TIME-LAPSE CINEMICROGRAPHIC STUDIES OF X-IRRADIATED HeLa S3 CELLS

II. Cell Fusion

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ABSTRACT Analysis of time-lapse cinemicrographs of X-irradiated HeLa S3 cells has shown that the incidence of cell fusion was increased from 0.9% (following 1267 divisions) in control cells to an average of 22% (following 655 divisions) in cells irradiated with 500 rad doses of 220 kv X-rays. The incidence depended on the stage of the generation cycle at which the parent cells were irradiated. It was nearly constant in the first three postirradiation generations. Fusion occurred at all stages of the generation cycle, but preferentially during the first 20%. Cells undergoing fusion progressed more slowly through the generation cycle and had a higher probability of disintegrating than did irradiated cells that did not fuse. The occurrence of fusion was clonally distributed in the population. It took place only between sister (or closely related) cells. Protoplasmic bridges were often visible between sister cells prior to fusion. Giant cells arose only as a result of fusion. The incidence of multipolar divisions, though higher than in unirradiated cells, was only 5.5% in cultures irradiated with 500 rads. Fusion occurred following 85% of the multipolar divisions and was often followed by a multipolar division.

INTRODUCTION

In the interval between irradiation of a culture of mammalian cells with several hundred rads of X-rays and the subsequent disintegration (necrosis; metabolic death) of the vast majority of the cells, a number of abnormal cytological processes may be detected. These include cell fusion, multipolar division, and the formation of giant cells. The mechanisms by which these abnormalities arise, and their relation to each other and to several additional manifestations of radiation damage—in particular to cell disintegration and, hence, to loss of the cell's capacity for sustained proliferation—are unclear.

We have previously presented a time-lapse cinemicrographic analysis of the effect of 220 kv X-rays on the progression, division, and disintegration of HeLa S3 cells during the first several generations following irradiation (Hurwitz and Tolmach, 1969). That analysis indicated that after 500 rad doses, radiation damage in this

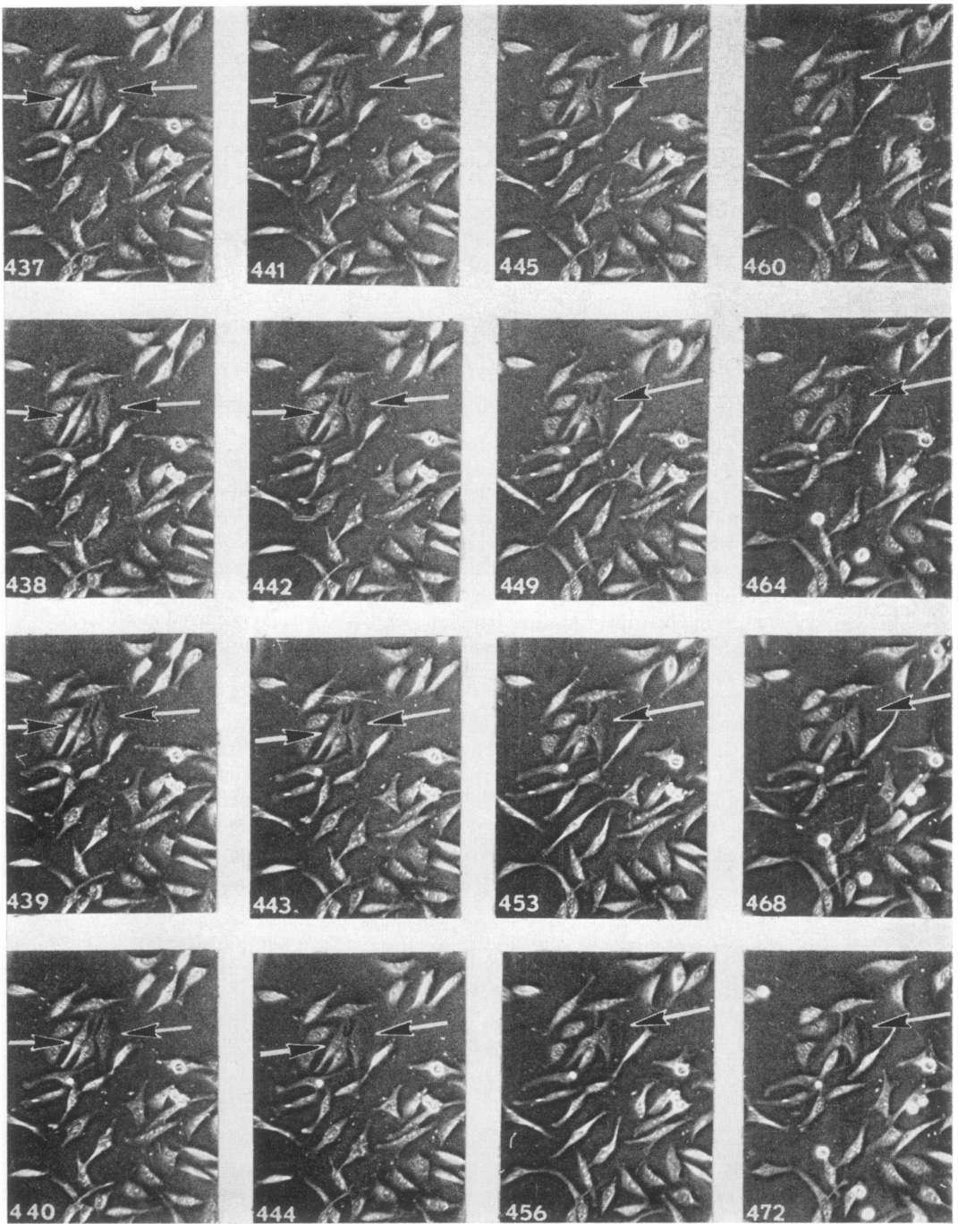

FIGURE 1 Film sequence illustrating fusion of two sister cells (arrows) in a population that had received 500 rads. Enlarged prints (6.5×) of the original film were cropped and numbered, and pertinent frames selected. Consecutive frame numbers represent exposures made 5 min apart. Contact between the cells is first visible in frame No. 439.

system is preferentially expressed during the mitotic process: three-quarters of all cell disintegrations were preceded by mitotic arrest. The present report is concerned with the characterization of cell fusion, and with the relation of this phenomenon to various other cellular consequences of exposure to ionizing radiation. Several of the results are in accord with those reported previously by Marin and Bender (1966), who drew their conclusions from the observation of 23 cell fusions in irradiated HeLa S3 cells. The data presented here were obtained from observation of six times as many fusions. The results are consistent with the hypothesis that cell fusion results from a derangement of mitosis.

MATERIALS AND METHODS

The present data were obtained from the films whose analysis was partially presented in paper I of this series (Hurwitz and Tolmach, 1969). In brief, the experimental procedure was as follows: synchronous populations (obtained by mitotic selection) of HeLa S3 cells in medium N16FCF were irradiated with 220 kv X-rays (constant potential; 15 ma; HVL 1.0 mm Cu, 83 rads/min) at selected times during interphase. Cells received 500, 1000, or 1500 rad doses (paper I, Table I), but most of the data presented here refer to the six cultures irradiated with 500 rads. Irradiated and control cultures were incubated in the same chamber. Single frames were exposed at 5 min intervals, using low-power phase optics, for up to 7 days. Analysis of the films included the construction of pedigrees and the scoring of both the occurrence and the time of cell rounding (R), division, disintegration, and fusion (F). The intervals between two events, $X_i \rightarrow Y_j$, were determined, and their mean, $\langle X_i \rightarrow Y_j \rangle$, calculated. The subscripts denote the generation number: the generation during which cultures were irradiated was designated 0, the first postirradiation generation 1, etc.

Cell fusion is illustrated in Fig. 1. Because the fusion process often spanned several frames, the designation of the time at which it occurred was imprecise, especially when a protoplasmic bridge was seen between the two cells prior to fusion (Marin and Bender, 1966). In the absence of a recognizable bridge, fusion was considered to have occurred at the frame in which a connection between the cells was first detectable (of course, only if the fusion process continued to progress; many presumptive incipient fusions failed to materialize), e.g., frame 439 in Fig. 1. When a bridge was visible, fusion was scored at the frame in which the two cells became joined over a region of periphery larger than that occupied by the bridge.

RESULTS

Incidence, Age-Dependence, and Generation-Dependence of Cell Fusion

A total of 142 fusions occurred following the 655 analyzable divisions (i.e. divisions yielding cells whose fate could be determined) observed in cultures irradiated with 500 rads, for an incidence of 22%. In unirradiated control cultures, the incidence was 0.9%: 11 fusions following 1,267 divisions. The comparable values reported by Marin and Bender (1966) were $^{23}\!/_{88} = 26\%$, and $^{3}\!/_{210} = 1.4\%$, respectively.

An increased incidence of fusion over that in controls was observed in each of the six irradiated cultures; however, as shown in Fig. 2, the incidence probably was not constant, but varied with the age of the cells at irradiation. The similarity of the shape of this curve to that relating the duration of mitosis to the age of the cell

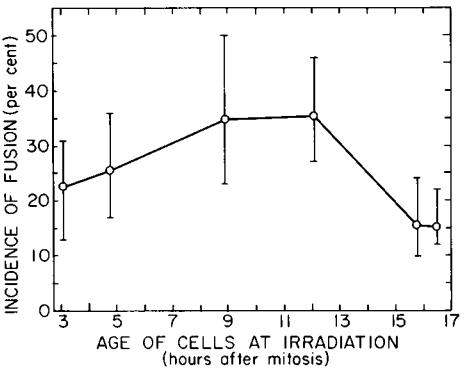

FIGURE 2 Incidence of cell fusion as a function of the age of the collected population at the time of irradiation with 500 rads. The bars indicate the 95% confidence intervals.

TABLE I

INCIDENCE OF FUSION AS A FUNCTION OF POSTIRRADIATION GENERATION*

Generation	Number of cells		Fusion incidence (with 95% confidence interval)
	Total	Fused	
			%
1	211	48	23 (17-30)
2	169	34	20 (13-27)
3	73	13	18 (11-31)

* Cells were irradiated with 500 rads in generation 0.

(paper I, Fig. 9 *B*) suggests that the cellular damage expressed by these two phenomena may be related.

The incidence of cell fusion as a function of the postirradiation generation is shown in Table I. No significant difference is apparent in the first three postirradiation generations. This result is not easily reconciled with the high correlation between cell fusion and cell disintegration (see below), inasmuch as there is a strong generation dependence of disintegration (paper I, Fig. 13). It may be recalled that dependence of mitotic prolongation on generation was unexpectedly absent also (paper I, Fig. 7).

The incidence of fusion after 1000 or 1500 rad doses could not be determined because of the small number of cells which were observable, even from the first division to the second.

Bridge Formation, Cell Fusion, and Giant Cell Formation

Examination by Marin and Bender (1966) of 23 fusions in HeLa S3 cells irradiated with 400 or 600 rads suggested that fusions take place most frequently between sister cells. The present study confirms this: only 13 of the 142 fusions observed in

populations irradiated with 500 rads occurred between nonsister cells, and these were either between aunt and niece or between first cousins. So high an incidence of involvement of sister cells in fusion suggests that a physical link exists between sisters prior to their fusing, and, indeed, protoplasmic bridges between the cells were often detectable during the period between cell division and fusion. (In at least one case, a bridge was detectable during all of interphase and division of one of the sisters; fusion between the aunt and one of the nieces occurred subsequently.) However, reliable detection of a fine bridge demands greater optical precision than was generally obtained in these films and, hence, we can make no quantitative statement about the incidence of intracellular bridges.

In general, fused cells were clearly larger than normal cells, and successive fusions yielded progressively larger cells. Conversely, the pedigrees of all cells which were obviously enlarged contained one or more fusions. For example, the pedigree of each of the seven cells whose area was more than twice that of normal cells included at least two fusions. Since no cell remained in interphase for longer than twice the average generation time (paper I, Fig. 11), and none remained in mitosis for longer than 5 hr before dividing (paper I, Fig. 8 *A*) [or 1 day before disintegrating (paper I, Fig. 10 *A*, *B*)], cell fusion would appear to be the sole route of formation of giant cells in this system.

Cell Age at Fusion

Determination was made of the time in the cell generation cycle at which fusion occurred, i.e. the interval from rounding of the mother cell, *R*, to fusion of the daughters, *F*. (It was more convenient to measure the interval from rounding than from division; subtraction of the mitotic times would not significantly change the distribution.) Fig. 3 shows the distribution of $R \rightarrow F$ for 127 sister cell pairs in cultures irradiated with 500 rads, and for 31 pairs in cultures receiving 1000 or 1500 rads. For the former group, a bimodal distribution is evident, about half the cells fusing in the first several hours after division (1–6 hr after rounding) and most of the remainder, following a virtual hiatus between 6 and 10 hr, fusing between 10 and 20 hr after rounding. For the cultures receiving the higher doses, a single peak is seen at early times, followed by an essentially flat distribution.

The initial peak might be related to the close proximity of sister cells after division; the origin of the later peak (500 rads) is not apparent. No differences were recognized between the fusions occurring during the two periods, nor was any significant correlation detected between the time of fusion and any other cellular property. Thus, the mean generation time and standard deviation for 40 members of the early-fusion group was 21.8 $\pm$ 5.3 hr for the generation in which fusion occurred (see below), while that for 53 members of the late-fusion group was 24.9 $\pm$ 8.8 hr. The difference, with 95 % confidence limits, was 3.3 $\pm$ 3.3 hr. In addition, comparison of the mean generation times for the generation preceding the one in which fusion occurred

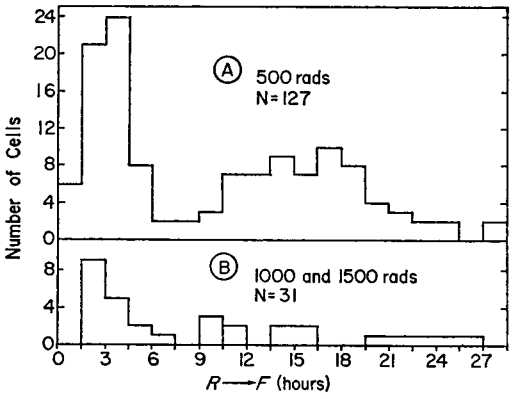

FIGURE 3 Frequency distribution of the interval in hours between cell rounding for mitosis, R, and fusion of the resulting sister cells, F, in 6 cultures irradiated with 500 rads at different times in the generation cycle (A) and in 4 cultures irradiated with either 1000 or 1500 rads (B). N is the sample size.

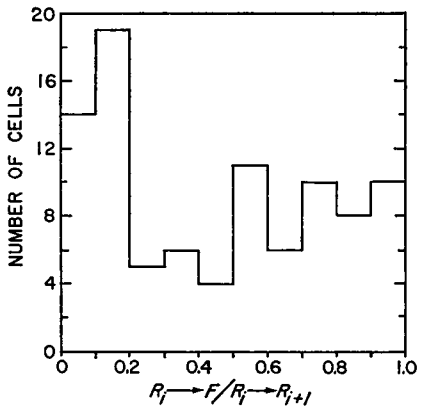

FIGURE 4 Data of Fig. 3 *a* expressed as the fraction of the generation cycle that had elapsed at the moment of fusion $(R_i \rightarrow F)/(R_i \rightarrow R_{i+1})$.

yielded no difference between the cells fusing in the early peak ($\langle R_{i-1} \rightarrow R_i \rangle$ = 21.5 hr) and in the later one ($\langle R_{i-1} \rightarrow R_i \rangle$ = 21.8 hr). Again, examination of the possibility that the cells fusing after 10 hr underwent disintegration with greater frequency than those fusing earlier revealed that the fraction of dividing cells which had fused within 7.5 hr of rounding (0.44 ± 0.13) was not significantly different from the fraction of disintegrating cells which had fused within this time (0.52 ± 0.13). Finally, attempts to distinguish between the members of the two groups on the basis of the generation during which fusion occurred were unsuccessful.

The foregoing indications that the bimodal distribution seen in Fig. 3 quite possibly has no significance suggested that clock time may not be the most suitable parameter against which to measure the occurrence of fusion. Accordingly, the frequency distribution was redetermined as a function of the fraction of the generation cycle that had elapsed at the moment of fusion, $(R_i \rightarrow F)/(R_i \rightarrow R_{i+1})$. Treated in this fashion, the data again show a peak in fusion activity during the first 20% of the generation cycle, but only small fluctuations thereafter (Fig. 4). We conclude that at least after the initial peak, fusion probably occurred randomly as cells progressed

through the generation cycle, and was not correlated with any particular stage of intracellular development.

Generation Time of Cells Undergoing Fusion

Further characterization of fusion was afforded by comparison of the mean generation time, $R_i \rightarrow R_{i+1}$, for fused cells (in the generation in which they underwent fusion) with that for the entire cell population. (Exclusion of fused cells from the population did not alter significantly either the mean value or the distribution of generation times.) The population of cells examined was limited to sisters which fused, and included those which disintegrated in the subsequent mitosis as well as those which divided. The mean generation time for 93 fused cells (500 rads), with standard deviation, was 23.6 $\pm$ 7.6 hr; that for the entire irradiated population of 828 cells (see paper I, Figs. 11 *A* and *B*) was 20.9 $\pm$ 4.7 hr. The difference, with 95% confidence limits, was 2.7 $\pm$ 1.1 hr. Although the incidence of disintegration among fused cells was appreciably greater than in unfused cells (see Table III below), and disintegrating cells had a longer than average generation time (paper I, Fig. 11), the 2.7 hr difference can be attributed only partially to this higher incidence of disintegration. That is, on the basis of mean generation times for dividing and disintegrating cells of 20.3 and 22.6 hr, respectively (paper I, Fig. 11), a difference of only 0.6 hr would be expected.

The increase in generation time in sister cells undergoing fusion was resolved into an increase in the preceding mitotic time and an increase in interphase time. Mitoses that were followed by fusion in the subsequent generation lasted 16.4 $\pm$ 14.5 min longer than did those not followed by fusion; however, the difference in the fraction of the generation time spent in mitosis between the two classes of cells, with 95% confidence limits, was 0.009 $\pm$ 0.011. Thus, mitosis and interphase were apparently increased proportionately.

Clonal Distribution of Cell Fusion

It has been shown by a number of workers that cell disintegration in irradiated populations is not a random event, but rather is clonally distributed (Froese, 1966; Thompson and Suit, 1967; paper I). To determine whether fusion similarly occurred preferentially in certain clones, the pedigrees of fused cells were grouped according to whether 2, 3, or 4 divisions could be detected. (Cell disintegration, migration, or obscuring debris always limited the number of divisions that could be scored in any given clone; more than 4 divisions could be scored in only a few clones. Clone initiation was considered to have occurred at any point from which observations could be made; in practice, this was usually generation 0.) The average frequency of fusion within each group was determined, and from this the expected frequencies of 0, 1, 2,$\cdots$ fusions were calculated according to the binomial distribution. The

TABLE II

CLONAL DISTRIBUTION OF FUSION*

Group‡	Number of clones	Average frequency of fusion		Number of clones, n_i, with i fusions					Dispersion test		
				n_0	n_1	n_2	n_3	n_4	χ^2	d.f.	P
2	51	0.34	Expected	22.0	23.0	6.0			64.29	50	<0.1
			Found	25	17	9					
3	33	0.15	Expected	20.2	10.8	1.9	0.1		52.34	32	<0.05
			Found	23	6	3	1				
4	20	0.27	Expected	5.5	8.4	4.8	1.2	0.1	42.38	19	<0.01
			Found	9	5	3	1	2			
Sum	104	0.23§							159.01	101	<0.001

* Cultures were irradiated with 500 rads.

‡ The group corresponds to the number of divisions detected in a clone.

§ Weighted mean.

number of clones, n_i, found to contain i fusions ($i = 0, 1, 2, \cdots$) was compared with the expected number of clones calculated from the corresponding expected frequencies. The results are listed in Table II. It would appear that for all three groups the number of clones in which either all or no divisions were followed by fusion is larger, and the number with intermediate numbers of fusions is smaller, than would be expected for random occurrence of fusion; that is, fusion appears to have been clonally distributed.

In an effort to assess the statistical significance of these data, a χ^2 dispersion test was applied. The values of χ^2 for each group, and the corresponding probabilities, P, that these or larger deviations from expectation in the observed distribution might arise by chance, are listed in Table II. For clones with 3 or 4 divisions, it may be concluded that fusion did not occur randomly, but for clones with 2 divisions, this test does not permit exclusion of a random occurrence of fusion. However, random occurrence appears to be strongly excluded by consideration of χ^2 for the three group aggregate. It should be noted, nevertheless, that a χ^2 dispersion test for homogeneity of the average fusion frequencies of the three groups gives a $\chi^2 = 11.31$ at 2 degrees of freedom, indicating that the average fusion frequency varies more than would be expected from random sampling effects alone. The variation might have arisen from the very different sorts of pedigrees that were (of necessity) included in scoring clones containing a given number of divisions.

Cell Fusion and Disintegration

It has been reported previously (see paper I for earlier references) that the probability of cell division, $p(i)$, defined as the ratio of the number of cells dividing at the end of generation i to the number entering generation i, is strongly dependent on i and on the X-ray dose. Because a fused cell contains twice the usual chromosomal comple-

TABLE III

PROBABILITY OF CELL DIVISION, $p(i)$, IN FUSED AND IN UNFUSED CELLS*

Film	Fused cells		Unfused cells	
	Number	$p(i)$‡	Number	$p(i)$‡
1 + 2	80	43 (30–53)	266	64 (58–72)
3 + 4	75	45 (34–56)	140	56 (47–64)
5 + 6	70	56 (44–67)	333	77 (71–81)
Total	225	48 (43–56)	739	68 (66–73)

* Cultures received 500 rads.

‡ The probabilities have been multiplied by 100. The mean values are given together with the 95% confidence limits.

ment and is otherwise probably abnormal, the present data have been examined to determine whether the probability of cell division was smaller in fused cells than in the remainder of the irradiated population. Marin and Bender (1966) have suggested that cell disintegration, which can be measured in this system by the failure to divide, $1\text{-}p(i)$ (paper I), is a common but not a necessary sequel to fusion.

The combined data for all generations and all cultures receiving 500 rads, shown in Table III, indicate that the incidence of disintegration among fused cells was indeed greater than among unfused cells, but that the difference, while almost certainly significant, is not large. Further indication that the difference is real is afforded by examination of the same data grouped into three subgroups, obtained, respectively, from films 1+2, 3+4, and 5+6; the same conclusion is reached from the data for the first and third groups, and the data for the second group are consistent with the conclusion. This finding suggests that cell fusion might be an early manifestation of the damage that leads to disintegration, though it is also possible that the two phenomena arise from separate kinds of damage. That is, fusion may result in such severe abnormalities that the fused cells have a high probability of disintegrating by a mechanism different from that by which unfused cells disintegrate.

Multipolar Division

The occurrence of multipolar divisions in irradiated cells was described many years ago (see Fetner and Porter, 1965, for references to early observations). More recently, Levis and Marin (1963) measured the incidence of multipolar mitoses in guinea pig RCP cultures sequentially fixed after irradiation, and Fetner and Porter (1965) made similar measurements on mitotic human KB cells harvested periodically from monolayers. Both groups reported that more than half of the mitoses were multipolar after 3 or 4 days of incubation.

TABLE IV

INCIDENCE OF MULTIPOLAR DIVISIONS AS A FUNCTION OF CELL AGE

Age of cells at irradiation*	Total number of divisions	Multipolar divisions	
		Number	Frequency
hr			
3.1	134	10	0.08
4.8	78	2	0.03
8.9	55	3	0.06
12.1	81	3	0.04
15.8	117	3	0.03
16.5	190	15	0.08
Total	655	36‡	0.055

* 500 rads.

‡ Of the 36 multipolar divisions, 35 were tripolar and 1 tetrapolar.

Among the 6 cultures irradiated with 500 rads in the present study, we observed between 3 and 8% multipolar divisions (Table IV); the mean was 5.5%. This incidence is similar to that ($^{7}/_{88}$ = 8%) reported by Marin and Bender (1966) for HeLa S3. Only three multipolar divisions were detected among more than 1200 divisions in unirradiated control cultures. Even allowing for possible bias arising from the longer duration of mitosis in cells which are about to disintegrate (paper I) and the strong correlation between multipolarity and cell disintegration (see below), it seems impossible to reconcile the observations of Levis and Marin and of Fetner and Porter with the incidence found by Marin and Bender and in the present study. An incidence as high as 50% at the second, third or fourth postirradiation generation would have resulted in a higher overall level of multipolarity, even if the incidence were zero in other generations. (In fact, multipolarity was observed in all generations, though analysis as a function of generation would mean little because of the small number of events observed.) The discrepancy can most conveniently be attributed to the different cell types examined in the two groups of investigations. However, because the RCP and KB cells were studied by examination of sequentially stained preparations, while the HeLa cells were subject to time-lapse analysis, we are hesitant to conclude that the differences are due only to the cell lines.

Marin and Bender (1966) have presented data suggesting that multipolar division tended to occur following cell fusion. The present study confirms those observations: 20 out of 36 multipolar divisions (in a total of 655 divisions which could be examined) occurred in cells which had undergone fusion. Conversely, the incidence of fusion following multipolar divisions was greater than it was after bipolar divisions: 18 of 33 multipolar divisions were followed by fusion of 2 daughter cells, and an additional 10 by fusion of 3 daughters, for a total incidence of 85%. Fusion in the irradiated cell population as a whole was only 22%.

Multipolar divisions gave rise to cells which exhibited a very high probability of disintegrating. Of the 46 such cells (either fused or not) whose histories could be determined, 33 (75%) disintegrated. This value is higher than that for fused cells (52%) or unfused cells (32%) in the total population of irradiated cells and their descendants.

DISCUSSION

As noted, many of the foregoing observations confirm those made by Marin and Bender (1966) on smaller populations of irradiated cells. We feel it very likely, therefore, that the results accurately reflect the behavior of HeLa S3 cells after irradiation with doses of several hundred rads. Interpretation of the results, however, remains highly speculative. Thus, while a number of the observations can be interpreted in terms of our previous conclusion (paper I) that damage from X-irradiation in this system, at the doses examined, is preferentially manifested in the mitotic process, direct evidence of mitotic involvement is not available. Indications that mitosis does

play an important part in the genesis of these responses to irradiation include (1) the restriction of fusion to cells that are closely related (usually sisters), suggesting that the phenomenon is dependent on the prior existence of a protoplasmic bridge, and hence on a persistent chromosomal bridge that was formed at anaphase; (2) the obligate role of cell fusion in the formation of giant cells, which similarly relates the latter process to mitotic abnormalities; (3) the age-dependence of fusion (Fig. 2), which resembles that of the duration of mitosis (paper I, Fig. 9 *B*); and (4) the finding that 85 % of the multipolar mitoses were followed by cell fusion.

While the data presented here provide no direct information concerning the mechanism of cell fusion, the aforementioned restriction of fusion to sister (or nearly as closely related) cells suggests that bridges like those often seen between fusing cells are in fact always present prior to fusion. It seems highly unlikely that some sort of long range attractive force might operate between sister cells; fusion occurs between cells separated by considerable distances, and there are often other cells lying between the sisters (Fig. 1). Evidence for the further assumption that the protoplasmic bridges contain nuclear material, and hence that they result directly from mitotic abnormalities, might be found on examination of these cells with improved optics; nuclear bridges have been reported by others in such irradiated cultures (Levis, 1962; Marin and Bender, 1966).

The clonal distribution of fusion, like that of cell disintegration (paper I), indicates that the radiation-induced damage responsible for the phenomenon is propagated; but whether the damage is the same as that which causes disintegration in the absence of fusion is unknown. If both phenomena result from mitotic abnormalities, as suggested, then they may well be identical or closely related. In any case, however, it is not possible to attribute a significant amount of cell disintegration to the previous occurrence of fusion; although $p(i)$ was somewhat smaller among fused cells than among cells that had not fused (Table III), most of the cells which disintegrated had not fused.

The observations reported here indicate that giant cells are formed only by way of cell fusion. That is, progressive cell enlargement, without the intervention of a mitotic event, was never observed. However, it should be noted that a process involving mitosis, cytokinesis with a residual protoplasmic bridge, and subsequent fusion, may not be fundamentally very different from an abortive mitotic event in which a cell fails to complete anaphase and subsequently reverts to interphase. The latter process has been observed by others in irradiated HeLa cells (P. I. Marcus, personal communication; Thompson and Suit, 1969).

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