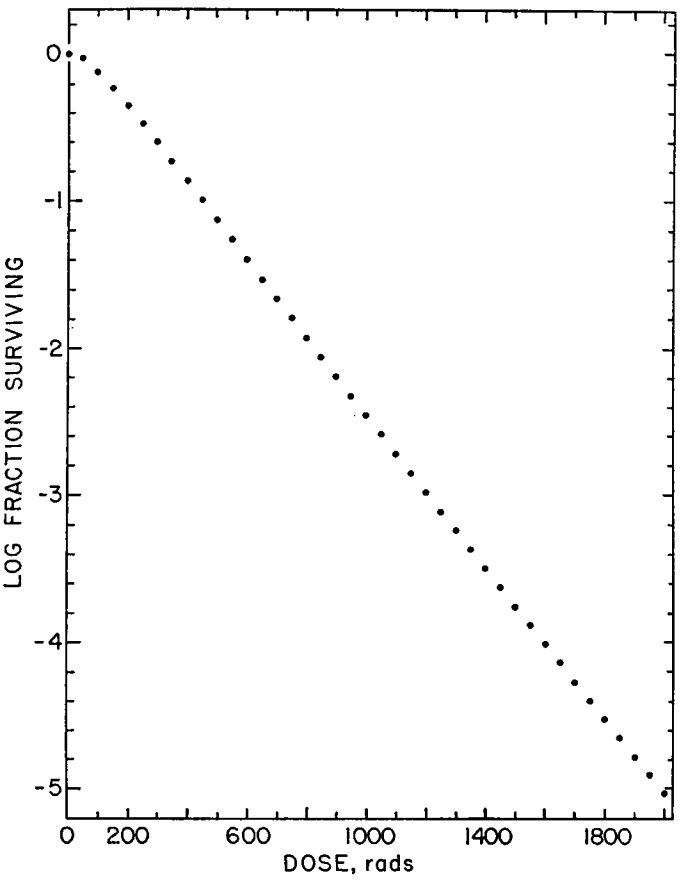

FIGURE 10 Calculated survival of a hypothetical cell population with the composition and survival characteristics given by Equation (1) and Table I.

been considered previously by Zimmer (1961) and again by Dewey and Cole (1962); cell populations which exhibit apparently homogeneous survival curves even to very low survival levels (Puck and Marcus, 1956; Hewitt and Wilson, 1959; Philpott *et al.*, 1962) may actually contain a number of components with differing survival characteristics.

B. *Division Delay.* The general pattern of division in irradiated synchronous cell populations found here, in which delay is minimal when cells are exposed to x-rays at 4 hours (or probably sooner) after mitosis, and progressively increases when they are irradiated later during interphase, is reminiscent of the situation which obtains with irradiated sea urchin eggs. Analysis of that system led Lea (1956) to postulate that recovery processes begin to operate at the time of radiation insult, and that the delay period represents the time required for recovery to be completed. As cells irradiated in G_2 have much less time available for repair

TABLE I

COMPOSITION OF HYPOTHETICAL POPULATION, BASED ON DATA OF FIG. 4, WITH THEORETICAL SURVIVAL GIVEN BY EQUATION (1) AND FIG. 10.

Time interval	Fraction of cells, C_i	Assumed mean lethal dose, D_i
hrs.		*rads*
0 to 1	0.045	90
1 to 4	0.137	180
4 to 5	0.045	170
5 to 6	0.045	160
6 to 7	0.045	150
7 to 8	0.045	137
8 to 9	0.045	125
9 to 10	0.045	115
10 to 11	0.045	111
11 to 12	0.045	122
12 to 13	0.045	133
13 to 14	0.045	145
14 to 15	0.045	155
15 to 22	0.323	165

before the next scheduled division, they exhibit maximal delay. However, delay in the succeeding division induced by irradiation of mitotic cells cannot be explained in terms of this model as it stands, because cells irradiated later in the cycle, in G_1, are delayed much less. Within the context of this model, we are forced to add the additional assumption that either mitotic cells suffer a greater amount of damage than do interphase cells, or the repair mechanism itself is damaged when mitotic cells are irradiated. We might invoke the highly condensed state of the mitotic chromosomes to rationalize the first of these assumptions, just as we have invoked it as a possible cause of greater reproductive killing, but there is little evidence that division delay results from chromosomal damage (*cf.* Yamada and Puck, 1961). Thus, although Sparrow's (1952) observations concerning mitotic inhibition in *Trillium*, and certain of Dewey and Humphrey's (1962) findings relating to division delay and chromosome damage are consistent with such a model, a possible role of centriolar damage (Rustad, 1959) or other reversible damage to cellular structures or functions in mitotic cells can not be dismissed.

C. *Effects on DNA Synthesis.* Table II summarizes our results concerning the effects of x-rays on the DNA synthetic cycle in HeLa S3.

The failure of cells irradiated during G_1 to suffer any large delay in the inception of DNA synthesis appears to be a general property of certain cell types. Thus, in agreement with others (Painter and Robertson, 1959; Yamada and Puck,

TABLE II

SUMMARY OF EFFECTS OF 300 RADS OF X-RADIATION ON THE DNA SYNTHETIC CYCLE OF HeLa S3

Cell Phase at time of irradiation	Duration of G_1	Duration of S	Rate of DNA synthesis	Duration of G_2	Total delay
M	Approximately normal	Slightly prolonged	Slightly depressed	Prolonged	Maximal
G_1	Slightly reduced?	Very slightly prolonged	Very slightly depressed	Minimally prolonged	Minimal (at 3 to 4 hrs. after mitosis)
S		Prolonged	Depressed	Prolonged	Long
G_2				Maximally prolonged	Maximal

1961), we find no delay in HeLa S3 cells irradiated with as much as 1000 rads, and Till (1961) has reported only a short delay after irradiation of mouse L cells with 2000 rads. In contrast, several other types of cells have been found to suffer a pronounced G_1 delay (Howard and Pelc, 1953; Holmes and Mee, 1954; Kelly *et al.*, 1957; Lajtha *et al.*, 1958*a*) on irradiation with doses of the order of a few hundred rads or less.

A large variability among cell types appears to exist also with respect to depression of DNA synthesis in cells that are irradiated when they are in the S period. Depression of synthesis has been reported in several cell types (Lajtha *et al.*, 1958*a*; van Lancker, 1959; Sherman and Quastler, 1960; Cattaneo *et al.*, 1960; Till 1961), as in the HeLa S3 system discussed here and by Painter (1962), but was not observed in others (Howard and Pelc, 1953; Kelly *et al.*, 1957; Dickson *et al.*, 1958).

Elucidation of the basis for these differences must await further investigation (Seed 1961; Quastler, 1962). It is not yet even known which cellular components suffer the radiation-produced lesion(s) responsible for these G_1 and S effects, although several reports (Ord and Stocken, 1958; Creasey and Stocken, 1959; Lajtha *et al.*, 1958*b*; Bollum *et al.*, 1960; Foster and Ord, 1962) tend to implicate both DNA itself and enzymatic processes concerned with the synthesis of DNA or its precursors.

In this connection, it is pertinent to inquire as to the reliability of labeled thymidine incorporation as a measure of DNA synthesis (Quastler, 1962; Hell *et al.*, 1960; Newton *et al.*, 1962). Acceptance of such reliability is implicit in the work reported here, and as stated above, experiment has shown no labilization of DNA following irradiation, in contrast with the findings of Newton *et al.* (1962) with

virus-infected HeLa cells. Nevertheless, the possibility that irradiation does indeed result in the breakdown of nucleic acids, with consequent dilution of the exogenous thymidine label, must be entertained. Also to be considered is the possibility that x-irradiation differentially affects the preformed and *de novo* pathways of thymidylic acid synthesis.

Hopefully, detailed analyses of a diversity of cell types, making use of synchronous cultures such as were employed here with HeLa S3, will provide the information needed to resolve these problems. Also to be determined is the relation, if any, that radiation-induced perturbations of the normal DNA synthetic cycle bear to the lethal effects of ionizing radiation and to division delay.

We are grateful to Mrs. Mary Hasler and Miss Mae Ellen Feldman for technical assistance, and to Mr. Don Franz of the Washington University Computer Center for calculating the values plotted in Fig. 10.

This investigation was supported by Research Grant No. C4483 from the National Cancer Institute, United States Public Health Service.

Received for publication, July 23, 1962.

REFERENCES

BARENDSEN, G. W., 1962, *Nature*, **193**, 1153.

BOLLUM, F. J., ANDEREGG, J. W., MCELYA, A. B., and POTTER, V. R., 1960, *Cancer Research*, **20**, 138.

BOZEMAN, M. L., and METZ, C. W., 1949, *Genetics*, **34**, 285.

CARLSON, J. G., 1954, *in* Radiation Biology, (A. Hollaender, editor), New York, McGraw-Hill, **1**, 763.

CATTANEO, S. M., QUASTLER, H., and SHERMAN, F. G., 1960, *Radiation Research*, **12**, 587.

CREASEY, W. A., and STOCKEN, L. A., 1959, *Biochem. J.*, **72**, 519.

DEWEY, W. C., and COLE, A., 1962, *Nature*, **194**, 660.

DEWEY, W. C., and HUMPHREY, R. M., 1962, *Radiation Research*, **16**, 503.

DICKSON, M., PAUL, J., and DAVIDSON, J. N., 1958, *Biochem. J.*, **70**, 18.

DONIACH, I., and PELC, S. R., 1950, *Brit. J. Radiol.*, **23**, 184.

ERIKSON, R. L., and SZYBALSKI, W., 1962, *Radiation Research*, in press.

FOSTER, R., and ORD, M. G., 1962, *Nature*, **194**, 883.

HAM, R. G., and PUCK, T. T., 1962, *in* Methods in Enzymology, New York, Academic Press, Inc., **5**, 90.

HARRINGTON, H., 1961, *Ann. New York Acad. Sc.*, **95**, 901.

HELL, E., BERRY, R. J., and LAJTHA, L. G., 1960, *Nature*, **185**, 47.

HEWITT, H. B., and WILSON, C. W., 1959, *Brit. J. Cancer*, **13**, 69.

HOLMES, B. E., and MEE, L. K., 1954, *in* Radiobiology Symposium (Z. M. Bacq and P. Alexander, editors), New York, Academic Press, Inc., 220.

HOOD, S. L., and NORRIS, G., 1961, *Radiation Research*, **14**, 705.

HOWARD, A., and PELC, S. R., 1953, *Heredity*, suppl. 6, 261.

KELLY, L. S., HIRSCH, J. D., BEACH, G., and PETRAKIS, N. L., 1957, *Proc. Soc. Exp. Biol. and Med.*, **94**, 83.

KIMBALL, R. F., 1961, *J. Cell. and Comp. Physiol.*, **58**, suppl. 1, 163.

KOHN, H. I., and FOGH, J. E., 1959, *J. Nat. Cancer Inst.*, **23**, 293.

LAJTHA, L. G., OLIVER, R., KUMATORI, T., and ELLIS, F., 1958*a*, *Radiation Research*, **8**, 1.

LAJTHA, L. G., OLIVER, R., BERRY, B., and NOYES, W. D., 1958*b*, *Nature*, **182**, 1788.

LEA, D. E., 1956, Actions of Radiations on Living Cells, 2nd edition, Cambridge University Press.
MARCUS, P. I., 1959, *Virology*, **9**, 546.
MARCUS, P. I., and PUCK, T. T., 1958, *Virology*, **6**, 405.
MESSIER, B., and LEBLOND, C. P., 1957, *Proc. Soc. Exp. Biol. and Med.*, **96**, 7.
NEWTON, A., DENDY, P. P., SMITH, C. L., and WILDY, P., 1962, *Nature*, **194**, 886.
ORD, M. G., and STOCKEN, L. A., 1958, *Nature*, **182**, 1787.
PAINTER, R. B., 1962, *Radiation Research*, **16**, 846.
PAINTER, R. B., MCALPINE, V. W. R., and GERMANIS, M., 1961, *Radiation Research*, **14**, 653.
PAINTER, R. B., and ROBERTSON, J. S., 1959, *Radiation Research*, **11**, 206.
PERSON, S. R., TOLMACH, L. J., and SATO, E., 1961, *Radiation Research*, **14**, 491.
PHILPOTT, G. W., SHAEFFER, C. W., and TOLMACH, L. J., 1962, *Radiation Research*, **17**, 508.
PUCK, T. T., and MARCUS, P. I., 1956, *J. Exp. Med.*, **103**, 653.
PUCK, T. T., MARCUS, P. I., and CIECIURA, S. J., 1956, *J. Exp. Med.*, **103**, 273.
QUASTLER, H., 1962, *in* Chemical and Biological Effects of Radiations, (Haissinsky, editor), in press.
RUSTAD, R. C., 1959, *Radiation Research*, **11**, 465.
SEED, J., 1961, *Nature*, **192**, 944.
SHERMAN, F. G., and QUASTLER, H., 1960, *Exp. Cell Research*, **19**, 343.
SPARROW, A. H., MOSES, M. J., and DUBOW, R. J., 1952, *Exp. Cell Research*, suppl. 2, 245.
STANNERS, C. P., and TILL, J. E., 1960, *Biochim. et Biophysica Acta*, **37**, 406.
SWANN, M. M., 1962, *Nature*, **193**, 1222.
TERASIMA, T., and TOLMACH, L. J., 1961, *Nature*, **190**, 1210.
TERASIMA, T., and TOLMACH, L. J., 1962, *Exp. Cell Research*, in press.
TILL, J. E., 1961, *Ann. New York Acad. Sc.*, **95**, 911.
TOLMACH, L. J., 1961, *Ann. New York Acad. Sc.*, **95**, 743.
TOLMACH, L. J., and MARCUS, P. I., 1960, *Exp. Cell Research*, **20**, 350.
VAN LANCKER, J. L., 1959, *Biochim. et Biophysica Acta*, **33**, 587.
WHITMORE, G. F., STANNERS, C. P., TILL, J. E., and GULYAS, S., 1961, *Biochim. et Biophysica Acta*, **47**, 66.
WOODARD, J., RASCH, E., and SWIFT, H., 1961, *J. Biophysic. and Biochem. Cytol.*, **9**, 445.
YAMADA, M., and PUCK, T. T., 1961, *Proc. Nat. Acad. Sc.*, **47**, 1181.
ZIMMER, K. G., 1961, Studies on Quantitative Radiation Biology, Edinburgh, Oliver and Boyd, Ltd., chapter 2.