X-RAY SENSITIVITY OF HeLa S3 CELLS IN THE G2 PHASE: COMPARISON OF TWO METHODS OF SYNCHRONIZATION

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ABSTRACT The sensitivity of HeLa S3 cells to 220 kv X-rays was measured in terms of cell survival (colony development) during the G2 phase of the cell generation cycle, employing two procedures designed to free G2 cultures from contaminating cells from other phases of the cycle. Treatment of synchronous cultures (obtained initially by mitotic selection) with high specific activity tritiated thymidine (HSA-^{3}HTdR) selectively eliminated S phase cells, while addition of vinblastine permitted removal of cells as they entered mitosis. It was found that HeLa S3 cells become increasingly sensitive as they progress through G2. The pattern of sensitivity fluctuations observed in synchronous HeLa S3 populations selected by the foregoing method was compared with that found in synchronous cultures prepared by the HSA-^{3}HTdR method of Whitmore. The latter method had been used previously with mouse L cells, which were found to undergo a different pattern of sensitivity fluctuations. The two methods yield similar results for HeLa cells in the S and G2 phases of the cycle. It may be concluded, therefore, that the discrepancies between HeLa and mouse L cells do not arise from methodological factors, but represent fundamental differences between the cell types.

INTRODUCTION

Cyclical fluctuations in the response of HeLa S3 cells to X-irradiation, as determined by measurement of cell survival (colony-forming ability) in synchronous populations irradiated at different times in the cell generation cycle, follow a defined pattern (Terasima and Tolmach, 1963 *b*). The relatively sensitive[1] mitotic cell population rapidly passes into a more resistant state early in the G1 (postmitotic) phase, and then again gradually acquires increased sensitivity during the remainder of that phase. During the subsequent S (DNA-synthetic) phase, sensitivity again decreases, and by blocking progression of cells from G1 to S by adding inhibitors of DNA

[1] Throughout this paper, the term "sensitivity" is employed as an abbreviation for the more precise "decrease in survival after irradiation with a specified dose of X-rays."

synthesis to synchronous cultures before any cells had reached S, it was shown (Terasima and Tolmach, 1963 *c*) that the latter decrease is dependent on DNA synthesis. Thus, the region of maximal interphase sensitivity was identified as the G1-S transition. While the asynchrony that develops in the system renders this identification somewhat uncertain (maximal sensitivity of individual cells might be reached before the end of G1), it is consistent with results reported by others for a number of different types of cultured mammalian cells, using a variety of techniques for obtaining synchronous populations (Sinclair and Morton, 1965; Erikson and Szybalski, 1963), though mouse L cells may behave differently (Whitmore et al., 1965).

However, the behavior of the system towards the end of the cycle, that is, in the G2 (premitotic) phase, could not be reliably determined because of the relatively brief duration of this phase and the loss of synchrony that develops during progression of the population through the earlier parts of the cycle. Although a large number of measurements (Tolmach et al., 1965, Fig. 10) suggested that cells become relatively sensitive again during G2, contamination with cells in M (mitosis), late G1, and early S could be responsible for this apparent sensitivity increase. Accordingly, experiments were carried out employing procedures designed to increase the purity of the cell population during G2, in order to describe the system more adequately.

The study received impetus with the publication of a report indicating that other mammalian cell systems undergo very different changes in sensitivity during the cell cycle. Indeed, concern not only about the G2 behavior of HeLa S3 cells, but also about possible influence of the method of cell synchronization on the observed pattern of sensitivity changes arose with description by Whitmore and coworkers (1965) of the cyclical changes in radiation response of mouse L cells. Those workers reported that L cells undergo fluctuations qualitatively different from those found with synchronous cultures of both HeLa S3 cells (Terasima and Tolmach, 1963 *b*) and Chinese hamster cells (Sinclair and Morton, 1965). The latter cell types had been prepared by the mitotic cell selection method (Terasima and Tolmach, 1963 *a*) or a modification thereof, while the synchronous L cell cultures, in contrast, though also obtained by a selection method, had been isolated by treating randomly dividing cells with tritiated thymidine of high specific activity in order to kill all cells that synthesized DNA during the treatment, and thereby yield a synchronous population of late G1 cells (Whitmore and Gulyas, 1966). The discrepancy between the behavior of L cells on the one hand and Chinese hamster and HeLa on the other is apparent during the latter part of the generation cycle in particular; L cells begin to increase in sensitivity about midway through S, while both HeLa and Chinese hamster cells continue to decrease throughout that phase. More dramatically, L cells decrease in sensitivity during G2, while Chinese hamster and HeLa (as will be shown here) both exhibit a sensitivity increase. In view of these differences, we have prepared synchronous HeLa cultures by the method of Whitmore and Gulyas (1966), and have examined their X-ray sensitivity pattern, particular attention being given to the latter part of the cycle.

MATERIALS AND METHODS

Cultural Procedures

HeLa S3 cells were grown in monolayer culture in medium N16HHF using conventional procedures (Ham and Puck, 1962). The technique of synchronization by selection of mitotic cells has been described previously (Terasima and Tolmach, 1963 *a*). Medium N16FCF (Pfeiffer and Tolmach, 1967) was used to grow cells for harvesting. It was replaced with N16HHF 3 hr after seeding mitotic cells.

Irradiation

Irradiation with 220 kv X-rays (constant potential; 15 ma; 83 rad per min; half-value layer 1.0 mm Cu) was carried out at 37°C in a controlled atmosphere of 4% carbon dioxide in air. Doses were measured with a suitably located ionization chamber.

Inactivation of S Phase Cells with Tritiated Thymidine

High specific activity tritiated thymidine (HSA-^{3}HTdR) was used to selectively kill (prevent colony formation from) cells in the S phase (Whitmore and Gulyas, 1966). Though not removed physically, the killed cells do not interfere with the assay of colony-forming ability used to measure radiation response (Whitmore et al., 1965). HSA-^{3}HTdR (12.5–14.25 c/mmole was applied at a level of 2 μc/ml in two types of experiments. In the first, the objective was to purify already partially synchronous cultures by killing the cells in S. Mitotically selected synchronous cultures were treated at times when the majority of cells were in G2. HSA-^{3}HTdR was applied for 1–3 hr in the presence of 10^{-6} M fluorodeoxyuridine (FUdR) which served to enhance thymidine incorporation. At termination of treatment, the medium was changed to N16HHF containing 2×10^{-5} M nonradioactive thymidine. Sinclair and Morton (1966) have independently employed a similar procedure to increase the homogeneity of partially synchronous cultures of Chinese hamster cells.

The second type of experiment involved the use of HSA-^{3}HTdR directly to produce synchronous cultures, following the method of Whitmore and Gulyas (1966). In this procedure, randomly dividing cultures are treated so as to kill all cells except those lying in a region of the generation cycle immediately preceding the S phase. Since the width of the region is determined by the duration of exposure to the HSA-^{3}HTdR, relatively large populations may be obtained, but with correspondingly lower degrees of synchrony. Treatment periods ranging between 7 and 9.5 hr were found useful; the synchronous populations thereby obtained constituted between 25 and 4.6% of the parent cultures. Treatment was initiated 24 hr after plating trypsinized cultures in Petri dishes and was terminated by removing the radioactive medium, rinsing the dishes with saline (Djordjevic and Szybalski, 1960), and adding fresh, nonsupplemented medium.

Arrest of Mitotic Cells with Vinblastine

Further purification of partially synchronous cultures was effected by treatment with vinblastine sulfate (VLB) (Eli Lilly and Co., Indianapolis, Ind.). This agent, when present at a concentration of 0.03 μg/ml, selectively arrests HeLa S3 cells in metaphase (Cutts, 1961; Palmer et al., 1960; Pfeiffer and Tolmach, 1966). As mitotic cells are only loosely attached to the dish, they may be readily flushed away with growth medium. After rinsing once with saline, fresh medium was added.

This procedure was also used to monitor the progression of synchronous cultures through the generation cycle. Cells were trapped as they reached M and subsequently removed, permitting construction of a plot of the number of colony-forming units remaining as a function of time. The fraction of cells progressing into M in any time interval could thereby be determined.

Alternatively, repeated microscopic observation was made of selected fields, in order to score the cells that remained attached to the dish, i.e., had not yet entered M.

Radioautography

Detection of DNA-synthetic activity was carried out by treating cultures with ^{3}HTdR (0.5 μc/ml; 0.5 c/mmole) for 15 min, rinsing with buffered saline, fixing for 30 min in acetic acid–ethanol (1:3), rinsing with fixative, and treating with 70% ethanol for 2 hr. After air drying, the plastic dishes were covered with nuclear track emulsion, dried for 1 hr, and exposed for 1 wk at 4°C. Monitoring of the HSA-^{3}HTdR synchronization procedure by radioautography was carried out by replacing the medium at the end of treatment with fresh medium containing ^{3}HTdR at 0.5 mc/ml, 0.5 c/mmole, and periodically preparing cultures for radioautographic analysis as above. The fraction of labeled cells was scored in a minimum sample of 500 cells. When microcolonies were scored for the presence of labeled cells, 250 colony-forming units were examined.

TABLE I

COMPARISON OF CELL SURVIVAL WITH CELL LABELING AFTER TREATMENT WITH HSA-^{3}HTDR

Period of treatment with HSA-^{3}HTdR	Survival	Colony-forming units containing at least one unlabeled cell
hr	%	%
19–20	56	59
19–24	57	60

RESULTS

X-Ray Sensitivity of HeLa S3 during the G2 Phase

The homogeneity of G2 populations was increased by simultaneous application of two procedures designed to avoid contamination by M, G1, or S cells. Cells still in S were effectively eliminated by incubation with HSA-^{3}HTdR, while contamination by more rapidly growing cells, i.e. those which would have progressed to M or G1, was prevented by arresting cells with VLB as they reached M, and subsequently removing them. Serial irradiation of the remaining cells permitted measurement of the X-ray response of G2 populations of progressively greater average age.

Preliminary experiments were carried out to ascertain that G2 populations subjected to these procedures behave in a fashion consistent with the known effects of the treatments. The result presented in Table I shows that the fraction of microcolonies

surviving treatment with HSA-^{3}HTdR corresponds closely to the fraction containing at least one unlabeled cell, as determined by radioautographic examination of the treated population. The agreement indicates that HSA-^{3}HTdR selectively kills those cells which incorporate it, i.e., those in the S phase. (Direct examination of the surviving colonies for the presence of low levels of tritium would not be feasible because of the severe dilution of label that occurs during colony development; see Dewey and Humphrey, 1963).

Table I also indicates that 5 hr of treatment kills no more cells in a nominally G2 culture than does 1 hr. This was confirmed in a number of similar experiments in which survival was compared after 1 hr and 4 or 5 hr exposures to HSA-^{3}HTdR (Table II). Although the survival level varied greatly from one experiment to another depending on the rate at which a particular culture was progressing through the

TABLE II

EFFECT OF DURATION OF TREATMENT WITH HSA-^{3}HTDR ON CELL SURVIVAL

Experiment No.	Period of treatment with HSA-^{3}HTdR	Survival
	hr	%
1	20–21	82
	20–25	85
2	20–21	21
	20–24	18
3	20–21	66
	20–25	62

cycle, in all cases maximal killing was obtained within 1 hr. A similar result has been reported for Chinese hamster cells (Sinclair and Morton, 1966) and is implied in the experiments of Whitmore and Gulyas (1966) with L cells.

The kinetic behavior of mitotically selected synchronous populations treated with VLB alone or with both VLB and HSA-^{3}HTdR was examined in the experiments whose results are shown in Figs. 1 and 2. In Fig. 1, the progression of untreated cells into S the phase, as assessed by radioautographic determination of the fraction of cells labeled by continuous treatment with low specific activity ^{3}HTdR beginning 5 hr after collection, is shown by the solid squares. The solid circles show how the untreated population divides, i.e., the number of cells scored in repeated microscopic observations of selected fields. The open squares represent the fraction of cells remaining spread when treated with VLB from 4 hr on, as determined by microscopic observation. The open circles show the relative fraction of colonies developing after treatment with VLB from 4 hr until the times indicated. In this experiment, cell division

achieved a significant rate about 18 hr after collection; decrease in the number of spread cells and of colony-forming units, in the presence of VLB, also began at about this time.[2] Hence, the treated cells apparently progressed at the normal rate.[3]

In Fig. 2, the open circles again show the disappearance of spread cells in the presence of VLB, and the open squares show the corresponding loss of colony-forming units. The triangles illustrate the effect of combined treatment with VLB and HSA-^{3}HTdR. The HSA-^{3}HTdR was present from 20 hr until the times shown.

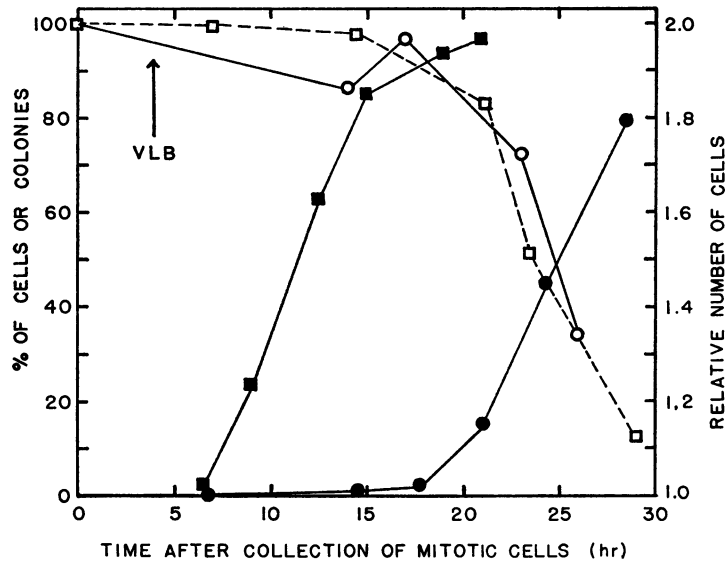

FIGURE 1 Cell progression in the presence of VLB. Mitotically selected synchronous cells were treated with VLB at 4 hr (arrow). The open circles show the per cent of colonies developing (zero time = 100%) as a function of time of incubation. The open squares show the per cent of cells remaining spread on the dish. The progression of parallel, untreated cultures is shown by the solid symbols: the squares indicate the fraction of cells labeled by ^{3}HTdR added at 5 hr, i.e. progression into S, and the circles show the increase in cell number as the culture underwent division.

Identical results were obtained if the HSA-^{3}HTdR was present from 20 until 21 hr only. Elimination of the S cells is seen to reduce somewhat the number of colonies; it does not appreciably affect the slope of the curve.

Before applying these procedures in radiation studies, it was necessary to show that they do not alter the radiation response of cells that are at stages of the cycle which are presumed to be insensitive to the selecting agent. The upper portion of Fig. 3 shows the progression of mitotically selected synchronous populations through

[2] In most experiments, disappearance of spread cells preceded loss of colony-forming units by 1–3 hr (compare circles and squares in Fig. 2). This may be attributed to the fact that the majority of cells occur as two-celled microcolonies; both of the cells must be removed before the colony is lost.

[3] The transient fall in the number of colony-forming units at 13 hr (Fig. 1, open circles) is anomalous.

the S period in the presence (open squares) of VLB (added at 3.5 hr) or its absence (circles), as measured by radioautographic determination of the fraction of cells labeled by 15 min pulses with ^{3}HTdR. The slightly elevated values for the treated cultures result from selective elimination of the most rapidly moving cells as they reach mitosis (Pfeiffer and Tolmach, 1966). The decrease in spread cells, reflecting the transition from G2 into M, is also shown for the VLB-treated population (solid squares) (see Figs. 1 and 2). Survival of these populations after irradiation with 500 rad is shown in the lower part of the figure. From the absence of any significant difference in the levels of survival of the untreated (circles) and VLB-treated (squares)

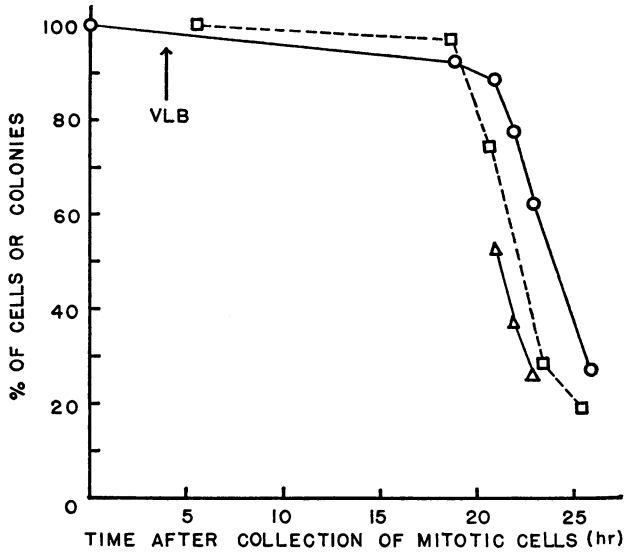

FIGURE 2 Cell progression in the presence of VLB and HSA-^{3}HTdR. Mitotically selected synchronous cells were treated with VLB at 4 hr (arrow). As in Fig. 1, the circles show the per cent of colonies developing (zero time = 100%) as a function of time of incubation, and the squares show the per cent of cells remaining spread on the dish. The triangles show the per cent of colonies developing when HSA-^{3}HTdR also was added at 20 hr.

cells (both of which have been corrected for colony multiplicity according to the method of Sinclair and Morton, 1965), it may be concluded that the X-ray response of HeLa cells is not altered by VLB.

It is more difficult to demonstrate that the radiation response of G2 cells subjected to HSA-^{3}HTdR treatment is undistorted; there are S cells present in mitotically selected populations at all times after 7 hr, and the treatment will therefore alter the population. However, the most likely effect of such treatment on the cells in question would be a sensitization to further insult, e.g. irradiation with X-rays or ultraviolet light, by virtue of having incorporated sublethal (and subdetectable) amounts of ^{3}HTdR. While the X-ray sensitivity of the treated population is indeed increased (Figs. 4 and

5), this is presumably the result of removal of relatively resistant S cells. A better test is provided by ultraviolet irradiation. With this agent, sensitivity is found to *decrease* after treatment with HSA-^{3}HTdR (Djordjevic and Tolmach, 1966). This observation speaks against a sensitization process, and we conclude that the radia-

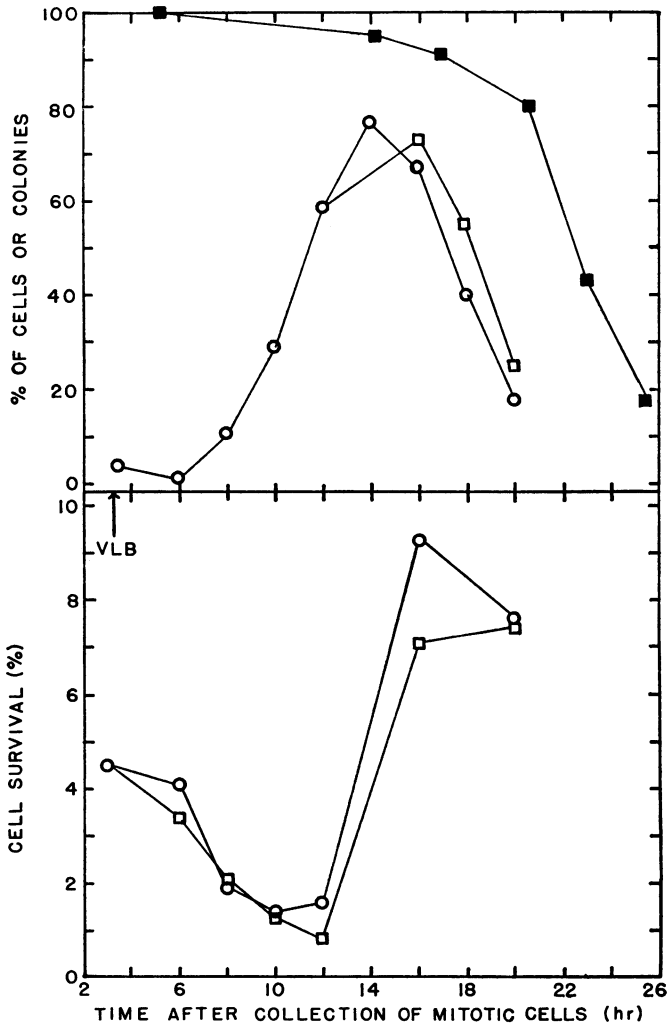

FIGURE 3 Survival after irradiation in the presence of VLB. The lower curves show survival (corrected for cellular multiplicity) after irradiation of mitotically selected synchronous cells with 500 rad. The squares refer to cells incubated in the presence of VLB from 3.5 hr on (arrow); the circles refer to untreated cells. The upper curves depict the progression of the cultures: the solid squares show how spread cells disappear, in the presence of VLB, as they enter M; the open squares show progression through the S phase in the presence of VLB, and the circles in its absence, as determined by autoradiographic examination of cells pulse-labeled (15 min) with ^{3}HTdR.

tion response of G2 cells is probably not altered by having been exposed to HSA-^{3}HTdR.

X-ray dose response curves of untreated and purified G2 populations 18.5 hr after collection of mitotic cells (uncorrected for colony multiplicity) are compared in Fig. 4. The treated cells (circles) were incubated in VLB from 4 hr, and in HSA-^{3}HTdR from 17 hr, until irradiated. It is clear that over the dose range tested, the selected cells are most sensitive. Since the main contaminants in an untreated culture at 18.5

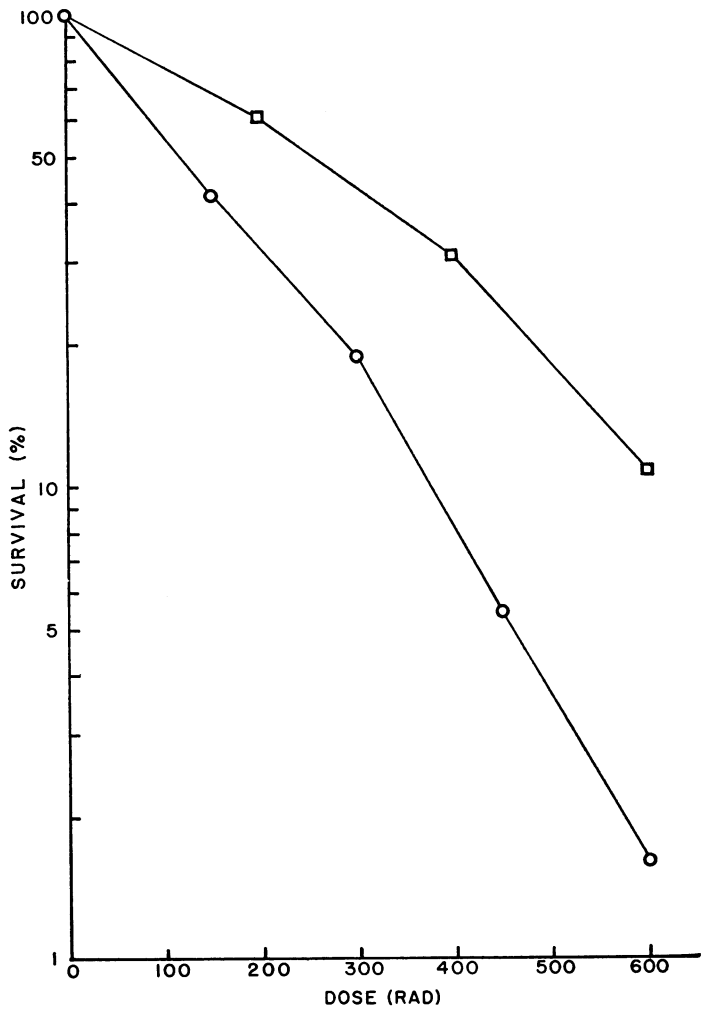

FIGURE 4 X-ray response of crude and purified G2 cells. The squares show the dose-response of a nominally G2 population irradiated 18.5 hr after collection of mitotic cells. The circles show the response of a similar population after elimination of non-G2 cells by treatment with both VLB (added at 4 hr) and HSA-^{3}HTdR (added at 17 hr). Survival values have not been corrected for colony multiplicity.

hr are late S phase cells, the increased sensitivity of the selected population indicates that G2 cells are more sensitive, on the average, than late S cells.

Confirmation of this conclusion is provided by the data shown in Fig. 5. which illustrates the change in survival observed after irradiation of progressively older

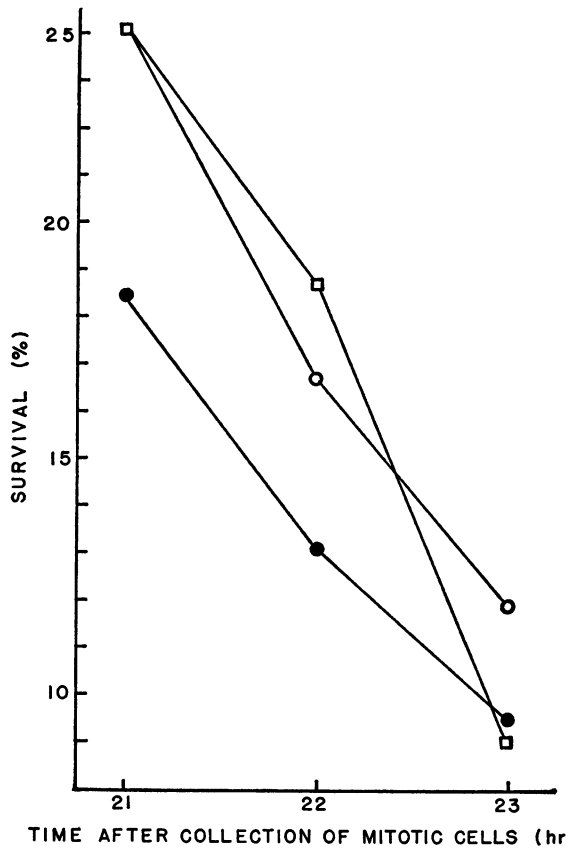

FIGURE 5 Survival of a purified G2 population after irradiation with 150 rad at three successive times. The population is the one whose progression is shown in Fig. 2. The open symbols refer to colony survival; the closed, to cell survival. VLB was added at 4 hr; HSA-^{3}HTdR was added at 20 hr and removed either at 21 hr (squares) or at the time of X-irradiation (circles).

selected G2 cells. The population tested was the one whose progression into M is shown in Fig. 2; the solid symbols show survival after exposure to 150 rad doses, corrected for colony multiplicity. It is clear that sensitivity increases as cells approach M. Furthermore, similar results (not corrected for multiplicity) were obtained whether the HSA-^{3}HTdR was applied only from 20 to 21 hr (squares) or continuously from 20 hr until irradiation (circles). Since in the former case all the G2 cells underwent the same exposure to ^{3}HTdR, the increase in sensitivity can be attributed

to increasing age, even if the HSA-^{3}HTdR treatment does perturb these cells (see above). Further confirmation is afforded by the results described in the next section.

Cyclic Fluctuations in the X-Ray Response of Synchronous HeLa S3 Populations Prepared by the HSA-^{3}HTdR Method

The growth of synchronous populations of HeLa S3 cells prepared by the HSA-^{3}HTdR method was characterized by measuring their progression from late G1 into S and M. Progression into S was followed by adding low specific activity ^{3}HTdR to cultures immediately after removing the HSA-^{3}HTdR. As shown in Fig. 6, and in accord with the findings of Whitmore and Gulyas (1966), the cells still unlabeled at the end of the HSA-^{3}HTdR treatment, 13.2% of the population in this experiment, soon entered S and became labeled. The fraction of unlabeled cells declined to 1%

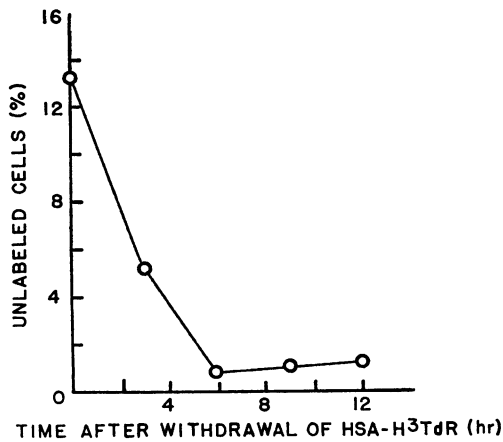

FIGURE 6 Progression of HSA-^{3}HTdR selected synchronous cells into the S phase. A randomly dividing culture was treated with HSA-^{3}HTdR for 9.5 hr. The medium was then removed and replaced with medium containing low specific activity ^{3}HTdR. The unlabeled cells were scored autoradiographically.

within 6 hr, and remained constant at that level for at least an additional 6 hr. The plateau at 1% presumably represents cells that are not progressing (or are progressing very slowly) through the generation cycle; Whitmore and Gulyas (1966) similarly found that 1% of their L cell populations failed to acquire label on prolonged incubation (33 hr) in the presence of HSA-^{3}HTdR. In several additional experiments in which HSA-^{3}HTdR treatment was applied for periods ranging from 7 to 9.5 hr, between 25 and 4.7% of the cells remained unlabeled at the end of treatment. In all cases, they rapidly entered S, in a fashion similar to that shown in Fig. 6.

Progression of synchronous populations through cell division could not be observed by microscopic examination because of the large background of moribund cells produced by the HSA-^{3}HTdR treatment. Instead, attempt was made to measure the number of viable (colony-forming) cells as a function of time, after removal of the HSA-^{3}HTdR. This procedure was satisfactory with L cells grown in suspension culture (Whitmore and Gulyas, 1966) where no trypsinization step is required, but

anomalous results were obtained in repeated experiments with HeLa cells, which, because they grow attached to the culture dish, require the successive trypsinization and replating of a series of replicate cultures.[4]

Hence, an alternative means of demonstrating synchronous growth was employed, based on the arrest of mitotic cells by VLB and their removal, as discussed in the previous section in connection with mitotically selected cells. Passage of cells from G2 into M was followed by measuring the loss of colony-forming units. The results shown in the upper portion of Fig. 7 are those expected for cell populations confined to the latter portion of G1 at the termination of treatment with HSA-^{3}HTdR. The number of colonies developing remains constant for at least 6 hr as incubation is continued in the presence of VLB, and subsequently falls to a low level during the next 12 hr. Thus, these populations behave in a fashion similar to that of mitotically synchronized cells (Figs. 1 and 2), except that the initial plateau is shorter because cells need traverse only the S and G2 periods, rather than all of interphase, before they begin to arrive at mitosis where they are arrested by VLB.

When replicate portions of a synchronous population prepared by the foregoing procedure were irradiated with a constant dose of X-rays (500 rad) at sequential times after removal of the HSA-^{3}HTdR, large changes in the survival level were detected. The data shown in the lower part of Fig. 7, which were obtained in two separate experiments covering different portions of the cycle, show that cells are relatively sensitive immediately after the completion of the selection process (zero time) when they are in late G1. Survival increases as cells progress through S, and then falls again as they enter G2 and M.[5] This behavior is consistent with that observed with mitotically selected synchronous HeLa S3 cells (see previous section and Terasima and Tolmach, 1963 *b*). It indicates that the method of cell selection does not influence the observed pattern of radiation sensitivity changes during the latter portion of the generation cycle. It may be concluded, therefore, that HeLa S3 cells and L cells (Whitmore et al., 1965) undergo qualitatively different sensitivity fluctuations during this part of the cycle.

However, in experiments extended to longer times, the pattern is apparently inconsistent with results obtained with mitotically selected synchronous cultures. The lower curves of Fig. 8 show the results of three experiments which were carried on

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[4] These difficulties may possibly arise from sensitization of G1 cells to trypsin treatment as a result of the incorporation of sublethal amounts of HSA-^{3}HTdR. If such sensitization does in fact occur, the HSA-^{3}HTdR is presumably incorporated from an acid-soluble precursor pool after removal of the radioactivity, since the fraction of labeled cells agrees closely with the fraction of colony-forming cells at the end of the treatment period. In a preliminary experiment, the number of unlabeled cells did in fact decrease by about 50% during a 5 hr posttreatment incubation period in the absence of ^{3}HTdR (see, however, Cleaver and Holford, 1965).

[5] The survival values shown in these plots have not been corrected to the single cell level. At the end of the HSA-^{3}HTdR treatment, 80% of the unlabeled (and presumably viable) cells are found in two-celled microcolonies and they divide during the period subsequent to 6 hr, so that the cellular multiplicity of the colonies must rise further. Accordingly, the cellular survival actually falls even faster than shown by the descending limb of the curve.

through the subsequent G1 period. The survival fluctuations during the first 18 hr after removal of the HSA-^{3}HTdR are similar to those shown in Fig. 7. During the succeeding 10 hr, survival again increases. By the end of this period most cells should be in the S phase, as indicated by the approximate scale (center of figure) showing the average position of cells in the generation cycle. This scale, which was constructed from data obtained in these and previous experiments (Terasima and Tolmach,

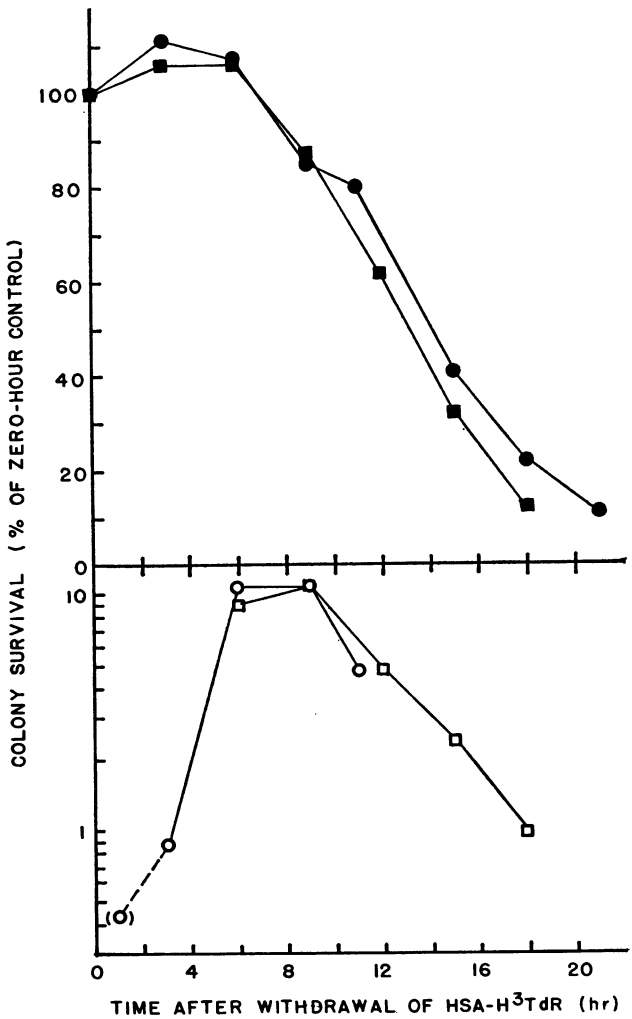

FIGURE 7 Survival of X-irradiated asynchronous populations selected by the HSA-^{3}HTdR method. The lower curves show survival of two synchronous populations after irradiation with 500 rad at different times following termination of the selection procedure. Exposure to HSA-^{3}HTdR was for 9.5 hr, selecting 4.6% of the population (circles), or 9.0 hr, selecting 11.1% (squares). The 1 hr point is unreliable. The upper curves show the progression of these cultures through the generation cycle, as determined by the loss of colony-forming units on continued incubation in the presence of VLB.

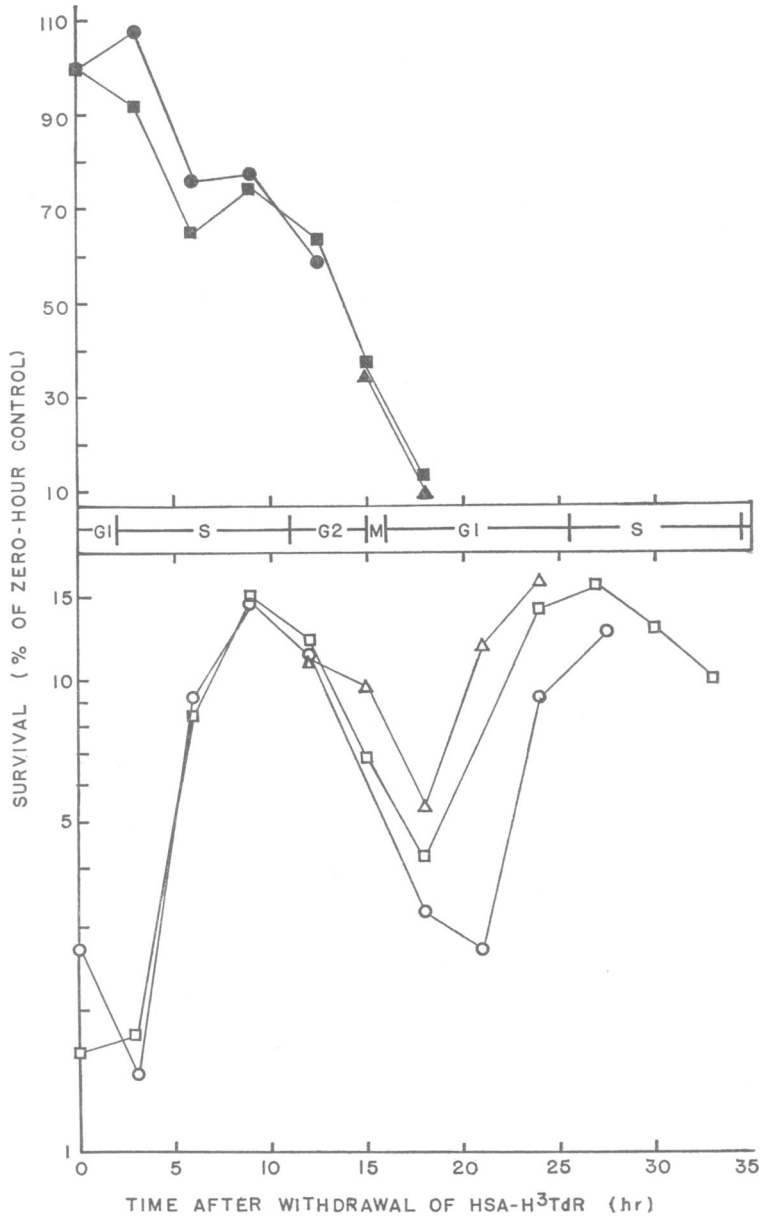

FIGURE 8 Survival of X-irradiated synchronous populations selected by the HSA-^{3}HTdR method. These experiments are similar to those whose results are shown in Fig. 7, but were carried on for longer times. The lower curves show survival of three synchronous populations after irradiation with 500 rad at different times following termination of the selection procedure. Treatment with HSA-^{3}HTdR lasted either 7 hr, selecting 18.7% of the population (circles), or 9 hr, selecting 12.0% and 12.5% respectively (squares and triangles). The upper curves show the progression of these cultures through the generation cycle, as determined by the loss of colony-forming units on continued incubation in the presence of VLB. The apparent early progression of a fraction of cells into M suggests that synchrony was less than optimal in these experiments. The scale showing the approximate location of the main body of cells in the generation cycle was deduced from these and previous experiments.

1963 *a*; Phillips and Tolmach, 1965), can be taken only as a rough guide to the position of any given cell, as identification of position becomes increasingly uncertain as the population becomes progressively more asychronous. Nevertheless, it might have been expected that the relatively resistant early G1 period and subsequent increase in sensitivity during the remainder of G1 (Terasima and Tolmach, 1963 *b*) would be apparent. Failure to detect the resistant period might be due to an inadequate degree of synchrony in the relatively broad bands of cells selected (12–18.7% of the parent populations). Furthermore, it is possible that the particular cultures used in these experiments suffered from exceptionally poor synchrony (the upper curves of Fig. 8 show an anomolous decrease in colony-forming units between 3 and 6 hr in the presence of VLB), even though X-ray survival fluctuated as expected during the first 18 hr. It should also be pointed out that in these experiments it was not possible to make correction for cell multiplicity, which was changing during the period in question. However, while such correction would lower the survival values at late times relative to those at earlier times, it is not apparent how it could make evident a period of resistance in early G1.

DISCUSSION

The foregoing results serve to characterize better the X-ray sensitivity fluctuations that mitotically selected HeLa S3 cells undergo during the generation cycle. The demonstration of progressively increasing sensitivity during G2 would appear to establish a general similarity in the behavior of HeLa S3 and Chinese hamster cells, except for the absence of a resistant period in the latter during early G1. This difference may be ascribed to the relatively short duration of G1 in Chinese hamster cells. As pointed out by Sinclair and Morton (1966), a resistant phase can be detected in Chinese hamster cultures that have extended G1 periods (Hahn and Bagshaw, 1966).

Differences between these two lines may exist, however, with respect to cyclical changes in the shape of dose-survival curves. Adopting the usual multitarget model for cell inactivation, Sinclair and Morton (1965) reported that if straight lines were fitted to the (semilogarithmic) plots of the data below approximately 10% survival, large apparent changes were observed in extrapolation number, while the slopes (mean lethal doses) were essentially constant, and although changes in slope also were later discerned when the mitotically selected populations were purified by treatment with HSA-^{3}HTdR (Sinclair and Morton, 1966), the extrapolation number still appeared to fluctuate during the cycle. On the other hand, Terasima and Tolmach (1963 *b*) reported that no large changes in extrapolation number were apparent when mitotically selected HeLa S3 cells were irradiated at different times in the cycle. However, it would appear that such observations must be interpreted with extreme caution, as in fact neither system yields data that are properly described by the usual multitarget formulation; at least at certain times in the cycle, the survival curves fail to exhibit limiting terminal slopes. Our original data (Terasima and Tol-

mach, 1963 *b*), though crude, suggested that this might be generally the case with HeLa S3; recent efforts to characterize the survival curves more precisely continue to be hampered by uncertainty as to the reliability of the lowest survival values. Sinclair and Morton (1966) and Sinclair (1965) have discussed the experimental difficulties that arise in measuring survival at low levels, and they too question the accuracy of the observed shapes of their survival curves. In fact, at the present time neither the most precise published data nor our own unpublished results provide experimental justification for assuming that any particular kinetic model is applicable, and accordingly, we have chosen to emphasize changes in survival level at a given dose, rather than in the dose-survival curve. Unfortunately, even this approach can lead to ambiguities, for with sufficiently large changes in shape, the pattern of sensitivity fluctuations can be qualitatively different, depending on the dose level chosen for comparison. This is the case with the results reported by Whitmore et al. (1965) for mouse L cells (see their Fig. 12). Nevertheless, if roughly comparable (1–10%) survival levels are examined in the three systems, it is clear that L cells behave quite differently from HeLa S3 and Chinese hamster during the latter part of the cycle. The experiments reported in the second part of this paper lead us to conclude that these differences are properties of the cells, not of the experimental methods. Hence, future investigations must be concerned not only with the complex sensitivity fluctuations that occur in any one of the systems, but also with the differences in radiation response between cell types.

In spite of several published descriptions of the general patterns of X-ray sensitivity fluctuations during the cell generation cycle, uncertainties still exist as to the precise locations in the cycle at which the increases and decreases in survival commence. (These will not, in general, correspond to the observed minima and maxima, which may vary with the degree of synchrony in a culture, as well as with the particular dose level chosen for comparison.) For example, the location in G1 at which maximal sensitivity is reached in HeLa S3 remains uncertain. Using FUdR to inhibit DNA synthesis (and taking the incorporation of ^{14}CTdR by the culture as a measure of its rate), Terasima and Tolmach (1963 *c*) had previously identified the point of maximal sensitivity as the end of G1, but the results of subsequent experiments (Phillips and Tolmach, 1965) are equally consistent with a location several hours before that. This is illustrated by the data of Fig. 9, which compare the fraction of cells labeled with ^{3}HTdR (solid circles), i.e. of cells that have entered S, with survival following irradiation with 500 rad in the presence or absence of FUdR (open symbols). It is seen that survival is already close to the minimum value (presumably a plateau; see Terasima and Tolmach, 1963 *c*), while the majority of cells is still in G1. If the transition from relative resistance to sensitivity is abrupt in the individual cells of the culture, this result would indicate that cells become sensitive some hours before DNA synthesis begins. Even the correlation that has been drawn between the start of the sensitivity decrease and the commencement of DNA synthesis (Terasima and Tolmach, 1963 *c*) has been questioned (Sinclair and Morton, 1966), although

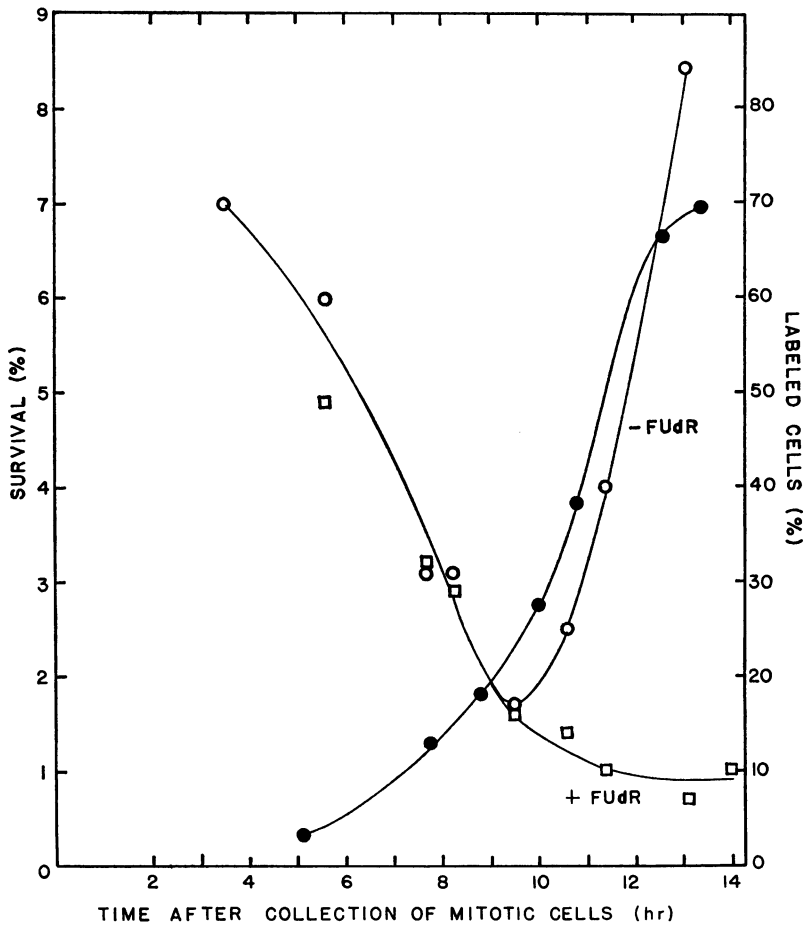

FIGURE 9 Comparison of X-ray survival with the fraction of cells that have started DNA synthesis, during the first part of the generation cycle. Mitotically selected synchronous cells were irradiated at the times shown with 500 rad in either the presence of 10^{-6} M FUdR from 3 hr on (open squares), or its absence (open circles). The fraction of untreated cells labeled by 10 min pulses with ^{3}HTdR is shown by the solid circles. That cells progressed through G1 normally in the presence of FUdR was shown by a close correspondence between the fraction of treated and untreated cells that were labeled.

there is uniform agreement that resistance does begin to rise sometime early in S and that late S is resistant in both HeLa S3 (Terasima and Tolmach, 1963 *b*) and Chinese hamster (Sinclair and Morton, 1965). Identification of the changes in radiation response with specific biochemical events occurring during the generation cycle is presumably important for elucidating the molecular basis of the cyclical radiation sensitivity fluctuations of mammalian cells. Further work along these lines would therefore appear warranted.

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