RADIOSENSITIVITY OF MAMMALIAN CELLS

I. TIMING AND DOSE-DEPENDENCE OF RADIATION-INDUCED DIVISION DELAY

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ABSTRACT The time of onset and duration of division delay induced by exposure to 250-kvp x-irradiation have been measured in several mammalian cell lines grown in suspension culture. Unique times of action (i.e. interval from irradiation to cessation of division) late in G_2 are characteristic for HeLa, L-5178Y, and Chinese hamster cells, and the time of action is independent of dose over the range 25–800 rads. The duration of delay was directly proportional to dose; all irradiated cells divided at least once and maintained their relative positions in the life cycle for periods exceeding one generation time. Neither random nor synchronous cultures exposed at varying times in the life cycle exhibited differences in radiation sensitivity measured either by onset or duration of the delay period. The time of action was experimentally indistinguishable from the point marking completion of protein synthesis essential for division, leading to speculation that division delay involves a translation defect.

INTRODUCTION

Radiation-induced division and mitotic delay have been extensively studied in several laboratories (Revesz, 1955; Kohn and Fogh, 1959; Elkind et al., 1961; Whitmore et al., 1961; Dewey et al., 1963; Elkind et al., 1963; Puck and Steffan, 1963; Terasima and Tolmach, 1963; Whitmore et al., 1967). Although it is conceded that duration of the division delay period is a dose-dependent property of the cell line employed, the nature of damage leading to division delay and of recovery therefrom is still unknown. Before any predictions can be made regarding the nature of division delay, the response to irradiation of cells in each phase of the life cycle must be characterized very precisely (Puck and Steffan, 1963). Subsequent correlation of this response with known biochemical events in the life cycle may provide some insight into the cellular processes involved in division delay.

Life-cycle analysis techniques made possible by high-precision, automated, cell-counting methods have permitted accurate description of several temporal markers in the G_2 segment of the life cycle (Tobey et al., 1966). The precise timing afforded

by these techniques has enabled us to examine division delay in terms of both time of onset and duration in several established mammalian cell lines. The results indicate that onset of the delay period (time of action) is dose independent, that the duration of delay is proportional to dose in the range 25–800 rads, and that the terminal point of action is coincident with the end of protein synthesis essential for division (Tobey et al., 1966). By employing synchronized cultures to segregate successive generations, it has been possible to demonstrate that Chinese hamster cells are equally sensitive to division delay throughout their life cycle—in contrast to several published reports of differential radiosensitivity depending upon position of the cell in the life cycle (Harrington, 1961; Dewey and Humphrey, 1962; Hsu et al., 1962; Mak and Till, 1963; Puck and Steffan, 1963; Terasima and Tolmach, 1963; Dewey et al., 1966; Firket and Mahieu, 1966; Froese, 1966; Whitmore et al., 1967)—and to speculate that the biochemical defect resulting in division delay is completely reversible and probably unrelated to the genetic defect ultimately resulting in demise of the irradiated cell. The defect may involve a process(es) in translation of functional protein molecules.

MATERIALS AND METHODS

Cell Culture

Three cell lines cultivated in suspension were employed for these studies: Chinese hamster (CHO) (Tjio and Puck, 1958), HeLa S-3 (Ham and Puck, 1962), and a murine lymphoma, L-5178Y (Fischer, 1958). Chinese hamster cells were grown in F-10 medium (Ham, 1963) supplemented with 10% calf and 5% fetal calf sera; HeLa S-3 were cultured in Eagle's minimum essential medium (MEM) plus 5% calf serum; and L-5178Y cells were grown in Manson's L-1 modification of Fischer's medium plus 5% calf serum. All growth media contained 100 units/ml of penicillin and 100 μg/ml of streptomycin. Cultures were routinely checked for PPLO contamination by the method of Chanock et al. (1962) and were demonstrated to be free from PPLO during the course of these studies.

Cell Counting Procedure

2 ml of cell suspension were diluted to 12 ml aliquots and counted in an electronic cell counter with a 100 $\times$ 100-μ aperture. All sampling and counting operations were performed with a 5 min cycle time in an automatic closed-system sampling device described in detail by Anderson et al. (1966). Total cell counts ranged from 0.5 to 0.8 $\times$ 10^5; therefore, errors were less than 0.5% for all measurements and analysis of the reproducibility showed that a precision of 0.5% or better was consistently obtained.

Irradiation of Cell Cultures

Jacketed spinner flasks containing 300 ml of exponentially-growing cells at a concentration of approximately 10^5 cells/ml were irradiated at 37°C. The cells were under continuous agitation during exposure. The exposures were delivered with a General Electric Maxitron X-ray therapy unit (General Electric Company, X-Ray Dept., Milwaukee 1, Wis.) operating at 250 kvp and 30 ma with Thoraeus II filtration, equivalent half-value layer 2.6 mm Cu. Exposures

were delivered at rates of 50 and 150 rads per min, and readings made with a 100-R Victoreen chamber (Victoreen Instrument Co., Cleveland, Ohio) were converted to absorbed dose at the center of each flask after correcting for attenuation of the beam by the solution.

Life-Cycle Analysis

A detailed discussion of the theory of life-cycle analysis by cell counting and a comparison with more conventional methods establishing its utility have been presented elsewhere (Tobey et al., 1966).

Synchronization of Chinese Hamster Cell Growth

Chinese hamster cells in suspension culture were partially synchronized by reversible inhibition of DNA synthesis with a single blockade in excess thymidine (Petersen and Anderson, 1964). Thymidine was added to logarithmically-growing cells to a final concentration of 10 mM. The cells were incubated 12 hr, centrifuged, and resuspended in normal medium. The resultant rate of division during the synchronous burst was three times greater than the division rate of the parent asynchronous population.

Quantities of up to 700 ml of metaphase mitotic cells (0.5–1.0 $\times$ 10^5 cells/ml) were prepared by mechanically detaching mitotic cells from monolayer cultures with a mechanical shaker (Tobey et al., 1967 *a*; Petersen et al., 1968). After stripping dead cells and debris from monolayers grown in six Pyrex Blake bottles (Corning Glass Works, Corning, N. Y.), detached cells collected at 10 min intervals over a period of 4 hr were cooled immediately after detachment to 0° in an ice bath. Upon resuspension of the pooled collections in warm medium, the cells divided synchronously. Fractions of mitotic cells of 0.9 or greater were routinely obtained.

RESULTS

Division Delay and Recovery in Exponentially-Dividing Cultures

Temporal relationships between irradiation and cessation of cell division for Chinese hamster, HeLa, and murine lymphoma cells are summarized in Fig. 1. The exponential growth rate was accurately established in each experiment before the cultures were exposed to doses of 250-kvp X-rays ranging from 25–800 rads. The populations were monitored with the particle counter at frequent intervals to establish both the times of cessation and spontaneous resumption of cell division. The time of onset of the division delay period (interval from irradiation to cessation of division) was 56 $\pm$ 4 min in the Chinese hamster cell, 71 $\pm$ 6 min in HeLa S-3, and 62 $\pm$ 4 min in L-5178Y cells. These times of action are highly reproducible, completely dose independent over the range studied, and all clearly in late G_2 rather than in the mitotic period. Thus, there exists a time late in G_2 when cells become insensitive to radiation and will divide at the *control rate* after irradiation. Cells past this point have evidently completed all preparation for division and will divide despite exposure to large doses of radiation, while younger cells will be inhibited from dividing.

The duration of delay measured as the interval between cessation and resumption

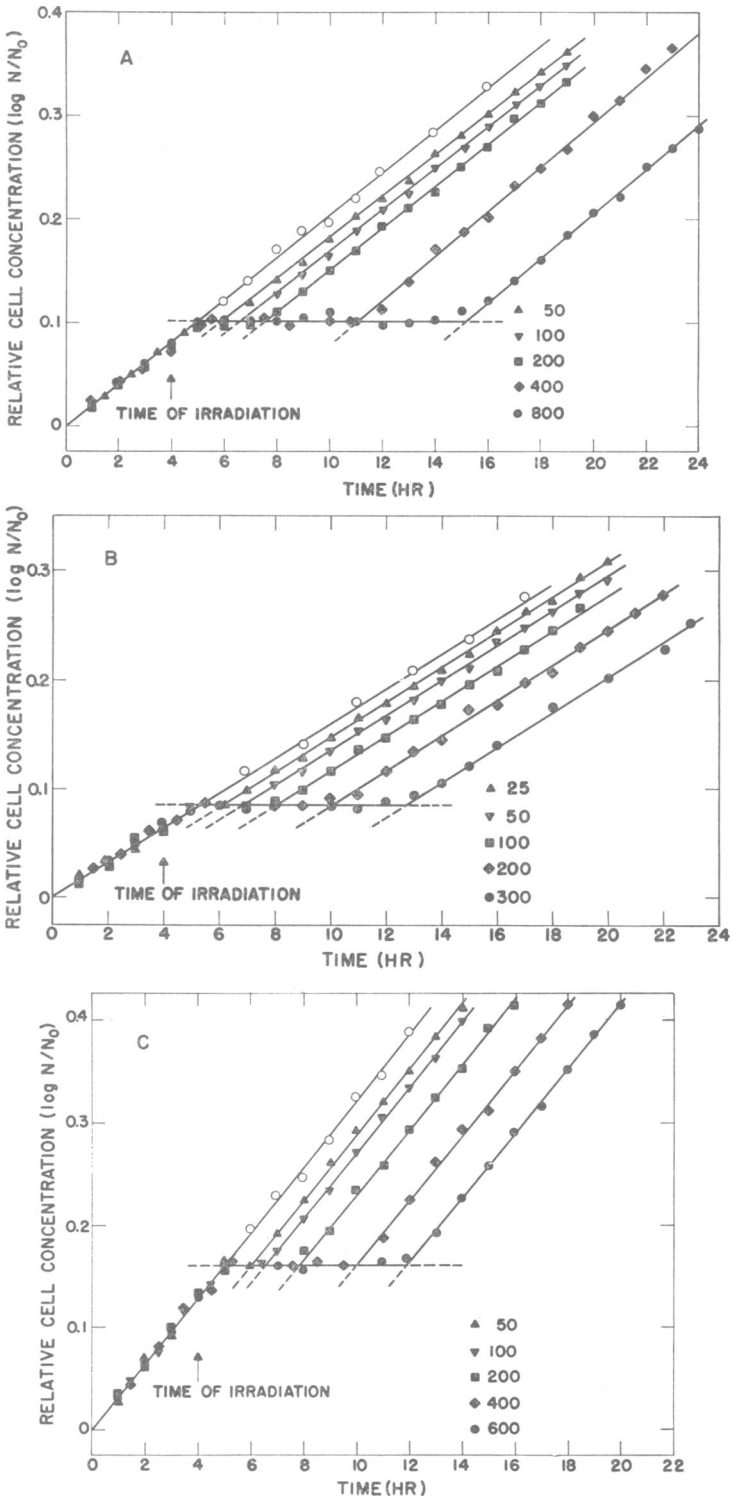

FIGURE 1 Effect of x-irradiation on asynchronous cultures of mammalian cells. Chinese hamster (*A*), HeLa S-3 (*B*), and L-5178Y (*C*) cells were irradiated at the times indicated by the arrows with the exposures (in rads) shown in the figures. The open circles denote control cultures, and the closed symbols represent irradiated cultures.

of division was obtained from the data in Fig. 1, and the data are plotted in Fig. 2 where the delay period is expressed as a function of dose. It is clear that there is a linear relationship between the induced delay period and the dose (Elkind et al., 1963). From these data it appears that the HeLa S-3 line, delayed approximately 0.024 hr/rad, is twice as sensitive as the two lines of rodent origin where the delay is 0.012 hr/rad. These delay values are in reasonable agreement with the results obtained by others (Puck and Yamada, 1962; Elkind et al., 1963; Puck and Steffan, 1963; Terasima and Tolmach, 1963).

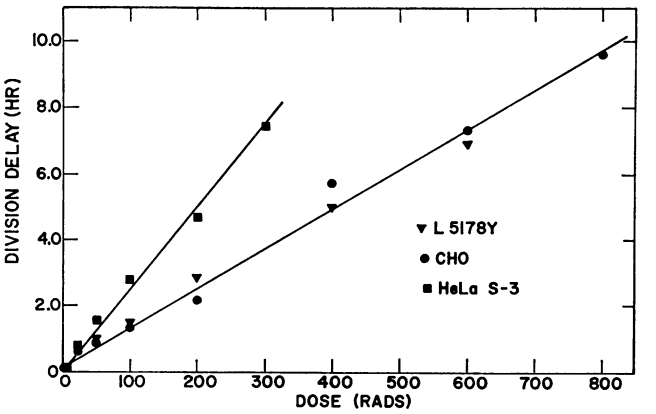

FIGURE 2 Dose dependence of radiation-induced division delay in three mammalian cell lines. The data were fitted by standard regression analysis techniques. Correlation coefficients for the linear fit to the data were 0.98 for all three cell lines.

Radiosensitivity as a Function of Position in the Life Cycle

Inspection of the growth curves of irradiated cells following spontaneous recovery, particularly in those experiments where the radiation doses were relatively small, suggests that an important aspect of the delay response (i.e. differential radiosensitivity) cannot be confirmed even in cell lines used in previous studies (Puck and Steffan, 1963; Terasima and Tolmach, 1963). The data summarized in Fig. 3 demonstrate that, following recovery from 25 rads, the growth curve of Chinese hamster cells never exhibits significant fluctuations from the previously established exponential growth curve. It is simply displaced by an interval equivalent to the delay period and never approaches the original growth curve. This observation is more consistent with the idea that all cells are equally affected by radiation than with models proposing differential radiosensitivity, in which different rates of traverse of the life cycle should result in parasynchronous bursts of division and nonexponential behavior of the growth curve.

The effect of position of the cell in the life cycle was examined by irradiating Chinese hamster cells synchronized by treatment with 10 mM of thymidine at various

intervals after synchrony induction. The results, shown in Fig. 4, indicate that the delay period was constant, regardless of the position of the cell in the life cycle at the time of irradiation, and that survival was essentially quantitative for at least two subsequent generations. It is also apparent that the cells maintained their relative position in the life cycle during the delay period. The growth curves of irradiated cells mimicked that of the control (including a synchronous wave of division preceded by division of cells originally trapped in S by thymidine) but were simply displaced in time trailing the control.

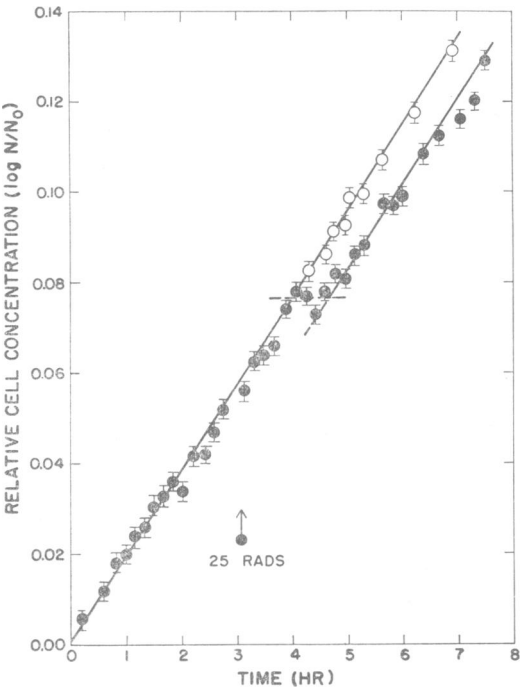

FIGURE 3 Effect of very small exposures on asynchronous Chinese hamster cells. Cells were irradiated with 25 rads at the time indicated by the arrow. The open circles denote the control, and the closed circles represent the irradiated sample. No tendency of the cells to return to the preirradiation growth curve was noted, nor was such the case at times much longer than those shown here ($1\ \sigma = 0.5\%$).

There was still the possibility that treatment with thymidine might alter the response of the population to radiation. Our modification of Terasima and Tolmach's detachment procedure (Tobey et al., 1967 *a*; Petersen et al., 1968) produces populations free of chemical perturbation. These mitotically-synchronized Chinese hamster populations were exposed to 150 rads at various times in the life cycle, and the delay in reaching the subsequent division was measured. The data are shown in Fig. 5, where two features are immediately obvious. First, regardless of the position of the cell in the life cycle, the duration of the delay period was constant and was also the same as that obtained with thymidine-synchronized cells. The last irradiation point represents a population in G_2 where a small fraction had passed the terminal point of sensitivity in late G_2. Those cells closer to division than 0.9 hr divided

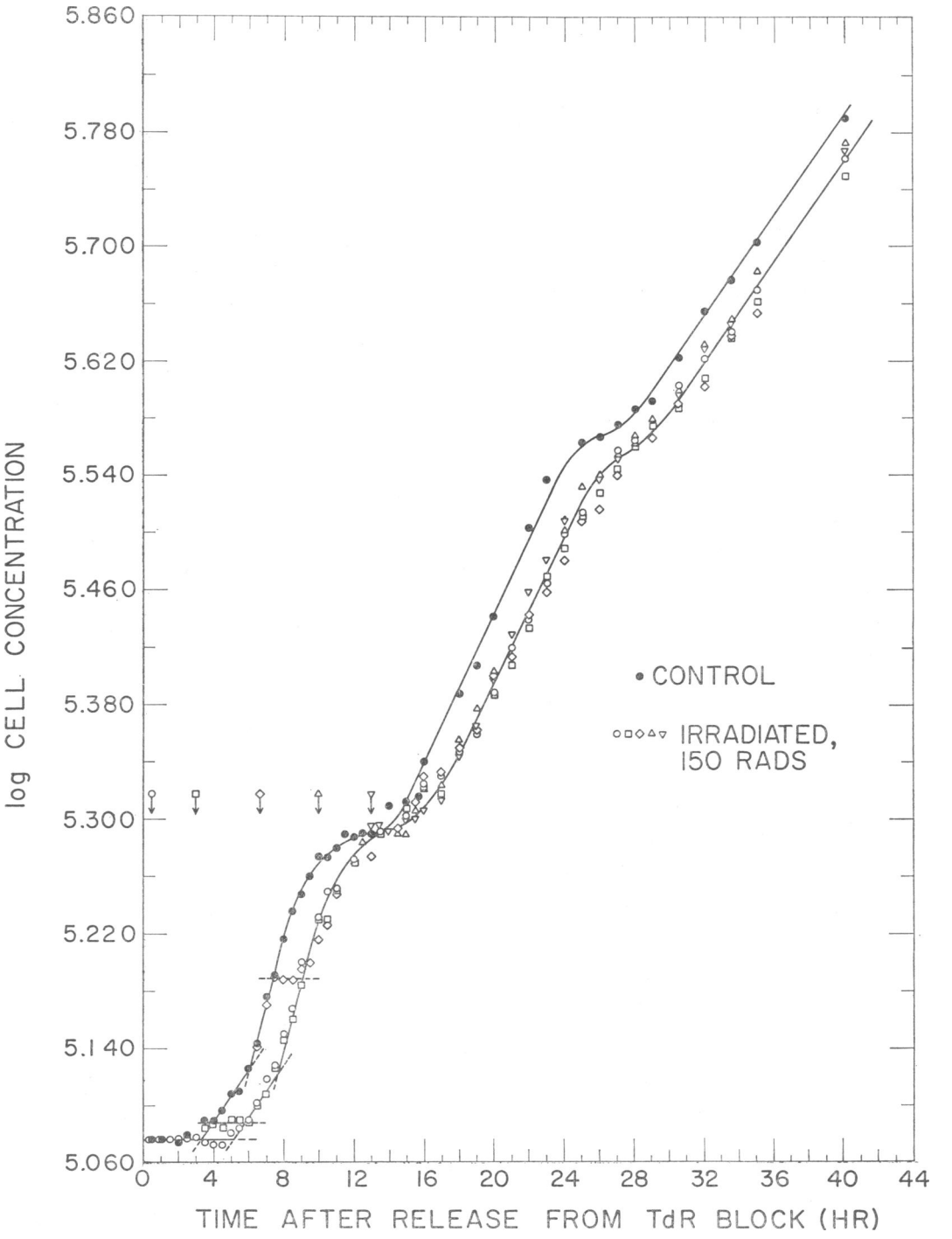

FIGURE 4 Effect of x-irradiation on thymidine-synchronized cultures of Chinese hamster cells. After removal of thymidine, cells were irradiated at the times indicated by the arrows. The approximate distribution of cells in the life cycle at each time of irradiation was: all in S (open circles); 68% in late S, 32% in G_2 (squares); 69% in late G_2, 28% in G_1 (diamonds). 95% in G_1, 2% in S (triangles); and 58% in late G_1, 42% in S (closed diamonds).

without delay, while the remainder was delayed for the same length of time as those cells exposed earlier in the cycle. Second, the total number of irradiated cells dividing was the same in all populations, indicating that again all cells survived the initial treatment and subsequently divided at least once, in agreement with Watanabe and Okada (1966). When traverse was resumed, cells proceeded at precisely the same rate as in the unirradiated culture.

Because cells irradiated 0.9 hr or less prior to division divide at the control rate in both random and synchronized populations it is evident that cells 0.9 hr or closer to division are resistant to radiation-induced division delay. However, the magnitude

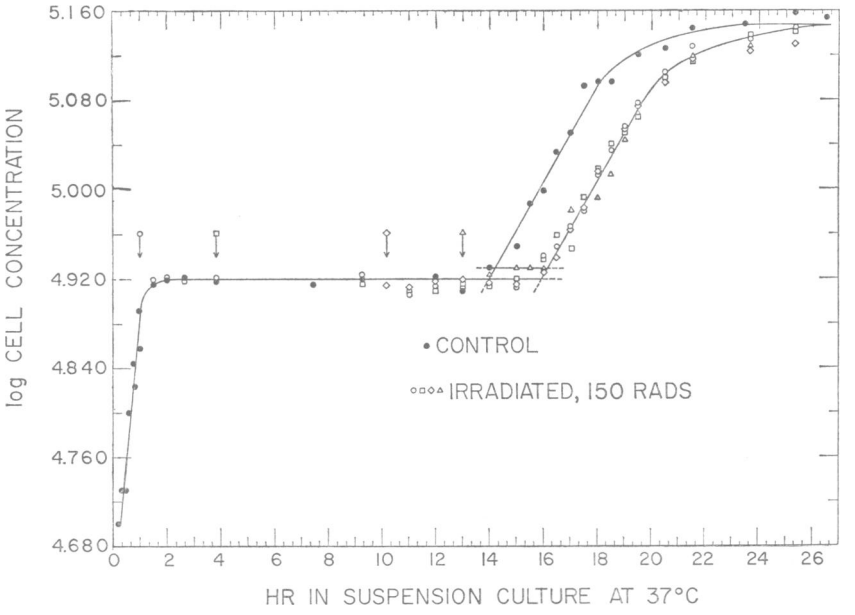

FIGURE 5 Effect of x-irradiation on mitotically-synchronized Chinese hamster cells. Cells were irradiated at the times after mitosis indicated by the arrows. Cells irradiated at 1 hr (open circles) and 4 hr (squares) were in early G_1 and late G_1, respectively. Cells irradiated at 10 hr (diamonds) were largely in S, and cells irradiated at 13 hr (triangles) were mostly in G_2.

of the effect of radiation, if any, on these cells after division is of interest. This was investigated by accumulating mitotic Chinese hamster cells in the cold, irradiating, and immediately warming them to 37°C. Data in Fig. 6 summarize the results from an experiment in which one-third of the selected mitotic culture was held as a control, one-third was irradiated in mitosis, and one-third was exposed in late G_2. As expected, the cells irradiated in mitosis divided at the control rate without delay but were delayed in completing subsequent division by exactly the same interval as cells irradiated in G_2, the period which has been reported as the most sensitive interval (Puck and Steffan, 1963; Terasima and Tolmach, 1963). Thus, in the Chinese

hamster cell cycle, there exists no period that is more radiosensitive than any other. Cells irradiated during the portion of the life cycle 0.9 hr or closer to division divided at the control rate but were delayed after cytokinesis by the same time as cells irradiated during interphase.

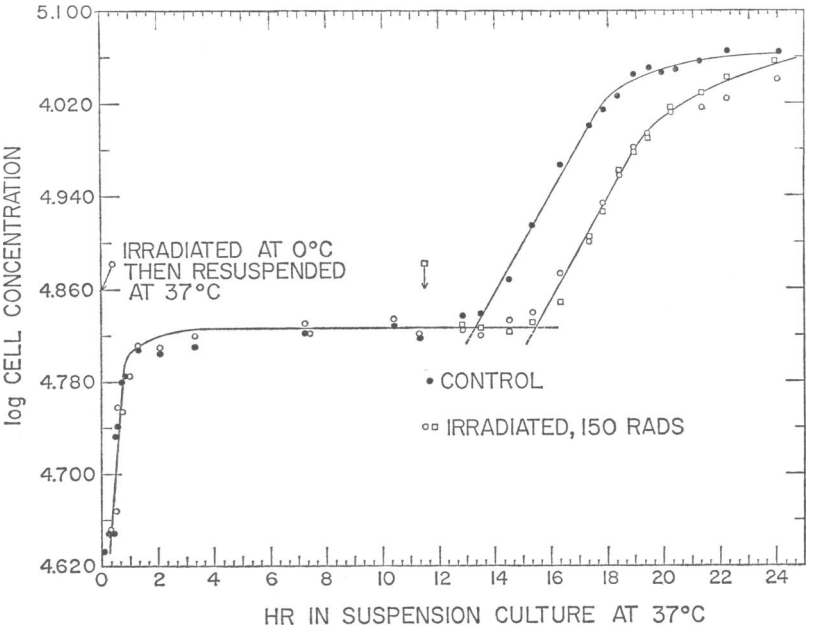

FIGURE 6 Effect of x-irradiation on mitotic Chinese hamster cells. Cells were accumulated in the cold, and an aliquot was irradiated while in mitosis (open circles). All cells, including the controls (closed circles) and those irradiated later in G_2 (squares), were then resuspended at 37°C.

DISCUSSION

The life-cycle analysis technique employed for these studies provides a means for establishing the terminal point of action of ionizing radiations in the cellular life cycle with a high degree of precision. The experiments have demonstrated that the terminal point of action of irradiation is temporally indistinguishable from the point locating completion of terminal protein biosynthesis essential for division (Tobey et al., 1966). The point is clearly in G_2 and occurs at precisely the same time in the life cycle following exposure to radiation doses ranging from 25–800 rads; the effects of irradiation are spontaneously reversible after times which are proportional to the radiation dose employed. These data are thus in agreement with the salient growth features of several previous studies (Elkind et al., 1963; Elkind and Whitmore, 1967) but differ with results of the latter in two important aspects: namely, the relative radiosensitivity of cells as a function of position in the life cycle, and the extent to

which recovery occurs as measured by division rate and number of cells which divide subsequent to spontaneous recovery.

Although the present technique provides a sensitive means for assessing the prompt response of cells exposed to ionizing radiations and their immediate fate, no clear picture can be deduced concerning their continued proliferative capacity, and no conclusions based on these observations can be drawn and compared with studies based on survival. Thus, we must emphasize that the results bear only on immediate biochemical consequences of radiation exposure relevant to division delay.

Discussions of experimental consequences of age-dependent delays have been presented for HeLa cells by Terasima and Tolmach (1963) and Puck and Steffan (1963) and for L cells by Whitmore et al. (1967). The delay per rad for 14 and 19 hr exposures of Terasima and Tolmach's synchronized HeLa cells is approximately 0.026 hr, and is in excellent agreement with the present figure for HeLa S-3 cells of 0.024 hr/rad; only in the case of cells 4 hr past mitotic selection in early G_1 was the delay per rad significantly less, indicating an insensitive peak. The present experiments agree with all points of the previous study except for the reduced radiosensitivity in G_1. This sensitive portion is not detectable either in (*a*) a random population as a parasynchronous burst of division in any of the cell lines employed, or (*b*) in the case of Chinese hamster cells as a shorter delay time compared to cells exposed at other times in the life cycle using cells synchronized by mitotic selection or by thymidine treatment. These data argue that no time in the life cycle is appreciably more sensitive than any other.

One possible explanation for the discrepancy between these data and previous results from several laboratories stems from a basic difference in technique which may have both numerical and biological implications. Essentially all of the division (or mitotic) delay experiments which have exhibited cycle position-dependent radiosensitivity (reviewed by Sinclair, 1968) have been performed on monolayer cultures. However, the present experiments and those of Elkind et al. (1963), which are in essential agreement, have employed suspension cultures. In the case of suspension cultures, mitotically- and thymidine-synchronized Chinese hamster cells exposed to x-irradiation yielded the same number of cells as the control cultures, showing that no cells dropped out of the population as a result of irradiation. Although Chinese hamster cultures routinely yield only an $\sim$ 70% cell number increase after synchronization with either thymidine or mitotic selection, this result can largely be explained by delayed cytokinesis (Tobey et al., 1967 *b*) and is not appreciably affected by either treatment with x-irradiation or a variety of antibiotic inhibitors (Tobey et al., 1966).

In a comparable experiment with synchronized HeLa S-3 on monolayer cultures (Terasima and Tolmach, 1963) only a fraction of the irradiated cells which exhibited a cycle position-dependent delay divided. Indeed, cell-cell interactions of the kind described by Froese (1966) may operate to a significant extent in monolayers to

affect the early behavior of an irradiated cell. The number of cells completing the first postirradiation division might very well affect the results obtained on measurement of the delay period. Whitmore et al. (1967) observed a differential delay of irradiated random cells about the life cycle but found that fluorodeoxyuridine-synchronized cells showed no cycle dependence. It is interesting that the yield of fluorodeoxyuridine-synchronized cells was higher than that obtained from random cells.

Thus, it is possible that immediate growth kinetics may be a result of as yet unknown complex interactions that may differ from cell line to cell line. Suspension cultures of specific cell lines selected for the absence of interphase inactivation may provide a way to eliminate interactions and to observe quantitatively the response of single cells. Another factor possibly affecting cell interactions is the presence of PPLO, and rigorous measures must be taken to ensure that cultures are PPLO-free (Levine et al., 1968). The amount of dispersion occurring as cells traverse the life cycle has been measured (Anderson and Petersen, 1964). For the cell lines considered here, dispersion probably does not exceed 30% per generation in any case and is not sufficient to conceal a defect of the magnitude reported in other cultures (Froese, 1966).

Results from synchronized Chinese hamster cells also indicate that all cells completed the first postirradiation division and grew at the control rate for long periods of time thereafter, in agreement with Elkind and his co-workers (Elkind and Sutton, 1960; Elkind et al., 1963). It appears that not only do all cells recover the ability to traverse the life cycle and divide after irradiation with relatively small doses but do so for significant periods of time without experiencing additional delays (see also Tolmach, 1961; Marin and Bender, 1966).

The data presented here suggest that the defect resulting in inability of the cell to traverse the life cycle and divide involves a process(es) common to all cells, regardless of their position in the life cycle. The observation that cells become insensitive to radiation at precisely the same time that they have completed all protein biosynthesis essential for division suggests that this defect may involve the ability of the cell to synthesize functional protein. The study of possible effects of irradiation on translation is continuing.

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