INFLUENCE OF DNA SYNTHESIS ON THE PRODUCTION OF CHROMATID ABERRATIONS BY X RAYS AND MALEIC HYDRAZIDE IN VICIA FABA

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IT is well established that the irradiation of cells at any stage in development during interphase results in the formation of chromosome aberrations, but that two basically different types of chromosome changes may be produced. Aberrations induced in roots of *Vicia faba* exposed to 50 rads of X rays are first seen in metaphase cells at about 2 hours after irradiation. In those cells which reach metaphase between 2 and 14 hours after irradiation, the chromosome structural changes found are exclusively of the chromatid-type in which the unit of rearrangement is the single chromatid (Figure 2). In cells observed at metaphase at later fixation times, most of the aberrations observed are of the chromosome-type, in which the unit of rearrangement is the two chromatids of a single chromosome which are broken or exchanged at identical loci (Figure 1).

In studies on chemically induced chromosome aberrations, particularly in cases where alkylating agents have been employed, there have been few, if any, reports of unambiguous chromosome-type changes observed in cells seen in their first posttreatment mitosis, all the aberrations reported upon being of the chromatid-type. Moreover, with many of the chemicals it has also been found that the first appearance at metaphase of the induced chromatid aberrations occurs at about 8 to 10 hr after the initiation of chemical treatment, i.e. there is a considerable delay relative to the time of appearance of similar aberrations after X-ray treatment. This delay in the appearance of aberrations at mitosis has been reported for a large number of chemical compounds tested on Vicia roots; these include nitrogen mustard (FORD 1949; REVELL 1953), beta-propiolactone (SMITH and SRB 1951; SWANSON and MERZ 1959), a diepoxide (REVELL 1953), maleic hydrazide (MCLEISH 1953; KIHLMAN 1956; MERZ, SWANSON and HEMALATHA 1961), triethylenemelamine (OCKEY 1960), ethyl alcohol, ethyl methane sulphonate and myleran (RIEGER and MICHAELIS 1960a and b) and N-nitroso-N-methylurethan (KIHLMAN 1960).

It must be pointed out that in the experiments on aberration induction by the chemicals referred to above, no information on the mitotic delay induced by these chemicals was obtained, and in many instances no detailed information on mitotic frequency and aberration yields found in the early hours after treatment has been presented. Nevertheless, the difference in time scale between the X-ray and chemical-induced effects has been interpreted (FORD 1949; REVELL 1953) as indicating that following chemical treatment, aberrations are induced only in

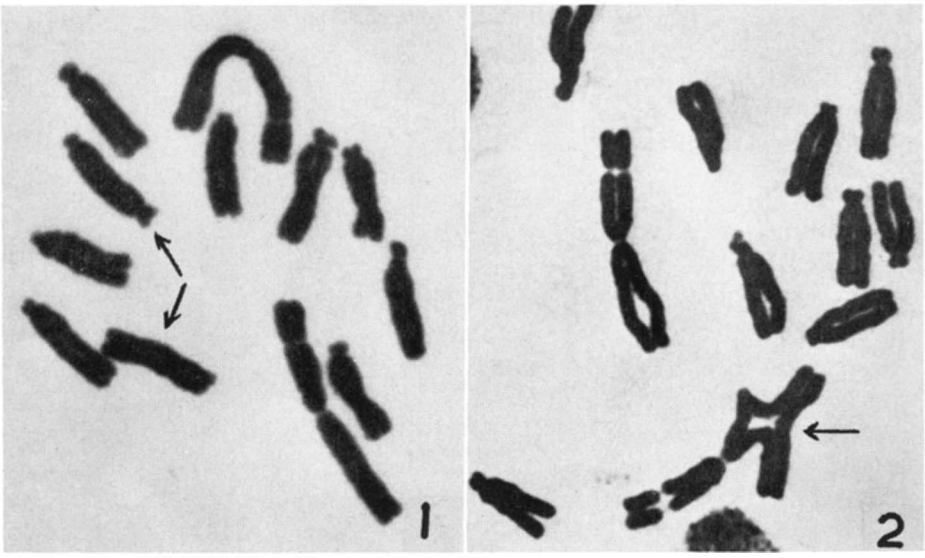


FIGURE 1.—Chromosome-type interchange in an X-irradiated G_1 cell showing a dicentric chromosome and its accompany acentric fragment. FIGURE 2.—Chromatid-type interchange in an X-irradiated G_2 cell showing a dicentric chromatid and acentric fragment (Both figures $\times 1800$).

those cells which were in the early stages of interphase at the time of treatment, in contrast with the findings with X rays where all stages of interphase are sensitive.

The autoradiographic studies of HOWARD and PELC (1953) showed that the interphase period in Vicia root-tip cells could be divided into three phases, the pre-DNA synthesis phase (G_1), DNA synthesis phase (S) and postsynthetic (G_2) phase. These authors stated that on the bases of their estimations of the durations of the phases of interphase, the results obtained by REVELL (1953) on the time of aberration induction by a diepoxide indicated that only cells in the G_1 phase were sensitive to the chemical. Furthermore, by analogy with the X-ray results where most of irradiated G_1 cells yield chromosome-type changes and chromatid-type changes are mainly induced in S and G_2 (EVANS and SAVAGE 1963; WOLFF and LUIPPOLD 1963), the finding that these chemically induced aberrations are of the chromatid-type has led to the suggestion that although the cell may be sensitive to the initiation of aberrations only in early interphase, the actual production of the aberrations occurs at some later stage in development (REVELL 1953).

The above findings are open to alternative interpretations in the absence of information on the rates of penetration of these chemicals used into the roots, and particularly in view of the knowledge that many of these chemicals are powerful mitotic inhibitors. It is possible that the observed results might be explicable on the idea that cells in S and G_2 are indeed sensitive to aberration induction, but that their appearance at mitosis is delayed because of a depression

in the rate of cell development. A similar line of argument might also account for the absence of chromosome-type aberrations if observations were not made at a sufficient time interval after treatment to allow G_1 cells to pass into mitosis.

In view of the above considerations we have carried out experiments designed to study the action of the chemical maleic hydrazide (hereafter designated by the symbol MH) in producing chromosome aberrations and in inducing mitotic delay; information on both these aspects being obtained from autoradiograph preparations from roots exposed to combined treatments with the chemical and with H^3-thymidine, a precursor of DNA. In addition to the maleic hydrazide experiments, for purposes of comparison, an experiment using a combination treatment with X rays and H^3-thymidine was carried out to obtain information on the sensitivity of the G_2 phase to the induction of chromatid-type aberrations by X rays.

MATERIALS AND METHODS

All treatments were carried out on the primary roots of eleven-day old seedlings of *Vicia faba* (var. Suttons' Prolific Longpod) which were cultured in tap water and randomised for experiment as previously described (EVANS, NEARY and TONKINSON 1957). For all experiments the roots were cultured at 19°C ± 0.1°C, and all treatment solutions were fully aerated and maintained at this temperature.

All roots, except those fixed at less than 2 hr after completion of H^3-thymidine, X-ray, or MH treatment, were pretreated with a 0.05 percent aqueous solution of colchicine for 2 hr prior to fixation. The roots were fixed in an osmic fixative, bleached, hydrolysed and stained using the Feulgen procedure as described elsewhere (EVANS 1961). Squash preparations were made from the terminal 2 mm of root, each root being used to make one slide. Stripping film autoradiographs were prepared with Kodak ARIO film, exposed in the dark for 10 to 20 days at 4°C, developed in Kodak D19b, and permantly mounted in euparal. For scoring purposes all slides were assigned code numbers and randomised. Two to four slides were scored at each treatment—fixation time and standard errors were calculated as errors of a binomial distribution, i.e. S.E. = $\pm \sqrt{pq/n}$.

Three series of experiments were carried out and details pertaining to each particular experiment are described together with the results.

RESULTS

Experimental series A: H^3-thymidine treatment only—The two experiments under this heading were carried out to obtain information on the timing of the various mitotic parameters, and on the frequency of chromosome aberrations induced by the endogenous radiation from tritium.

In Experiment 1, roots were exposed to H^3-thymidine (nominally 6-T, s.a. 1.9c/mmole, Amersham) for 3 hr in a vessel containing 1 ml of solution per root, at a concentration of 1μc/ml. In Experiment 2, roots were exposed to H^3-thymidine (5-methyl-T, s.a. 4.7c/mmole, Amersham) in the manner described above, but for 2 hr as a direct control to the experiment with MH (series C). Following H^3-thymidine treatment, roots were washed in water, returned to water, and fixed at intervals up to 32 hr posttreatment. The presence or absence of silver grains overlying the chromosomes in 150 mitotic cells was determined on each of two slides at each fixation time: the cells were classified as prophases or metaphases, later stages of mitosis being absent due to the action of the colchicine used as a pretreatment.

The results of these experiments are summarised in Figures 3 and 4. The duration of the mitotic cycle phases G_1, S, G_2 and mitosis (M), may be deduced by studying the relative changes in the proportions of mitotic cells which are labelled at various time intervals, spanning one complete mitotic cycle, after H^3-thymidine treatment (cf. QUASTLER and SHERMAN 1959). If thymidine enters the roots rapidly, and we have shown elsewhere (EVANS 1964) that significant nuclear labelling occurs 5 min after exposure of roots to H^3-thymidine, then the *minimum* duration of G_2 is given by that time interval between the commencement of H^3-thymidine treatment and the appearance of the first labelled dividing cells. This time interval, from Experiments 1 and 2, is 3 and 4 hours, respectively (Figure 3). An estimate of the *average* duration of G_2 is given by that time interval between the beginning of H^3-thymidine treatment and the time when the mitotic labelling index reaches one half of its maximum value; in both experiments this value is about 5 hr 40 min. It is to be remembered, however, that these roots showed considerable metaphase accumulation as they received colchicine treatment prior to fixation. A better estimate of the average duration of the G_2 period would therefore be given by the time taken to reach one half of the maximum *prophase* labelling index (Figure 4), which gives a mean value of 4.9 hr. Separation of the prophase and metaphase data also allows us to estimate the duration of prophase. The displacement of the prophase and metaphase labelling index curves in these experiments (Figure 4) is between 1 hr and 1½ hr. Similarly, since the decline in the frequency of unlabelled prophases in the first few hours after H^3-thymidine treatment is exponential, this rate of decline also gives an estimate of the mean duration of prophase and averages about 1 hr 20 min between experiments.

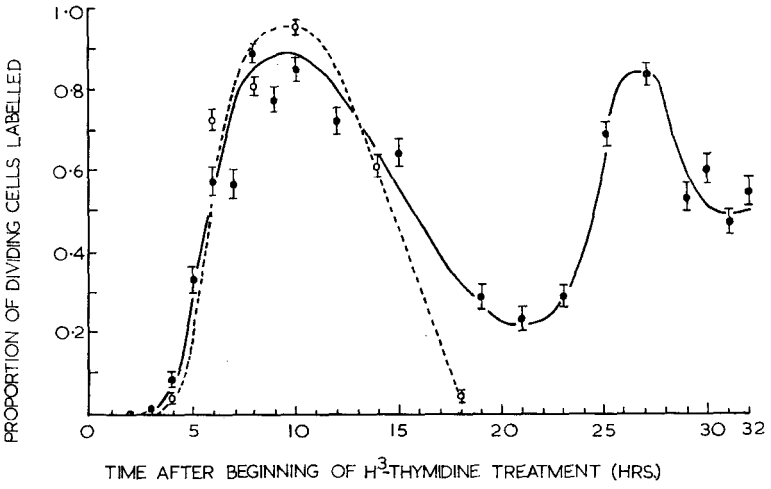


FIGURE 3.—Labelling index curves showing relation between proportion of mitotic cells labelled and time after commencement of H^3-thymidine treatment. Experiment 1. 3 hr. H^3-thymidine (—●—); Experiment 2. 2 hr H^3-thymidine (– –O– –).

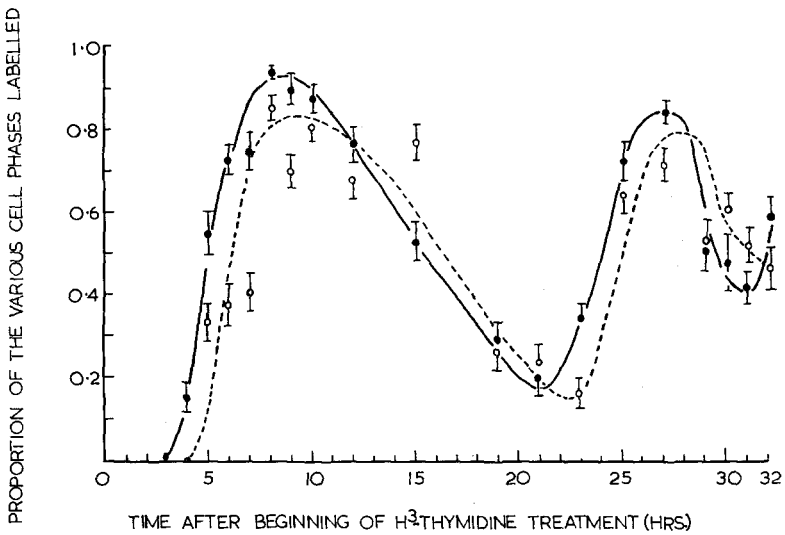


FIGURE 4.—Proportion of prophases (—●—) and metaphases (– –O– –) labelled at various times after a 3 hr H^3-thymidine treatment.

The first ascending limbs of the labelling index curves correspond to those cells that were near the end of the S phase at the time of commencement of H^3-thymidine treatment, and the descending limbs to those cells which were at the beginning of S at the end of H^3-thymidine exposure. Thus, the average duration of S corresponds to that time interval between the one-half maximum labelling index on the first ascending and descending limbs of the curves, minus the H^3-thymidine treatment time. It has been shown elsewhere that all the labelled precursor in the roots is used up within 10 min of the removal of the roots to water (EVANS 1964), so that cells in G_1 at the time of removal of roots from H^3-thymidine remain unlabelled and do not contribute to delaying the decline of the labelling index. The average durations of S taken from the prophase labelling index curves in the two experiments are respectively 8 hr and 7 hr. We shall therefore take the mean value of S as 7.5 hr.

The second peak in the mitotic labelling index curve (Experiment 1, Figure 3) represents those cells that were in S at the time of H^3-thymidine treatment, but which were in their second post-treatment mitosis at the time of fixation. The time intervals between the first and second labelling index peaks gives an estimate of the duration of the total mitotic cycle (T). However, as the second peak is unusually well defined, a better estimate is that time interval between the half-maximum labelling index point on the ascending limb of the first peak and the corresponding index value on the ascending limb of the second peak. This time interval gives a value of T = 19.3 hr which is similar to the values of T = 18 hr to 24 hr obtained in this system by the use of a colchicine technique for measuring mitotic rate (EVANS *et al.* 1957; EVANS and SAVAGE 1959).

In roots fixed immediately on completion of the 3 hr H^3-thymidine treatment

in Experiment 1, 48.5 percent of the 2,000 interphase cells scored were labelled. The proportion of cells which are labelled at this time allows us to make another estimate of S. Since the fraction of cells labelled was 0.485, and assuming a total interphase duration of 17.3 hr (see below), then $S = 17.3 \times 0.485$ or 8.4 hr. This value is in reasonable agreement with the value of 7.5 hr obtained from the labelling index curves, but we consider it to be less accurate, especially as it assumes that the number of cells passing into S from G_1 during the 3 hr H^3-thymidine treatment is equal to the number of unlabelled cells passing into G_1 from mitosis and G_2 during this period.

Estimates of the duration of mitosis (M) may be obtained in two ways. In the first fixations following H^3-thymidine treatment, about 10 percent of the cells were in mitosis so that M is roughly one tenth of T, or about 2 hr. A second estimate may be derived from the fact that 60 percent of the mitotic figures observed are prophases, thus, as the average duration of prophase is 1.3 hr then M is about 2 hr.

Summarising the above information, the mean durations of the various parameters of the mitotic cycle in these 'control' roots, which were not exposed to X rays or MH, is:

$$\begin{aligned} M &= 2.0\,\text{hr} \\ G_2 &= 4.9\,\text{hr} \\ S &= 7.5\,\text{hr} \\ T &= 19.3\,\text{hr} \\ G_1 = T - (M + G_2 + S) &= 4.9\,\text{hr}. \end{aligned}$$

The spontaneous aberration frequency, under our conditions of culture, in the cells used in the experiments is extremely low and can only be estimated as being at most one in 10^3 cells. On the other hand, there have been numerous reports (e.g. WIMBER 1959) of chromosome aberrations induced by the endogenous beta radiation from tritium. In our experiment where roots were subjected to a 2 hr treatment with H^3-thymidine an attempt was made to determine the induced aberration frequency, but no aberrations were observed in the 358 labelled metaphase cells which were scored. It is clear therefore that the frequency of any tritium-induced aberrations in the experiments in which roots were subjected to X rays or maleic hydrazide would be insignificant and would not influence the aberration yield results.

Experimental series B: H^3-thymidine followed by X rays—In this second experimental series, roots were exposed for 30 min to H^3-thymidine (nominally -6-T,s.a. 3.1c/mMole, Amersham) at a concentration of 3μc/ml of solution, with 1ml of solution per root, and then to X rays, fixations being made at 0, 1½, 2, 3, 5, 7 and 8 hr after X-irradiation. Twenty-five metaphase cells on each of four slides were scored at each fixation time for aberrations and for the presence or absence of labelling. The results are summarised in Figure 5.

The first labelled cells to appear at metaphase were seen at 5½ hr after the beginning of H^3-thymidine treatment. Since the fixation times did not extend beyond 8½ hr and as at this time the labelling index had attained a value of only 0.27, we cannot give a good estimate of the duration of G_2. Nevertheless it

is evident from the data that some mitotic delay occurs in irradiated G_2 cells.

The first cells carrying unambiguous chromatid-type aberrations were observed at metaphase at 1½ hr after X-irradiation, the aberration yield increasing rapidly to a peak at 5 hr post-irradiation (Figure 5). Details of the aberration yields are given in Table 1, achromatic lesions or gaps being excluded from the data. The fact that the maximum aberration yield occurs at 5 hr, and in unlabelled G_2 cells, indicates that cells in mid G_2 are most sensitive to the induction of chromatid aberrations, cells in late S and early G_2 being less sensitive. It is of interest to note that a considerable proportion of this increased sensitivity in G_2 is due to an increase in the frequency of interchange aberrations (cf. EVANS and SAVAGE 1963).

The relative proportions of aberrations which were incomplete were 0.222 and 0.175 for interchange and isochromatid aberrations respectively. No isochromatid aberrations showing complete nonunion were found. The class of aberrations under the heading 'other intrachanges' (Table 1) contained some true chromatid breaks, some of which were associated with an intercalary deficiency yielding a minute fragment. As has been pointed out previously (REVELL 1959; NEARY and EVANS 1958; EVANS 1962), such true chromatid breaks are relatively rare, and in these data the ratio of chromatid breaks to incomplete isochromatid breaks is 3.66:2, a ratio somewhat lower than the 5:2 ratio predicted on the exchange hypothesis for aberration formation (REVELL 1959).

Experimental series C: H^3-thymidine and MH—Experiment 4. Roots were exposed to a mixture of H^3-thymidine (details as in Experiment 2) and MH (obtained from Light Limited, Colnbrook, England) for 2 hr, and samples were fixed at intervals up to 36 hr after treatment: the final concentration of MH was 0.25mM and the pH of the solution was 5.5. At each fixation 50 metaphase cells on each of two or three slides were scored, both for aberration yield and presence of labelling, although in a few cases the required number of metaphase cells could not be obtained due to the mitotic depression resulting from MH treatment. A further complication encountered during scoring was the fact that in some slides

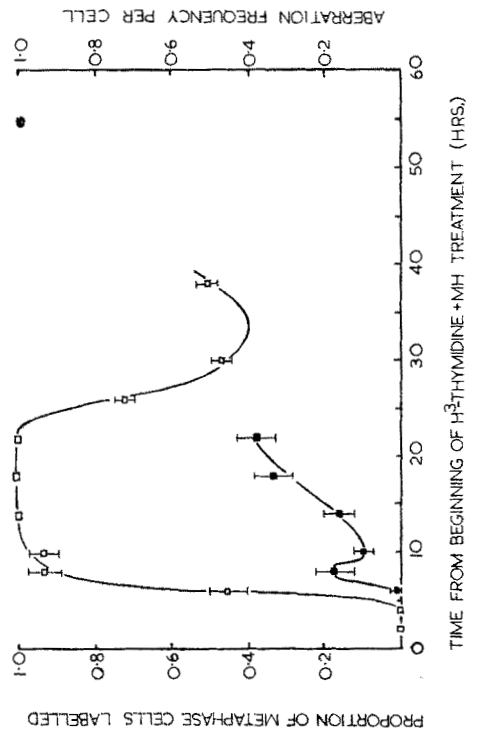
TABLE 1

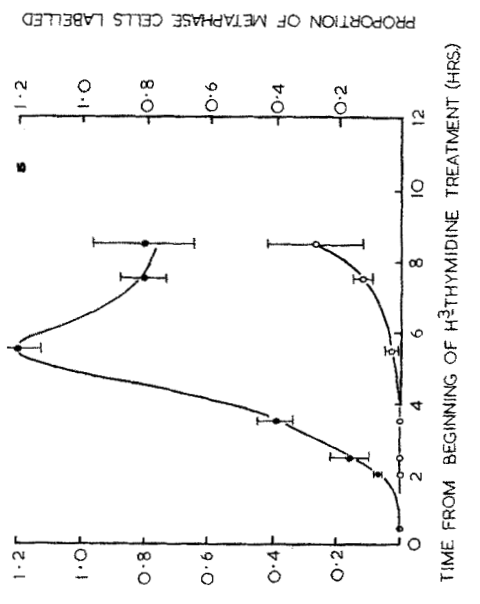
Yields of the various aberration types after 100 rads of X rays

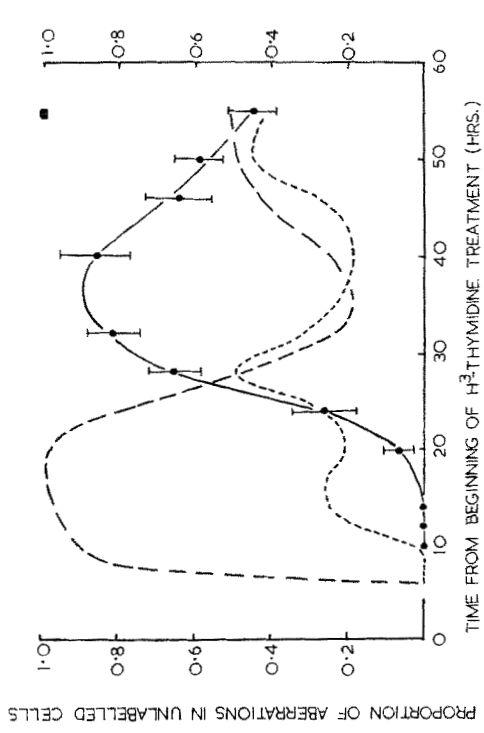
Time post-irradiation (hr)	Number of metaphase nuclei		Number of interchanges		Number of isochromatid		Number of other intrachanges*	
	U	*L*	*U*	*L*	*U*	*L*	*U*	*L*
0	100	0	0	.	0	.	0	.
1½	100	0	6	.	0	.	1	.
2	100	0	6	.	4	.	6	.
3	100	0	22	.	7	.	10	.
5	97	3	56	0	34	0	29	1
7	88	12	35	2	22	6	14	2
8	73	27	24	6	23	8	12	8
Totals	658	42	149	8	90	14	72	11

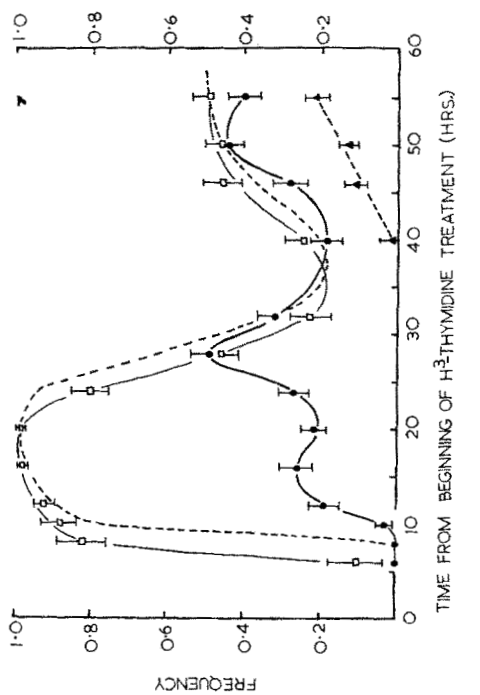
* 40 percent of the total "other intrachanges" were incomplete, appearing as chromatid breaks either with or without an associated minute fragment.

L and *U* refer to labelled and unlabelled nuclei respectively.


ABERRATION FREQUENCY PER CELL
TIME FROM BEGINNING OF H³-THYMIDINE+MH TREATMENT (HRS.)
PROPORTION OF METAPHASE CELLS LABELLED


ABERRATION FREQUENCY PER CELL
TIME FROM BEGINNING OF H³-THYMIDINE TREATMENT (HRS.)
PROPORTION OF METAPHASE CELLS LABELLED


PROPORTION OF ABERRATIONS IN UNLABELLED CELLS
TIME FROM BEGINNING OF H³-THYMIDINE TREATMENT (HRS.)


FREQUENCY
TIME FROM BEGINNING OF H³-THYMIDINE TREATMENT (HRS.)

the intensity of labelling was too great for accurate unbiassed scoring of the aberrations in labelled cells. For this reason no aberration scores were obtained from certain slides, all of which if transpired were from fixations made later than 20 hr post-treatment. The results on labelling index and aberration frequency are summarised graphically in Figure 6.

(a) *Mitotic cycle timing:* The frequency of labelled metaphases (Figure 6) is seen to reach a 50 percent value at 6.25 hr and since the normal average duration of prophase is 1.3 hr, then G_2 must be about 5 hr and is therefore the same as found in control roots. The fact that the first labelled cells are not delayed in their development towards mitosis indicates that cells in very late S are but little affected although the S phase as a whole is considerably protracted relative to controls and has an average duration of 20.5 hr (Figure 6). No reliable information on the duration of the total mitotic cycle and G_1 phase can be obtained from the data in Figure 6 as the latest fixation times did not appear to completely span one average mitotic cycle.

(b) *Aberration yields:* The data on aberration yields show clearly that no aberrations are observed at the first posttreatment mitosis of those cells that were in G_2 at the time of MH treatment. The first aberrations to be observed occurred in labelled cells and the results in general indicate that there is an increase in sensitivity with increasing fixation time, i.e. cells in early S are more sensitive than cells in late S. There is, however, a notable exception to this general trend in that a small peak in aberration yield occurs between 8 and 10 hr after the beginning MH treatment, clearly involving those cells in late S (Figure 6). No data on aberration yields are available in this experiment from cells exposed in the G_1–early S phases.

Details relating to the aberration yields (Table 2) are similar to those found in the experiment where MH and H^3-thymidine were given as separate treatments and will be discussed below.

Experiment 5: In this experiment, in which roots were exposed for 2 hr to H^3-thymidine (details as for Experiment 2) and then for 2 hr to MH (0.25 mM at

FIGURE 5.—Proportion of metaphases labelled (—○—) and frequency of aberrations (—●—) after exposure to H^3-thymidine followed by 100 rads of X rays. Note that aberrations are of the chromatid-type and that the peak in yield occurs in unlabelled G_2 cells. FIGURE 6.—Proportion of metaphases labelled (—□—) and aberration frequency (—■—) in roots exposed to a mixture of H^3-thymidine and MH for 2 hr. Note the absence of aberrations in unlabelled G_2 cells, 0 to 6 hr post-treatment, the increased duration of S, and the small peak in aberration yield in late S cells. FIGURE 7.—Labelling index and aberration yield results for roots exposed for 2 hr to H^3-thymidine followed by 2hr in MH (Experiment 5). (—□—), proportion of metaphases labelled; (—●—), aberration yield; (- -▲- -), frequency of unambiguous second cycle, or T_2, cells which sustained damage at the first posttreatment mitosis (see text and Table 3). The dashed line represents the labelling index curve displaced by 2 hr as explained in the text. Note the minor peak in aberration yield in late *S* cells (cf. FIGURE 6) and the relation between labelling index and aberration yield. FIGURE 8.—Proportion of aberrations which are found in unlabelled cells in roots exposed first to H^3-thymidine and then to MH (Experiment 5); (— — —), proportion of metaphases labelled; (- - - - -) aberration yield; (—●—), proportion of aberrations which are in unlabelled cells.

TABLE 2

Yields of the various aberration types after treatment with a mixture of MH and H^3-thymidine

Time from beginning of *MH* treatment (hr)	Number of metaphase nuclei		Number of interchanges		Number of isochromatid		Number of other intrachanges*	
	U	*L*	*U*	*L*	*U*	*L*	*U*	*L*
2	50	0	0	.	0	.	0	.
4	83	0	0	.	0	.	0	.
6	42	35	0	0	0	0	0	1
8	3	39	0	0	0	7	0	0
10	6	76	0	2	0	6	0	0
14	0	100	.	1	.	13	.	2
18	0	72	.	4	.	18	.	2
22	0	100	.	7	.	27	.	6
Totals	184	422	0	14	0	71	0	11

* Four of the aberrations in these classes were true chromatid breaks either with or without an accompanying minute fragment.
L and *U* refer to labelled and unlabelled nuclei respectively.

pH 6.5), fixations were made at intervals up to 55 hr from the beginning of H^3-thymidine treatment and information on both aberration yield and labelling index was obtained at all fixation times. Fifty metaphase cells were scored on each of two or three slides at each fixation time, the slides being coded and scored interspersed with the slides from Experiment 4. The results obtained in Experiment 5 are summarised in Figure 7 and Table 3. Since H^3-thymidine was applied for 2 hr prior to MH treatment, the labelling index curve in Figure 7 has been displaced 2 hr to the right, for ease in relating aberration yield to stage in cell development. It may be noted, however, that a displacement of 2 hr in the labelling index curve between 20 and 30 hr assumes a delay in entry of G_1 cells into S equivalent to the delay in the rate of progress of cells through S.

(a) *Mitotic cycle timing:* During H^3-thymidine treatment two hours' "worth" of labelled cells would have passed from S into G_2 so that if the development of G_2 cells was delayed by MH, an accurate estimate of the duration of this phase would be difficult to obtain. It may be seen from Figure 7 that 50 percent of the metaphase cells are labelled at 7 hr after the beginning of H^3-thymidine treatment and, again allowing 1.3 hr for the duration of prophase, this result is in agreement with the finding in Experiment 4 indicating that MH causes little interference with the development of G_2 cells. The average duration of S is 19 hr, in good agreement with the 20.5 hr obtained from Experiment 4.

The second labelling index peak lies between 50 and 55 hr post-H^3-thymidine treatment, and at this time there is appreciable evidence of dividing cells which are marked by the presence of chromosome damage and micronuclei sustained at the first mitosis, but which are now in their second posttreatment or T_2 mitosis. Examination of the ascending regions of the two peaks yields an estimate of an average T of about 38 hr, although the fastest cells clearly pass through a complete cycle in about 30 hr, i.e. the time interval between the first appearance of aberrations at metaphase (10 hr) and the first appearance of metaphase T_2 cells (40 hr).

If we assume that MH does not produce any change in the order of progression of cells through the mitotic cycle, then the first of the unlabelled metaphase cells observed between the two labelling index peaks would comprise those cells which were at the very beginning of S, or were in G_1, at the time of MH treatment and which are appearing at their first posttreatment mitosis. Those unlabelled metaphase cells observed shortly before the time of onset of the second labelling index peak, i.e. roughly between 40 to 50 hr post-H^3-thymidine treatment, would consist mainly of those cells which were in G_2 and M at the time of treatment and are in their second posttreatment mitosis at this time of observation. It is clearly very difficult to estimate the average duration of the G_1 phase after MH treatment for we have no knowledge of the time taken by the cells which were in G_2 or M, at the time of treatment, to proceed through their second mitotic cycle.

(b) *Aberration yields:* The aberration yield data confirm the results obtained in Experiment 4 in showing that no aberrations are found in G_2 cells in their first posttreatment mitosis. The general pattern of the results shows that the first aberrations to appear at mitosis are in those cells that were in S at the time of MH treatment, cells in early S showing a higher aberration yield than cells in late S. As in Experiment 4, there is evidence of a peak in aberration yield involving those cells that were in late S at the time of treatment. In general, throughout S the aberration yield increases to a maximum in the early S cells and then drops markedly between 26 and 30 hr reaching a minimum at about 38 hr from the beginning of MH treatment. This fall at 38 hr is followed by a further rise, giving a third aberration peak which coincides almost exactly in time with the time of entry of labelled cells into their second posttreatment mitosis (Figure 7).

The average aberration frequency in those cells that were in S at the time of treatment in Experiment 4 is 0.227/cell and a similar value, 0.243/cell, is found for equivalent labelled cells in Experiment 5 (data in Table 3). From the data in Experiment 5 we cannot, with any measure of confidence, distinguish between cells which were in early S and cells which were in late G_1 at the time of treatment with MH. This is because no H^3-thymidine would be expected to be incorporated into DNA in the 2 hr after the roots had been removed from the radioactive solution, so that the cells which pass into early S from G_1 in the presence of MH will be unlabelled. It seems probable that these early S cells are indeed the most sensitive to aberration induction for a considerable proportion of the aberrations observed at the time of peak aberration yield, 26 hr after the beginning of MH treatment, are in unlabelled cells (Figure 8). The actual aberration yields observed in labelled and unlabelled cells respectively at 26 to 30 hr after the beginning of MH treatment are 0.333 and 0.443 per cell. All the aberrations observed at the first posttreatment mitosis in cells which were in S at the time of treatment were of the chromatid-type.

Cells which would have been in the G_1 phase at the time of MH treatment would be unlabelled and might be expected to arrive at mitosis at around 38 hr posttreatment. Unlabelled cells observed at this time contain aberrations and, significantly, these aberrations are also of the chromatid-type. It is clear therefore that no chromosome-type changes are induced in the first cell cycle following

TABLE 3

Yields of the various aberration types after a 2 hour treatment with H^3-thymidine followed by a 2 hour treatment with MH

Time from beginning of *MH* treatment (hr)		Number of metaphase nuclei		Number of interchanges		Number of isochromatid		Number of other intrachanges*	
		U	*L*	*U*	*L*	*U*	*L*	*U*	*L*
4		17	2	0	0	0	0	0	0
6		8	36	0	0	0	0	0	0
8		7	52	0	1	0	1	0	0
10		8	88	0	2	0	14	0	2
14		2	98	0	2	0	22	0	2
18		2	142	1	5	1	14	0	10
22		20	80	2	4	2	11	3	5
26		54	46	9	2	13	13	10	2
30		77	23	9	1	14	3	3	2
38	T_1	57	19	4	1	7	1	1	0
	T_2	1	0	0	.	0	.	0	.
44	T_1	54	35	3	3	10	4	5	0
	T_2	0	11	.	1	.	0	.	2
48	T_1	70	60	6	4	20	14	7	4
	T_2	10	9	2	3	2	1	2	1
53	T_1	62	55	7	5	11	18	1	6
	T_2	14	19	3	1	3	2	2	1
Totals	T_1	438	736	41	30	78	115	30	33
	T_2	25	39	5	5	5	3	4	4

* Seven of the aberrations in these classes were true chromatid breaks either with or without an accompanying minute fragment.
U and *L* refer to unlabelled and labelled nuclei respectively, and T_1 and T_2 to first and second posttreatment mitoses.

MH treatment, although duplication of chromatid-type structural changes may occur during the second posttreatment mitotic cycle, yielding "derived" chromosome changes at the second or T_2 mitosis. The presence of such "derived" changes almost invariably accompanied by a deficiency (and sometimes a duplication) of part of the chromosome complement, is a reliable marker indicating that these cells are in their second mitosis following the induction of structural change.

Cells which were undamaged at the first mitosis and were in the mitotic or G_2 phase at the time of MH treatment must make up the bulk of the unlabelled cells observed in mitosis shortly prior to the appearance of the second labelling index peak, i.e. at about 40 to 44 hr post-MH-treatment (Figure 7). These cells are in their second posttreatment mitosis and also contain chromatid-type aberrations, at a frequency of 0.33/cell, which must have developed during the posttreatment mitotic cycle. It would thus appear that all stages of the mitotic cycle may be sensitive to the induction of chromatid-type aberrations by MH, but that the aberrations are only produced when cells pass through a DNA synthesis phase.

At 44 hr post-treatment (Table 3) 10 percent of the metaphase cells observed were T_2 cells with "derived" chromosome changes, and all these cells were labelled. A most significant finding is the fact that these labelled T_2 cells contained, in addition to the derived chromosome changes, chromatid-type aber-

rations which must have developed, or been induced, during the second post-treatment mitotic cycle. It was pointed out above that the mean aberration frequency, at the T_1 mitosis, in labelled cells that were in S at the time of treatment was 0.227/cell. At 44 to 53 hr post-MH-treatment, 39 out of the 189 labelled cells observed (Table 3) were T_2 cells marked by their "derived" chromosome changes, i.e. a frequency of 0.201. Since many of the chromatid aberrations are isochromatid types which result in the formation of "derived" chromosome changes in both daughter cells, then the close correspondence between these two frequencies indicates that there is no preferential loss, due to premature differentiation, of those cells which suffered deficiency at the first mitosis as a consequence of chromatid structural change. In these 39 labelled and marked T_2 cells 12 chromatid-type aberrations were observed, i.e. 0.301/aberration/cell, which must have arisen during the second posttreatment interphase.

The labelled cells observed at metaphase between 44 and 53 hr which do not contain "derived" chromosome changes are nevertheless T_2 cells which were exposed to H^3-thymidine and to MH whilst in the S phase, but remained undamaged at least in their development to their first posttreatment mitosis. At their second division these cells are seen to contain aberrations which are again of the chromatid-type. The mean aberration frequency in these cells is slightly higher, 0.386/cell, but not significantly different from the yield of 0.308 chromatid-type aberrations/cell found in the labelled T_2 cells carrying "derived" chromosome changes. Since in this latter cell type there is almost invariably a deficiency of chromosome material, this slightly lower yield in this cell type is not unexpected. These results thus indicate that both cell types are equally susceptible to aberration induction in their second or posttreatment cycle, all the induced aberrations being of the chromatid-type.

The unlabelled T_2 cells which contain chromatid aberrations at 48 and 53 hr (Table 3) are cells which were in the late G_1–early S phase at the time of MH treatment. These cells which were most sensitive to aberration induction at the time of MH treatment in the first mitotic cycle, are also most sensitive to breakage during the second division cycle, the mean aberration yield induced posttreatment being 0.58/cell.

Structurally the aberrations induced by MH are similar to, and in most instances are indistinguishable from, the chromatid-type aberrations induced by X rays. However, as is well known (McLEISH 1953) the aberrations induced by the chemical treatment tend to be localized to particular chromosome zones and a considerable proportion of the aberrations observed in the present experiments occurred at the site of the heterochromatin adjacent to the centromere of the nucleolus-bearing arm of the metacentric chromosome.

Details of aberration structure and relative frequencies were similar between Experiments 4 and 5 (cf. Tables 2 and 3) and analysis of the pooled data yielded values for the frequencies of incompleteness of 0.103 for interchanges, 0.092 for isochromatid aberrations and 0.139 for other intrachanges. The relative frequency of chromatid breaks was therefore much lower following chemical treatment than was found in the X-ray experiment. The ratio of true chromatid breaks

to incomplete isochromatid breaks was 0.88:2, which is significantly less than the 5:2 ratio expected on the exchange hypothesis (REVELL 1959) and the 3.66:2 ratio obtained from the X-ray data (Table 1).

A further significant difference between the X-ray aberration data and the MH data is that the yield of interchanges, relative to other aberration types, is much lower following MH treatment. For example, with a mean aberration yield of 0.40/cell observed at 22 hr after MH treatment in Experiment 4 (Table 2; Figure 6), the mean yield of interchange aberrations is 0.07/cell, whereas at 3 hr after X-irradiation where the mean aberration yield is similar, 0.39/cell (Table 1), the mean yield of interchanges is three times as high at 0.22/cell.

DISCUSSION

Sensitivity of the G_2 phase to X-ray-induced aberrations: The results obtained from control roots show that the average duration of the G_2 phase is 4.9 hr with a minimum time of about 3 hr. This average value is very different from the 8 hr estimated by HOWARD and PELC (1953) from their original studies on primary roots cultured under similar conditions. Moreover, our results show that up to 10 percent of the cells observed at metaphase 4 hr after H^3-thymidine treatment would have been in the late S phase at the time of thymidine treatment. This result thus suggested that the peak in the yield of X-ray-induced chromatid aberrations, which after 100 rads is observed at about 5 hr post-irradiation, could have been due to the high sensitivity of cells in late S and not to the high sensitivity of G_2 cells as is commonly supposed. The results of the experiment using a combined H^3-thymidine and X-ray treatment, however, show quite conclusively that the highest aberration yield occurs in cells which were in mid G_2 at the time of irradiation, a significant fall in aberration yield being observed in those cells that were in the late S or early G_2 phases (c.f. EVANS and SAVAGE 1963). These results conflict with the conclusion arrived at in a recent paper by DAS and ALFERT (1962) who state that the sensitivity of interphase cells of Vicia to chromosome breakage by X rays is at its maximum in cells which undergo DNA synthesis at the time of irradiation. Their conclusion is based on comparisons between the relative proportions of abnormal anaphases in labelled and unlabelled cells observed after exposure of roots to H^3-thymidine and 200 rads of X rays. No distinction was made in their experiments between unlabelled G_2 and G_1 cell types—the latter type being very much less sensitive (REVELL 1961; WOLFF 1961; EVANS and SAVAGE 1963)—and, of more importance, no observations were made between 4 and 12 hr post-irradiation, so that their cell samples may not have included the well-known peak in chromatid aberration yield.

The production of aberrations by MH: Maleic hydrazide is a potent plant growth inhibitor (SCHOENE and HOFFMAN 1949) and, in parallel with our earlier observations (SCOTT and EVANS 1964), the present results show that the inhibiting effects of MH on cell development results in at least a doubling of the time taken to proceed through a complete mitotic cycle. The simplest explanation of the results illustrated by the labelling index curves in Figures 6 and 7 would be that much of the delay in cell development is due to a severe depression in the rate

of DNA synthesis, the treated cells requiring about 20 hr to complete a synthesis which normally takes about 7.5 hr. Similarly, cells may suffer a delay in their second mitotic cycle after treatment and it seems likely that much of this delay may also occur when these cells are proceeding through an S phase. In both MH experiments little or no delay in development occurred in those cells that were in G_2 at the time of treatment, at least in so far as their development to the first post-treatment mitosis was concerned. Furthermore, no aberrations were found in these cells at this mitosis although chromatid-type aberrations were present at their second posttreatment mitosis.

The absence of any immediate effects on G_2 cells prompts the question of whether the apparent insensitivity of these cells is merely a reflection of a slow rate of penetration of the MH in solution into the root cells or into the cell nuclei. No direct evidence on this point is available, but since MH is an isomer of uracil (Figure 9) and as purine and pyrimidine precursors are incorporated into nuclear RNA within a few minutes of exposure of roots to the compounds (Woods 1959), it seems likely that the compound is rapidly incorporated. Experiments with Vicia roots treated with C^{14} labelled MH (Callaghan and Grun 1961) have shown that MH becomes selectively concentrated in nuclei, and in some cases nucleoli, but unfortunately the earliest observations in these experiments were made at 24 hr after treatment so that no direct information on rate of penetration is available.

All the aberrations observed in cells at their first mitosis following MH treatment are of the chromatid-type. This finding is of particular significance in that if aberrations were induced in G_1 cells at the time of treatment then, by analogy with the X-ray results, these aberrations would be expected to be of the chromosome-type, characteristic of damage induced in unduplicated chromosomes. The fact that chromatid-type aberrations are observed in cells treated with MH in G_1, together with the finding that the aberration yield is highest in those cells that were in early S at the time of treatment, suggests that the aberrations are produced at the time of chromosome duplication and that MH acts by interfering in some way with the normal process of duplication.

Howard and Dewey (1961) have obtained results which indicate that during the S phase in Vicia there are two peaks in the rate of DNA synthesis, one in early and one in late S. Our own studies have shown (Evans 1964) that DNA synthesis in the heterochromatic chromosome zones occurs largely in late S and

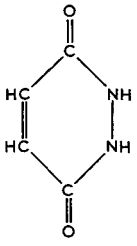


Figure 9.—Structural formula of maleic hydrazide (*MH*).

it has been suggested that the peak in DNA synthesis rate observed at this time is due to the late replication of heterochromatin. In both of the MH experiments a small peak in the yield of aberrations (Figures 6 and 7) was found in those cells that were in late S at the time of treatment. These peaks in aberration yield coincide in time with that phase in S during which H^3-thymidine is incorporated at high rate into heterochromatin, and the majority of the aberrations observed at these times are localised to heterochromatic chromosome zones, the amount of localisation being less in cells in early S. These results thus support the conclusion that MH produces aberrations in chromosome regions at the times of replication of those regions.

From the point of view of chromosome duplication in has been shown that each chromatid observed at mitosis consists of two units, only one of which was synthesized during the preceding interphase (TAYLOR, WOODS and HUGHES 1957). The G_1 chromosome, which is represented by a single chromatid, therefore consists of two old units whereas each of the two chromatids of the G_2 chromosome is made up of one old and one new unit. The absence of aberrations in G_2 nuclei observed in their first posttreatment mitosis would thus imply that MH cannot break both old and new strands of the chromatid at homologous or near homologous loci at this stage. Similarly, the absence of chromosome-type aberrations in G_1 nuclei also implies that breakage at homologous loci of both old strands of the G_1 chromosome does not occur. In the formation of an aberration during S, however, breakage of an old strand must occur, and it is possible that this breakage may precede and be the cause of a similar breakage at the same locus during the synthesis of the new strand. In terms of the mechanics of aberration formation we can consider two hypotheses: (a) that MH may produce breaks only in single strands in both G_1 and G_2, each break being independent of any others so that the chance of producing double breaks at identical loci would be very small, or (b) that the chemical does not produce breaks in half chromatids at G_1 or G_2 but that breakage of both old and new strands occurs only in S.

On hypothesis (a) aberrations produced during S would be the result of misreplication at the points of breakage and such errors would result in chromatid-type aberrations. Similarly the breakage of half chromatid units in G_2 chromosomes would result in the formation of chromatid-type aberrations in these nuclei at the following S period and these aberrations would be observed at the second posttreatment mitosis. However, on this hypothesis it is to be expected that the half chromatid breaks in G_2 cells should appear at the first mitosis as sub-chromatid aberrations of the type observed following X-irradiation or treatment with 8-ethoxycaffeine (KIHLMAN 1955; SCOTT and EVANS, unpublished). No such aberrations have been observed following MH treatment and this we consider to be an important objection to hypothesis (a). We might also point out that, on hypothesis (a), if at the time of duplication of a strand which was broken in G_1 or G_2 such a break either results in misreplication or is repaired, then no aberrations will be produced in G_1 cells in their second posttreatment cycle. In our experiments observations have been made up to seven days after treatment with MH and we have found that chromatid-type aberrations are produced even

in cells in their third or fourth posttreatment mitosis. It seems very likely, however, that much of this delayed breakage is not simply a reflection of an initial change produced in the chromosome at the time of MH treatment, but may well reflect the continued presence of the chemical in an active state in the cell nuclei (cf. CALLAGHAN and GRUN 1961).

If we reject hypothesis (a), then to account for the sensitivity of G_1 cells and for delayed breakage we must postulate that the MH either becomes an integral part of, or is in some way bound to, the chromosome or the extrachromosomal RNA-protein of the nucleus, and that breakage or exchange only occurs during S (hypothesis b). It is possible that at the time of treatment the chemical may produce some sort of lesion on the G_1 or G_2 chromosome which is not a break, but which results in a breakage at that locus during synthesis. However, the mode of action may be rather more complicated for if the aberrations are a consequence of lesions in, or attachments to, unduplicated chromosome zones, then the aberration yield should be highest in cells in early S at the time of treatment and should show a continuous fall with increasing development stage in S. The results show a small but marked increase in aberration yield in cells in late S (Figures 6 and 7) and the aberration yield is higher in early S than in G_1, indicating that the rate of DNA synthesis (chromosome duplication) may be a most important factor.

A number of investigators (references in CALLAGHAN and GRUN 1961) have shown that MH tends to be stable and there is only a relatively small amount of breakdown in plant tissue. Moreover, CALLAGHAN and GRUN showed that some three weeks after exposure to C^{14} MH, chromosomes of root tip cells were still heavily labelled. The fact that the compound is an isomer of uracil suggests the possibility that it could become incorporated into DNA in place of thymine. However, LOVELESS (1953) has shown that various treatments with an equivalent of ten times the amount of uracil, thymine or orotic acid does not reduce the yield of MH-induced aberrations: similarly, we have found that high concentrations of thymidine have no protective effect. It seems more likely that MH could become incorporated in place of uracil in RNA, and the findings that the compound may become selectively concentrated in nucleoli and in some cases causes an increase in nucleolar volume and persistence of nucleoli through mitosis (CARLSON 1954; GRAF 1957) lends some support to this possibility. However, as pointed out above, uracil and orotic acid appear to have no effect on aberration yield suggesting that the possible presence of MH in RNA might in any case be ineffective in inducing chromatid structural changes. Furthermore, in the autoradiographic work of CALLAGHAN and GRUN their cytological preparations were made from roots hydrolysed in N HCl at 60°C for 4 min—a procedure which would remove most of the RNA. From these considerations it would therefore seem unlikely that MH is incorporated in place of a normal base into DNA or RNA and it seems more plausible to suggest that it may be bound in some unspecific manner to nuclear proteins and possibly also to the nucleic acids.

In the work with C^{14} labelled MH in acid-hydrolysed roots there was no specific localisation of label to heterochromatic chromosome zones although, as mentioned

above, many of the aberrations induced by this compound are localised in heterochromatin. In molecular terms therefore we can offer no reasonable explanation for the mechanism of action of the compound, but can merely speculate that the errors in duplication which result in chromatid aberrations may be due to alterations in the protein component of the chromosome or to an antimetabolite action of MH loosely linked to the DNA-RNA-protein complex, as is perhaps indicated by the considerable lengthening of the DNA synthesis phase.

Possible relations between the effects of X rays, of MH and of other chemicals: There has been no systematic study relating the time of action of alkylating agents and other chemical mutagens in inducing chromatid aberrations to the time of chromosome duplication. However, the information that no aberrations are observed in the first 6 to 8 hr after treatment with alkylating agents (see the introduction), and the general shapes of the aberration yield curves, strongly parallels the results we have obtained with MH. On the other hand a considerable amount of information is available on the mutagenic efficiency of these compounds in Drosophila, particularly in treated sperm, and it is of interest to compare some of the general findings in these experiments with the results obtained in the present work. In much of the work on chemical mutagenesis in Drosophila comparisons have been drawn between the effects of X rays and of chemicals such as various alkylating agents (AUERBACH 1946, 1951; HERSKOWITZ and SCHALET 1954; FAHMY and FAHMY 1956; BROWNING and ALTENBURG 1961), urethane (VOGT 1950), formaldehyde (AUERBACH and MOSER 1953; SLIZYNSKA 1957, 1963a) etc. Despite the likelihood that the rather large variety of chemicals tested may act by different mechanisms, in all these reports some consistent differences have been observed between the physical agent and the chemical agents taken as a group. Two of the most significant differences are (1) the relatively reduced frequency of large rearrangements, particularly translocations following chemical treatment, and (2) the very high incidence of fractional or mosaic mutants, in which only one half of the fly is usually mutant, relative to the very low frequency of mosaic changes found after X-irradiation.

(1) Following exposure to ionizing radiations breakage is induced over all chromosome zones at about the same time, and the formation of a translocation requires interaction between two breaks in different chromosomes, exchange occurring only at chromosome zones which are in close association. Because of the production by certain chemicals of potential breaks, or delayed breakage, it was suggested that part of the shortage of translocations in Drosophila following chemical treatment might be connected with the delayed effect of the mutagens (AUERBACH 1951). More specifically, from SLIZYNSKAS' (1957) study on formaldehyde-induced rearrangements, it was proposed that potential breaks in the same chromosome tend to open simultaneously and hence favour the formation of intrachromosomal changes. In Drosophila males, following treatment of cells in equivalent stages with either formaldehyde or X rays, the ratio of inter- to intrachromosomal rearrangements following chemical treatment was found to be 1:4, whereas following X-irradiation this ratio approximated to 1:1 (SLIZYNSKA 1963b). In the present experiments on the effects of MH in Vicia roots similar

ratios have been obtained; 1:1 in the case of X rays and 1:4 for MH when comparisons are drawn at equivalent aberration yield levels.

Our conclusion that MH produces aberrations at the time of replication, immediately suggests a simple explanation for the favouring of intra- over interchange rearrangements. Clearly for interchange (translocation) formation following MH treatment the respective chromosome regions to be involved in exchange must not only be close together, but must also be undergoing replication at the same time (cf. SLIZYNSKA 1957). In other words chromosome regions which undergo replication at different times are unable to interact with one another to give structural rearrangements so that exchange, not only between but also within chromosomes, may only occur between chromosome zones undergoing simultaneous replication. We would suggest that this is the main reason for the observed reduced yields in translocation and high yield of intrachanges, particularly isochromatid aberrations, following chemical treatment. It should be pointed out that this dependence on replication may also be an important influence in affecting the *distribution* of aberrations both within and between chromosomes.

(2) The finding that MH produces only chromatid-type changes and that such aberrations may be produced in cells which have undergone a number of mitoses since treatment parallels some of the findings on the production of mosaic individuals following treatment of Drosophila sperm, or auxocytes, with certain chemicals. Only a small percentage of mutant individuals produced by X-irradiated sperm are half/half mosaics for the mutation (or structural change), whereas if sperm are treated with alkylating agents about 50 percent of the mutants are usually mosaic. Since sperm are in an unduplicated G_1 phase, X rays will induce chromosome-type structural changes which will, after fertilisation, be present in all cells of the offspring. However, if the alkylating agents have a delayed action directly analogous to that of MH, then treatment of sperm should result in aberrations only at the time of duplication of the male chromosomes in the fertilised egg, the aberrations being of the chromatid-type. Chromatid-type changes, other than isochromatid aberrations, in the fertilised egg would result in mosaic individuals in which one half of the cells contained a structural change. Smaller fractions of tissue containing a given rearrangement would of course occur if aberrations were induced in later cell cycles. If we can extrapolate our results with MH to Drosophila then the above observations suggest the possibility that, at least in so far as structural changes are concerned, many of the alkylating agents may produce most (but perhaps not all) of their aberrations at the time of duplication. Studies on the effect of certain alkylating agents on the Vicia system are in progress and a better comparative evaluation may be possible in the future.

In conclusion we might refer to the fact that despite the very much lower frequency of incomplete aberrations with MH relative to X rays, many of the MH-induced aberrations are asymmetrical and will lead to genetic deficiency. The majority of the MH-induced aberrations are isochromatid breaks which show complete sister reunion and usually result in the loss of a whole chromosome arm. This type of aberration is in some cases known to be, and is often supposed to be, lethal. In our experiments it has been possible to study the survival capacity of

cells carrying such aberrations for a limited period of time, and the results show that isochromatid aberrations are not lethal and are not selected against, at least in so far as the first cell cycle following their induction is concerned.

SUMMARY

Using H^3-thymidine as a marker for DNA synthesis, and autoradiographic techniques, the average durations of the various parameters of the mitotic cycle in Vicia roots were determined as follows: presynthesis phase (G_1) 4.9 hr, DNA synthesis phase (S) 7.5 hr, postsynthesis phase (G_2) 4.9 hr, and mitosis (M) 2 hr. The duration of these phases and their sensitivity to the production of chromosome aberrations following X-ray or maleic hydrazide (MH) treatment was then studied in experiments using combined treatments with these agents and with H^3-thymidine. (1) Cells exposed to 100 rads of X rays whilst in G_2 were delayed in development and contained chromatid-type aberrations. The aberration yield was highest in cells exposed whilst in mid-G_2, cells in late S and early and late G_2 being less sensitive. (2) In contrast with the X-ray results, with MH it was found that G_2 cells were not delayed in their development to the first mitosis and did not contain any aberrations. However, these cells contained chromatid-type aberrations when observed at the second posttreatment mitosis. (3) Cells exposed to MH whilst in the S phase were considerably retarded in development and carried chromatid-type aberrations at the first and later posttreatment mitoses. The yield of aberrations in these cells was found to be directly correlated with the amount and rate of DNA synthesis occurring during exposure. (4) No chromosome-type aberrations were induced by MH; cells in G_1 were sensitive but contained only chromatid-type changes. From this observation and from (3) it was concluded that the aberrations induced by MH are consequences of errors in chromosome duplication occurring only during the DNA synthesis phase, and possible mechanisms of action were discussed. (5) Comparisons were made between the relative frequencies of the various chromatid aberration types after X rays and MH and it was concluded that the reduced frequency of translocation following chemical treatment was a consequence of the dependence of aberration formation on duplication. (6) The absence of chromosome-type breaks and delayed production of aberrations with MH in Vicia parallels some of the effects of other chemical agents which produce structural changes and mutations in Drosophila. These parallels were discussed, particularly with reference to the production of mosaic individuals.

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