STUDIES ON THE FREQUENCIES OF CHROMATID ABERRATIONS INDUCED BY X RAYS AT DIFFERENT TIMES OF THE CELL CYCLE OF VICIA FABA

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IT has been known for some time that there are differences in the radiation-induced chromosomal aberrations observed at metaphase, according to the stage of the cell cycle the cells were in at the time of irradiation (SWANSON 1957). The observed differences are not only quantitative (changes in frequency per cell), but are also qualitative in that, depending on the stage and the chromosomal structural unit involved, three different classes of aberrations can be induced. In the pre-DNA-synthesis stage (G_1) of interphase, chromosome aberrations are induced. These involve the single, pre-split chromosome. During the DNA-synthesis (S) and post-DNA-synthesis (G_2) periods of interphase, chromatid aberrations are induced. These aberrations involve the double, post-split chromosomes. During prophase subchromatid aberrations that presumably involve half-chromatids are formed. In addition to these, two different groups of aberrations (one-break and two-break) are observed; these vary with respect to the kinetics of their induction. With sparsely ionizing radiations, one-break aberrations result from single ionizing clusters and show a linear increase with dose, whereas two-break aberrations involve the interaction of breaks that are produced by two independent ionizations (or clusters) and increase approximately as the square of the dose (see reviews by WOLFF 1961; EVANS 1962).

In the G_1 cells the chromosome has been found to exhibit constant sensitivity to radiation-induced breakage (see SAX 1938), whereas as the cell approaches metaphase the sensitivity changes and the frequency of chromatid aberrations increases (see SWANSON and SCHWARTZ 1953). Very close to metaphase the sensitivity drops (WOLFF and LUTHPOLD 1960). EVANS and SAVAGE (1963) and EVANS and SCOTT (1964) have attempted to find the relationship between the changes in sensitivity that occur when chromatid aberrations are induced and the stage of the cell cycle. They have shown that at the onset of S the sensitivity of the chromosome to breakage doubles and then increases slightly through S until G_2 , when another increase in sensitivity is observed. Since the aberrations induced during S and subsequently scored at metaphase are chromatid aberrations, they have suggested that the apparent gradual increase in sensitivity through S is probably caused by an increase in the number of doubled chromosomes produced as S progresses, and not to an actual increase in the sensitivity of the chromosome material during this period.

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In regard to the apparent increase in sensitivity that occurs between G_1 and S, WOLFF (1961) has pointed out that this may only be a reflection of the method used to score aberrations. When chromosomal aberrations induced in G_1 are scored, only asymmetrical exchanges (dicentrics) and rings are scored as two-hit aberrations; this is because the symmetrical exchanges are impossible to score accurately. When chromatid aberrations induced in S and G_2 are scored, however, both asymmetrical and symmetrical exchanges can be scored accurately because sister-strand attraction makes it possible to classify each type unequivocally. In addition, many breaks that are induced in pre-split chromosomes normally rejoin and so do not form visible aberrations. When induced in post-split chromosomes, however, these breaks are able to undergo sister-chromatid rejoining and form scorable isochromatid aberrations (WOLFF 1961).

Although the above changes can contribute to the difference between cells irradiated in G_1 and S, the increase in the frequency of aberrations from S to G_2 as observed by EVANS and SAVAGE (1963) and EVANS and SCOTT (1964) cannot be attributed simply to such a bias in scoring, in that chromatid aberrations are induced in both stages. It seemed probable that such an increase would be explainable on the site hypothesis of WOLFF and ATWOOD (see WOLFF, ATWOOD, RANDOLPH, and LUIPPOLD 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) have found that the number of sites appears to increase in G_2 .

The present experiments were designed to determine if the change in yield of aberrations induced as the cell proceeds from S to G_2 is actually caused by a change in the sensitivity of the chromosomes to ionizing radiation or a change in the probability of the interaction of broken ends to form aberrations observable at metaphase, as would be expected on the site hypothesis.

MATERIALS AND METHODS

Seeds of *Vicia faba* were soaked in tap water for 24 hours, the seed coats peeled, and the beans grown according to the method of GRAY and SCHOLES (1951). When the primary roots attained a length of 3 to 4 cm, the meristem was cut off and the beans were skewered on a stick and grown with their roots immersed in continuously aerated spring water. Lateral roots appeared within 3 to 4 days and were allowed to grow until they reached a length of about 1 cm. At this point the roots were placed in a solution of 5-amino uracil (5-AU) at a concentration of 750 ppm. SMITH, FUSSEL and KUGELMAN (1963) have shown that if the beans are left in 5-AU for 24 hours the mitotic index falls to zero. If the roots are then removed and washed thoroughly, the mitotic index will rise from zero to a peak in which approximately 40 to 50 percent of the cells are observed in metaphase. They believe the synchronization is caused by a 5-AU induced block of DNA synthesis (S). The cells in G_1 , G_2 , and mitosis are thought to continue through the cell cycle until they reach S. The cells are, therefore, all more or less in the same stage of the cell cycle and so upon removal of the 5-AU will proceed fairly synchronously to metaphase.

In the present experiments the beans were left in 5-AU for 24 hours, washed in running water for 30 minutes, and then returned to aerated spring water. At various intervals after removal from 5-AU (see Tables 1 and 2), groups of four beans were placed in tritiated thymidine with a specific activity of 1.9 curies/mole at a final concentration of 0.0175 mc/ml and pulse labeled for 10 minutes in order to label those cells that were in S. The beans were then washed in distilled water for 10 minutes. Immediately after washing, two of the four beans were irradi-

ated with 25r of X rays at 50r/min from a General Electric Maxitron X-ray machine operated at 250 kvp and 30 ma with 3 mm aluminum filtration added ($Hv1 = 0.45$ mm Cu).

After irradiation lateral roots were tested periodically to determine when the majority of the cells in the meristem were entering prophase. This was done by heating a root tip in nine drops of aceto-carmin and one drop of normal HCl and then making a temporary aceto-carmin squash preparation. Nine and one-half hours after removal from 5-AU the test showed that the cells were entering prophase, and at this point the remainder of the lateral roots were placed in a solution of 0.2 percent colchicine for 2 hr to accumulate metaphases. The root tips were then fixed and stained according to the method of C. E. Ford, i.e., fixed with a modification of Flemming's fixative, bleached in ammonium oxylate and hydrogen peroxide, hydrolyzed in 1 N HCl, and stained with Feulgen (see WOLFF 1963, for details).

Autoradiographs were prepared by dipping several slides of both the irradiated roots and the unirradiated control roots from each interval in Kodak Nuclear Track Emulsion, Type NTB. These slides were dried, exposed for 10 days, developed in Kodak D-19 Developer for 2 min, washed, fixed in Kodak Acid Fixer and then made permanent by passing through an alcohol series to 100 percent alcohol and mounting with euparal. These slides were scored only for the presence or absence of label in the metaphase chromosomes. On the other slides from the same intervals, terminal deletions, isochromatid deletions, and chromatid exchanges were scored as aberrations. Achromatic lesions, or "gaps," were not scored.

RESULTS

Tables 1 and 2 summarize the results obtained in the irradiated roots and the control roots. It may be seen that when cells are treated 3 hours after removal from 5-AU, the subsequent peak of mitosis contains more than 70 percent of heavily labeled cells. This indicates that at 3 hours the majority of the cells that would appear in the peak were in S. The frequency of labeled mitoses falls steadily with increasing time between removal from 5-AU and exposure to tritiated thymidine. This indicates that over the time span represented in the tables the cells progress from S to mitosis. By 7 hours only an occasional cell was labeled, showing that all the cells to be observed in the peak were in G_2 . The major part of the transition occurred between 3.5 and 5 hours.

It should be noted that although only 70 percent of the cells observed at 3 hours

TABLE 1

Control chromatid aberrations at various times after removal from 5-amino uracil

Time (hours)	Number cells scored	Deletions (percent)	Isochromatid (percent)	Exchanges (percent)	Total breaks (percent)	Heavily labeled metaphases (percent)
3	150	0.0 $\pm$ 0.0	2.0 $\pm$ 1.1	2.0 $\pm$ 1.1	6.0 $\pm$ 2.0	70.0
3½	150	0.0 $\pm$ 0.0	0.7 $\pm$ 0.7	1.3 $\pm$ 0.9	3.3 $\pm$ 1.5	56.6
5	150	0.0 $\pm$ 0.0	2.7 $\pm$ 1.3	1.3 $\pm$ 0.9	5.3 $\pm$ 1.9	9.7
6	150	0.0 $\pm$ 0.0	0.7 $\pm$ 0.7	2.0 $\pm$ 1.1	4.7 $\pm$ 1.7	5.3
7	150	0.0 $\pm$ 0.0	1.3 $\pm$ 0.9	1.3 $\pm$ 0.9	4.0 $\pm$ 1.6	0.0
7½	150	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	2.0 $\pm$ 1.1	4.0 $\pm$ 1.6	0.0
8	150	0.0 $\pm$ 0.0	0.7 $\pm$ 0.7	1.3 $\pm$ 0.9	3.3 $\pm$ 1.5	1.0
8½	150	0.7 $\pm$ 0.7	1.3 $\pm$ 0.9	1.3 $\pm$ 0.9	4.7 $\pm$ 1.7	0.0
9	150	0.0 $\pm$ 0.0	0.7 $\pm$ 0.7	1.3 $\pm$ 0.9	3.3 $\pm$ 1.5	1.0
Total	1,350	0.07 $\pm$ 0.07	1.1 $\pm$ 0.027	1.6 $\pm$ 0.034	4.3 $\pm$ 0.056	...

TABLE 2

Irradiation-induced chromatid aberrations at various times after removal from 5-amino uracil

Time (hours)	Number cells scored	Deletions (percent)	Isochromatid (percent)	Exchanges (percent)	One-hit (percent)	Breaks (percent)	Heavily labeled metaphases (percent)
3	300	1.0 $\pm$ 0.6	5.3 $\pm$ 1.3	2.0 $\pm$ 0.8	6.3 $\pm$ 1.5	10.3 $\pm$ 1.9	71.0
3½	200	1.0 $\pm$ 0.7	4.0 $\pm$ 1.4	4.0 $\pm$ 1.4	5.0 $\pm$ 1.6	13.0 $\pm$ 2.6	58.0
5	300	1.7 $\pm$ 0.7	5.7 $\pm$ 1.4	7.3 $\pm$ 1.6	7.3 $\pm$ 1.6	22.0 $\pm$ 2.7	11.7
6	300	0.3 $\pm$ 0.3	5.3 $\pm$ 1.3	9.7 $\pm$ 1.8	5.7 $\pm$ 1.4	25.0 $\pm$ 2.9	6.0
7	300	3.0 $\pm$ 1.0	5.0 $\pm$ 1.3	8.0 $\pm$ 1.6	8.0 $\pm$ 1.6	24.0 $\pm$ 2.8	...
7½	300	4.7 $\pm$ 1.2	3.3 $\pm$ 1.1	8.3 $\pm$ 1.7	8.0 $\pm$ 1.6	24.7 $\pm$ 2.9	0.3
8	300	3.0 $\pm$ 1.0	6.0 $\pm$ 1.4	8.7 $\pm$ 1.7	9.0 $\pm$ 1.7	26.3 $\pm$ 3.0	0.0
8½	300	3.7 $\pm$ 1.1	4.7 $\pm$ 1.2	11.0 $\pm$ 1.9	8.3 $\pm$ 1.7	30.3 $\pm$ 3.2	0.0
9	250	1.6 $\pm$ 0.8	2.4 $\pm$ 1.0	5.6 $\pm$ 1.5	4.0 $\pm$ 1.3	15.2 $\pm$ 2.5	1.0

are heavily labeled when they reach metaphase, probably considerably more than 70 percent of the cells were in S at this time. The reasons for this are: (1) In addition to the 70 percent of heavily labeled cells, some 15 to 20 percent were very lightly labeled, i.e., had less than 10 grains/nucleus and were scored as unlabeled; and (2) those cells in late S at the time of treatment would not be expected to pick up enough label to give a positive autoradiograph under the present experimental conditions.

On the other hand, the peak should not consist entirely of cells from the S period. WOLFF and LUIPPOLD (personal communication) have found that 5-AU blocks not only S, but also early G₂. At the time of removal from 5-AU, therefore, there would be some cells from G₂ in the roots. In addition, it would be expected that cells which were in late S at the time of the addition of 5-AU would move from late S into G₂ during the course of the 3 hours between the removal of the roots from 5-AU and the first treatment.

Since the labeling pattern in the control cells (Table 1) is the same as in the irradiated cells (Table 2), it may be concluded that if the cells are given a low dose of 25r and the metaphases are accumulated in the peak by treating the beans with colchicine, there is no mitotic delay that changes the relative number of cells from the various parts of the cell cycle in the peak.

In Figure 1 the percentage of breakage is plotted against the time after removal from 5-AU at which the cells were exposed to tritiated thymidine and irradiation performed. The same figure also shows the percentage of cells that had tritiated thymidine incorporated into their chromosomes at each interval and were subsequently scored as labeled metaphases. The labeling pattern indicates the stage of the cell cycle in which the cells were at the time of irradiation; i.e., at the 3 hr interval the cells were in S, at 3.5 hr the cells were progressing from S to G₂, from 5 to 8.5 hr a great majority, if not all the cells, were in G₂, and at 9 hours the cells were entering mitosis. In essence, then, Figure 1 shows the frequency of aberration production in the various stages of the cell cycle from S to prophase. It may be seen that there is an increase in aberration yield at the time that the frequency of labeled metaphases drops. This is the time the cells are passing from S to G₂.

DISCUSSION

The data show that as the cell progresses from S to G_2 the frequency of observed aberrations increases by a factor greater than two. Although in the present data this increase takes place over a period of from 1 to 2 hours, it is possible that the shift is a sudden one. This is because the cells scored in these experiments were collected by colchicine over a 2-hr period, and therefore at the 3.5 and 5-hr intervals cells that were in both S and G_2 at the time of irradiation were scored at the subsequent metaphase. Unfortunately, uncolchicined metaphases of *Vicia faba* are impossible to score accurately because the chromosomes are not well contracted and there is considerable overlapping. Similarly, visibly labeled cells from autoradiographs are also difficult to score accurately because of the overlying silver grains. Therefore aberrations and labeling patterns were obtained from separate slides.

The duration of G_2 , as estimated from the time between the disappearance of labeled metaphases and the onset of prophase (Figure 1), is found to be 4.5 to 5 hours. This agrees well with EVANS and SCOTT's determination (1964) in the primary roots of *Vicia faba*. The sudden decrease in observed aberrations as the cell leaves G_2 and enters mitosis also agrees with the results of EVANS and SAVAGE (1963), EVANS and SCOTT (1964), and WOLFF (1960).

If the observed increase in aberration production as the cells progress from S to G_2 were a result of increased sensitivity of the chromosome strand to the X rays, then the frequency of all types of aberrations would increase. It is observed, however, that this is not the case. The frequency of one-break aberrations remains relatively constant, with only minor fluctuations, from S through G_2 , whereas

FIGURE 1.—The relation between frequency of aberrations and time of cell cycle the treatment was given is shown here. O --- O aberrations observed after 24 hours of 5-AU, H^3 -thymidine, and 25r of X rays; ● --- ● aberrations observed after 24 hours of 5-AU and H^3 -thymidine; □ — □ labeled metaphases after 24 hours of 5-AU, H^3 -thymidine, and 25r of X rays; ■ — ■ labeled metaphases after 24 hours of 5-AU and H^3 -thymidine. The arrow at 9.5 hours indicates the onset of visible prophase.

the frequency of two-break aberrations increases by a factor greater than four. While the frequency of terminal deletions is higher in G_2 , the increase is not significant because of the small numbers observed. Therefore, it appears that the observed increase in aberration production in G_2 can be explained by an increase in the number of sites for exchange. Such an increase in site number in G_2 cells as compared to G_1 cells was found by GARCIA-BENITEZ and WOLFF (1962) to occur in *Vicia faba*. WOLFF (1961) has hypothesized that the increased yield in aberrations observed after the chromosomes split, i.e., the increase in yield from G_1 to S, comes from an increased number of places where chromosomal strands are within the rejoining distance of one another. The main increase would come from sister chromatids that, if broken, could rejoin to form isochromatid deletions.

After the chromosomes are already split, however, any increase that now occurs in site number is expected to affect exchanges and not isochromatid deletions. EVANS and SAVAGE (1963) and EVANS and SCOTT (1964) have noted that the yield of exchanges in G_2 cells is higher than that in S cells, indicating that the number of exchange sites can increase as the cell proceeds from S to G_2 . The present data show just such a relationship and support the view that the increased yield of aberrations observed in cells irradiated in G_2 are a result of an increase in the number of sites at which an exchange can be formed. [The similarity between the present data and that of EVANS and SAVAGE (1963) and EVANS and SCOTT (1964) indicates that the 5-AU is not modifying the results.]

Figure 1 shows that there is a slight increase in the number of aberrations observed at the 8.5 hr interval after removal from 5-AU. Although the slight increase observed at the end of G_2 (8.5 hr point) is not significantly higher than any other point in G_2 , the possibility exists that the increase is real and reflects another change in site number as the chromosomes contract and spiralize. The decrease in aberration production at the time the cell enters mitosis (9-hr point) occurs for all types of aberrations, although the number of one-break aberrations is not significantly different from the number at 8.5 hours. From these observations it can be postulated that there is probably a decrease in the site number as the cell enters mitosis. PARKER (1963) has noticed such a decrease in sites in *Drosophila* oocytes when the synapsed chromosomes of stage 7 oocytes separate and proceed to diakinesis (stage 14). The present evidence is not critical enough, however, to distinguish between the possibility of a decrease in site number and a possible decrease in the chromosome's sensitivity to X rays.

SUMMARY

Cells of *Vicia faba* lateral root tips were mitotically synchronized and irradiated with 25r of X rays at various times during the cell cycle. The stage of the cell cycle in which the cells were at the time of irradiation was determined by their ability to incorporate tritiated thymidine into their chromosomes. It was found that as the cells go from the period of DNA Synthesis (S) to the post-DNA synthesis period (G_2) the number of aberrations induced increases by a factor greater than two. This increase is a result of an increase in only two-break aberra-

tions, the frequency of one-break aberrations remaining constant from S through G_2 . It is concluded from these data that the increase in aberration yield as the cells go from S to G_2 is the result of an increase in the number of sites within which an exchange can occur, and not of an increase of the sensitivity of the chromosome strands to X rays. In very late G_2 or early prophase a sudden decrease in aberration yield occurs. This is interpreted as a decrease in the number of sites and also a possible decrease in the sensitivity of the chromosome strand to X rays, since both one- and two-break aberrations decrease in frequency.

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