X-RAY AND ULTRAVIOLET SENSITIVITY OF SYNCHRONIZED CHINESE HAMSTER CELLS AT VARIOUS STAGES OF THE CELL CYCLE

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ABSTRACT Populations of Chinese hamster cells, synchronized by selecting for cells at or close to division, were exposed to 250 kvp x-rays and to ultraviolet light at different stages of the cell cycle and colony-forming ability examined thereafter. These cells were found to be most resistant to x-rays during the latter part of the DNA synthetic period (S) and to be about equally sensitive before (G_1) and after (G_2) this period. Multitarget type curves of the same slope ($D_0 \sim 200$ rad) only approximately fitted the survival data at different stages in the cycle. The changes in response were primarily due to variations in the shoulders (or extrapolation numbers) of the curves however. The response to ultraviolet light differed from that to x-rays. Resistance was greatest in G_2 and changes in both shoulder and slope of the survival curves occurred throughout the cell cycle. The x-ray and ultraviolet responses for component stages of the cell cycle were respectively compounded into expected survival data for a log phase asynchronous population of hamster cells and found to agree well with direct experiment.

INTRODUCTION

Techniques of culturing single mammalian cells initiated by Puck *et al.* (1) offer unique opportunities for the quantitative study of radiation response. Recently these techniques have been used to study the relative radiosensitivity of various parts of the intermitotic cycle, the DNA synthetic phase S, the pre-DNA synthetic phase G_1, and the post-DNA synthetic phase G_2 (2). Although such studies are possible in asynchronous cultures and *in vivo* using radioactive compounds, they can be greatly extended with synchronized populations. Two investigations of this type have been reported (3, 4) in which the radiation response in two different mammalian cell lines has been studied. This report is concerned with the response of partially synchronized Chinese hamster cells to x- and ultraviolet radiation delivered at various stages of the intermitotic cycle.

MATERIALS AND METHODS

1. *Method of Synchronization.* Cells cultured on plastic (or glass) dishes are flattened out and securely attached to the surface, except during mitosis, when the cells round up and may be removed without disturbing many interphase cells. Terasima and Tolmach (5) obtained a high percentage of mitotic cells from HeLa cell cultures using a simple washing-off technique. In our hands this method has proved unsuccessful with Chinese hamster cells, but with a combination of methods a very substantial degree of synchrony has been obtained (6).

The cell line used was derived from the V79 clone subcultured by Elkind and Sutton (7). The cells are normally grown on plastic Petri dishes (85 mm diameter) using a medium similar to HU-15 (7) in a humid atmosphere of 2 per cent CO_2 and air at 37°C. An increased percentage (25 per cent) of fetal calf serum has sometimes improved the percentage yield of mitotic cells. Single cells grown in log phase for some 16 to 40 hours until the titer ranged from 1 to 4×10^6 cells per plate were refrigerated for 1 hour at 4°C and reincubated at 37°C for 2 to 4 hours. The medium was then removed and without washing, 4 ml of 0.03 per cent trypsin solution was added to each plate. The plates were shaken under controlled conditions on a bench shaker for 4 minutes. The trypsin suspension was removed and the product of many plates pooled to provide the source of cells for the experiments described below. The concentration of cells was 4 to 8×10^3 per ml, a yield of about 1 per cent.

Cooling cells to 4°C either for 1 hour or 24 hours provided, at best, only a slight pulse of mitosis (from $\sim$ 5 to $\sim$ 8 per cent maximum) in the population after reincubation at 37°C and no marked synchrony resulted (8). However, cooling was found desirable to provide a better differential attachment between interphase cells (which become more difficult to trypsinize) and dividing cells.

2. *Degree of Synchrony.* Several methods were used to determine the degree of synchrony obtained in a given experiment.

(*a*) *Mitotic index.* The percentage of cells initially in mitosis was obtained from slides prepared by fixation, staining, and squashing cells from an aliquot of the suspension.

(*b*) *Cell size distribution.* The size distribution of the initial cell suspension was obtained by counting an aliquot of the suspension on a Coulter electronic cell counter (Model B, 100 μ aperture) and size distribution plotter which enabled sizing to be carried out automatically.

(*c*) *Cell number (growth).* Two methods were used. In the first, plates were inoculated with about 1.5×10^4 cells per plate and incubated. Subsequently, at each time interval, the total number of cells per plate on two plates was determined by trypsinizing and counting the resulting suspension on the Coulter counter. In the second method, stained colonies from plates used for DNA studies with H^3-thymidine (see below) were analyzed microscopically for the mean number of

cells per colony as a function of fixation time. These two methods measure essentially the same quantity, increase in cell number.

(*d*) *Pulse labeling with H^3-thymidine.* The percentage of cells in the S phase at a given time was measured by pulse labeling with tritiated thymidine. Plates inoculated with about 1.5×10^4 cells each were incubated and at intervals exposed to 0.2 μc/ml of H^3-thymidine (3 c/mM) for 15 minutes. The medium already contained about 0.38 μg/ml of unlabeled thymidine. Thereafter, the medium was removed, the cells fixed, and a thin layer of liquid film emulsion added. After storage for 3 to 4 weeks at 4°C the plates were developed and the cells stained through the film with Mayer's hematoxylin and eosin. Cells with more than 12 grains/nucleus were scored as labeled cells,[1] but it was rarely necessary to apply this criterion.

3. *X-Ray Exposure.* For exposure to a constant dose of x-radiation, a series of plates inoculated with about 10^3 cells per plate were incubated. At intervals, two plates were removed and exposed at room temperature on a rotating platform to 750 röntgen (710 rad) of 250 kvp x-radiation HVL = 0.9 mm Cu, dose rate, 110 R/min.). The exposure was measured with a calibrated Victoreen r-meter in air at the level of the cells. Measurements made with various volumes of ferrous sulfate solution in the plastic Petri dishes established the absorbed dose to the cells as 0.945 rad/R. After 710 rad, the single cell surviving fraction in the asynchronous population is about 0.12 (9). The plates were restored to the incubator less than 10 minutes later and incubated for 10 to 12 days. The plates were then stained with methylene blue and all visible colonies counted. Other plates at 1/6 the cell concentration were used as plating efficiency controls.

For x-ray survival curves, cells were inoculated at levels between 2×10^4 cells per plate and 2×10^2 cells per plate. Inocula were chosen so that about 150 colonies appeared on each plate for doses ranging up to 1700 R (9). Plates were at room temperature for less than 20 minutes, which, in asynchronous populations, does not affect either plating efficiency or survival. Immediately after exposure, medium was added to the plates containing the highest inocula.

4. *Ultraviolet Light Exposure.* Exposure to a single dose and survival curves over a range of doses of UV light were conducted in a manner similar to exposures to x-rays. The source of UV was a germicidal lamp (Westinghouse sterilamp G15-T8) emitting primarily 2537 A. At the exposure level, the intensity was 4.0 ergs/mm^2/sec. measured[2] with an Eppley thermopile calibrated at the

[1] This figure was obtained from control experiments with the asynchronous population. With this type of film the background is rather high and cells not exposed to radioactivity usually average 2 to 3 grains/nucleus for an equivalent storage time. Thus the probability of a non-synthesizing cell having greater than 12 grains/nucleus is very small. This criterion is probably too severe, but in fact very few cells between 4 and 12 grains/nucleus were observed in any of these experiments.

[2] We are indebted to Dr. R. H. Haynes, University of Chicago, for this measurement.

National Bureau of Standards. The fixed single dose used was 200 ergs/mm^2, for which the single cell survival in the asynchronous population ($\sim$ 0.12) is the same as for 710 rad of 250 kvp x-radiation. For survival curves, adjusted inocula were used as for x-rays. All exposures were conducted in room light, as no photoreactivation was observed in the asynchronous population under these conditions.

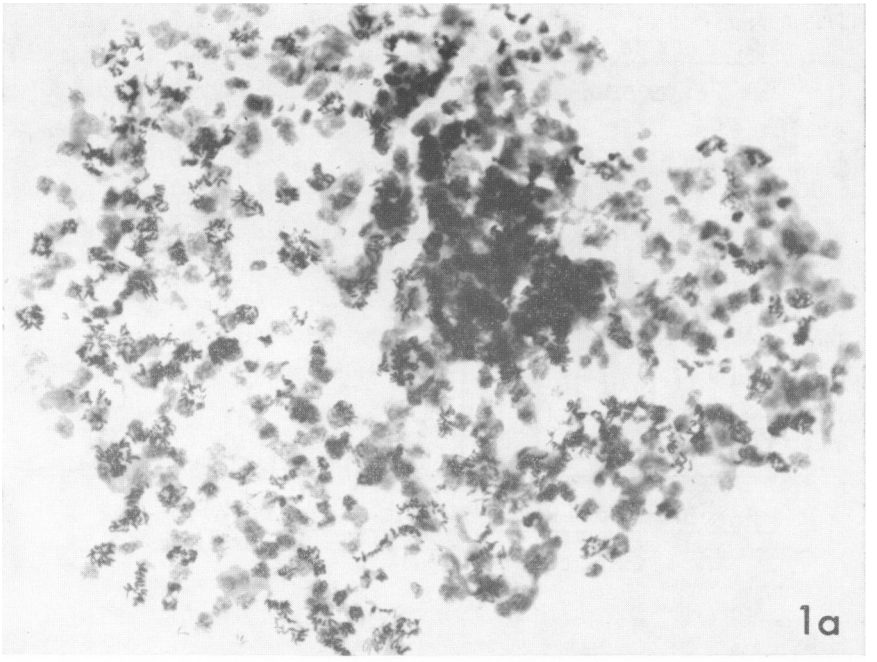
Two departures from the x-ray procedure were necessary for ultraviolet radiation. First, because of UV absorption, the cells were exposed with the lid off the dish and the medium removed. The medium was replaced immediately after exposure. Controls received the same treatment. Second, in the counting of colonies after staining, only those on the flat bottom of the dish were counted, as some cells survive in the bevelled rim where they are protected from UV by dregs of medium. These two modifications reduce the accuracy of the UV data.

RESULTS

1. *Degree of Synchrony.* (*a*) *Mitotic index.* The percentage of cells in mitosis was determined from stained slides. The time during which cells are rounded up ($\sim$ 30 minutes) is less than 5 per cent of the division cycle. Rounding-up commences after mitosis begins and extends beyond the completion of mitosis; consequently the degree of synchronization may be better than indicated by the mitotic index. The mitotic index has ranged from 30 to 80 per cent in our experiments. In Figs. 1a and 1b pictures of portions of slides from two experiments are shown. In these examples, although some 25 to 50 per cent of the cells could be in interphase, it is probable from our other data that the majority of these cells have just divided.

(*b*) *Size distribution.* In Figs. 2A and 2B the size distribution of a harvested population with a mitotic index of about 50 per cent is compared with the population of cells left on the plate after the separation procedure. In the "synchronized" population the modal relative volume is 14 (*i.e.*, channel 14 on the plotter under the counting conditions chosen); there is a larger proportion of small cells; and very few cells have the volume characteristic of the average interphase cell (*i.e.*, modal relative volume, channel 9). In experiments in which the mitotic index is higher, the relative amount of smaller cells is decreased and the peak around 14 is increased. The reverse is true when the mitotic index is lower. In all cases very few cells appear in the average interphase region at channel 9. The subsequent history of populations with lower mitotic indices indicates that they were initially almost as well synchronized as those for which the mitotic index was initially very high (Figs. 4 and 6 below).

(*c*) *Cell number.* Growth curves obtained by Coulter counting and by counting cells per colony from stained plates were shown in an earlier report (6). Examples from a later experiment are shown in Figs. 3A and 3B. Zero time on this graph and for all other data presented in this paper is the time of incubating the "synchro-



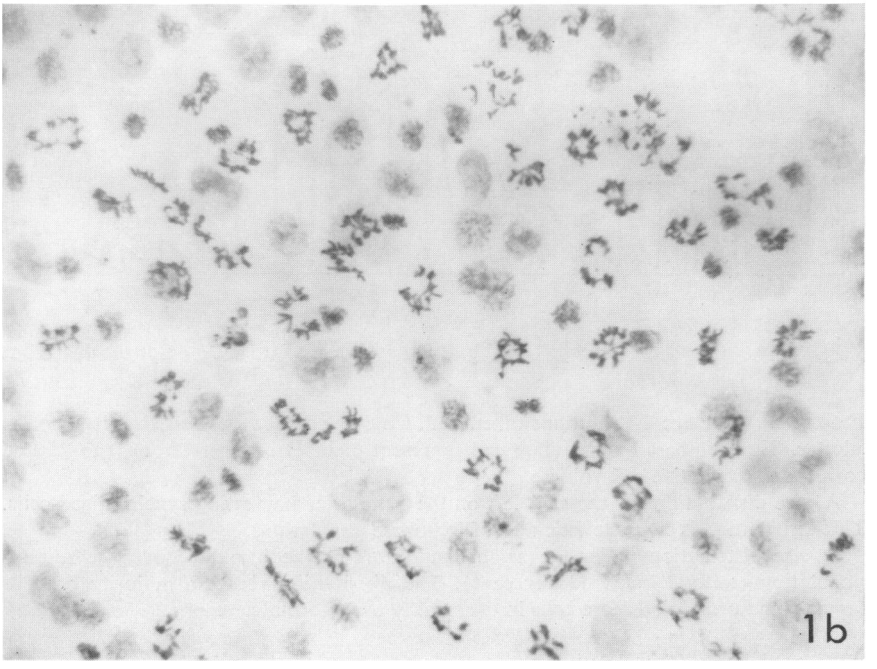


FIGURE 1 Stained preparations of partially synchronized populations. a, Exp. S-12, mitotic cells 75 per cent of the total. b, Exp. S-18, mitotic cells 47 per cent of the total.

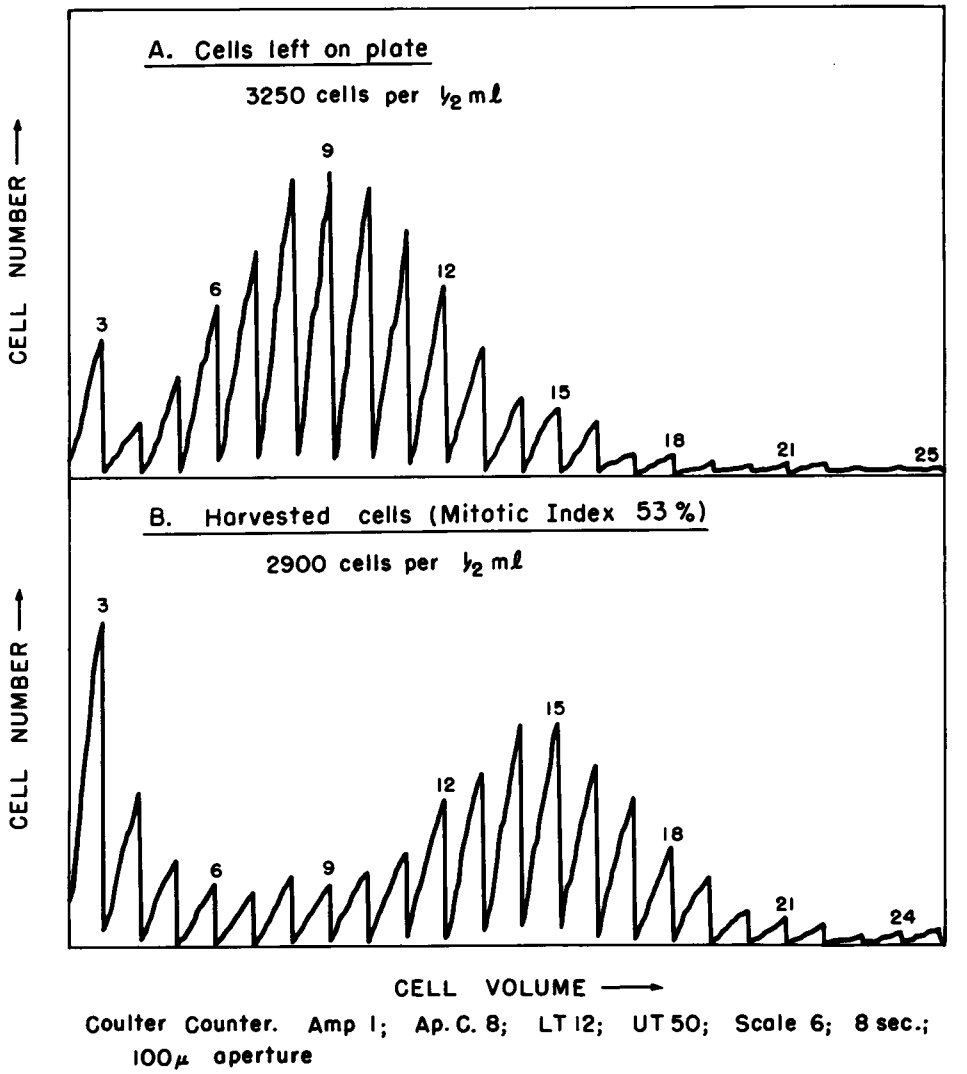


FIGURE 2 Cell size distributions taken with Coulter counter and cell size distribution plotter. The numbers on the diagrams represent channel numbers from 0 to 25 and correspond to relative cell volume.

A, size distribution for the cells left on the plate after harvesting synchronized cells. This distribution is characteristic also of log-phase populations.

B, size distribution under the same conditions for the harvested or synchronized population (mitotic index 53 per cent). The distribution shows a majority of cells larger than normal and more cells in the smaller than normal category.

nized" population. The application of the Blumenthal and Zaler index (10) to some of our earlier growth data has already been described (6). For the curves of Figs. 3A and 3B, this index is $\sim + 0.35$ to $+ 0.40$ during division, but in other

experiments, values at division of up to + 0.80, and often more than + 0.50, have been found. Another index of synchrony based on growth data, used by Zeuthen (11) and by Burns (12), is the *per cent phasing*

$$= \left(\frac{T}{2} - D\tfrac{1}{2}\right) \Big/ \frac{T}{2} \qquad (1)$$

where T = generation time

$D\frac{1}{2}$ = time for 50 per cent of the cells to divide.

For the curves shown in Fig. 3 the per cent phasing is between 50 and 60 per cent although often higher values, between 60 and 80 per cent, have been found. Growth indices of synchronization are lowered by the presence of non-viable cells (see Discussion).

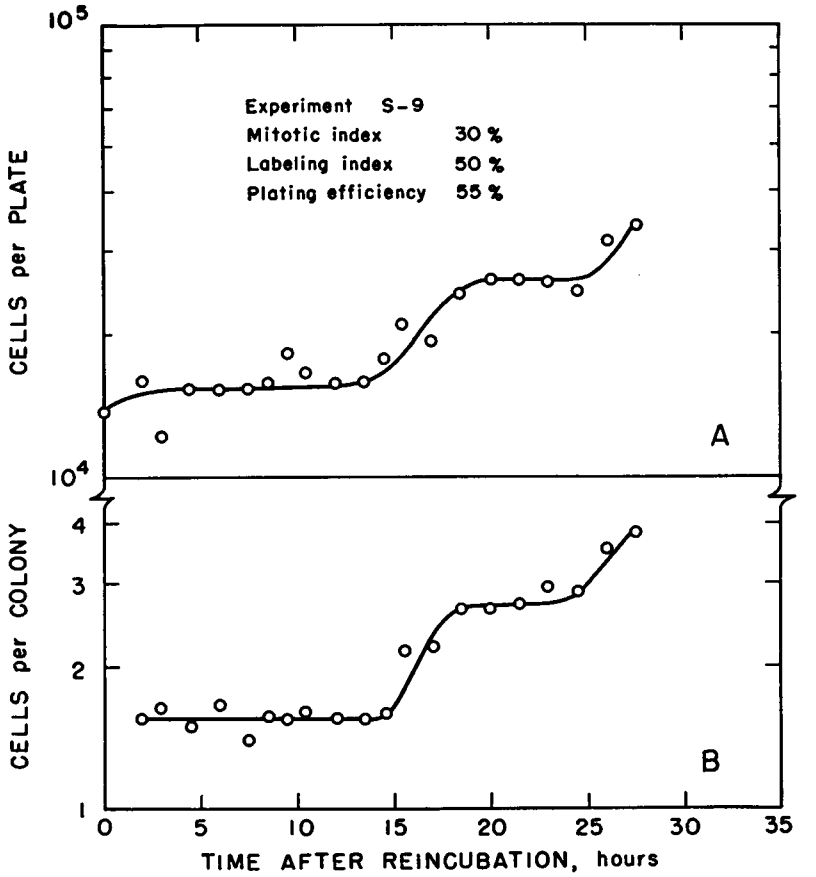


FIGURE 3 Growth of synchronized hamster cell population. A, growth determined by Coulter counting (1/Amp · 1, 1/Ap. C. · 8, LT 12, UT > 100).* B, growth determined by cells per colony on stained plates.

* Ap. C., aperture current; LT, lower threshold; UT, upper threshold.

Fig. 3 also shows that under our conditions, the first interdivisional period is longer than the second. This difference, which may include a lag due to the detachment procedure, is also affected by the frequency of sampling altering incubator conditions. For this reason, many of our experiments were carried throughout two full division cycles or more.

(*d*) *Percentage of cells labeled by an H³-thymidine pulse.* The results of three experiments in which the percentage of cells pulse-labeled with tritiated thymidine at different times was determined, are shown in Fig. 4. In one experiment (S-7)

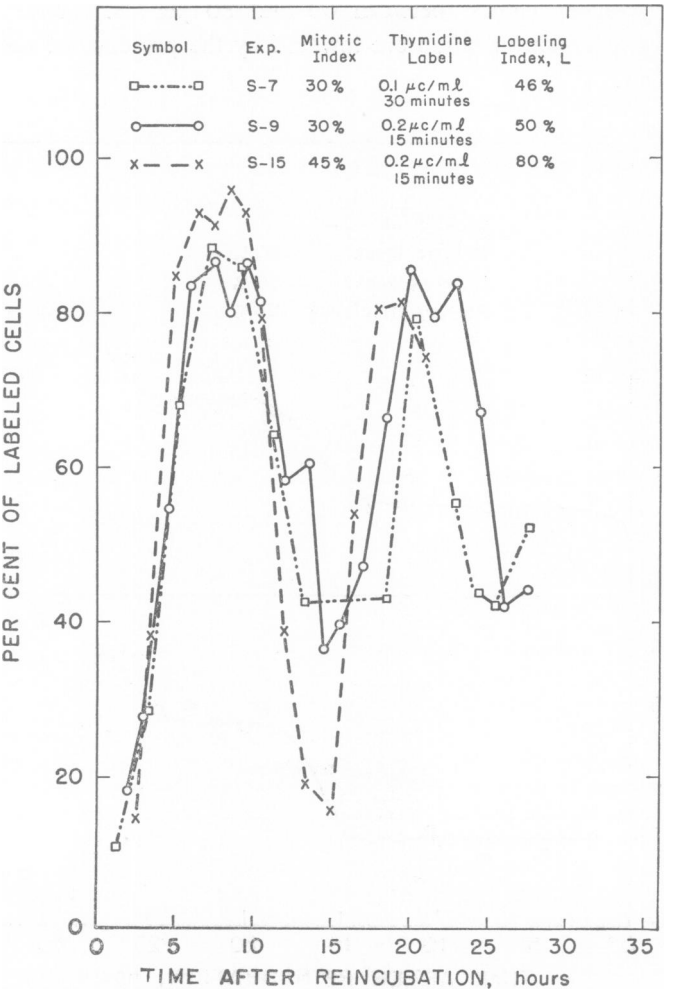


FIGURE 4 Per cent of cells labeled with tritiated thymidine *versus* time after incubation of the synchronized population. From these curves a labeling index of synchrony, *L*, can be determined. *L* is the difference between the maximum per cent labeled cells and the following minimum (see text).

the exposure was to 0.1 μc/ml for 30 minutes while in recent experiments, of which S-9 and S-15 are examples, the exposure was to 0.2 μc/ml for 15 minutes.

It is convenient in comparing the results of these experiments to have an index of population synchrony determined by such a curve. We define a labeling index of synchrony

$$L = L\text{max} - L'\text{min} \tag{2}$$

where L = labeling index of synchrony

Lmax = fraction of cells labeled at maximum of such a curve as Fig. 4.

L'min = fraction of cells labeled at minimum (following the peak percentage of labeled cells).[8]

We have most frequently found Lmax to be 85 to 95 per cent and L'min to be 15 to 30 per cent. Thus L is usually between 80 and 55 per cent. Precise measurements of L however, require reliable determinations of Lmax and L'min. In some of our experiments L'min was not determined precisely because the period between samples was too long. In these cases L will have been underestimated (*e.g.*, see Fig. 4, Exp. S-7).

2. *X-Radiation Response.* The response of the synchronized population to a constant dose, 710 rad, of 250 kvp x-radiation is shown for two experiments (one with initial mitotic index 70 per cent, the other, 30 per cent) in Fig. 5. As previously reported (6), the survival of cells rises sharply during the S period only to fall again before division. The peak of x-ray resistance at this dose occurs while the maximum number of cells are in the latter part of the S period. This response has been consistently observed in many experiments in both first and second cycles.

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[8] This index may be justified as follows. In a population of perfectly synchronized identical cells the percentage of cells labeled by a brief H^3-thymidine pulse will be zero from the beginning of the experiment until the end of G_1, 100 per cent during the S period, and zero again during G_2. Thus $L = 100$ per cent. If a certain fraction α of cells is not synchronized but spread uniformly throughout the interphase cycle, they will contribute a constant labeled fraction 100 S/T α per cent during G_1, S, and G_2, where S is the length of the S period and T the generation time. The synchronized remainder (1-α) contributes zero labeled cells during G_1 and G_2 and 100 (1-α) per cent during S. The total cells labeled are therefore 100 S/T α per cent in G_1 and G_2 and [100 (1-α) + 100 $S/T \cdot \alpha$] per cent in S. Therefore, for such a population $L = 100$ (1-α) per cent and to a first approximation L is the percentage of cells which are perfectly synchronized. The final minimum period, in G_2, should be chosen rather than the initial period in G_1 because harvested cells may have a short lag.

A more sophisticated analysis should take into account the fact that cells do not have identical G_1, S, and G_2 periods and a distribution of such periods among members of the population would lead to a decrease in the observed value of L. Also, in actual experiments the fraction α is probably not distributed uniformly throughout the cycle. A value of $L = 100$ per cent is therefore not to be expected in practice even for cells initially "perfectly" synchronized.

The "colony surviving fraction" is the number of colonies on the x-irradiated plates divided by six times the average number of colonies on the plating efficiency plates (see earlier). Our technique of cell synchronization yields a mixture of primarily one- and two-cell colonies; therefore radiation responses (*e.g.*, Fig. 5) obtained for these populations are not those for single cells. Thus the magnitude of the peak in Fig. 5 depends not only on the average variation of x-radiation survival among single cells during the cell cycle and the degree of synchronization, but also on the average number of cells per colony. To obtain the single cell response, the surviving fraction must be corrected for the average number of cells per colony

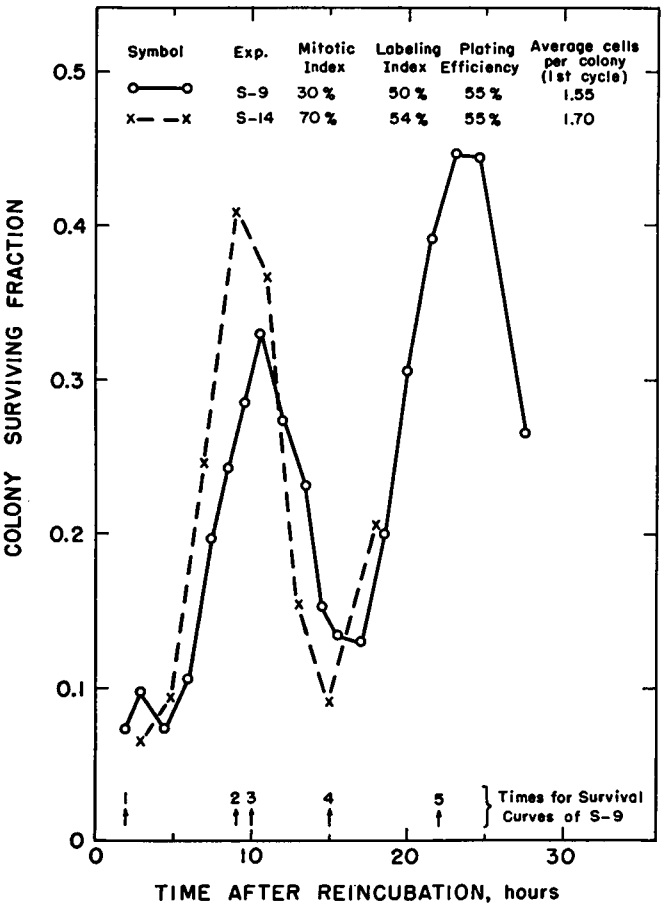

FIGURE 5 The response of synchronized cells to a constant dose of x-rays (710 rad) as a function of time after incubation. Note that the response for S-14 (70 per cent mitotic index) is only a little sharper than that for S-9 (30 per cent mitotic index). The times at which survival curves (Fig. 7) were measured (Exp. S-9) are indicated.

as follows: The fractional survival, f, for colonies of average multiplicity, $\bar{N}$, is related to the average single cell survival, s, by

$$f = 1 - (1 - s)^{\bar{N}} \tag{3}$$

Therefore,

$$s = 1 - (1 - f)^{1/\bar{N}} \tag{4}$$

In deriving this equation it is assumed that each cell of a colony survives independently, but this has already been established for this cell line (7). Equation 4, however, is only an approximation to a more exact equation relating f and s (see Appendix). Values of $\bar{N}$ are obtained from data such as those shown in Fig. 3B, although strictly, $\bar{N}$ should be for viable cells only (see Discussion). The single cell response obtained by applying equation 4 to the data of Fig. 5 is shown in Fig. 6. The difference in response for cells with initial mitotic indices of 70 and 30 per cent is quite small.

Survival curves over a range of doses at various times have also been obtained.

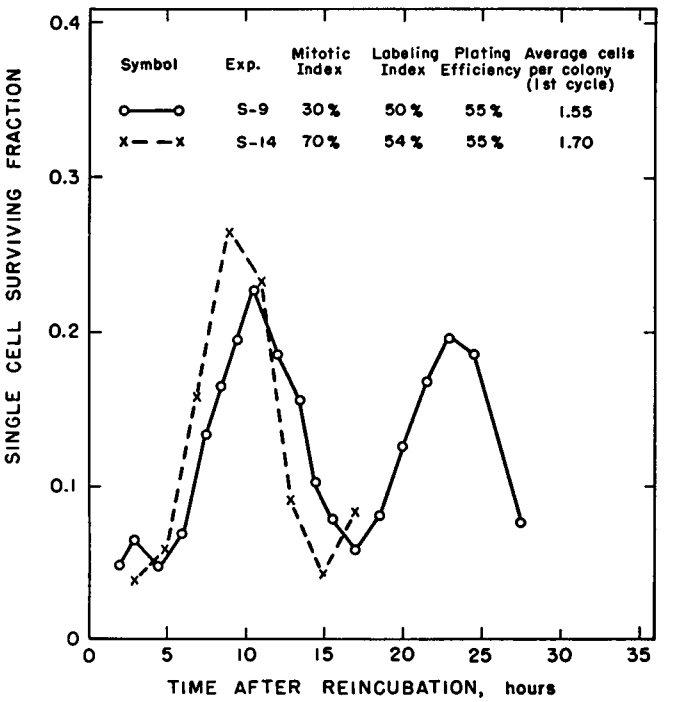

FIGURE 6 The single cell response to 710 rad of x-rays obtained by applying $s = 1 - (1 - f)^{1/\bar{N}}$ to the data of Fig. 5 (see text). $\bar{N}$ is the average cells per colony (Fig. 3). The difference in response between S-14 (70 per cent mitotic index) and S-9 (30 per cent mitotic index) is now even smaller.

In experiment S-9, for example, survival curves were taken at 2, 9, 10, 15, and 22 hours after incubation of synchronized cells. The results are shown in Fig. 7.

The relation

$$s = 1 - (1 - e^{-D/D_0})^n \tag{5}$$

where s = surviving fraction for single cells

D = dose (rad)

D_0 = reciprocal of the slope of the exponential part of the curve (rad)

n = extrapolation number for single cells

is a satisfactory fit to the survival data for the asynchronous cell population (9), for which n should be replaced by $\bar{n}$, the average extrapolation number for a heterogeneous population of single cells. In Fig. 7, the data at 2 and 15 hours and even at 22 hours appear to fit equation (5) quite well (except perhaps close to the origin) but at 9 and 10 hours, the fit is rather poor and a definite change in

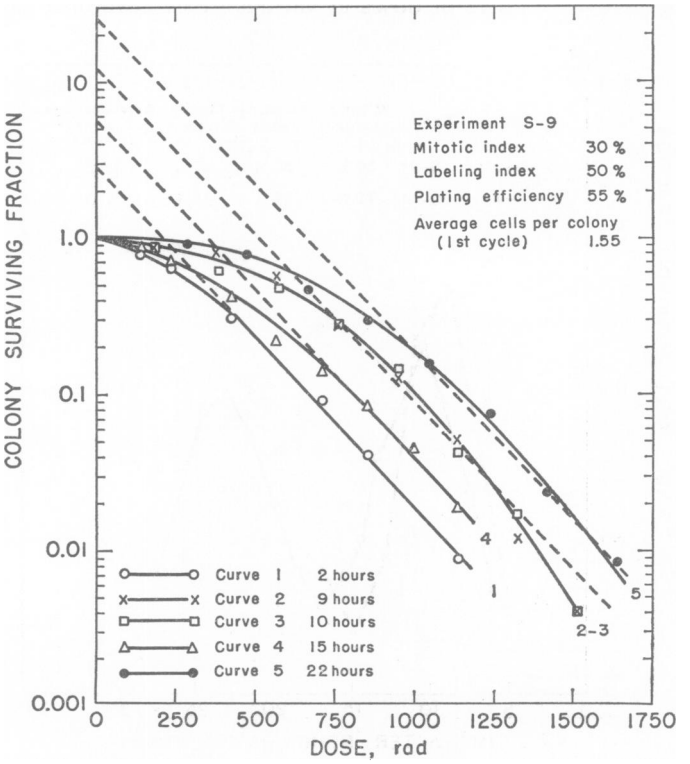

FIGURE 7 X-Ray (colony) survival curves for a synchronized population at different times after incubation (Experiment S-9, see Fig. 5). The solid lines have been drawn through the points and the dashed lines have all been drawn for a D_0 of 200 rad.

the shape of the curve appears to have taken place. At the present time, however, possible alternative mathematical analyses of the data for the curves at 9 and 10 hours do not seem warranted. To a first approximation useful for calculation purposes and valid for about two decades, all five curves of Fig. 7 will be assumed to fit equation (5), with a single D_0 value of about 200 rad (dashed lines) and the changes observed may be ascribed essentially to changes in extrapolation number, n.

The data shown in Fig. 7, in which the ordinate is the colony surviving fraction f, may be corrected to the single cell survival, s, using equation (4). The corrected data are shown in Fig. 8. Curves 1 and 4 are similar and curves 2, 3 (cycle 1), and 5 (cycle 2) for cells in the S phase are almost indistinguishable. The extrapolation numbers for the dashed lines of Fig. 7 and the estimated single cell extrapolation numbers are listed in Table I.

Later experiments (*e.g.*, S-16, Fig. 9) have confirmed that over two decades the

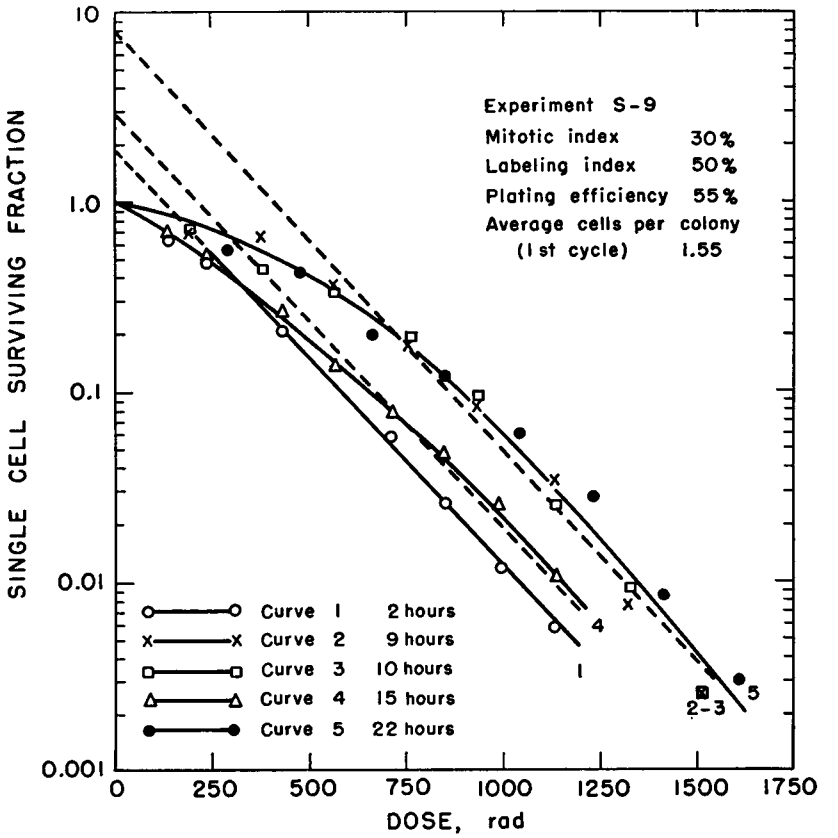

FIGURE 8 X-Ray survival curves for single cells. The values of Fig. 7 modified using $s = 1 - (1 - f)^{1/\bar{N}}$. $\bar{N}$ is the average cells per colony at the time stated (Fig. 3). The value of $\bar{N}$ for curve 4 is less certain because cells are beginning to divide at this time.

TABLE I

EXTRAPOLATION NUMBERS FOR SURVIVAL CURVES OF EXPERIMENT S-9

Time after reincubation	Cell cycle	Phase (approx.)	Observed extrapolation No.	Cells/colony $\bar{N}$	Single cell extrapolation No., n
hrs					
2	1st	G_1	3	1.55	2
9	1st	S	12	1.55	8
10	1st				
15	1st-2nd	G_2	5	2.1*	2.5
22	2nd	S	25	2.7	8

* Actual value measured at 15 hours.

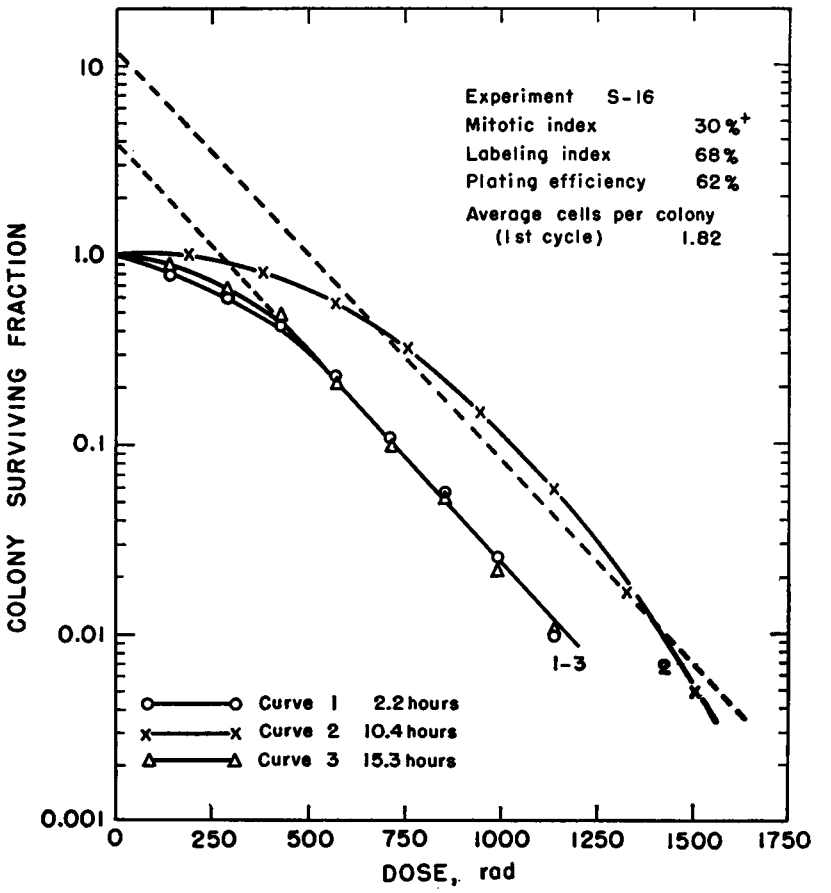

FIGURE 9 X-Ray (colony) survival curves for another synchronized cell population at different times after incubation (Experiment S-16). The dashed lines have been drawn for a D_o of 200 rad.

changes are associated principally with variation in n. In Experiment S-16, D_0 is again about 200 rad and the corrected value of n for a single cell is again $\sim$ 2 at 2 and 15 hours and $\sim$8 at 10 hours. In each of 3 sets of experiments, the same change in shape of the survival curve at 10 hours was observed and consequently equation (4) is only an approximate fit to the data for cells in the S phase. The trend in curves 2 and 3 of Fig. 7 is such that if our data could be extended to lower fractional survivals, a fit to equation (5), if possible at all, would require appreciably higher values of n and lower values of D_0 than those indicated. This appears to be the case in recent experiments by Whitmore *et al.* (13) using *L* cells, in which both

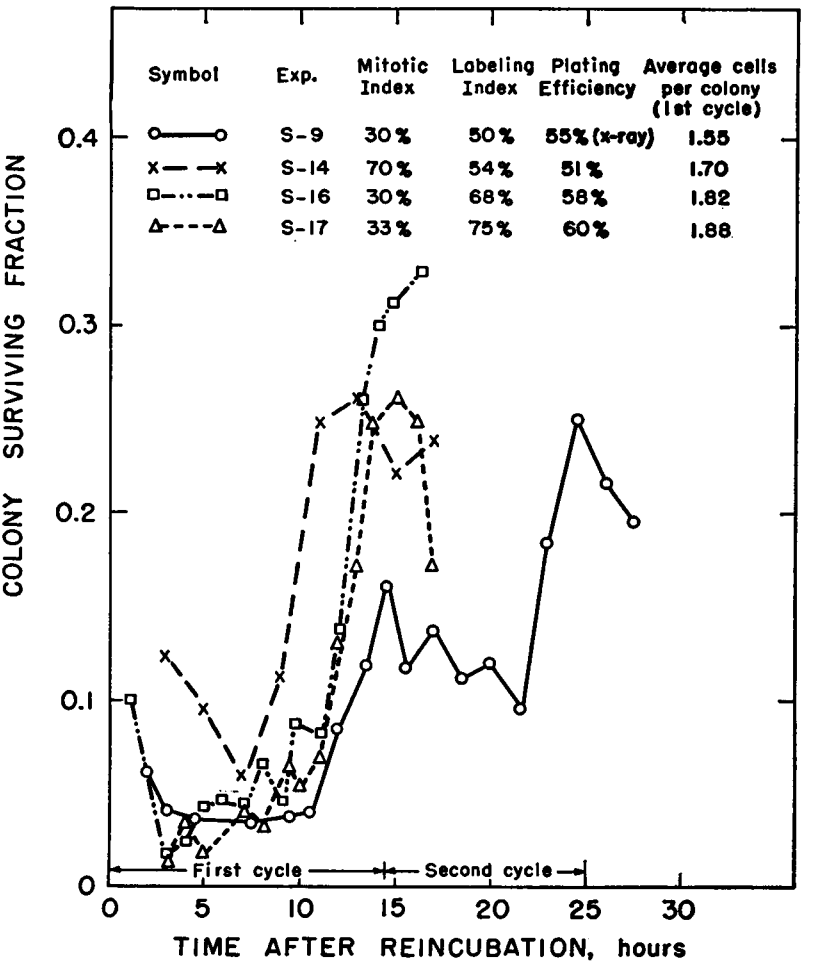

FIGURE 10 Response of synchronized populations to a constant dose of UV (200 ergs/mm^2) as a function of time after reincubation. The approximate length of the first and second cycles in indicated.

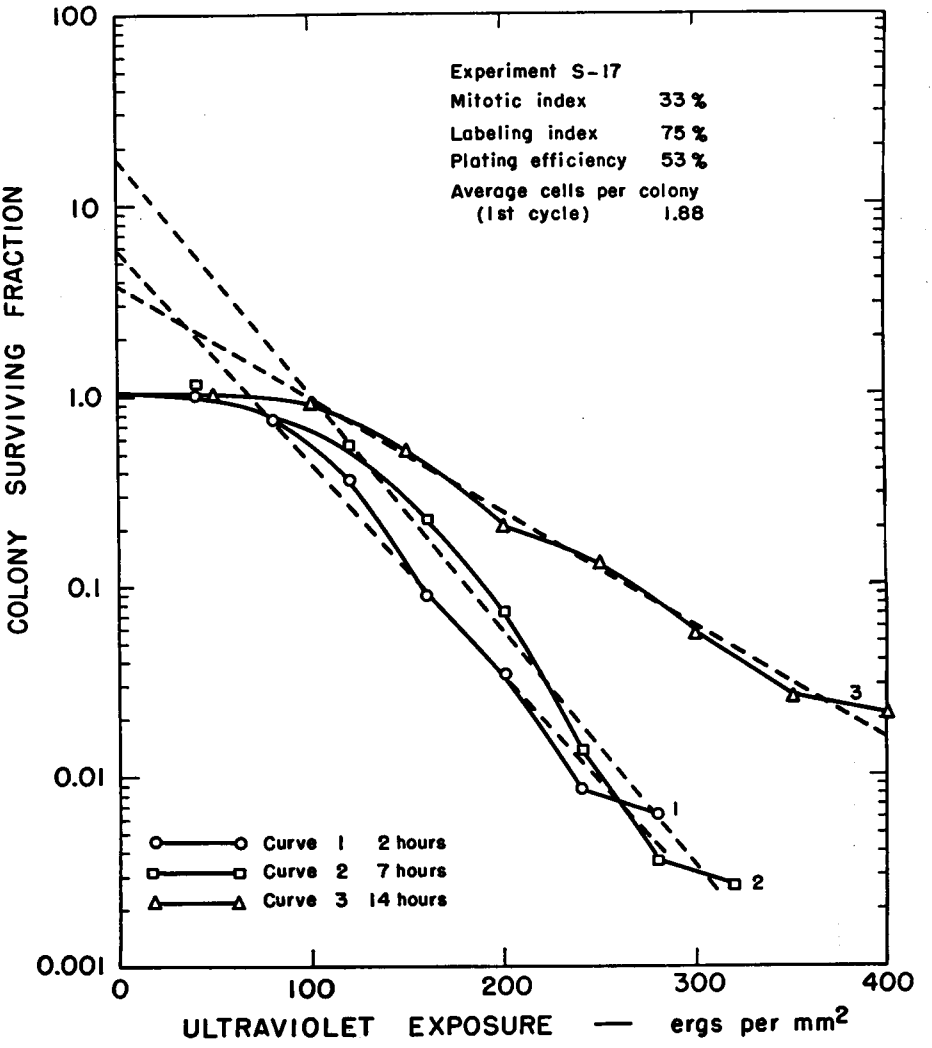

FIGURE 11 Colony survival curves of a synchronized cell population for ultraviolet exposure at different times after reincubation. Survival values below 0.01 were determined from plates having less than 20 colonies and are therefore uncertain. Dashed lines have nevertheless been fitted by eye to the points and the parameters pertaining to these lines are listed in Table II.

n and D_0 were observed to change during the cell cycle. At lower survival levels changes in the shape of our curves at stages of the cell cycle other than S are also possible.

3. *Response to Ultraviolet Light.* The response of the cell population to a constant exposure (200 ergs/mm^2) of UV throughout the cycle differs markedly

from that for x-rays. The results of four experiments are plotted in Fig. 10. Although the data vary, the general pattern is clear. During G_1, the number of survivors falls but some time after DNA synthesis begins, the surviving fraction starts to rise, continues to rise after S is completed, and drops only about the time of division. This pattern is repeated in the second cycle. Again the magnitude of the change depends not only on changes in the response of individual cells, but also on the average number of cells per colony and the degree of synchronization.

Survival data over a range of doses have been obtained in two sets of experiments at 2, 7, and 14 hours. The results for one (S-17) are shown in Fig. 11. Table II summarizes the observed parameters obtained by fitting equation (5) to the data.

TABLE II

MULTITARGET PARAMETERS FOR ULTRAVIOLET SURVIVAL CURVES OF EXPERIMENT S-17

Time after reincubation	Cell cycle	Phase (approx.)	Observed extrapolation No.	Cells/colony $\bar{N}$	Single cell extrapolation No., n	D_o
hrs						(*ergs/mm²*)
2	1st	G_1	6	1.88	3	39
7	1st	Early S	18	1.88	10	34
14	1st	G_2	4	1.88	2	72

The changes observed in a constant dose response curve such as those in Fig. 10 are due at least in part to changes in D_o and their magnitude is therefore dose-dependent. There also appear to be changes in n which are somewhat similar to those observed with x-rays but are more difficult to quantitate because of the changes in D_o. Also, values of D_o for UV are expressed in terms of incident energy while D_o for x-radiation is expressed as absorbed energy. For x-rays the conversion factor from exposure to absorbed dose (0.945 rad/R) is constant whereas for UV the energy is dissipated non-uniformly and the conversion factor may not be constant throughout the intermitotic cycle. Shape and size changes of the cells during attachment and throughout the cycle may affect their UV response, although studies with asynchronous populations have not shown any clear dependence on such changes (14).

DISCUSSION

Experiments on the variation of radiation sensitivity during the intermitotic cycle yield the function s (τ,D), the probability of an average cell surviving a dose D. The position of the cell in the cycle is represented by the variable τ, the time since the cell was born at a previous mitosis. Implicit in such a definition is the assumption that all the cells have a reasonably definite interdivisional or generation time,

T, and that definite biochemical events take place in a sequence between birth and the next mitosis. If the function $s(\tau,D)$ is known it can be compared with the biochemical events defining the intermitotic cycle and the variable τ.

To obtain cells having a given value of τ, we have used a partially synchronized population. As described above, cells are removed from a log-phase population in a well defined metabolic state and allowed to grow. If cells in the log population have a distribution of intermitotic cycle properties (*i.e.*, length of G_1, S, G_2), cells in the synchronized population are expected to have the same distribution. Under ideal conditions the cells are "synchronized" only at the beginning of an experiment, if the selection procedure has been successful. The cells remain synchronized only if the rate of movement towards mitosis is identical. Since this is not the case, the next division is not expected to be closely synchronized, but to be spread out over a time interval determined by the distribution of generation times in a normal log-phase culture. For mammalian cells this spread may be quite large, perhaps of the order of ± 20 per cent (15). However, such differences in rate of movement result in only a small correction to the response, $s(\tau,D)$.

Differences in rate of movement of cells through the intermitotic cycle may not be the only source of difficulty in interpreting the results of synchronization experiments. The process of selecting a uniform population may lead to metabolic changes. In our experiments there appears to be an initial adjustment of the synchronized population during the first interdivisional period. This is clearly seen in Fig. 3 where the mean time until the first division is about 16.5 hours and the period between the first and second divisions only 12 hours. (It is usual in our experiments for the first division to take about 15 hours. In log-phase growth, the generation time for these cells is $\sim$ 10 hours with S $\sim$ 6 hours, $G_1 \sim$ 1.5 hours, $G_2 \sim$ 1.5 hours, and mitosis $\sim$ 1 hour.) However, such responses as are observed in the first cycle are repeated in the second, the difference being as expected for an increased number of cells per colony. Consequently, our data are believed to yield reliable quantitative information upon the variation of radiation response with τ.

The number of cells per colony ($\bar{N}$) is a source of uncertainty in the determination of the single cell radiation response since the observed value of $\bar{N}$ includes both viable and non-viable cells, and for the application of equation (4), we require the effective value of $\bar{N}$ for viable cells only. The plating efficiency for the synchronized population is usually lower (45 to 65 per cent) than for asynchronous populations of the same cell line (65 to 90 per cent) and is estimated from the ratio of the cells which form visible colonies to the particle concentration assessed by Coulter counting. The latter count includes material which will not be identified as attached stainable cells. This material is a small percentage of the count for an asynchronous population but is relatively larger in the synchronized population because the harvesting procedure removes any loosely held material and the yield of cells is low. However, in many of our experiments, the observed $\bar{N}$ does

not double during the first division and an analysis of the distribution of cells per colony before and after division shows that a non-viable portion of cells is included in $\bar{N}$, which is therefore an overestimate of the effective $\bar{N}$. An examination of the data in one case showed that the maximum difference between the observed and effective $\bar{N}$ was about 15 per cent. Values of s derived from f using equation (4) and $\bar{N}$ observed may be too low by up to this percentage. In fact, although observed values of $\bar{N}$ have varied from experiment to experiment, we have found quite good agreement for s, which supports the view that the difference between $\bar{N}$ observed and effective is either not large or rather constant. This uncertainty will affect mainly the extrapolation number in survival curves, and therefore values of n given in Tables I and II must be recognized as approximate.

One way of testing the validity of the data is by comparison of the observed dose response of the asynchronous population with that predicted by a summation of its components. Good agreement here not only supports the view that the selection procedure (*e.g.*, temperature shock)[4] has not affected the subsequent behavior of the cells but also confirms that the small fraction of the population selected is representative of the whole population. For x-radiation the procedure is quite simple if we assume that the function $s(\tau,D)$ is given by

$$s(\tau, D) = 1 - (1 - e^{-D/D_0})^{n(\tau)} \tag{6}$$

where D_0 is about 200 rad throughout and $n(\tau)$ is the observed variation of extrapolation number during the interdivisional cycle (see Table II).

The composite population of subfractions obeying equation (6) will have a survival curve practically indistinguishable from equation (4) with $D_0 = 200$ rad and an average extrapolation number, $\bar{n}$. To obtain $\bar{n}$ for a random population we may first take a simple time average of the data in Fig. 6, *i.e.* S (τ, 710 rad), from 0 to 17 hours which yields an average survival for 710 rad of 0.11. If this dose is large enough to reach the exponential region, the population average survival will be given by:

$$\bar{s} = \bar{n}e^{-D/D_0} \tag{7}$$

thus $\bar{s} = 0.11$ at 710 rad would correspond to $\bar{n} = 3.8$ (for $D_0 = 200$ rad). Since the first cycle is somewhat longer than the usual log-phase doubling time, it is advisable to repeat this process for the second cycle of Fig. 6. This gives a value, $\bar{n} = 4.5$. In an asynchronous population, cells are not distributed equally at all interphase times τ, but have an exponential distribution (16) given by:

$$\frac{dN}{N} = \frac{\ln 2}{T} e^{(\ln 2/T)(T-\tau)}\, d\tau \tag{8}$$

[4] *Note Added in Proof.* Experimental data on the absence of effects of trypsin or cooling on the x-ray age response and time course of labeling with H^3-thymidine are included in a more recent publication (25).

where dN/N is the fraction of the asynchronous population having values of τ between τ and $\tau + d\tau$. Weighting the survival values of Fig. 6 by equation (7) and integrating, we obtain (for the second cycle) $\bar{s} = 0.12$ and from this, $\bar{n} = 4.2$. Our best estimates of the multitarget parameters for an asynchronous population would therefore be: $D_o = 200$ rad, $\bar{n} = 4$. These are typical values for many of our experiments with asynchronous populations; D_o is usually between 195 and 210 rad and $\bar{n}$ between 3.5 and 4.5 (9).

Our results with synchronized cells predict that the value of $\bar{n}$ found in an asynchronous experiment should depend on the fraction of cells synthesizing DNA at the time of irradiation. Since asynchronous cells are frequently irradiated in a post-trypsinization lag period, this fraction might be expected to vary. Variation in $\bar{n}$ from experiment to experiment is quite often observed. While cells are synthesizing DNA, however, not only is $\bar{n}$ increased, but there is a significant decrease in the slope of the survival curve, which we have already commented upon. Thus, a small decrease in $\bar{D}_o$ should accompany an increase in $\bar{n}$, which may be responsible for the frequently noted inverse correlation between $\bar{n}$ and $\bar{D}_o$ in asynchronous populations (17).

One final observation concerning x-ray responses should be made. Although we have referred throughout to G_1 and G_2 cells at the appropriate times, and certainly the majority of the cells may be considered to be in these categories at those times, a few labeled cells are usually also present. Since cells in S are generally more resistant, their contribution to the over-all survival response will be greater relatively than that of G_1 or G_2 cells. Thus, the true survival characteristics of "uncontaminated" cells in G_1 and G_2 cannot be as well described as for S cells.

A comparison between the expected UV survival curve which may be derived from our results for $s(\tau,D)$, with that observed for an asynchronous population is more difficult than for x-rays. No simple expression can be written down for the asynchronous survival because D_o is dependent on τ. An approximate estimate has been made as follows. From the curves on Fig. 10 the cells were considered to spend the first 0.3 of the cycle with $n = 3$ and $D_o = 39$ ergs/mm^2, 0.5 [0.3 − 0.8] of the cycle with $n = 10$, $D_o = 34$ ergs/mm^2, and the last 0.2 of the cycle with $n = 2$, $D_o = 72$ ergs/mm^2 (Table II). In an asynchronous population the actual proportion of cells in these different sensitivity states was found, using the Stanners and Till distribution (16), to be 0.375, 0.511, and 0.114 respectively. Combining these components graphically yields a multitarget curve for which $\bar{n} = 3.2$ and $\bar{D}_o = 45$ ergs/mm^2. In view of the relatively crude approximations made these values are in reasonable agreement with those normally found for an asynchronous population experimentally ($D_o \sim 50$ ergs/mm^2 and $\bar{n} \sim 4$–5) (14).

It was initially hoped that a comparison between the UV and x-radiation response would lead to some understanding of underlying mechanisms. Although the x-radiation response is different from that for UV, there are two similarities. One

is an increase in survival soon after DNA synthesis begins, although the interval is appreciably longer in the case of UV. This observation has been made by others using synchronized cultures both for UV (4) and for x-radiation (3, 4). The other is the similarity in the changes in extrapolation number, n, during the cycle. For both radiations, n is initially low in G_1 ($\sim$ 2 for x-radiation and $\sim$ 3 for UV), rises to about four times this value during S, and then falls back to about the G_1 value during the G_2 phase. Whether or not these changes represent a process inherent in all mammalian cells cannot yet be stated.

Our studies on cell survival may be compared with results obtained for chromosome damage following UV exposure (100 ergs/mm^2) reported by Humphrey, Dewey, and Cork (18). By labeling with H^3-thymidine in an asynchronous population of Chinese hamster cells, they found chromosome damage to be greatest in early S. Total chromosome damage in G_1 and G_2 was approximately the same but the proportion of different types of damage differed. We have found G_1 to be more sensitive than G_2 with respect to cell death and thus different mechanisms may be principally responsible for cell death in the G_1 and G_2 periods. However, more information is needed on the variation of chromosome damage with dose at each stage of the cell cycle and on the relationship between chromosome damage and cell survival.

In the case of x-radiation most observers agree that the response is in some way related to DNA synthesis. Erikson and Szybalski (4) using Detroit 98 cells synchronized by 5-fluorodeoxyuridine, found a temporary period of resistance to x-rays during DNA synthesis, and from 2 to 8 hours after the release of the inhibitor the changes seemed to be mostly in the extrapolation number. Terasima and Tolmach (3) have suggested that for synchronized HeLa cells the increase of resistance during the S phase was due to a change in D_o. However, it is doubtful in view of the changes in curve shape apparent in their data that the changes can be associated with one parameter only. Terasima and Tolmach (19) have also shown that if the DNA synthetic period in synchronized HeLa cells is blocked with an inhibitor, the x-ray resistance is similarly delayed.

In contrast to these results, Dewey and Humphrey (20) by labeling asynchronous populations, find that L cells are most sensitive to x-radiation in the S phase both with regard to cell death and chromosome damage (21). However, they observe that Chinese hamster cells (20) may be slightly more resistant to x-radiation delivered in the S phase, agreeing with their observations on chromosome damage for these cells (22). A possible explanation for such differences may be that the subdivision of the intermitotic cycle into G_1, S, and G_2 may not be very suitable for describing the x-radiation response, for example, early S in many cell lines appears to be more sensitive than late S. In each cell line studied however, except perhaps for L cells, resistance to x-radiation seems to be associated with DNA synthesis or to some other process intimately related to it.

A phenomenon observed by Terasima and Tolmach (3) but not observed in this work, is the early (G_1) resistance to x-rays of synchronized HeLa cells. The difference may be because the short G_1 of hamster cells provides no opportunity for a resistant period to show itself. It has been pointed out (23, 24) that cells of widely different generation times differ primarily in the length of the G_1 period, the period from the beginning of S until the end of mitosis being relatively constant. During this latter period the response to x-rays of Detroit 98 cells (4) and Chinese hamster cells reported here appears to be quite similar, but in HeLa cells (3) no appreciable decrease in survival during G_2 was apparent. Thus in spite of the similarities in x-ray response noted above, some differences also appear to exist among different cell lines. It may be too early to speculate on mechanisms giving rise to the observed changes until these differences are reconciled or better understood. However, if changes in both n and D_o are possible at different stages of the cycle, especially during the S period, and if the causes of these changes are independent, it is not difficult to see how the interplay of these two parameters in different cell lines may lead to different constant dose response and to different survival curves. The constant dose response is also, to some extent, dependent on the level of that dose if D_o varies. Furthermore, it should be emphasized that there appear to be in our data and also in those of Terasima and Tolmach (3) changes in the shape of the survival curves not easily described by changes in the parameters of the multitarget relation expressed in equation (5).

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APPENDIX

Derivation of single cell survival s from the survival f of a mixed population of multiple cell colonies.

Assuming that each cell survives exposure independently of the presence of others, the true average single cell survival s is related to the survival f for a mixed population of multiple cell colonies by the equation

$$f = \sum_{i=1}^{\infty} \phi_i \{1 - (1 - s)^i\} \tag{1'}$$

where ϕ_i is the fraction of the total colonies having i cells, see, for example reference 7.

The approximation used in the text is

$$s_a = 1 - (1 - f)^{1/\bar{N}} \tag{2'}$$

where $\bar{N}$ is the average viable cell multiplicity $= \Sigma \phi_i \cdot i$, s_a is the approximate single cell survival derived from (2'), and f is the observed colony surviving fraction.

A complete discussion of the validity of this approximation is beyond the scope of this paper; however, it is comparatively simple to make an estimate of the error involved in

using the approximation. We might anticipate that the magnitude of the error will depend on the magnitude of f and $\bar{N}$ and that an expression for the error involving these parameters will be a useful guide to the applicability of equation (2'). Although in many practical circumstances, high values of i are not encountered, the evaluation of equation (1') is time-consuming even in simple cases when the cell distribution is known, and thus a knowledge of the error involved in applying equation (2') is particularly useful. Setting the error, $\Delta s = s - s_a$ and expanding $f(s)$ in a Taylor's series yields

$$\begin{aligned} f(s) &= f(s_a + \Delta s) \\ &= f(s_a) + \Delta s \frac{df}{ds_a} + \frac{(\Delta s)^2}{2!} \frac{d^2 f}{ds_a^2} + \cdots \end{aligned} \tag{3'}$$

where $f(s)$ is equation (1') evaluated for s

$f(s_a)$ is equation (1') evaluated for s_a

df/ds_a is the first derivative of equation (1') evaluated for s_a, etc.

If s_a is a sufficiently close approximation to s, we need retain only the first two terms of equation (3') and thus

$$\Delta s = \frac{f(s) - f(s_a)}{\frac{df}{ds_a}} \tag{4'}$$

or

$$\Delta s = \frac{f - \Sigma_i \phi_i \{1 - (1 - f)^{i/\bar{N}}\}}{\Sigma_i \phi_i \cdot i(1 - f)^{(i-1)/\bar{N}}} \tag{5'}$$

substituting the approximation of equation (2') for s_a.

If f is sufficiently small so that the binomial expansion of $1 - (1 - f)^{i/\bar{N}}$ may be limited to $i \cdot f/\bar{N} - (i/2\bar{N})(i/\bar{N} - 1)f^2$, when this substitution is made in equation (5'), Δs may be reduced to

$$\Delta s \approx \frac{f^2}{2(\bar{N})^3} \{\overline{N^2} - (\bar{N})^2\} \tag{6'}$$

where $\overline{N^2} = \Sigma \phi_i \cdot i^2$, and terms in f^3 and higher powers are neglected. Equation (6') always yields positive values for Δs and thus s is always greater than s_a. Furthermore, the error Δs is proportional to the square of the standard deviation ($= \overline{N^2} - (\bar{N})^2$) of the colony distribution. Consequently, in our experiments the error will always be greater during the second cycle than during the first.

Also, as $f \rightarrow$ zero, s tends to $f/\bar{N}$ and the fractional error in using equation (2') becomes

$$\frac{\Delta s}{s_{f \to 0}} = \frac{f}{2(\bar{N})^2} \{\overline{N^2} - (\bar{N})^2\} \tag{7'}$$

To illustrate these points we have calculated for several values of f, values of s_a using equation (2'), values of s using equation (1') and the method of successive approximations, and the error $s - s_a$ in using approximation (2'). Representative data from Experiment S-9 have been used for this purpose and are listed in Table I'. For comparison, the error calculated according to equation (6') is included in the last column and yields values smaller than the actual values when f is large because of the approximations made in the derivation. Equation (6') is nevertheless a reasonable guide to the true situation. At smaller values of f, calcu-

TABLE I′

CALCULATION OF ERRORS IN s_a FOR DATA OF EXPERIMENT S-9

Dose	Observed survival f	Single cell survival s_a*	Single cell survival s‡	Actual error $s - s_a$	Actual error $s - s_a/s \times 100$	Estimated errors from equation (6′)§
rad					*per cent*	*per cent*
142	0.790	0.632	0.672	0.040	5.9	4.2
236	0.627	0.468	0.491	0.023	4.7	3.6
426	0.303	0.204	0.211	0.007	3.3	2.0
710	0.089	0.058	0.058	<0.001	<1.7	0.6
990	0.0181	0.0116	0.0116	<0.0001	<0.9	0.1

*s_a calculated from equation (2′), with $\bar{N} = 1.56$.

‡s calculated from equation (1′), with the following parameters

$$\phi_1 = 0.475, \phi_2 = 0.496, \phi_3 = 0.021, \phi_4 = 0.008.$$

§Δs calculated from equation (6′), with

$$\bar{N} = \Sigma \phi_i \cdot i = 1.56 \qquad \bar{N}^2 = 2.44$$

$$\overline{N^2} = \Sigma \phi_i \cdot i^2 = 2.78 \qquad \overline{N^2} - \bar{N}^2 = 0.34.$$

lations of s and s_a are not sufficiently precise for accurate values of $s - s_a$ and the errors determined from equation (6′) are probably more reliable. The difference between s and s_a when f is less than 0.1 is clearly less than 1 per cent. These errors refer, of course, only to the use of the approximation and do not include experimental errors such as previously discussed for $\bar{N}$.

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