EFFECTS OF ULTRAVIOLET RADIATION ON MAMMALIAN CELLS.

II. DIFFERENTIAL UV AND X-RAY SENSITIVITY OF CHROMOSOMES TO BREAKAGE IN 5-AMINOURACIL SYNCHRONIZED CELL POPULATIONS¹

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X-IRRADIATION of cells at various stages of the mitotic and meiotic cycles induces varying types and frequencies of chromosome aberrations (see reviews: SPARROW 1961; GILES 1954; KAUFMANN 1954; EVANS 1962; BREWEN 1964a). Since mammalian cells in tissue culture can be sufficiently flattened for low-penetrating radiation to reach the nucleus, it is also possible to compare the cell-stage radiosensitivity to ultraviolet light (UV). For example, differential cell killing by UV or X rays during the mitotic cycle of cultured mammalian cells has been reported (ERIKSON and SZYBALSKI 1963; SINCLAIR and MORTON 1965). HUMPHREY, DEWEY and CORK (1963) showed significant differences in the frequencies of chromosome aberrations induced by UV or γ rays at different cell stages. Although such studies are possible in asynchronous cultures, they can be extended with synchronized cell populations. This report is concerned with the variation in the frequencies of chromatid aberrations induced by UV or X rays in partially synchronized Chinese hamster cells *in vitro*. A portion of this work has been previously summarized (CHU 1964).

MATERIALS AND METHODS

A clonal cell line of Chinese hamster was used. The morphological characteristics and the duration of the mitotic cycle of the cells, as well as the procedures for cell culture and cytological preparations, have been described (CHU 1965). A standard cell suspension (2×10^5 cells/ml) was inoculated into quartz, glass, or plastic Petri dishes. Twenty-four hours after inoculation the growth medium was replaced with one containing 3×10^{-3} M 5-aminouracil (5-AU). After an additional 24 hours of incubation, the medium containing 5-AU was removed. The cells were rinsed twice with warm Hanks' balanced salt solution (BSS) before reincubating in fresh medium. In the text, figures and tables, this time is referred to as "0 hours" after removal from 5-AU. The control cells were handled identically except for the chemical treatment.

Successive samples of the chemically synchronized cells were pulse-labeled with H³-thymidine (H³-TdR, 1.9 c/mmole, 1 μ c/ml of medium for 15 min) rinsed with BSS, and returned to non-radioactive medium just prior to X- or UV irradiation at every hour for 7 hours. Cells were irradiated on the hour except the 0-hour series, in which cells were irradiated about 20 minutes after removal from 5-AU. X rays (150r) were delivered at 100r/min from a General Electric Maxitron tube operated at 250 kvp and 30 ma with 3 mm aluminum filtration added (HVL = 0.45 mm copper). The source of UV was a water-cooled capillary mercury arc (Phillips) operat-

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ing at 3 amp and 115 v. Monochromatic incident radiation at 2650 Å was used (100 ergs/mm², dose-rate approximately 5 ergs/mm²/sec).

Both irradiated and control cell-populations were treated with 5×10^{-7} M colchicine for 3 hours beginning at the 7th hour after their removal from 5-AU. The cells were then collected, exposed to a 0.7% sodium citrate solution for chromosome spreading, fixed in 3:1 (v/v) ethanol-glacial acetic acid mixture, air-dried on wet slides and acetic-orcein stained. Metaphases were studied and scored for chromatid aberrations. From the same colchicine-treated cell populations, the relative degree of mitotic inhibition induced by UV or X rays during various cell stages was compared.

In order to determine the mitotic activity of cells in the presence of 5-AU and after removal from the chemical, 3×10^5 cells in 1.5 ml of medium were inoculated into each 35-mm plastic dish containing a 22×22 mm coverslip. Duplicate cultures were sampled periodically by fixing the coverslips. The mitotic index is expressed as the percentage of metaphase cells per 1000 counted in each culture.

The sequence and rate of DNA synthesis in synchronized cell populations were studied both by the autoradiographic technique and by counting the incorporated radioactivity. The autoradiographic procedure consisted of the following steps: tritium-labeled cells were fixed and air-dried on slides which were coated with Kodak NTB3 nuclear track emulsion exposed for 7 to 10 days at 4°C, developed, and stained with toluidine blue. The proportion of labeled cells (1000 cells per point) and the average number of silver grains per cell (200 cells per point) were determined.

For assaying the DNA-synthetic activity on the basis of incorporated radioactivity, duplicate cultures were periodically pulse-labeled with H³-TdR before the cells were ruptured by sonication. For this particular experiment, 1.5 ml of the radioactive growth medium was placed for 15 minutes in each 35 mm plastic culture dish. Immediately after labeling, the dishes were kept on ice and cells sonicated for 30 seconds in the radioactive medium. No intact cell was left after sonication. A known quantity (0.1 ml) of the sonicate was dispensed to a filter paper disc. The disc was washed with 5% cold trichloroacetic acid and its radioactivity measured in a liquid-scintillation counter (BOLLUM 1959; REGAN and CHU 1965).

RESULTS

Mitotic activity: Data on mitotic activity of the hamster cells treated with 5-AU are presented in Figure 1. During the first two days after inoculation, about 2 to

FIGURE 1.—The frequency of metaphases in Chinese hamster cell populations at various times in the presence and after the removal of 5-aminouracil. No colchicine was added to any sample. ○—○, control; ●—●, 5-AU-treated.

3% of the control cells were in metaphase. After the cells were rinsed and the growth medium was changed, there was an immediate drop in mitotic activity, but the activity increased for several hours until the normal rate was again reached. In the presence of 5-AU, mitotic activity was considerably reduced but not completely suppressed. After the chemical was removed and the cells were reincubated in fresh medium, only a few dividing cells were seen in the first 4 or 5 hours. This pattern differs from that of the untreated cells. Five hours after cells were removed from the chemical, mitotic activity quickly increased and reached a peak at the eighth hour. The peak was of short duration, indicating a high degree of synchronization of the cells that were capable of arriving at division. There was no evidence for a second peak 80 hours after inoculation, when these cells would have been expected to divide again. Instead, the synchrony appears to have decayed as the cells began to divide randomly at the normal control rate.

In subsequent experiments (some with slight modifications such as collecting cells by trypsinization), the mitotic index at the peak varied from experiment to experiment (6 to 14%) although the timing was the same in all experiments. It is possible that variation in growth activity in different experiments affected the maximum activity.

The number of metaphases accumulated by a 3-hr colchicine treatment was determined from experiments in which successive cell populations of the chemically synchronized cells were pulse-labeled and X- or UV-irradiated. The results are summarized in Table 1. In unirradiated populations (except for exposure to H^3 -TdR at various hours), approximately 21 to 27% of cells were accumulated at metaphase. With the doses used, mitotic activity was more severely inhibited by UV than by X rays. Furthermore, in terms of mitotic inhibition, cells at S seemed to be more sensitive to UV than those at G_2 although the reverse was true for X rays. The incorporated tritium might also have inhibited mitosis to a certain extent, but the above comparison remains valid since both unirradiated and irradiated populations were treated alike.

DNA synthesis: Figure 2 shows the results by liquid-scintillation counting of the total incorporated H^3 -TdR in various hamster-cell populations. The peaks of

TABLE 1

Mitotic inhibition in 5-aminouracil-synchronized Chinese hamster cells induced by UV (100 erg/mm² at 2650 Å) or X rays (150r) during S and G_2

Time of irradiation*	Percentage of cells in metaphase†		
	Unirradiated	UV	X rays
1 (S)	21.0	6.9	24.2
2 (S)	26.9	7.5	...
4 (S)	21.1	7.9	24.6
7 (G_2)	21.8	13.4	4.2

* In hours after removal of cells from 5-AU. The corresponding cell stages are indicated.

† Accumulated by colchicine from the seventh to the tenth hour after removal of cells from 5-AU. From 2000 to 4000 cells were counted in each series.

FIGURE 2.—Total DNA-synthetic activity in Chinese hamster cell populations at various times in the presence and after the removal of 5-aminouracil. The activity was determined by liquid-scintillation counting of incorporated H^3 -thymidine immediately after 15-minute pulse-labeling at each interval. ○—○, control; ●—●, 5-AU-treated.

DNA-synthetic activity in control populations probably resulted from changing medium. In the presence of 5-AU there was practically no DNA synthesis. The synthetic activity quickly resumed, however, and reached a maximum 5 hours after removal of the chemical. The results have not been corrected for cell number. As compared to controls, the lower level of total radioactivity in 5-AU-treated cells may be due primarily to a lower number of cells per culture, since it has been shown that there was little mitotic activity during the 24-hour exposure to 5-AU. There were twice as many cells in the control groups after the second day of growth.

The data demonstrate that DNA synthesis was either blocked or slowed down by 5-AU and that at least a portion of the cell population could recover from the chemical treatment and engage in and complete DNA synthesis before entering G_2 and division. The shape of the curve indicates that the portion of cells which was synchronized at division probably also had a rather uniform timing for DNA synthesis.

Similar results have been obtained with autoradiographic techniques (Table 2). In the first part of this experiment, hourly samples of cells were fixed immediately after pulse labeling and the percentage of labeled interphase cells in each population was determined. Control populations were handled in the same manner but without the 5-AU treatment. On the average, approximately one third of the control population was in synthesis any time during the sampling period. The result agrees with our previous determination for this cell line during logarithmic growth (CHU 1965). The reduced frequency of labeled interphase at 0 hours and the apparent increase in frequency at hours 4 and 5 may reflect a slight degree of synchronization owing to the change of medium. The majority of the 5-AU-treated cells, on the other hand, was engaged in active synthesis during the first few hours following the removal of the chemical. This pattern differs significantly from that of the untreated populations.

TABLE 2

Proportion of Chinese hamster cells in DNA synthesis at various times after removal from 5-aminouracil

Hours after removal from 5-AU	A* Percentage of labeled interphase cells		B† Percentage of labeled metaphase cells‡		
	Control	5-AU treated	Unirradiated	X rays	UV
0	20.8	76.6	100	...	...
1	29.9	75.5	100	97.3	...
2	29.6	84.3	98.0	90.5	91.0
3	27.5	66.6	97.5	80.0	81.0
4	39.9	52.1	85.0	55.8	55.5
5	38.7	33.8	51.5	27.3	...
6	26.5	30.8	18.3	4.0	4.5
7	35.5	24.8	3.1	0	0

* Part A: cells were fixed immediately after a 15-minute pulse-labeling with H³-thymidine.

† Part B: Different cell populations were pulse-labeled just prior to X(150r) or UV(100 ergs/mm² at 2650 Å) irradiation at various hours.

‡ Metaphases were collected with colchicine at the subsequent mitotic peak (7 to 10 hours).

The percentage of labeled metaphases among those accumulated by 3-hours exposure to colchicine is shown in Table 2 and plotted in Figure 3. Different cell samples had been pulse-labeled at successive hours just prior to X- or UV irradiation. One experiment with each type of radiation was performed; the data from the unirradiated controls from both experiments were essentially identical and have been combined. It is clear from the data that immediately after cells were removed from 5-AU, all the unirradiated cells that reached metaphase between hours 7 and 10 were in synthesis. The percentage of labeled metaphases dropped abruptly by hours 6 and 7, when most cells had apparently arrived at G₂.

FIGURE 3.—The frequency of tritium-labeled metaphases accumulated with 3-hour colchicine treatment around the mitotic peak of the chemically synchronized Chinese hamster cell populations. At various hours after removing cells from 5-aminouracil, different populations were pulse-labeled just prior to UV or X-irradiation. ●—●, unirradiated; △—△, X-irradiated; ■—■, UV-irradiated.

At the doses employed, neither X rays nor UV seemed to affect the sequence of cell stages. It is interesting to note, however, that the overall frequency of labeled metaphases in the irradiated populations was lower than in the unirradiated. In other words, fewer irradiated cells were capable of reaching metaphase during the 3 hours in colchicine. Since the cells were pulse-labeled prior to irradiation, it seemed reasonable to assume that radiation would not affect the amount of labeled DNA already synthesized. The lower curve in Figure 3 represents the change in frequency of labeled metaphases when different cell populations were irradiated at various times during S. This curve is approximately parallel to that of the unirradiated populations. Thus, among the surviving irradiated S cells, the time of reaching metaphase was delayed for about 1 hour.

The amount of DNA synthesis during different hours of S was studied by counting the number of silver grains per metaphase cell (Table 3). Both the range and mean of grain numbers are given. There is a wide distribution of numbers, but the mean approaches the mode and is thus indicative of the average synthetic activity at a particular hour. If the total number of grains per cell was 10 or less, the cell was considered unlabeled although the background was usually below five grains per unit cell area.

The amounts of synthesis in the unirradiated populations from the two experiments were the same. It appears that DNA synthesis in the hamster cell is not uniform throughout the S period. It must be emphasized, however, that these cells had been treated with 5-AU, which might have modified the rate of synthesis. The results also show that the synthetic activity was the highest at the third hour after the cells' removal from 5-AU. Only a small fraction of the new DNA remained to be synthesized toward the very end of S (hour 7). Since the

TABLE 3

Relative amounts of H^3 -thymidine incorporation in X- or UV-irradiated and unirradiated Chinese hamster cells during S and G_2

Treatment	Hours after removal from 5-AU	Number of silver grains per metaphase cell			
		Unirradiated		Irradiated	
		Range	Mean	Range	Mean
X rays (150r)	1	111-207	118.3	97-220	192.0
	2	103-203	143.6	14-313	187.8
	3	20-297	191.8	61-290	196.4
	4	18-292	170.9	16-278	137.0
	5	11-219	121.1	12-293	109.6
	6	11-253	114.4	11-170	45.4
	7	11-118	33.7	12-19	13.2
UV, 2650 Å (100 ergs/mm ²)	2	28-216	152.7	66-204	144.0
	3	39-345	201.3	13-228	124.5
	4	67-293	161.5	15-287	111.5
	6	11-235	91.9	13-137	64.6
	7	11-221	25.2	11-137	40.1

time of colchicine treatment was longer than the average time for G_2 , the 7-hour sample includes 3.1% of late S cells (Table 1).

The radiation-induced mitotic delay results in a shift in the temporal distribution of average numbers of silver grains, but this shift seems to be uniform and an orderly completion of synthesis is seen.

Chromosome aberrations: Duplicate experiments with each type of radiation were performed in which chemically synchronized cell populations were irradiated at successive hours of S and G_2 . Statistical analysis indicates that the results from duplicate experiments are not significantly different from each other. Therefore, the results have been pooled.

At the level of concentration used, 5-AU appears to have induced an appreciable frequency of aberrations (Table 4). About 5% of the cells were abnormal, containing at least one chromatid aberration. Among these, all types of chromatid aberrations (terminal deletions, isochromatid breaks, and exchanges) were observed. The control populations which were pulse-labeled at different times showed only slight variation in aberration frequencies. In 2600 cells examined, the average frequency of chromatid aberrations in cells exposed to 5-AU for 24 hours was 0.1 break/cell. This rate is about five to ten times the spontaneous rate (CHU 1965). DUNCAN and WOODS (1953) have shown that 5-AU induces cytological aberrations in root meristems of *Allium cepa*. Since all cell popula-

TABLE 4

Frequencies of chromatid aberrations in unirradiated populations of Chinese hamster cells at various times after removal from 5-aminouracil

Time (hours)	Cells scored	Percentage normal	Aberrations per 100 cells			
			Terminal deletions	Isochromatid breaks	Exchanges	Total breaks
0	100	97.0	0	0	3.0 (0.8-8.1)*	6.0 (2.6-12.8)
1	300	94.0	2.3 (1.0-4.4)	0.7 (0.1-2.2)	6.0 (3.7-9.2)	15.0 (10.7-19.6)
2	300	94.7	2.0 (0.9-4.3)	0.7 (0.1-2.2)	3.7 (1.8-6.3)	11.0 (6.7-13.9)
3	400	95.3	1.3 (0.5-2.5)	0	4.0 (2.4-6.3)	9.5 (6.7-12.8)
4	400	95.5	1.3 (0.6-3.2)	0.3 (0.0-1.3)	2.0 (0.8-3.7)	5.5 (3.4-8.1)
5	300	95.0	2.3 (1.1-4.5)	1.3 (0.1-2.2)	4.0 (2.2-6.4)	11.0 (7.6-15.0)
6	400	94.0	2.7 (1.3-4.8)	0.3 (0.0-1.3)	5.6 (3.9-8.5)	14.5 (11.8-18.5)
7	400	96.3	0.3 (0.0-1.3)	0	3.8 (2.0-5.9)	7.8 (5.3-10.8)
Total	2600	95.1	1.7 (1.2-2.2)	0.3 (0.1-0.6)	4.1 (3.3-4.9)	10.1 (8.9-11.3)

* Values in parentheses indicate the 95% confidence limits for the expectation of a Poisson variable.

tions in the present studies were pulse-labeled with H^3 -TdR, it is probable that some of the aberrations were induced by the radioisotope (e.g. WIMBER 1959; BENDER, GOOCH, and PRESCOTT 1962).

With X rays, the frequencies of all types of chromatid aberrations appear to be more or less constant throughout S (Table 5, Figure 4). As the cell goes from S to G_2 , the frequency of isochromatid breaks remains the same, that of exchanges increases significantly, and that of terminal deletions increases very significantly.

TABLE 5

Frequencies of chromatid aberrations in X-irradiated populations of Chinese hamster cells at various times after removal from 5-aminouracil

Time (hours)	Cells scored	Percentage normal	Aberrations per 100 cells			
			Terminal deletions	Isochromatid breaks	Exchanges	Total breaks
1	400	75.7	10.8 (7.8-14.2) *	5.5 (3.4-8.1)	17.8 (13.8-22.3)	51.8 (44.7-58.8)
2	400	77.3	10.5 (7.5-13.9)	2.5 (1.4-4.4)	22.8 (18.3-27.5)	58.0 (50.8-65.5)
3	400	77.8	10.8 (7.8-14.2)	2.3 (1.0-4.4)	18.0 (13.9-22.3)	49.0 (42.3-56.0)
4	400	80.5	10.5 (7.5-13.9)	2.5 (1.4-4.4)	13.3 (9.9-17.0)	39.5 (33.3-45.7)
5	400	68.5	19.3 (15.0-23.7)	3.0 (1.7-5.1)	15.6 (11.9-19.9)	44.8 (38.0-51.3)
6	400	65.5	30.0 (24.8-36.0)	5.0 (3.2-7.5)	21.0 (16.7-25.8)	77.3 (66.1-85.8)
7	400	48.3	68.3 (60.5-76.4)	7.8 (5.3-10.8)	39.0 (33.0-45.4)	151.5 (139.4-163.6)

* See footnote, Table 4.

FIGURE 4.—Total frequencies of chromatid breaks in synchronized hamster cell populations UV- or X-irradiated at various times during S and G_2 . ●—●, unirradiated; △—△, X-irradiated; ■—■, UV-irradiated.

TABLE 6

Frequencies of chromatid aberrations in UV-irradiated populations of Chinese hamster cells at various times after removal from 5-aminouracil

Time (hours)	Cells scored	Percentage normal	Aberrations per 100 cells			
			Terminal deletions	Isochromatid breaks	Exchanges	Total breaks
0	200	76.0	21.5 (15.8–28.3)*	9.0 (5.5–13.8)	19.0 (13.1–25.6)	68.5 (57.2–80.2)
1	200	68.5	27.0 (20.2–34.8)	9.5 (5.5–14.4)	19.5 (13.9–26.0)	75.5 (64.2–88.1)
2	200	82.5	9.5 (5.5–14.4)	5.0 (2.6–8.8)	8.0 (4.8–12.7)	30.5 (23.5–38.5)
3	400	79.0	14.3 (10.8–18.1)	2.8 (1.3–4.8)	9.5 (6.6–12.8)	36.0 (30.0–42.3)
4	400	84.8	9.3 (6.6–12.6)	2.5 (1.3–4.4)	8.0 (5.3–11.0)	27.8 (22.6–33.0)
5	200	86.5	12.5 (8.3–18.0)	3.0 (1.3–6.4)	7.5 (4.0–12.8)	30.5 (23.5–38.5)
6	400	88.5	7.3 (4.8–10.2)	1.8 (0.8–3.4)	6.0 (3.7–8.4)	21.0 (16.7–25.8)
7	400	88.5	8.8 (5.9–11.9)	2.5 (1.3–3.9)	3.8 (2.0–5.9)	18.8 (14.5–23.3)

* See footnote, Table 4.

In fact, terminal deletions contribute largely to the overall increase of breakage frequency in G_2 .

With UV, on the other hand, the frequencies of all types of chromatid aberrations were highest when cells were irradiated during the first hour after removal from 5-AU (Table 6, Figure 4). This was presumably the beginning of S. The chromatid aberration frequencies induced during the remaining periods of S were significantly lower than those in early S, but not significantly different from each other. There is some indication of a further reduction in UV-induced aberration yield during G_2 , especially in the frequency of chromatid exchanges.

DISCUSSION

The pyrimidine analogue 5-aminouracil (5-AU) has been shown to cause partial synchronization of nuclear division in *Vicia faba* root tip meristemic cells (SMITH, FUSSELL and KUGELMAN 1963). The induced synchrony was the result of a depression in the rate of DNA synthesis, which brought about an accumulation of cells in the S phase of the cell cycle (PRENSKY and SMITH 1965).

Further studies in the same material indicate, however, that DNA synthesis and mitotic synchronization can occur while the seedlings are in 5-AU (JAKOB and TROSKO 1965; E. MATTINGLY 1965, personal communication). Furthermore, synchronization could not be eliminated by the addition of thymidine to 5-AU, as would have been expected if interference with thymidine synthesis were a cause of synchronization (JAKOB and TROSKO 1965). MATTINGLY (personal com-

munication) further noted that different types of cells and different regions of the same root respond differently to 5-AU. We have shown that there was practically no incorporation of thymidine in hamster cells grown in the presence of 5-AU. After removal of cells from the chemical, DNA synthesis quickly resumed. These results clearly indicate a pronounced effect of 5-AU on DNA synthesis in mammalian cells, although its mode of action remains to be elucidated. To explain the difference in response between mammalian and certain root cells, it is conceivable that the clonal line of hamster cells in culture is more homogeneous in cell type. Moreover, the chemical in the growth medium is easily accessible to all cells or can be quickly removed and diluted to an ineffective level.

The hamster cell line used in the present studies has an average generation time of 24 hours, which includes 8 hours for S, 2 to 3 hours for G_2 , and 13 to 14 hours for G_1 and mitosis (CHU 1965). In another recent study in which 5-AU treated hamster cells were rinsed with BSS and reincubated without the chemical but in the continued presence of H^3 -TdR, the accumulated radioactivity was found to reach a maximum by the 7th hour (CHU, unpublished data). This can be interpreted to mean that 5-AU effectively blocked the majority of cells at the onset or at the very beginning stage of S, and that, upon removal of 5-AU, cells could complete DNA synthesis within normal time though not necessarily at the normal rate.

It is possible that cells at different stages of S and G_2 were also stopped or slowed down by 5-AU, as evidenced by the low mitotic activity during the first few hours after cells were removed from the chemical. A delay in the rate of exit of Vicia root cells from the G_2 period has been reported (PRENSKY and SMITH 1965; WOLFF and LUIPPOLD 1963, unpublished data). Even if the hamster cells were blocked by 5-AU in late S or G_2 of the previous mitosis, they were not accumulated at metaphase by colchicine in our experiments. Data on DNA synthesis (Tables 2, 3) support this view. Almost all metaphases which had been briefly exposed to H^3 -TdR during the first few hours were heavily labeled, indicating they were not late S cells. Moreover, if any blocked G_2 cells of the previous cycle were X-irradiated at hours 1 or 2 and delayed mitotically, they were not found in this accumulated metaphase population, because all cells were labeled.

The data on the incorporated radioactivity obtained by liquid scintillation counting indicate the total synthetic activity of the population during the pulse-labeling period at a particular stage of S; the interphase labeling indices represent the proportion of cells in synthesis during each such period. From these determinations it is concluded: (1) the great majority of cells (75 to 85%) proceeded to synthesize DNA during the first few hours after removal from 5-AU. Considering that even in actively growing normal populations there is senescence and cell death, the degree of synchrony in 5-AU-treated cells was remarkably high. The shapes of curves in terms of total DNA synthetic activity (Figure 2) and mitotic index (Figure 1) give additional support to the above conclusion. (2) The data from silver grain counts per metaphase (Table 3) indicate that the DNA-synthetic activity after the 5-AU treatment is not uniform throughout the S period. This is probably true for normal cells as well, but

it is not known how 5-AU modified the rate of synthesis. DENDY and CLEAVER (1964) have shown that in L cells the average rate of DNA synthesis varies during the S phase.

The colchicine-accumulated metaphase cells constitute a selected (on the basis of reproductive capacity) population in which a very high degree of synchrony was reached (Figure 3). Our results from the tritium-labeling experiments demonstrate the synchronous progression of cells through S and G₂ before arriving at metaphase. Five-aminouracil apparently can synchronize this part of the mitotic cycle, but does not alter either the order or duration of stages. After UV or X-irradiation, cells with chromatid aberrations induced at each designated hour during S and G₂ also appeared to reach metaphase in an orderly fashion.

With the doses used, mitotic activity was more severely inhibited by UV than by X rays (Table 1). UV-irradiation of cells at S reduced the mitotic index to about one third of the unirradiated level. Cells irradiated at early S seemed to be more severely affected. The mitotic index was reduced to about one half of the control level when cells were irradiated at G₂. Attempts were made to collect metaphases in two successively later 3-hour periods than the 7- to 10-hour period. There were very few dividing cells in these populations, demonstrating the permanent sterilizing effect of UV (CHU 1965). Nevertheless, it is remarkable that a sizable proportion of the UV-irradiated S cells still could reach metaphase.

Most cells which had been X-irradiated during S appeared to be capable of entering division in time to be collected during the 3 hours in colchicine. In fact, the delay was found to be about 1 hour. The frequencies of accumulated metaphases in these populations are similar to the average control frequency. In contrast, cells in G₂ were extremely sensitive to X rays. This last observation agrees with the finding of WHITMORE *et al.* (1961) in L cells, and that of TERASIMA and TOLMACH (1963) in HeLa cells. It is possible that the duration of delay in X-irradiated G₂ cells was longer than that in the irradiated S cells, but it is also possible that G₂ cells are more sensitive to X rays in terms of survival.

What is scored in the present experiments as simply an inhibition of mitosis includes both mitotic delay and the lack of ability to reach mitosis (interphase death). In a synchronized culture of another line of Chinese hamster cells, SINCLAIR and MORTON (1965) found that, in terms of the ability to form colonies, cells were least sensitive to X rays during the latter part of S and were about equally sensitive at G₁ and G₂. With UV, on the other hand, sensitivity was the lowest at G₂. Since both interphase death and genetic death (partly resulting from chromosome aberrations) reduce the total surviving fraction of cells and are directly and intimately related to the colony-forming capacity, our findings on differential stage radiosensitivity, both in terms of mitotic inhibition and in terms of chromosome aberrations, are in agreement with that on cell killing, at least for hamster cells during S and G₂.

In general, the time of mitotic delay with doses of either type of radiation at S was found to be about 1 hour (Figure 3). This result was obtained only from cells irradiated at various phases of S and may not be applicable to other cell stages. For example, the radiation-induced delay at G₂ was not measurable by our

criterion of labeling index. It is entirely possible that a much longer delay can be induced by X rays at G_2 . Very few cells which were UV-irradiated at G_1 reached mitosis (CHU, unpublished data).

Differences in the duration of mitotic delay when cells were irradiated at S or G_2 might lead to a certain bias in the selection of cells for chromosome aberrations analysis. Since UV radiation at various times in S induces the same extent of mitotic delay, a significantly higher frequency of aberrations induced at early S may represent a real difference in sensitivity of chromosomes at this stage. On the other hand, if most of the X-irradiated G_2 cells were either killed or mitotically delayed, the observed frequency of aberrations induced at this period might be a minimum estimate of the radiation damage.

The majority of data on stage sensitivity to ionizing radiations in plant species shows that more aberrations can be induced per unit dose in S than in G_1 , and that a still higher frequency of aberrations can be induced in G_2 ($G_1 < S < G_2$). WOLFF (1961) pointed out that the apparent difference in sensitivity between presplit (G_1) chromosomes and postsplit (S and G_2) chromosomes, is not caused by a change in the sensitivity of the chromosome to breakage but by (1) a bias in scoring procedure (only asymmetrical exchanges, i.e., dicentrics and rings, are scored as two-hit aberrations induced in G_1 ; both symmetrical and asymmetrical exchanges in S and G_2 are scored); and by (2) the formation of a new type of aberration (isochromatid aberration) in postsplit chromosomes that is not usually found in the presplit chromosomes.

The increase in frequency of aberrations from S to G_2 cannot, however, be attributed to such a bias in scoring since chromatid aberrations are induced in both stages. It seemed probable that such an increase would be explicable on the site hypothesis of WOLFF and ATWOOD (WOLFF *et al.* 1958; WOLFF 1959). They have suggested that in the nucleus there is a limited number of sites available, within which the broken ends of chromosomes can interact to form exchanges. GARCIA-BENITEZ and WOLFF (1962) found that the number of sites appears to increase in postsplit chromosomes. Similar observations have been made on *Vicia* and *Tradescantia*, which indicate that the frequency of chromatid exchanges increases as the cells approach mitosis (SWANSON and SCHWARTZ 1953; REVELL 1961; EVANS and SAVAGE 1963; EVANS and SCOTT 1964). BREWEN (1964a, b) observed in both 5-AU-synchronized and nonsynchronized cells in lateral root tips of *Vicia* that, as the cells go from S to G_2 , the number of aberrations induced by X rays increases by a factor of two. This increase comes only from the two-break aberrations; the frequency of isochromatid breaks remains constant from S through G_2 , and that of terminal deletions is too low to show a clear-cut tendency. BREWEN too interpreted his results as an increase in the number of sites within which an exchange can occur, and not as an increase of the sensitivity of the chromosome strands to X rays.

In the present experiments, the increase of the X-ray-induced chromatid exchanges and terminal deletions, but not of isochromatid breaks, as the cell proceeds through S to G_2 , is consistent with all of the published data. The increase in exchanges can probably be attributed to an increase in the number of sites within

which an exchange can occur. The increase in terminal deletions could be explained by the hypothesis that the broken ends move apart, possibly as a result of coiling, before restitution of the broken ends can occur (BREWEN 1965). The rejoining of broken ends resulting from isochromatid breaks would not be seriously affected by chromosome movement. Similarly, the frequency of chromatid exchanges may not be reduced by chromosome movement, and in fact may even be increased (see discussion in WOLFF 1961). BENDER and GOOCH (1961), working with bone marrow of the Chinese hamster, demonstrated that cells closest to metaphase (presumably G_2 cells) when X-irradiated have more aberrations, mostly terminal deletions, than those cells farther from metaphase. BREWEN (1965) also noted in human leukocytes in culture that there are more terminal deletions when the cells are irradiated in G_2 . All in all, it appears that for both the plant and mammalian materials studied, there is a uniformity of chromosome sensitivity to X-ray-induced breakage during the mitotic cycle, and that the apparent changes in sensitivity reflect changes in rejoining rather than breakage.

From our present data, the frequencies of all types of chromatid aberrations induced by UV in early S were significantly higher than those induced in the rest of S and G_2 . The variation in frequency of UV-induced aberrations during S and G_2 is drastically different from those induced by ionizing radiations. By pulse-labeling of a randomly growing population of Chinese hamster cells just prior to irradiation and by scoring metaphases during various postirradiation periods, a similar conclusion has been reached earlier that cells at S are more sensitive to UV and those at G_2 more sensitive to γ rays (HUMPHREY *et al.*, 1963; HSU, DEWEY and HUMPHREY 1962). Independently, using UV and X rays, similar observations have been made from essentially the same type of experiments (CHU, unpublished data). The synchronized populations, however, offer a much better opportunity for quantitative study of the relative radiosensitivity during various stages of S and G_2 . Since there was no mixture of cell populations which were irradiated at different parts of the cell cycle, certain difficulties in interpreting results can be resolved.

The basic causal mechanism(s) of aberration-induction by different kinds of radiations may be different. The interaction of X-ray photons, or ionizing particles, with protoplasm is generally considered to be mediated by excitations and/or ionization. The effects of UV on cellular macromolecules are probably more selective or specific. Our earlier results (CHU 1965) suggest that the initial damage to DNA by UV may contribute considerably to the induction of chromosomal breakage. We also have evidence that UV irradiation of Chinese hamster cells inhibits DNA synthesis (CHU, unpublished data) and induces, among other possible photoproducts, thymine dimer formation in chromosomal DNA (TROSKO, CHU, and CARRIER 1965), but the relationships between thymine dimerization, DNA synthesis, and production of chromosome aberrations in mammalian cells remain to be established.

The present results show that UV induces a significantly higher frequency of chromatid aberrations in hamster cells soon after their removal from 5-AU, presumably in early S, when active DNA synthesis has just resumed but has not

yet reached the maximum rate. However, the data do not permit definite conclusions to be drawn on the possible relationship, if any, between DNA synthesis and the observed differential UV sensitivity of chromosomes in different stages of S and G₂.

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SUMMARY

Five-aminouracil (5-AU), a pyrimidine analogue, is an effective agent for partial synchronization of mitosis in Chinese hamster cells *in vitro*. In its presence, DNA synthesis and mitotic activity were heavily suppressed. After removal of cells from the chemical, both activities proceeded synchronously and reached peaks at hours 5 and 8, respectively. The majority of cells seemed to be blocked at the beginning of S. It is possible that cells at different stages of S and G₂ were either stopped or slowed down by 5-AU. The treated cells could apparently recover, then resume and complete DNA synthesis before entering G₂ and division in an orderly manner. There is no evidence, however, for mitotic synchrony in subsequent cycles. Under the experimental conditions, 0.1 chromatid break per cell was observed in 5-AU-treated populations.

If cells arriving at metaphase around the mitotic peak (from hour 7 to hour 10) were accumulated at metaphase with colchicine, this selected metaphase population showed a high degree of synchrony. The chemical apparently can synchronize this portion of the mitotic cycle (early S to M) without altering either the order or duration of stages. Different populations of cells which had been UV- or X-irradiated at successive hours during S and G₂ appeared to reach metaphase in an orderly fashion.

On the basis of the rate of H³-thymidine incorporation in 5-AU-synchronized cells, it is concluded that in these cells DNA-synthetic activity is not uniform throughout S.

In terms of mitotic inhibition, cells were more sensitive to ultraviolet light (UV) in S than at G₂, but the reverse was true for X rays. The relationship between mitotic inhibition, chromosome aberrations, and cell killing are discussed.

The frequency of the X-ray-induced chromatid aberrations reached a peak after DNA synthesis (G₂), whereas that of the UV-induced aberrations was highest during S, particularly early S. Although the frequencies of the X-ray-induced aberrations vary, depending on the cell stage at which the cell was irradiated, the X-ray sensitivity of chromosomes to breakage during the mitotic cycle is believed to be uniform throughout the cycle. On the other hand, the observed variation in UV sensitivity of chromosomes to breakage cannot be explained with data at hand.

LITERATURE CITED

- BENDER, M. A., and P. C. GOOCH, 1961 Spontaneous and X-ray-induced somatic chromosome aberrations in the Chinese hamster. *Intern. J. Radiation Biol.* **4**: 175-184.

- BENDER, M. A., P. C. GOOCH, and D. M. PRESCOTT, 1962 Aberrations induced in human leukocyte chromosomes by H^3 -labeled nucleosides. *Cytogenetics* **1**: 65-74.
- BOLLUM, F. J., 1959 Thermal conversion of nonpriming deoxyribonucleic acid to primer. *J. Biol. Chem.* **234**: 2733-2734.
- BREWEN, J. G., 1964a Studies on the frequencies of chromatid aberrations induced by X rays at different times of the cell cycle of *Vicia faba*. *Genetics* **50**: 101-107. — 1964b Kinetics of X-ray-induced chromatid aberrations in *Vicia faba* and studies relating aberration frequencies to the cell cycle. *Mutation Res.* **1**: 400-408. — 1965 Cell cycle and radio-sensitivity of the chromosomes of human leukocytes. *Intern. J. Radiation Biol.* (In press.)
- CHU, E. H. Y., 1964 Variations in the frequency of chromatid aberrations in mammalian cells induced by X rays and ultraviolet light during different stages of the cell cycle. *Mammalian Chromosome Newsletter* **14**: 68-69. — 1965 Effects of ultraviolet radiation on mammalian cells. I. Induction of chromosome aberrations. *Mutation Res.* **2**: 75-94.
- DENDY, P. P., and J. E. CLEAVER, 1964 An investigation of (a) variation in rate of DNA-synthesis during S-phase in mouse L-cells (b) effect of ultra-violet radiation on rate of DNA synthesis. *Intern. J. Radiation Biol.* **8**: 301-315.
- DUNCAN, R. E., and P. S. WOODS, 1953 Some cytological aspects of antagonism in synthesis of nucleic acids. *Chromosoma* **6**: 45-60.
- ERIKSON, R. L., and W. SZYBALKI, 1963 Molecular radiobiology of human cell lines. IV. Variation in ultraviolet light and X-ray sensitivity during the division cycle. *Radiation Res.* **18**: 200-212.
- EVANS, H. J., 1962 Chromosome aberrations induced by ionizing radiations. *Intern. Rev. Cytol.* **13**: 221-321.
- EVANS, H. J., and J. R. K. SAVAGE, 1963 The relation between DNA synthesis and chromosome structure as resolved by X-ray damage. *J. Cell. Biol.* **18**: 525-540.
- EVANS, H. J., and D. SCOTT, 1964 Influence of DNA synthesis on the production of chromatid aberrations by X rays and maleic hydrazide in *Vicia faba*. *Genetics* **49**: 17-38.
- GARCIA-BENITEZ, C., and S. WOLFF, 1962 On the increase of sites for chromosome exchange formation after chromosome duplication. *Science* **135**: 438-439.
- GILES, N. H., JR., 1954 Radiation-induced chromosome aberrations in Tradescantia. pp. 713-761. *Radiation Biology*. Vol. 1, Part 2. Edited by A. HOLLAENDER. McGraw-Hill, New York.
- HSU, T. C., W. C. DEWEY, and R. M. HUMPHREY, 1962 Radiosensitivity of cells of Chinese hamster *in vitro* in relation to the cell cycle. *Exptl. Cell Res.* **27**: 441-452.
- HUMPHREY, R. M., W. C. DEWEY, and A. CORK, 1963 Relative ultraviolet sensitivity of different phases in the cell cycle of Chinese hamster cells grown *in vitro*. *Radiation Res.* **19**: 247-260.
- JAKOB, K. M., and J. E. TROSKO, 1965 The relation between 5-aminouracil-induced mitotic synchronization and DNA synthesis. *Exptl. Cell Res.* (in press).
- KAUFMANN, B. P., 1954 Chromosome aberrations induced in animal cells by ionizing radiations. pp. 627-711. *Radiation Biology*. Vol. 1, Part 2. Edited by A. HOLLAENDER. McGraw-Hill, New York.
- PRENSKY, W., and H. H. SMITH, 1965 The mechanism of 5-aminouracil-induced synchrony of cell division in *Vicia faba* root meristems. *J. Cell Biol.* **24**: 401-414.
- REGAN, J. D., and E. H. Y. CHU, 1965 A convenient method for periodic assay of DNA synthesis in synchronous human cells in suspension culture. *Excerpta Medica* (in press).
- REVELL, S. H., 1961 An attempt at continuous metaphase estimation of chromatid and chromosome aberration frequencies in broad-bean root meristem cells in the period 2-23 h after 50 r of X rays. pp. 229-242. *Proc. Intern. Symp. on the Effects of Ionizing Radiations on Seeds*. International Atomic Energy Agency, Vienna.

- SINCLAIR, W. K., and R. A. MORTON, 1965 X-ray and ultraviolet sensitivity of synchronized Chinese hamster cells at various stages of the cell cycle. *Biophys. J.* **5**: 1-25.
- SMITH, H. H., FUSSELL, C. P., and B. H. KUGELMAN, 1963 Partial synchronization of nuclear divisions in root meristems with 5-aminouracil. *Science* **142**: 595-596.
- SPARROW, A. H., 1951 Radiation sensitivity of cells during mitotic and meiotic cycles with emphasis on possible cytochemical changes. *Ann. N. Y. Acad. Sci.* **51**: 1508-1540.
- SWANSON, C. P., and D. SCHWARTZ, 1953 Effect of X rays on chromatid aberrations in air and nitrogen. *Proc. Natl. Acad. Sci. U. S.* **39**: 1241-1250.
- TERASIMA, T., and L. J. TOLMACH, 1963 Variations in several responses of HeLa cells to X-irradiation during the division cycle. *Biophys. J.* **3**: 11-33.
- TROSKO, J. E., E. H. Y. CHU, and W. L. CARRIER, 1965 The induction of thymine dimers in ultraviolet-irradiated mammalian cells. *Radiation Res.* **24**: 667-672.
- WHITMORE, G. F., C. P. STANNERS, J. E. TILL, and S. GULYAS, 1961 Nucleic acid synthesis and the division cycle in X-irradiated L-strain mouse cells. *Biochim. Biophys. Acta* **47**: 66-77.
- WIMBER, D. E., 1959 Chromosome breakage produced by tritium-labeled thymidine in *Tradescantia Paludosa*. *Proc. Natl. Acad. Sci. U.S.* **45**: 839-846.
- WOLFF, S., 1959 Interpretation of induced chromosome breakage and rejoining. *Radiation Res.* (Suppl. 1): 453-462. ——— 1961 Some postirradiation phenomena that affect the induction of chromosome aberrations. *J. Cell. Comp. Physiol.* **58**: (Suppl. 1): 151-162.
- WOLFF, S., K. C. ATWOOD, M. L. RANDOLPH, and H. E. LUIPPOLD, 1958 Factors limiting the number of radiation-induced chromosome exchanges. I. Distance: Evidence from non-interaction of X-ray- and neutron-induced breaks. *J. Biophys. Biochem. Cytol.* **4**: 365-372.