RADIOSENSITIVITY OF MAMMALIAN CELLS

II. Radiation Effects on Macromolecular Synthesis

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ABSTRACT Radiation effects on macromolecular synthesis essential for the Chinese hamster cell to traverse the life cycle and to divide have been investigated. Life-cycle analysis techniques employing inhibitors of macromolecular synthesis were used in determining the kinetics of cell growth for specific segments of the population following spontaneous recovery from radiation-induced division delay. The results indicated that recovery does not occur in the absence of functional protein synthesis. Under conditions which inhibit normal RNA and DNA synthesis, irradiated cells can recover the capacity to traverse the life cycle and to divide. The stability of mRNA species coding for proteins essential for division in irradiated cells was also measured. The mean functional lifetime of these mRNA species was 1 hr. The data demonstrate the existence of a specific segment of the population consisting of cells which have completed transcription related to division but not concomitant translation and which can recover from the radiation injury without synthesis of additional RNA. Thus, initial recovery of the ability to divide has an obligate requirement for protein synthesis but no corresponding requirement for nucleic acid synthesis during the period when original messenger remains intact.

INTRODUCTION

Little is known of the biochemical nature of radiation-induced lesion(s) ultimately resulting in a delay of division in mammalian cells. Although there very likely exists a causal relationship between radiation damage to DNA and ultimate cell death, genetic death generally occurs after the cell has successfully completed one or more divisions. In the preceding report (Walters and Petersen, 1968) it was shown that the radiation-induced division delay period was independent of position of the cell in the life cycle at the time of irradiation and that, after recovery, cells divided at the control rate for several generations. These observations suggested that the radiation defect involved a process(es) common to all cells throughout the entire life cycle. From previous studies, it is known that continued progress around the mammalian life cycle is dependent upon concomitant RNA and protein synthesis (Tobey et al., 1966 *a*) and, accordingly, radiation might be damaging to the cell's ability to syn-

thesize these macromolecules. Direct implication of these macromolecules in recovery may be demonstrated by determining the cell's ability to divide in the presence of specific inhibitors of macromolecular synthesis.

The results presented in this paper indicate that spontaneous recovery from the induced delay cannot occur in the presence of the inhibitor of protein synthesis, cycloheximide; only *after* removal of the inhibitor can the recovery process continue. From studies of populations of G_2 cells having completed transcription of all essential division-related RNA species but not concomitant translation of these species into functional protein prior to treatment with actinomycin D and irradiation, it was shown that recovery occurred in the absence of RNA synthesis. The mean lifetime of mRNA coding for proteins essential for division in irradiated cells was 1 hr. Furthermore, when G_2 cells were simultaneously treated with 10 mM thymidine (to inhibit DNA replication) and irradiated, the cells were delayed but ultimately divided at the pretreatment rate suggesting that DNA synthesis is not a necessary requirement in the recovery process.

Thus, initial recovery has an obligate requirement for protein synthesis but no corresponding requirement for nucleic acid synthesis during the period when original messenger remains intact. These results suggest that in irradiated mammalian cells defects leading to a delay in division differ substantially from those leading to genetic death (Phillips and Tolmach, 1966; Elkind et al., 1967; Sinclair, 1967; Weiss and Tolmach, 1967). The radiation-induced delay period appears to result from a defect in the ability of the cell to translate mRNA into functional protein, rather than from direct damage to the nucleic acid species.

MATERIALS AND METHODS

Cell Culture and Synchronization

Propagation in suspension of Chinese hamster ovary (CHO) cells in F-10 medium and synchronization of cell growth with excess thymidine or by selectively detaching mitotic cells from monolayer cultures have been described in detail elsewhere (Tobey et al., 1967; Walters and Petersen, 1968). Cell concentrations were measured with the automated cell counter previously employed by Walters and Petersen (1968). Cells were irradiated with a General Electric Maxitron X-ray therapy unit (General Electric Company, X-Ray Dept., Milwaukee 1, Wis.) The details of irradiation have been described previously (Walters and Petersen, 1968). The mitotic index was determined by the method of Tobey et al. (1966 b). Autoradiographs of ^{3}H-thymidine labeled cells (0.1 μc/ml) were prepared by the method of Puck and Steffan (1963) with the exception that cells were stained after the liquid emulsion was applied.

Chemicals

Cycloheximide (Acti-dione) was purchased from The Upjohn Co. (Kalamazoo, Mich.); actinomycin D was a gift from Merck, Sharp, & Dohme (West Point, Pa.); and tritiated thymidine (6 μc per mmole) was purchased from Schwarz Bio Research Inc., Orangeburg, N.Y.

RESULTS

Effect of Cycloheximide on CHO Cells

Cyclocheximide was chosen for this study because in addition to being an effective inhibitor of protein synthesis it reduces the rate of breakdown of polyribosomes (Noll et al., 1963; Wettstein et al., 1964). Its effect on protein synthesis is immediately reversible and cell viability is unaffected (Colombo et al., 1965; DeKloet, 1966; Tobey et al., 1966 *a*).

Cycloheximide (2 μg/ml) grossly reduces incorporation of ^{14}C-leucine within minutes and reduces the rate of incorporation of ^{3}H-uridine in CHO cells after 60

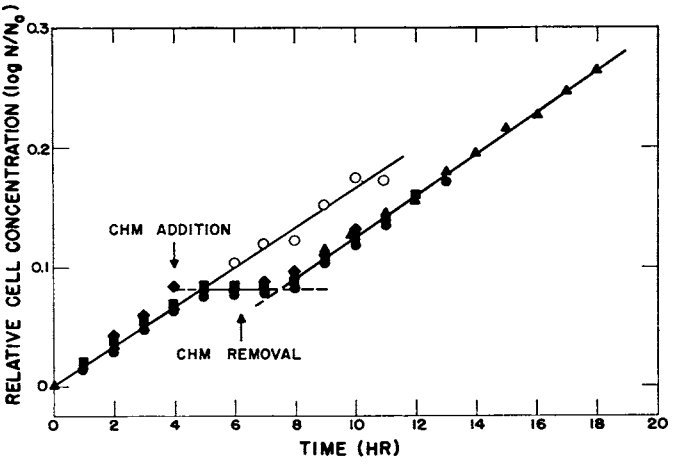

FIGURE 1 Effect of cycloheximide (CHM) on division in asynchronous Chinese hamster ovary cells. The open circles denote the drug-free control, and the solid symbols represent cycloheximide-treated cells.

min (Tobey et al., 1966 *a*). Concentrations greater than 2 μg/ml produce the same effect on incorporation of precursor into protein and RNA, while lower concentrations fail to inhibit cell division completely.

The time delay between cycloheximide addition and cessation of division (time of action) was measured (Fig. 1) and found to be 56 ± 3 min. The situation is indistinguishable from that described in detail in the preceding paper for radiation effects (Walters and Petersen, 1968); cells beyond the point of drug action have completed protein biosynthesis essential for division and will divide despite presence of the drug, while younger cells will be prevented from dividing.

The conditions for removal of cycloheximide were critical in fixing the time interval between removal of the drug and resumption of cell division and in producing minimal perturbation in the pattern of cell growth after resumption of division. Optimal release from the cycloheximide effect was obtained by washing the centrifuged cell pellets twice in resuspension medium and then resuspending the cells in

"conditioned medium" (e.g., medium which had previously supported cell growth from 1.0–1.3 $\times$ 10^5 cells/ml). All operations were performed at 37°C.

Reversibility of cycloheximide in these cells was established by measuring the time interval between resuspension and resumption of division. Cells incubated for 2 hr in cycloheximide were resuspended in drug-free, conditioned medium. If the effects of cycloheximide are completely reversible, division should resume approximately 56 min after resuspension. Cells closer to division have already divided in the presence of the drug, resulting in an emptying of that 56 min portion of the life cycle immediately preceding division. Cells resumed division from 60 to 73 min after cycloheximide removal, in good agreement with the predicted time. Division continued at the pretreatment rate for long periods of time after drug removal (Fig. 1), indicating that (*a*) progress of each cell about the life cycle was halted when cycloheximide was added, and (*b*) essentially all cells treated with cycloheximide divided for periods of time long in comparison to the duration of these experiments.

Effect of Protein Synthesis Inhibition on Recovery of the Ability of Irradiated Cells to Divide

Chinese hamster cells were exposed to 200 rads (a dose from which all cells will subsequently recover and divide a number of times) and incubated in cycloheximide (2 μg/ml) for 2 hr at various times after irradiation. Three control cultures were included in each experiment: (1) an unirradiated drug-free control; (2) a drug-free irradiated control; and (3) an unirradiated cycloheximide-treated control. The results are summarized in Table I, where the values presented were measured from controls run concurrently with each determination; Fig. 2 illustrates a typical

TABLE I

EFFECT OF INHIBITION OF PROTEIN SYNTHESIS ON RECOVERY OF THE ABILITY OF IRRADIATED CELLS TO DIVIDE

Time of addition of CHM* after irradiation	Time required for resumption of division after CHM removal	Total delay time minus time in CHM (col. 1 + col. 2)	Delay time of untreated cells
hr	*hr*	*hr*	*hr*
0	3.8	3.8	3.3
0	4.2	4.2	3.3
0.5	2.8	3.3	3.3
0.5	3.7	4.2	3.3
1.0	3.7	4.7	3.3
1.0	2.3	3.3	3.0
1.2	2.9	4.1	3.5
1.3	1.4	2.7	3.1
1.6	3.0	4.6	3.7
		$\bar{x}$ = 3.8	$\bar{x}$ = 3.3

* CHM denotes cycloheximide.

experiment. It is apparent that irradiated cells treated with cycloheximide subsequently recovered and divided at the control rate for long periods of time.

The total delay period, including incubation time in cycloheximide, is additive under the described conditions. Clearly, the radiation-induced defect could not be repaired *in the absence of protein synthesis.* It should be noted that the effect of cycloheximide on recovery is independent of time of drug addition after irradiation, indicating that, although recovery processes begin immediately after irradiation, unimpaired protein synthesis must continue throughout the entire recovery period.

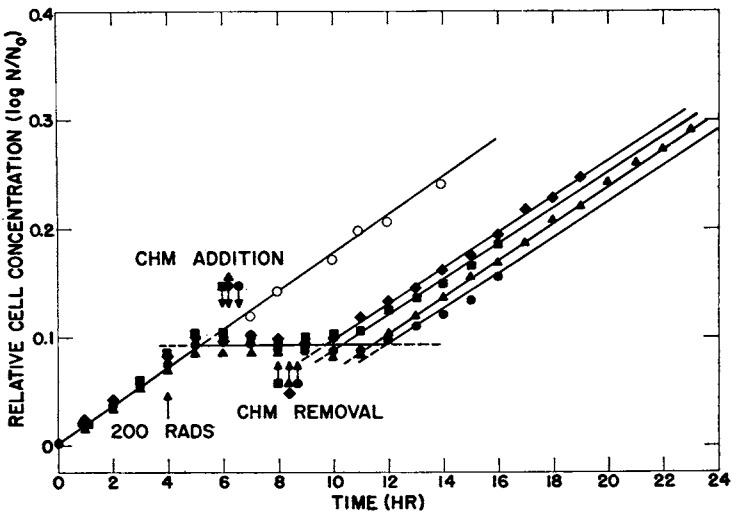

FIGURE 2 Effect of inhibition of protein synthesis on recovery of the ability of irradiated cells to traverse the life cycle and to divide. Cycloheximide (CHM) was added to irradiated cultures (solid symbols), then removed 2 hr later. The times of respective treatments are shown by the arrows, and the open circles denote the unirradiated, drug-free control.

Effect of RNA Synthesis Inhibition on Recovery of the Ability of Irradiated Cells to Divide

The effect of x-irradiation on division mimics, in many respects, the effect of inhibitors of protein synthesis. Indeed, it has been shown that cells did not recover the ability to divide in the absence of protein synthesis. This observation suggested that irradiation affected in some way the ability of the cell to synthesize functional protein (i.e., impaired transcription or translation). To test for possible effects on transcription, a series of experiments was designed to measure the functional stability of mRNA related to division in irradiated cells.

It has been reported previously that the actinomycin D time marker (i.e. end of RNA synthesis essential for division) was 1.9 hr prior to division in the Chinese hamster cell (Tobey et al., 1966 *b*). Actinomycin D, an irreversible inhibitor of DNA-dependent RNA synthesis (Reich et al., 1961; Collins, 1965), grossly reduces

uridine-^{3}H incorporation into RNA of CHO cells within minutes, while only slightly affecting leucine-^{14}C incorporation (Tobey et al., 1966 *b*). The effect of adding actinomycin (2 μg/ml) to both thymidine-synchronized and asynchronous populations of Chinese hamster cells is shown in Fig. 3. Division was inhibited 2 hr after addition of actinomycin in each case, in good agreement with Tobey et al. (1966 *b*).

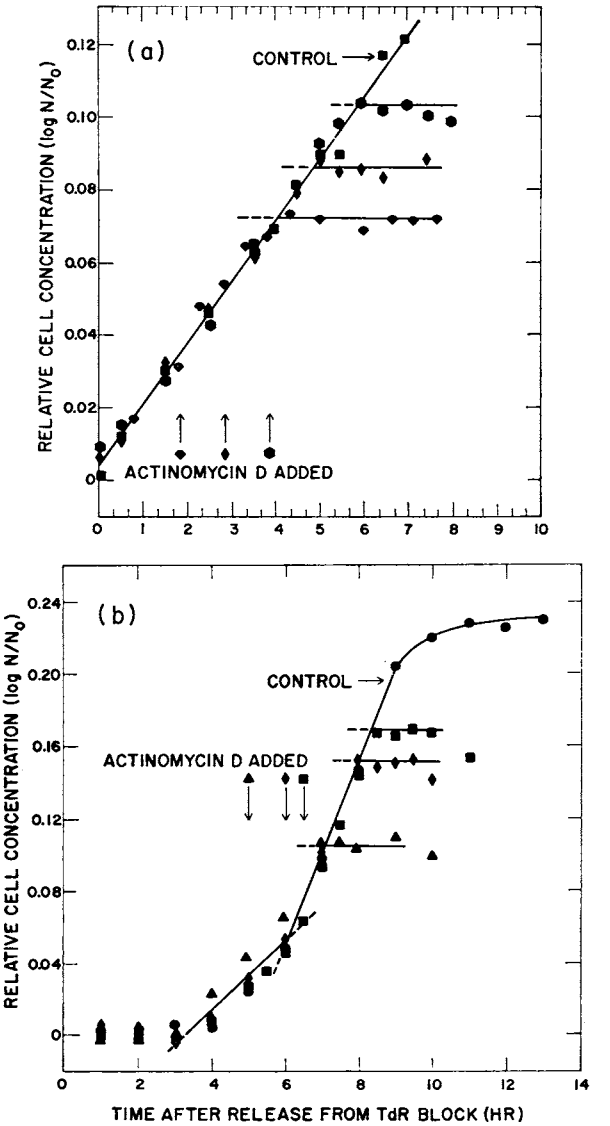

FIGURE 3 Effect of actinomycin D (2 μg/ml) on division in Chinese hamster ovary cells. Actinomycin D was added at times indicated by the arrows to asynchronous (part *a*) and thymidine-synchronized (part *b*) cells.

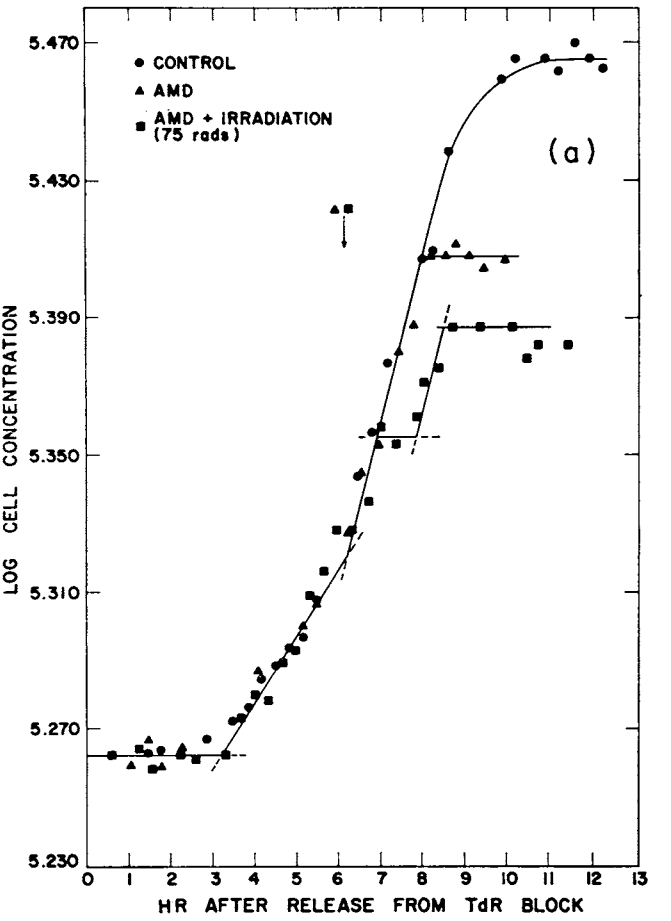

FIGURE 4 *a*

If actinomycin is added simultaneously with irradiation, three distinct portions of the population can be classified: (1) cells which have passed both the actinomycin and X-ray markers, where actinomycin-sensitive and radiosensitive division-related synthesis is complete; (2) cells which have passed the actinomycin marker but not the X-ray marker, have completed all transcription related to division but not concomitant translation, and are reversibly inhibited from dividing by irradiation (hereafter referred to as *A-X* cells); and (3) cells which are located before the *A* marker and are lacking RNA essential for division. In the presence of both actinomycin and X-ray inhibition, cells of class 1 will complete division and leave a 0.9 hr "gap" (i.e. time of action of x-irradiation) in the population distribution, since their ability to divide is not affected by either treatment. Since actinomycin inhibition is irreversible, cells of class 3 will never be able to divide. The only cells of class 2 capable of division will be those which have recovered from radiation effects in the absence of additional RNA synthesis.

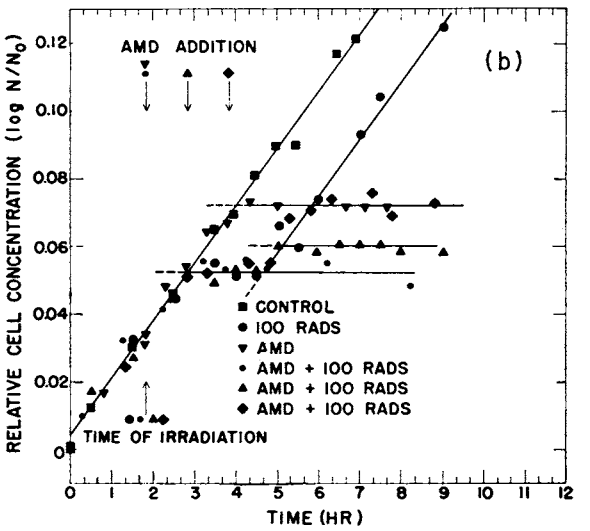

FIGURE 4 Effect of an inhibitor of RNA synthesis on recovery of the ability of irradiated *A-X* cells to divide (see text for the details). A culture synchronized with thymidine (Fig. 4 *a*) was irradiated (75 rads) and treated simultaneously with actinomycin D (squares), while a second culture was treated with actinomycin alone (triangles). A drug-free, unirradiated culture served as a control. A series of asynchronous cultures (Fig. 4 *b*) was irradiated (100 rads) and at varying times thereafter during the delay period was treated with actinomycin D (AMD).

The fraction of *A-X* cells capable of division after exposure to both inhibitors was determined using cells synchronized with 10 mM of thymidine to increase the proportion of *A-X* cells in the population. Exposure doses ranging from 25 to 200 rads were used to produce increasingly longer delay periods while ensuring that all cells would divide a number of times after irradiation. Cells were irradiated and simultaneously treated with actinomycin (one such experiment shown in Fig. 4 *a*). The number of trapped *A-X* cells was determined from the appropriate control, and the number of these actinomycin-treated cells actually dividing after irradiation was measured. A similar experiment was performed with asynchronous cells, where actinomycin was added at several times after irradiation so that the drug was present for varying times during the delay period (Fig. 4 *b*). Fig. 5 summarizes the results obtained in a number of experiments like those of Fig. 4 *a* and *b*, where the fraction of trapped cells dividing is plotted as a function of division delay time. The fraction of *A-X* cells recovering the ability to divide can thus be expressed as a function of time during the radiation-induced delay period in which actinomycin was present. This fraction dropped to 50% for a 1 hr delay period. The data clearly demonstrate the existence of a specific segment of the population consisting of cells (*A-X*) which have completed transcription related to division but not concomitant translation and which can recover from radiation injury without additional RNA synthesis.

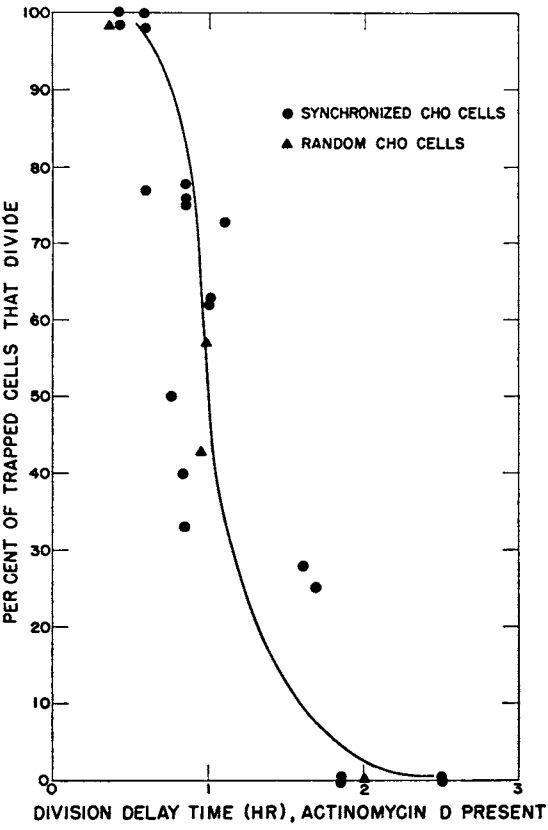

FIGURE 5 Fraction (%) of trapped *A-X* cells that divided in the presence of actinomycin. Cells were irradiated with exposures ranging from 25 to 200 rads. The fraction of trapped *A-X* cells capable of recovery in the absence of RNA synthesis is expressed as a function of the induced division delay period for thymidine-synchronized (circles) and random (triangles) Chinese hamster ovary cells.

Thus, cells which have been irradiated and treated with actinomycin can recover the ability to divide if the radiation-induced delay time following RNA inhibition is less than the lifetime of the messengers coding for division proteins.

Effect of DNA Synthesis Inhibition on Recovery of the Ability of Irradiated Cells to Divide

It appears that the initial recovery process has an obligate requirement for protein synthesis but no corresponding requirement for RNA synthesis during the period when original messenger remains intact. This suggests, of course, the corollary that damage to the genome and/or repair of genetic material need not necessarily be involved in the processes leading to initiation of or recovery from the radiation-induced delay period. The ability of cells to recover under conditions where normal semiconservative DNA replication is inhibited can be examined by simultaneously

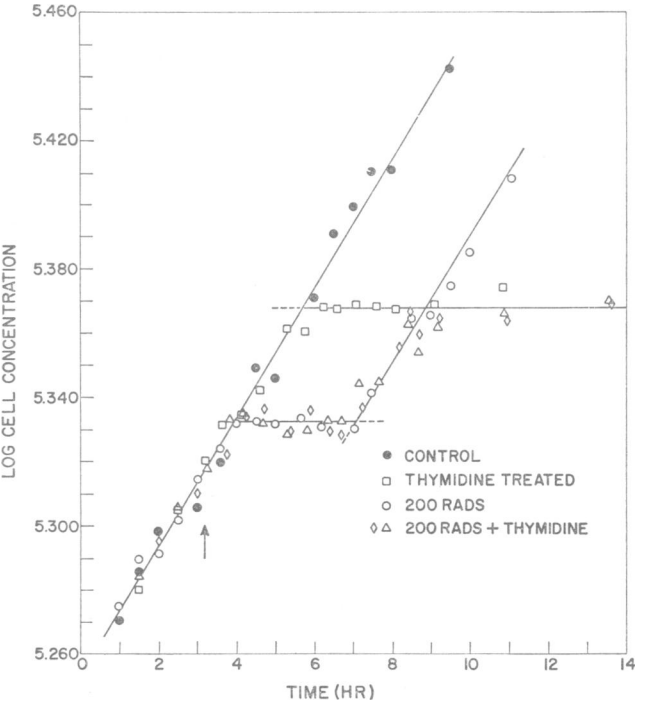

FIGURE 6 Effect of inhibition of DNA synthesis on recovery of the ability of irradiated G_2 cells to traverse the life cycle and to divide. Asynchronous cells were irradiated and simultaneously treated with excess thymidine (10 mM) at the time indicated by the arrow (diamonds and triangles). The open circles denote cells that were irradiated but not treated with the drug, and the squares represent cells that were treated with thymidine alone.

irradiating and treating exponentially growing cells with excess thymidine (10 mM). In the presence of excess thymidine, cells initially in G_2 and M divide and eventually accumulate at the G_1/S boundary, while cells in the other portions of the life cycle are unable to divide. The data obtained by irradiating cells with and without thymidine are shown in Fig. 6. Cells treated with thymidine alone continued dividing for 3 hr (the duration of G_2 + M in these cells), then division ceased. Cells irradiated with 200 rads but not treated with thymidine exhibited a delay period of 3 hr, followed by resumption of division at the control rate. Cells that were irradiated and treated with thymidine gave an identical delay period and resumed division at the control rate. The number of cells dividng was equivalent to that of the unirradiated thymidine control. Thus, cells in G_2 that were prevented from dividing by x-irradiation recovered the ability to traverse the life cycle and divide under conditions which inhibit normal DNA replication.

Time after Irradiation that Cellular Delay is Expressed

Fixed temporal biochemical events about the life cycle enable one to determine the point in the life cycle at which the delay of an irradiated cell is expressed. Synchronization by treating with excess thymidine or by selectively detaching mitotic cells from a monolayer culture was employed to produce cells in close temporal proximity to existing biochemical markers. Cells irradiated in different phases of the life cycle were then examined for their ability either to synthesize DNA (i.e. time of entry into S) or to complete transcription of essential division-related RNA species (i.e. to cross the actinomycin marker).

Thymidine-synchronized cells were irradiated with 150 rads to produce a mean division delay period of 120 min. In this case, rather than simultaneously irradiating and treating with actinomycin as before, actinomycin was added at varying times (0.6–1.4 hr) *before* resumption of division. Such an experimental protocol enables one to examine (*a*) the ability (or inability) of an irradiated G_2 cell to traverse a portion of the life cycle during the delay period, and (*b*) the ability of *A-X* cells to resynthesize messenger RNA species during the delay period. Determination of the cell number increase after addition of actinomycin at different times before resumption of division can establish when, after irradiation, the cell was delayed. If progress of the cell about the life cycle was inhibited immediately upon irradiation and remained inhibited during the entire delay period, then the number of *A-X* cells capable of dividing after recovery should be constant. If, on the other hand, cells younger than the actinomycin marker were either not inhibited or gained the ability to traverse a portion of the life cycle during the delay period, then the number of *A-X* cells should be greater than that calculated from the control. This is a necessary corollary since, during the interval from irradiation to addition of actinomycin, cells would have been capable of crossing the actinomycin marker and becoming *A-X* cells insensitive to treatment with actinomycin during the period when messenger RNA remains intact. The only limitation on such an experiment is a consideration of the messenger stability. Actinomycin D must be present for less than 1 hr (0.7 hr) during the delay period to avoid a reduction in number of *A-X* cells capable of division (see Fig. 5).

Theoretically, actinomycin can be added up to 1.6 hr prior to resumption of division with no loss of *A-X* cells due to messenger instability. Although, at first glance, 1.6 hr appear to be inconsistent with the data on messenger stability, such is not the case. Because cells continue dividing for 0.9 hr after irradiation, the delay period ends, in fact, $\sim$0.9 hr before division is resumed. This is the time required for cells to traverse the "gap" emptied of cells after irradiation. If actinomycin is added to these cells at any time up to 1.6 hr before resumption of division, the drug

will at most be present during only 0.7 hr of the actual delay period and would not be expected to affect the number of *A-X* cells capable of division (Fig. 5). The results of the study are presented in Table II. When actinomycin was added for 0.6–1.4 hr prior to resumption of division, the cell number increase was constant and division continued at the control rate for ~1.3 hr in all cells. In addition, all of the *A-X* cells divided (~99%). These results are consistent only with the interpretation that upon irradiation cells were immediately inhibited from proceeding about the life cycle and remained inhibited during the entire division delay period. Thus, irradiated cells did not cross the actinomycin marker during the delay period, even in the absence of actinomycin. These results also indicate that irradiated cells maintain

TABLE II

CELL NUMBER INCREASE AFTER TREATING IRRADIATED CELLS WITH ACTINOMYCIN D AT VARYING TIMES BEFORE RESUMPTION OF DIVISION

Time before resumption of division that AMD* was added	Time during which cell increase was observed after resumption of division	Cell increase observed	Cell increase expected from control (A-X Cells)	A-X cells dividing
hr	*hr*	%	%	%
0.6	1.25	18	17	106
0.6	1.17	19	17	111
0.8	1.33	16	17	94
0.9	1.33	14	17	83
0.9	1.25	15	17	88
1.2	1.33	19	17	111
1.4	1.33	17	17	100
1.4	1.42	17	17	100
	$\bar{x}$ = 1.30			$\bar{x}$ = 99

* AMD denotes actinomycin D.

a capacity to resynthesize mRNA during the delay period. Had actinomycin been added simultaneously with a radiation exposure sufficient to induce a 2 hr delay period, messenger instability would have precluded the recovery of all *A-X* cells. It appears that irradiated cells synthesized mRNA during the interval from irradiation to subsequent addition of actinomycin D.

To test for similar effects on cells irradiated in another portion of the life cycle, mitotic cells were selected by mechanical detachment from monolayer cultures. As shown previously (Walters and Petersen, 1968), cells irradiated in mitosis completed mitosis at the control rate but were delayed in reaching the subsequent division point. The question of where in the life cycle the delay occurred becomes pertinent. To obtain such information, synchronized mitotic cells were irradiated with 150 rads at 0°, then resuspended at 37°C. After completion of mitosis (~0.5 hr), ^{3}H-thymidine (0.1 μc/ml) was added to both irradiated and control cultures. Samples were taken simultaneously from the same culture for both autoradiography and cell

counting. Results obtained from the continuous labeling procedure are shown in Fig. 7, where the fraction of labeled cells is a measure of rate of entry of cells into S. It is obvious that irradiated cells entered S at the same rate as control cells and that essentially all of the cells incroporated label; however, the irradiated cells trailed the controls by 1.4 hr. These same cells were delayed 1.6 hr in reaching division. Thus, it appears that delay occurred before the cells entered S and that the cells traversed S, G_2, and M at the same rate as control cells. These results, coupled with those

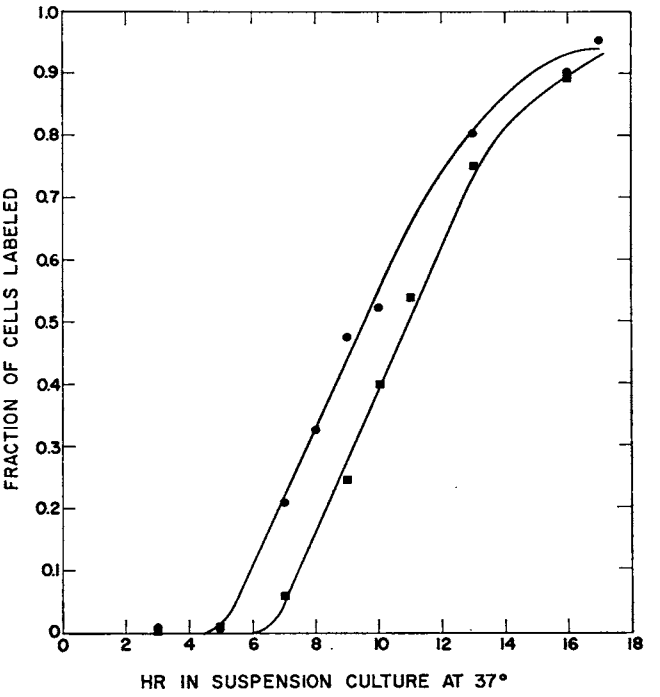

FIGURE 7 Effect of irradiation of synchronized mitotic cells on the rate of entry of the cells into S. Cells were treated with thymidine-^{3}H (0.1 μc/ml) 0.5 hr after completion of mitosis. The circles denote the unirradiated control, and the squares represent cells irradiated in mitosis (at 0°) with 150 rads.

obtained from the actinomycin marker in G_2, indicate that (*a*) irradiated cells are delayed at the time of irradiation, (*b*) cells do not age biochemically during the induced delay period, and (*c*) upon resumption of progress about the life cycle, cells proceed at the same rate as control cells and experience no additional delays in other portions of the life cycle.

DISCUSSION

The initial effect of x-irradiation on Chinese hamster cells is a nonspecific one in which the duration and location of the radiation-induced division delay period are independent of the position of the cell in the life cycle at the time of irradiation

(Walters and Petersen, 1968). The final point of X-ray inhibition occurs in late G_2 but is temporally well in advance of initiation of prophase, and it has been proposed that the final point of inhibition coincides with the time at which synthesis of a product essential for division is completed. At this time, the cell has completed all preparation for division and will complete division at the control rate despite exposure to increasingly larger doses of x-irradiation (Walters and Petersen, 1968). The process of synthesis, rather than the product, clearly is the radiosensitive component.

Results reported here from the study of effects of selected antibiotics on recovery of the ability of irradiated mammalian cells to divide suggest that the biosynthetic defect is intimately associated with the ability of the cell to translate mRNA into functional proteins essential for continued traverse of the life cycle and ultimate division. Studies with cycloheximide, an inhibitor of protein synthesis, indicate an obligate requirement for protein synthesis throughout the recovery period. Since additivity of the total delay period of irradiated cells treated with cycloheximide was independent of time of drug addition, recovery apparently began immediately after irradiation (see also Lockart et al., 1961) and continued until inhibited by cycloheximide. Upon removal of the drug, the recovery process resumed at the point attained when cycloheximide was added. This eliminates the possibility that the observed effect was due to the drug itself, rather than an effect of the drug on the recovery process.

The results also argue against the possibility that inhibition of protein synthesis allows a recovery process to repair defects before they are expressed in the cell. Inhibition of recovery by cycloheximide apparently occurs in all phases of the life cycle, since division continued in a smooth, exponential function at the control rate, approaching one generation time after recovery (Fig. 2). Had cells recovered in the presence of cycloheximide, one would expect to see no additional delay in resumption of division after drug removal. If recovery only in specific segments of the population were inhibited by cycloheximide, deviations from exponential growth should have resulted when uninhibited cells divided at the same time as cells in which recovery was inhibited. Such responses were never seen. As noted earlier, the final point of X-ray inhibition coincides with the time of action of cycloheximide 0.9 hr prior to division. These observations suggest that irradiation interferes with translation of functional proteins in cells distributed about the life cycle. When cells become insensitive to cycloheximide, having completed all protein synthesis essential for traverse of the life cycle and division, they also become insensitive to x-irradiation.

It is difficult to separate effects on transcription of RNA from those on translation, since the end result is likely to be the same in either case (i.e. inhibition of functional protein synthesis). However, this is not the case for cells which have completed all transcription related to division but not concomitant translation; the *A-X* cells.

Irradiating these cells with increasingly larger doses in the presence of actinomycin led to a decreased fraction of *A-X* cells capable of division (Fig. 5). In view of the known instability of mRNA, additional synthesis of which was prevented by actinomycin, the decreased fraction of *A-X* cells capable of division is interpreted as a measure of instability of mRNA species coding for essential division proteins. Using this criterion, a mean functional lifetime of 1 hr was obtained for division-related mRNA's in irradiated cells. This value is in agreement with other estimates of the mean lifetime of mammalian mRNA (Villa-Trevion et al., 1964; Trakatellis et al., 1965 *a*; Trakatellis et al., 1965 *b*) and suggests that irradiating the cells does not affect the stability of mRNA. Thus, messengers synthesized at the time of irradiation (as established by simultaneous irradiation and actinomycin addition) are functional in coding for division proteins if the radiation-induced delay period following RNA inhibition is less than the lifetime of the messengers. Therefore, the recovery process has no obligate requirement for RNA synthesis during the period when original messenger remains intact. However, once the messengers have been degraded by processes within an actinomycin-free cell, it is apparent that resynthesis of the messengers must occur if the cell is to recover the ability to divide.

The absence of any recovery requirement for RNA synthesis during the period when original messengers remain intact suggests that damage to or repair of the genetic material may not be initially involved in the recovery process. The data (Fig. 6) show clearly that irradiated G_2 cells recovered the ability to traverse the life cycle and to divide under conditions which inhibit normal DNA replication (10 mM of thymidine). Although this does not preclude the possibility that "unscheduled" reparative DNA synthesis is different from normal synthesis (Painter and Cleaver, 1967; Evans and Norman, 1968), it does place an added restriction on such a process. Excess thymidine is thought to inhibit the reductive conversion of cytidylic acid to deoxycytidylate (Morris and Fischer, 1963; Whittle, 1966), thereby preventing DNA synthesis by depriving the cell of necessary precursors. Since deoxynucleotide triphosphates would presumably be needed for repair synthesis as well as for semiconservative replication, it seems that any DNA synthesis would be affected by depriving the cell of precursors, regardless of possible differences in the enzyme systems responsible for polymerization of deoxynucleotide triphosphates into DNA molecules.

It has been shown that Chinese hamster cells in G_2 did not cross the actinomycin marker during the division delay period, even in the absence of actinomycin (Table II). In addition, cells irradiated in mitosis were delayed in entry into S (Fig. 7) by the same time that they were delayed in reaching division, although the two events are separated by about 8 hr. These data support not only our previous prediction that cells irradiated at any phase of the life cycle are delayed immediately upon irradiation and not at some later time (Walters and Petersen, 1968) but also show that cells do not age biochemically during the delay period (e.g. do not traverse the life cycle).

Although these results differ from observations by Terasima and Tolmach (1963), where HeLa cells irradiated in mitosis were not delayed in entering S but were delayed in the following G_2 period, they are consistent with the observation in a number of other cell lines that initiation of DNA synthesis is delayed as a consequence of irradiation (Bollum et al., 1960; Lieberman et al., 1963; Mak and Till, 1963; Little, 1968). It is apparent that x-irradiation affects the ability of the cell to traverse the life cycle in a highly reproducible manner. An irradiated cell cannot proceed to the next biochemical step in the life cycle until recovery is complete. Once recovery is complete, the cell can proceed around the life cycle at the control rate. This is true for all cells in the population—both the surviving and nonsurviving fractions.

Data presented here suggest that the initial radiation effect is on some process(es) involved in translating a coded message into functional protein. Although ionizing radiations are thought to affect the ability of some systems to transcribe mRNA from DNA (Novelli et al., 1961; Clayton and Adler, 1962; Uchiyama et al., 1965; Pollard and Barrone, 1966; Yatvin and Lathrop, 1966), such an interpretation here seems insufficient for a number of reasons. If transcription were completely eliminated by irradiation, as suggested by Pollard (1964) in *E. coli*, then the end of the recovery period should be fixed by the ability of the cell to reinitiate synthesis of functional mRNA. In this event, adding actinomycin at any time before resumption of division might then be expected to inhibit all *A-X* cells from regaining the ability to divide, which is obviously not the case here. Furthermore, if transcription alone were affected, one might expect that cells should divide for 2 hr after irradiation, the time at which all RNA synthesis essential for division is complete. Instead, we found that (*a*) irradiated cells divided 0.9 hr after irradiation (the time corresponding to the end of protein synthesis essential for division), and (*b*) messengers present in the irradiated *A-X* cells were capable of coding for division proteins if the induced delay period were sufficiently short (less than 1 hr). Irradiation must, therefore, interfere with a process basic to normal cellular control, possibly synthesis of specific proteins. Doida and Okada,[1] employing a different cell line and different analytical techniques, have also concluded that the block involves interference with the synthesis of a protein or proteins necessary for entry into mitosis. We are in agreement that protein synthesis is somehow involved, but these data and the results of the preceding paper (Walters and Petersen, 1968) make the synthesis of a specific protein unique to a particular part of the life cycle unattractive.

It is thought that progress about the life cycle occurs as a series of sequential events (Halvorson et al., 1964; Halvorson et al., 1966; Tobey et al., 1966 *a*, 1966 *b*; Petersen et al., 1968). If RNA is sequentially transcribed from DNA at a rate tightly coupled to or controlled by the translation of functional proteins (Bautz et al., 1966; Cline and Bock, 1966) any interference (or uncoupling) with the operation

[1] Doida, Y., and S. Okada. Personal communication.

of one might be expected to affect the other secondarily. If, indeed, the initial radiation effect is on a translational process and the cell must successfully complete each biochemical step before it can proceed to the one following, then one would predict that during the radiation-induced delay period the cell would be prevented from aging biochemically and traversing the life cycle, even though it may still be capable total macromolecular synthesis. Further, one might also expect the cell to proceed at the normal rate about the life cycle after recovery from the radiation-induced block. We find that such is, indeed, the case with irradiated Chinese hamster ovary cells.

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