THE RELATION BETWEEN DNA SYNTHESIS AND CHROMOSOME STRUCTURE AS RESOLVED BY X-RAY DAMAGE

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ABSTRACT

Vicia faba root tip cells were treated for short periods with tritiated thymidine, either immediately before or after exposure of roots to x-rays, and autoradiograph preparations were analysed in an attempt to test the hypothesis that chromatid type (B') aberrations are induced only in those chromosome regions that have synthesized DNA prior to x-irradiation, whereas chromosome type (B'') aberrations are induced only in unduplicated chromosome regions. Studying the relation between presence or absence of label at loci involved in aberrations, in cells irradiated at different development stages, and the pattern of labelling in cells carrying both types of aberration leads to the conclusion that B'' aberrations are induced only in unreplicated chromosome regions. Following replication, only B' aberrations are induced, but these aberrations are also induced in chromosome regions preparing to incorporate DNA. It is suggested that the doubled response of the chromosome to x-rays prior to DNA incorporation might reflect a physical separation of replicating units prior to replication. The aberration yields in damaged cells which were irradiated in G_1, S, and early G_2 were in the ratio of 1.0:2.0:3.2. The data indicate that the increased yield of B' in early G_2 relative to S cells may be a consequence of changes in the spatial distribution of the chromosomes within the nucleus.

INTRODUCTION

Following the irradiation of a somatic or meiotic cell population, three principal types of chromosome structural change are induced, which differ according to the unit of breakage or exchange which is involved. These are (*a*) the chromatid type aberration, where the unit of structural rearrangement is the single chromatid, (*b*) the chromosome type aberration, where the unit of rearrangement is the two chromatids of a single chromosome which are broken or exchanged at identical loci, and (*c*) the subchromatid aberration, which involves a subunit of a chromatid—probably a half chromatid (1).

In some of the early studies on radiation-induced chromosome aberrations in mitotic cell populations it was found that cells irradiated in late interphase yielded chromatid type aberrations, whereas cells irradiated in early interphase produced chromosome type aberrations. At intermediate fixation times, mixtures of cells containing chromosome type and chromatid type aberrations were observed and occasionally both aberration types were found in one and the same cell (2). This pattern of events following irradiation was interpreted as indicating that chromosome type aberrations resulted from effects on chromosomes prior to splitting, and that chromatid type aberrations resulted from effects on postsplit chromosomes. The evidence for this, although highly suggestive, was not conclusive, for it was

realised that a break in a presplit chromosome could give rise to a chromatid break if, after splitting, one of the resultant chromatids restituted. Furthermore, it was also possible that aberrations that appeared as simple chromosome breaks might be produced from split chromosomes, if the chromatids were very closely associated so that both reacted to the radiation as one unit.

The finding that in *Tradescantia* microspores most of the radiation-induced breaks underwent restitution or rejoining within less than an hour after irradiation (3–5), supported the contention that the chromatid type aberrations resulted from the irradiation of postsplit chromosomes. However, some controversy existed over the status of those aberrations which were induced in late interphase cells, but which involved both chromatids of the same chromosome at apparently identical loci. These isochromatid (6) or isolocus (7) breaks usually show rejoining between sister chromatids, and they were believed by some to be induced in unsplit chromosomes (8). However, these aberrations are induced in cells which carry unambiguous chromatid type aberrations, and the evidence from studies on their dose response relation (6, 9), and on their time of appearance at the first metaphase following irradiation (10), leaves no doubt that most of these aberrations are indeed true chromatid type aberrations.

Following the discovery that the incorporation of P^{32} into the DNA of *Vicia faba* root tip meristem cells occurred at some time during interphase (11), it soon became apparent that a correlation existed between the time of DNA synthesis, as determined by Howard and Pelc (12), and the time at which a transition from the chromosome type to chromatid type aberration occurred (10). Later observations on the timing of DNA synthesis in uninucleate *Tradescantia* microspores (13, 14) were also shown by Sax and King (15) to correlate with their data on the transition from chromosome to chromatid type aberrations, and a further correlation is seen to exist between Bishop's (16) data on aberration types in *Tradescantia* generative nuclei and the data of Moses and Taylor (14) on the timing of DNA synthesis in these nuclei.

DNA synthesis in meiotic cells has been shown to occur prior to, or at the very beginning of, the prophase stage in both *Tradescantia* (13) and *Lilium* (17, 18), and in both these species the time of DNA synthesis seems to be closely related to the time at which x-rays discriminate between a single and a doubled chromosome state (15, 19). The evidence relating the time of DNA synthesis to the time at which the chromosome responds as a double structure to x-rays is thus, in a general sense, reasonably conclusive. However, no information is available from experiments specifically designed to study the relation between DNA synthesis and aberration type where information on both parameters is obtained in one and the same cell. With the advent of high resolution autoradiography for studying chromosome structure and DNA replication, such experiments are now possible, and the need for a more critical evaluation of this sort is evident when one considers that the period during which DNA is synthesized may be a considerable fraction of the total mitotic cycle time. For instance, in *Vicia faba* root meristem cells having a total mitotic cycle time of 18.5 hours, the duration of the synthesis or *S* phase is about 7.5 hours (20) and thus occupies 40 per cent of the total interphase time. Furthermore, it has become increasingly clear, from autoradiographic studies on chromosomes exposed to tritiated thymidine during the *S* phase, that all chromosomes or chromosome regions do not undergo DNA synthesis at the same time (21–25). This asynchrony in DNA replication within a chromosome complement might of course be in keeping with the observation that a small proportion of irradiated interphase cells may contain both chromosome and chromatid aberration types.

In view of these considerations we have carried out a number of experiments in which cells were exposed to x-rays and to tritiated thymidine, in various combinations, to test the hypothesis that chromatid aberrations are induced only in those chromosome regions that have synthesized DNA prior to x-irradiation, whereas chromosome type aberrations are induced only in chromosome regions which are unduplicated, at least in so far as their DNA content is concerned.

MATERIALS AND METHODS

In all the experiments, primary root tips of 11-day-old *Vicia faba* seedlings, cultured as described previously (26), were used. In the first experiment roots were exposed to a solution of tritiated thymidine (Schwartz, nominally 6-T) with a specific activity of 1.9c/mmole, at a concentration of 1 μc/ml, for 3 hours. Fifty seedlings were exposed to H^3-thymidine in an

annular vessel containing 100 ml of solution. Following completion of the H^3-thymidine treatment the roots were thoroughly washed and returned to water, and 15 minutes later some of the roots were exposed to 200 rad of x-rays given in air (250 kv, 10 ma, HVL, 2 mm of Cu, at a dose rate of 49.6 rad/min.). Fixations were carried out at 2-hour intervals between 22 and 28 hours after irradiation, the times being selected so that some of the cells observed in metaphase would have been in the late presynthesis (late G_1) or early synthesis (early S) stage at the time of x-irradiation. Both control and x-irradiated roots were exposed to a 0.05 per cent solution of colchicine for 2 hours before fixation in an osmium tetroxide fixative, and were bleached, hydrolysed, and stained by the Feulgen procedure as previously described (27). Root tip squashes were made on 2 × ⅞ inch coverslips which were filmed with Kodak AR10 stripping film, dried, and exposed in the dark at 4°C. The terminal 2 mm of root tip was used for each preparation, and the films were exposed for 3 weeks to allow maximum silver grain density without obscuring chromosome structural details. Autoradiographs were developed in D19b (Kodak), the preparations dehydrated, and the coverslips permanently mounted, inverted on 3 × 1 inch glass slides, so that both the film and the cells were sandwiched between two glass surfaces (28).

In the second series of experiments roots were exposed to H^3-thymidine (Amersham, 5-methyl-T) of a higher specific activity, 4.7 c/mmole, for 1 hour at a concentration of 2 μc/ml. The H^3-thymidine treatment was given either immediately before or immediately after exposure to 300 rad of x-rays. Cytological technique was as described above, and fixations were made at 10, 14, 26, 28, 32, 36, and 40 hours after x-irradiation. These fixation times were selected to allow metaphase observation of those cells which were in early G_2 and late S and late G_1 and early S at the time of x-irradiation.

For scoring purposes the slides were coded and randomised, and details of the scoring procedures for each particular experiment are given with the results.

RESULTS AND DISCUSSION

In order to facilitate presentation and discussion of the results, labelled and unlabelled nuclei will be referred to as L and U cells respectively, and chromosome type and chromatid type aberrations will be designated by the symbols B'' and B'. The symbols B'' and B' were introduced by Darlington and Upcott (29) to denote breakage in presplit and postsplit chromosomes. As mentioned in the Introduction, Darlington and La Cour (8) interpreted certain chromatid type aberrations, particularly isochromatid aberrations, as being derived from chromosome type breaks, whereas in our presentation isochromatid aberrations showing sister reunion and all other chromatid aberrations will be referred to as B'.

Before discussing the distribution of aberration types between L and U cells in the experiments, it is necessary to consider the duration and relative position of the S phase in the mitotic cycle. Under our conditions of culture, the duration of the mitotic cycle in *Vicia* root meristem cells is on average around 17 to 24 hours (26, 30), but considerable variation is found, in that the fastest cells may complete a cycle in about 12 hours, whereas some of the slower cells may take many days. The results of a typical experiment using H^3-thymidine to determine the average duration of the various parameters of the mitotic cycle (unpublished data of Evans and Scott) are shown in Fig. 1 *A*.

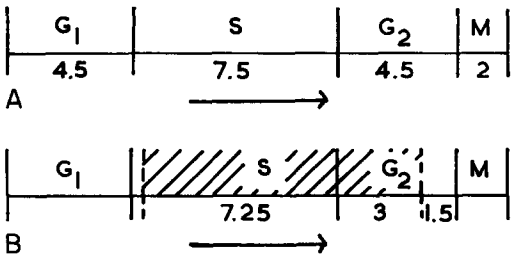

FIGURE 1 *A*. Average values for various phases of the mitotic cycle in *Vicia* root meristem cells having a mean total mitotic cycle time of 18.5 hours (data from (20)). G_1, presynthesis phase; S, DNA synthesis phase; G_2, postsynthesis phase; M, mitotic phase.

B. The expected distribution of labelled cells (shaded area), with respect to development phase, 15 minutes after an exposure to H^3-thymidine for 3 hours, *i.e.* the condition that pertains at the time of x-irradiation in experiment 1.

Experiment 1

The roots were exposed to H^3-thymidine for 3 hours and then for a 15-minute period to water before exposure to 300 rad of x-rays. Thus at the time of x-irradiation 3 hours' "worth" of cells in G_2 and all, or almost all, the cells in S would have incorporated H^3-thymidine and would appear as L cells (see Fig. 1 *B*). About one-third of the cells in G_2, which will include some of the slowly developing cells as well as those cells which had completed DNA synthesis shortly before exposure to H^3-thymidine, will be

U cells. If H^3-thymidine is rapidly utilised by the roots—and there is evidence that this is so in *Tradescantia* roots (31)—then cells that are in the first 10 minutes of *S* at the time of x-irradiation, and all the G_1 cells, will be *U*. We cannot rule out the possibility, however, that some label may be incorporated into DNA after the removal of the roots from H^3-thymidine, in which case all the cells in *S* would be labelled and possibly a small proportion of the late G_1 cells would pick up label after exposure to x-rays.[1]

Three slides were used at each of the four fixation times, and 30 *L* and 30 *U* cells on each slide were scored for aberrations. True breaks, intrachanges, and interchanges were scored, each break or exchange being scored as a single aberration, and the results are summarised in Table I. The over-all frequencies of the aberrations illustrate the typical finding at this dose-fixation combination, that *B′* is about three times as frequent as *B″*, and that the frequency of *B′* decreases with increasing fixation time whereas the frequency of *B″* is roughly constant. That the selected fixation times encompass the transition period from *B″* to *B′* is evident from the fact that 10 out of the 720 cells scored contained both *B′* and *B″*. However, at each of the fixation times cells which were quite clearly in their second mitosis following irradiation, *i.e.* X_2 cells, were observed (Fig. 2). This observation illustrates the fact that considerable variation in cell development rate exists in the meristem, and after irradiation this variation is likely to be increased owing to differential mitotic delay.

In spite of the observation of considerable variation in the rate of mitotic cycle development, let us first consider the expected results on the basis of minimal variation in cell development rate and on the hypothesis that *B′* are induced only in chromosomes that have undergone DNA replication before x-irradiation, whereas *B″* are induced only in unreplicated chromosomes. On this basis, cells observed in the earliest fixation times would, at the time of irradiation, have been in early G_2 and *S* and will therefore be labelled and contain *B′*; G_1 cells will be unlabelled and contain *B″*. The results in Table I show that no such clear-cut demarcation occurs, in that both *L* cells and *U* cells contain *B′* or *B″*. The general trend of the results is, however, as expected, as the majority (82 per cent) of the cells containing only *B′* aberrations were labelled, whereas the majority (82 per cent) of the 91 cells containing *B″* aberrations were unlabelled.

The presence of a small proportion of *L* cells containing only *B″* aberrations does not imply that *B″* aberrations can be induced in duplicated

TABLE I

Frequencies of Chromosome (B″) and Chromatid (B′) Type Aberrations in Labelled and Unlabelled Cells after 3 Hours' H^3-Thymidine Treatment Followed by 200 Rad of X-Rays (Experiment 1)

Time after irradiation (hours)	Labelled (*L*) or unlabelled (*U*) cells	Normal cells	Damaged cells	No. of cells containing aberrations			Total no. of aberrations	
				B′	*B″*	*B′* + *B″*	*B′*	*B″*
22	*L*	26	64	55	6	3	89	9
	U	56	34	12	20	2	20	26
24	*L*	43	47	43	4	—	69	4
	U	62	28	9	19	—	13	25
26	*L*	48	42	35	3	4	64	7
	U	59	31	6	25	—	6	30
28	*L*	54	36	32	3	1	46	4
	U	69	21	10	11	—	11	12
Totals	*L*	171	189	165	16	8	268	24
	U	246	114	37	75	2	50	93

[1] We have recently found that, under experimental conditions similar to those described above, 10 minutes after removal of roots from H^3-thymidine to water there is no further incorporation of tritium into DNA.

chromosomes, and it also seems unlikely that their presence is due to incorporation of tritium some 20 minutes after removal of the roots from H^3-thymidine, *i.e.* after x-irradiation. A more likely explanation for *L* cells with *B″* suggests that they consist of a mixture of *L* cells of three different types:

a) Cells which were entering the *S* phase at the end of H^3-thymidine treatment. In these cells only part of the chromosome complement would become labelled, part would undergo replication in the absence of label, and some would remain unduplicated before x-irradiation.

b) Cells which were leaving the *S* phase shortly after the commencement of H^3-thymidine treatment and which were in mitosis 3.25 hours later, at the time of irradiation. We have shown elsewhere (20) that in unirradiated roots fast-developing cells may pick up label in *S* and be observed as *L* cells at mitosis between 3 and 4 hours later. Such cells, if irradiated whilst in mitosis, are believed to yield *B″* aberrations at the succeeding division.

c) It seems probable that the bulk of the *L* cells carrying *B″* are in fact X_2 cells. A proportion of the X_2 cells in a population may be recognised on the basis of altered chromosome morphology arising from deficiency, etc., occurring at the X_1, or first postirradiation, mitosis. In the present experiment some 5 to 15 per cent of the labelled metaphases observed (Fig. 2) were of this type, and these were of course not included in the analysis. However, a proportion of the X_2 cells will be indistinguishable from X_1 cells, in that they will not be deficient and will contain aberrations identical in structure with some of the *B″*

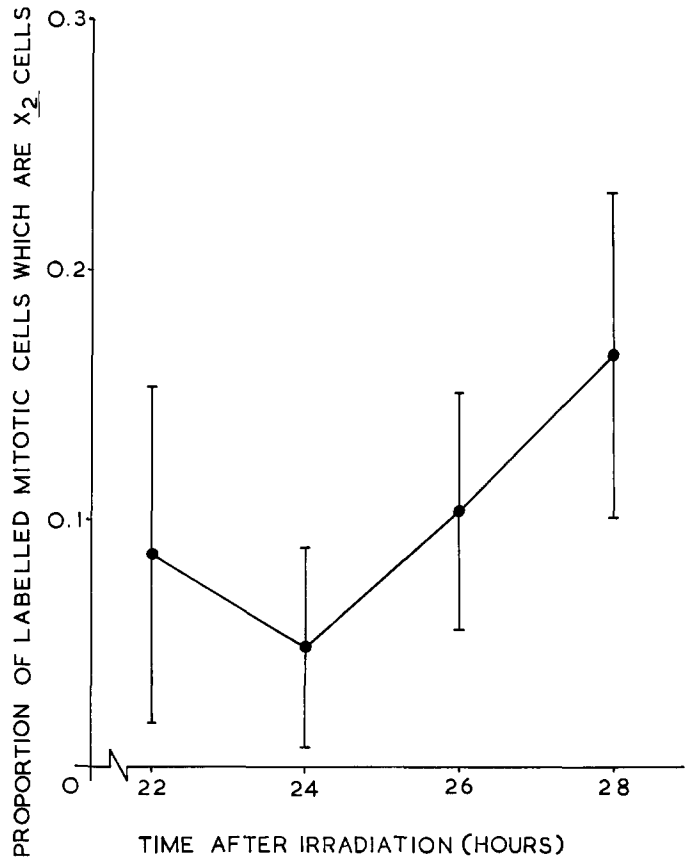

FIGURE 2 Proportion of labelled dividing cells which are in their second mitosis after x-irradiation (experiment 1).

aberrations found in X_1 cells. It seems likely, then, that most of these 8 cells (Table I), all of which were only lightly labelled, were indeed X_2 types.

In the *U* cell category the bulk of the cells (65 per cent) contain *B″* aberrations which must have been induced in G_1, although again a small proportion of the *U* cells with *B″* may have been X_2 cells derived from unlabelled G_2 cells carrying *B′* at the X_1 division. The presence of *U* cells carrying *B′* is unexpected on the simple hypothesis, but whereas the presence of label in a chromosome may be a definite indication of its incorporation into DNA, the failure to detect label is equivocal and could be due to physical factors such as the cells being too far from the film, or more probably to the amount of label's being too small for detection with the film exposure times used. This latter factor might be important particularly with respect to those cells which passed from G_1 into *S* during the 15 minutes between the completion of H^3-thymidine treatment and x-irradiation, although these cells would constitute only a very small fraction of the total cell population. In fact, *U* cells carrying *B′* make up some 18 per cent of the total cells carrying *B′*, and this suggests that the absence of labelling in these cells is not simply a consequence of physical factors.

Two other explanations might be advanced to account for the presence of the *U* cells with *B′*: (*a*) that these cells are slowly developing cells which were in G_2 at the time of x-irradiation, but had completed DNA synthesis before H^3-thymidine treatment, and (*b*) that the hypothesis that *B′* can be induced only in duplicated chromosomes is incorrect and that the chromosome reacts to x-rays as a double structure for some period of time before DNA incorporation. Suggestion (*a*) seems perhaps the most plausible explanation in that some of the *U* cells in G_2 at the time of irradiation might be expected to suffer considerable mitotic delay following exposure to 200 rad of x-rays. Explanation (*b*) is possible, but there is little or no evidence to support it.

Ten of the damaged cells were found to contain both *B′* and *B″* aberrations, and of these 8 were *L* cells, in line with the expectation that *B′* would occur in labelled chromosome zones and *B″* in unlabelled chromosome zones. The 2 *U* cells carrying *B′* + *B″* must have been irradiated in the late G_1–*S* region of the mitotic cycle, so that the absence of label in these cells must be due either to failure to detect the tritium which was incorporated into their DNA or to the fact that no such incorporation had occurred, *i.e.* that the cells were in late G_1 and that *B′* was induced in unduplicated chromosomes.

From this first experiment it was evident that the bulk of the cells irradiated in *S* and all the cells in G_2 yielded only *B′* aberrations; that most, and possibly all, of the cells irradiated in G_1 yielded *B″* aberrations; and that a transition from *B″* to *B′* occurred sometime between late G_1 and early *S*. The experiment does not allow us to determine the time of chromosome doubling, as detected by x-rays, with any degree of precision, and the results leave open the possibility that the chromosome may react as a double structure in the late G_1 phase. A second series of experiments was therefore carried out in an attempt to obtain more precise data on this transition phase.

Experiments 2 and 3

Relating aberration type to presence or absence of label in the whole nucleus is inefficient, since DNA duplication within the nucleus is an asynchronous process. More precise data, and better resolution, would be obtained by studying the relation between the aberration type and the distribution of label with reference to the points of location of the aberrations. Such a procedure was adopted in experiments 2 and 3, note being taken of the presence or absence of label in the nucleus and of the presence or absence of silver grains overlying chromosome zones involved in aberrations.

In both experiments x-irradiation doses of 300 rad were used in an attempt to increase the frequency of cells carrying both *B′* and *B″* aberrations. These cell types are of cardinal importance in attempting to pin-point the time of transition from *B″* to *B′*, and although the high x-ray dose makes scoring of heavily damaged *B′* cells rather difficult, it was thought that this would be more than offset by the increased yield of cells carrying both aberration types.

In experiment 2, roots were exposed to H^3-thymidine for 1 hour immediately before x-irradiation, whereas in experiment 3 the order was reversed, 300 rads of x-rays being followed by 1 hour's treatment with H^3-thymidine. The interval between x-irradiation and H^3-thymidine treatment in both experiments was less than 5 minutes. In each experiment 30 aberrant cells were analysed,

usually from two slides, and all aberrations, excluding achromatic lesions or gaps, were scored; again each break or exchange was classed as a single aberration. In addition, in experiment 3 the frequency of labelling in metaphase cells was determined from 50 metaphases scored on each of three slides at all fixation times. The expected distribution, at the time of x-irradiation, of those cells which appear as L cells at metaphase is shown in Fig. 3 *A*.

Experiment 2 is similar in design to experiment 1 and the general pattern of the results obtained is much the same (Table II). The majority of cells (75 per cent) containing only B' aberrations were L cells, whereas almost all

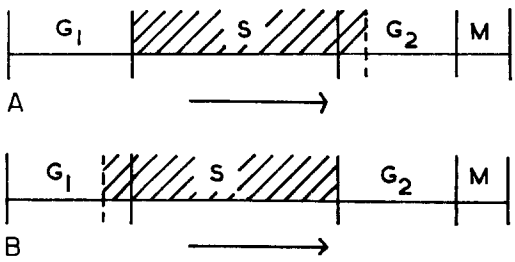

FIGURE 3 *A*. Expected distribution of labelled cells after 1 hour's H^3-thymidine treatment, *i.e.* at the time of x-irradiation in experiment 2.

B. Expected distribution, at the time of x-irradiation, of those cells which appear labelled when 1 hour's H^3-thymidine treatment is given after x-irradiation, *i.e.* experiment 3.

(98 per cent) of the cells containing only B'' were U cells. The relative frequency of L cells with B'' was smaller than in the first experiment, possibly as a consequence of the higher dose, giving a greater mitotic delay, and of the shorter time period between the beginning of H^3-thymidine treatment and x-irradiation. However, although in each of the 2 L cells containing only B'' the aberrations occurred in labelled chromosome zones, in the analysis of these cells both were considered as possible X_2 cells. Furthermore, unambiguous X_2 cells which were L were observed at all fixation times. The suggestion that these 2 cells were X_2 cells is also strongly supported by the finding that in the 2 L cells containing both B' and B'', the B'' aberrations occurred in U chromosome zones, as would be expected if B'' occurs only in unduplicated chromosome regions. In these two cells B' aberrations were found in both L and U chromosome zones. Since $B' + B''$ cells are found only in late fixation times—*i.e.* the cells must have been irradiated in late G_1 to S—then the presence of 6 $U\,B'$ aberrations as opposed to 3 $L\,B'$ points to the possibility that the chromosome reacts as double to x-rays for some time prior to the incorporation of DNA—a conclusion which is supported by the finding that 3 out of the 5 cells carrying $B' + B''$ were unlabelled (Table II).

In experiment 3, where H^3-thymidine is applied after x-irradiation (see Fig. 3 *B*), the possibility that label is incorporated into chromosomes for some time after removal from H^3-thymidine does not arise as a complication in the analysis of B' aberrations. All the cells in G_2 at the time of irradiation will be U, and all those chromosome regions, of cells in S, which had already undergone DNA replication at the time of irradiation will be unlabelled. *Thus if B' can occur only in chromosome regions that have completed replication, it might be expected that all the B' would be confined to U cells or to the U zones in L cells.* The results on the relation between aberration type and presence or absence of label are summarised in Table III.

The early fixation times between 10 hours and 16 hours after irradiation, in experiment 3, were carried out in order to study cells which were in late S at the time of irradiation, whereas the later fixations covered cells in late G_1 and early S. The first L cells to be observed in metaphase cfter 300 rad of x-rays arrive at about 10 hours after irradiation, and the rate of increase of L metaphase cells and their distribution with time are shown in Fig. 4. It may be seen from this figure that the maximum proportion of L metaphases is about 40 per cent and that the labelling index varies between 20 and 40 per cent between 16 hours and 40 hours after irradiation. In unirradiated roots the maximum labelling index attains a level of about 90 per cent; this high value is reached about 6 hours after the beginning of H^3-thymidine treatment and is maintained over a period of about 4 hours (20). The reduced labelling index, the increased period over which the maximum labelling index is maintained, and the increased variability observed in irradiated roots are primarily the result of x-ray–induced mitotic delay and increased variability in cell development rate. Variability is also evidenced by the fact that unambiguous X_2 metaphase cells were observed at 26 hours after irradiation.

It may be seen from Table III that in the first four fixation times, between 10 hours and 16 hours, only B' aberrations were observed; moreover, as expected, the bulk of the cells (75 per cent) were U cells which were in G_2 at the time of irradiation. The L cells observed in these early fixation times are amongst the first of those cells which arrived at mitosis following exposure to irradiation whilst in the S phase. On the simple hypothesis, it is to be expected that B' aberrations in these cells would be confined to U chromosome zones. *Such an expectation is however, not realised, for a considerable proportion of the aberrations in these cells occurred in chromosome zones which were quite definitely labelled.* Similarly labelled B' aberrations were also observed in the later fixation samples (Table III).

The absolute frequency of B' aberrations in L cells decreases from 3.4 to 2.0 per cell with increasing fixation time (Table III), *i.e.* the yield increases with increasing development into S. This change in B' yield accords with the idea that duplication proceeds progressively throughout S, so that the probability of inducing B' relative to B'' increases with degree of development into S. On this basis the rate of change in the total yield of B' in these cells would be a measure of the rate of chromosome duplication. Since H^3-thymidine incorporation can occur at or near to a chromosome zone that reacts as double to x-rays, then a trend towards a decreasing proportion of the B' aberrations occurring in L chromosome zones, with increasing stage of development in S at the time of irradiation, is not unexpected. In Fig. 4 we have expressed the relative frequency of B' aberrations which are definitely labelled in those regions where breakage or exchange had occurred, as a proportion of the total B' aberrations observed in L cells which contained only B'. The samples are small, the number of B' in these cells at each fixation time ranging from 17 to 54. However, the data show a general tendency towards an increasing proportion of the aberrations occurring in L chromosome zones with increasing fixation time, but with marked depressions in the curve occurring at around 12 to 14 hours and 28 to 32 hours (Fig. 4). Similar but less pronounced depressions are also observed in the total aberration yields in these cells at these same fixation times (Table III).

TABLE II

Frequencies of Chromosome (B'') and Chromatid (B') Type Aberrations in Damaged Labelled and Unlabelled Cells after 1 Hour's H^3-Thymidine Treatment Followed by 300 Rad of X-Rays (Experiment 2)

Time after irradiation (hours)	No. of aberrant cells scored	Labelling status of cells	Cells containing one aberration type						Cells containing both B' and B''				
			B'			B''				No. of aberrations*			
			No. of cells	No. of aberrn.*		No. of cells	No. of aberrn.*		No. of cells	B'		B''	
				L	U		L	U		L	U	L	U
28	30	L	15	33	12	—	—	—	1	3	2	—	2
		U	3		11	10		13	1	—	1	—	1
32	30	L	11	13	10	—	—	—	—	—	—	—	—
		U	6		23	13		19	—	—	—	—	—
34	30	L	7	12	7	1	1	—	—	—	—	—	—
		U	1		2	20		24	1	—	6	—	2
36	30	L	6	13	1	1	2	—	1	—	4	—	1
		U	5		27	17		27	—	—	—	—	—
40	30	L	10	10	17	—	—	—	—	—	—	—	—
		U	1		4	18		23	1	—	2	—	1
Totals	150	L	49	81	47	2	3	—	2	3	6	—	3
		U	16		67	78		106	3		9		4

* The aberrations in L cells are separated into those aberrations observed in labelled chromosome zones (L) and those in unlabelled zones (U).

The shape of the curve relating the relative frequency of B' aberrations in L chromosome zones to stage in cell development is not simply a reflection of the *amount* of DNA incorporated into the chromosomes prior to irradiation, but must also reflect the *rate* of DNA synthesis. H^3-thymidine was available for only a short time, 1 hour, in the incorporation of precursor into DNA does not occur at a constant rate throughout S. In their experiments it was found that incorporation was more rapid in early and late S than in the mid S period. Our results are thus in accord with this finding of a differential rate of incorporation in different stages in S, although our data indicate

TABLE III

Frequencies of Chromosome (B'') and Chromatid (B') Type Aberrations in Damaged Labelled and Unlabelled Cells after 300 Rad of X-Rays Followed by 1 Hour's Treatment with H^3-Thymidine (Experiment 3)

Time after irradiation (hours)	No. of aberrant cells scored	Labelling status of cells	Cells containing one aberration type						Cells containing both B' and B''				
			B'			B''				No. of aberrations*			
				No. of aberrn.*			No. of aberrn.*			B'		B''	
			No. of cells	L	U	No. of cells	L	U	No. of cells	L	U	L	U
10	30	L	5	9	8	—	—	—	—	—	—	—	—
		U	25		106	—	—	—	—	—	—	—	—
12	30	L	8	10	16	—	—	—	—	—	—	—	—
		U	22		83	—		—	—		—		—
14	30	L	12	8	24	—	—	—	—	—	—	—	—
		U	18		75	—		—	—		—		—
16	30	L	5	12	6	—	—	—	—	—	—	—	—
		U	25		122	—		—	—		—		—
26	30	L	14	30	14	2	1	2	3	4	1	4	—
		U	8		26	2		6	1		1		1
28	30	L	9	11	10	2	—	2	—	—	—	—	—
		U	12		47	6		7	1		3		1
32	30	L	18	33	21	—	—	—	1	3	1	—	1
		U	2		4	9		9	—		—		—
36	30	L	15	28	7	1	1	1	—	—	—	—	—
		U	8		45	4		8	2		5		2
40	30	L	8	14	2	1	1	—	1	1	1	—	2
		U	14		59	6		8	—		—		—
Totals	270	L	94	155	108	6	3	5	5	8	3	4	3
		U	134		567	27		38	4		9		4

* The aberrations in L cells are separated into those aberrations observed in labelled chromosome zones (L) and those in unlabelled zones (U).

relation to the total duration of S, so that if the amount of DNA incorporated per hour was constant throughout S, then the proportion of B' aberrations occurring in L chromosome zones should show a smooth decline with increasing development through S. Howard and Dewey (32) have obtained evidence, from grain count studies on autoradiographs of *Vicia* cells exposed to H^3-thymidine, which can be interpreted as showing that the possibility of two phases rather than one phase with relatively low incorporation rates.

A similar partitioning of the B' aberrations in L cells into two classes, on the basis of the presence or absence of label at the aberrant chromosome loci, was carried out in experiment 2. In this experiment H^3-thymidine was applied before irradiation, and the proportion of B' aberrations which are labelled in those cells which were

around the early S period at the time of irradiation contrasts sharply with the result obtained in experiment 3 (Fig. 4). In experiment 3, at 40 hours after irradiation, 88 per cent of the B' aberrations in L cells were themselves labelled, the label being incorporated after the aberrant chromosome regions had responded as double structures. However, in equivalent cells in experiment 2, the proportion of B' in labelled chromosome zones shows a dramatic and significant fall from 93 per cent at 36 hours to 37 per cent at 40 hours after irradiation, and this fall is not accompanied by a decrease in the B' aberration yield in these cells. Such a fall can be explained if the L population seen at 40 hours contains a considerable number of cells which have spent only a small proportion of the H^3-thymidine treatment time in synthesis and thus carry the majority of B' in U zones.

In the case of B'' aberrations in experiment 3, the presence of L cells carrying only B'' is to be expected, because of cells passing into S from G_1 in the presence of H^3-thymidine, the precursor being applied after x-irradiation. In this experiment there were 33 cells carrying only B'' aberrations and of these 18 per cent were L cells. Thus, in those cells containing both B' and B'' aberrations it was expected that some of the B'' would occur in L chromosome zones. On the other hand, if B' could be induced only in duplicated chromosome zones in these cells, then they should occur in U chromosome regions. Of the 9 $B' + B''$ cells observed, 5 were L cells, and in 4 of these cells B' aberrations were observed in L chromosome zones, 2 of the cells having both aberration types in L chromosome regions. These results are thus in line with the findings made on those cells containing only B' aberrations. If the absence of

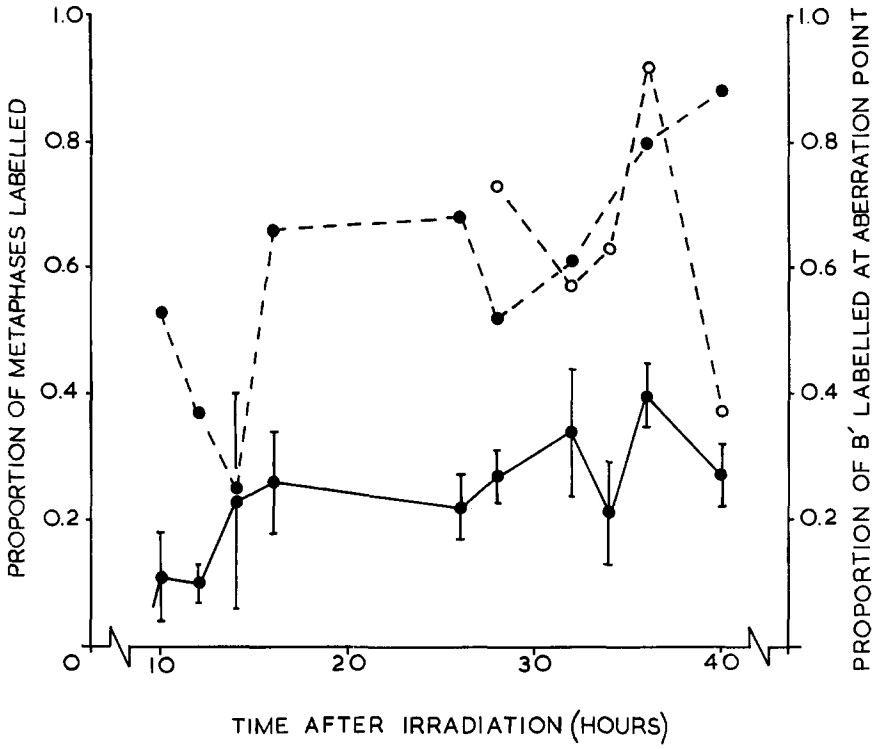

FIGURE 4 Lower (solid) line, closed circles, proportion of metaphases which are labelled at various times after exposure to 300 rad of x-rays (experiment 3).

Upper (dashed) lines, closed circles, proportion of B' aberrations in labelled cells which are labelled at the aberration point (experiment 3); open circles, proportion of B' aberrations in labelled cells which are labelled at the aberration point (experiment 2).

label is not simply the result of physical factors, then the presence of a high proportion of U cells amongst those cells containing $B' + B''$, 4 out of 9, suggests that the double nature of a chromosome region prior to DNA incorporation may exist for a period of more than 1 hour. On the other hand, the occurrence of a significant proportion of B'' aberrations in L chromosome zones (Table III), and, moreover, the finding of cells containing both B' and B'' aberrations in L chromosome zones, suggests that this period when a chromosome zone reacts as double to x-rays before DNA incorporation lasts, on an average, for very much less than 1 hour.

In addition to information on the time of transition from B'' to B', the data summarised in Tables II and III also yield information on the relative sensitivities of cells irradiated in G_1, S, and G_2. These data are somewhat limiting, in that only aberrant cells were scored, no account being taken of cells which appeared to be morphologically normal or of those which contained only gaps or achromatic lesions. Furthermore, with the high radiation dose and consequent high aberration yield, cytological analysis of the more heavily damaged cells was difficult. Bearing in mind these limitations of the data, it is nevertheless instructive to look at the relative frequencies of aberrations in the various aberrant cell types.

The frequencies of B' aberrations in L cells are similar as between experiments 2 and 3 (Tables II and III), the pooled mean frequency being 2.73 aberrations per cell as compared with a mean frequency of 4.23 per cell for B' aberrations in U cells. In the case of B'' aberrations, averaging the data for both L and U cells gives a mean frequency of 1.34 per cell. Since in both experiments the majority of the L cells carrying B' aberrations would have been in the S phase at the time of irradiation, whereas the bulk of the U cells carrying B' would have been in G_2, then the relative frequency of aberrations in those damaged cells irradiated whilst in G_1, S, and early G_2 is respectively 1.0:2.0:3.2. We have previously shown that in *Vicia* roots exposed to 100 rad of x-rays the yield of B' aberrations in cells irradiated in early G_2 is higher than the yield found in cells irradiated in late G_2 and S (20): the present results are thus in line with this observation. We might point out that in experiment 1 there is no difference in the B' aberration yield between L cells and U cells, which is of course due to the fact that in this experiment a large proportion of the L cells were in G_2 at the time of irradiation.

The B' aberration yield in G_2 cells is about 55 per cent higher than in S cells, and it would seem probable that some of this increased yield in G_2 may be due to an increase in the number of sites in the nucleus where the chromatids of different chromosomes are sufficiently close together to undergo interchange. This possibility is strongly suggested by the fact that out of a total of 150 U cells containing 634 B' aberrations, 12 cells contained 5 interchange aberrations, 5 cells contained 6 interchanges, and 3 cells contained 7, 8, and 9 interchanges. On the other hand, amongst the 143 L cells containing 391 B' aberrations no cell was found to contain more than 4 interchanges. It has been shown previously that the distribution of interchange aberrations amongst cells irradiated in G_1 (33) or late G_2 (27) may not be in accord with the Poisson distribution, owing to the occurrence of too few cells having multiple exchanges. Wolff (34) has suggested that this non-randomness is due to a limitation on the number of interchange sites in the nucleus, the data on B' and B'' aberrations in *Vicia* indicating that about two such sites occur in each nucleus. However, it has recently been reported (35) that the B' interchange aberrations observed 24 hours after exposure of *Vicia* roots to 200 rad of x-rays are distributed randomly amongst cells, and this has been interpreted to mean that the interchange site number increases as a result of chromosome duplication. Our results, however, suggest that the increase in site number may not be a simple consequence of chromosome duplication, in that the increased yield of interchange aberrations appears to occur sometime after DNA incorporation has been completed. Moreover, as nuclei irradiated in late G_2 show evidence of site limitation, the increased yield of interchanges in early to mid G_2 must be a reflection of some transient change in the structure or physiology of the G_2 nucleus.

GENERAL DISCUSSION AND CONCLUSIONS

The main object of the experiments described was to test the hypothesis that B' aberrations are induced only in chromosome regions that had synthesized DNA prior to exposure to x-rays, B'' aberrations being induced only in those regions which had not synthesized DNA. The results suggest that this hypothesis is only partly correct,

there being a very strong indication that B' aberrations may also be induced in unduplicated chromosome zones for a short time prior to DNA incorporation. The evidence leading to this conclusion is of three sorts:

a) the occurrence of B' aberrations in strongly labelled chromosome zones in experiments where H^3-thymidine is applied after exposure to x-irradiation;

b) the pattern of labelling observed in cells carrying both B' and B'' aberrations in experiments where H^3-thymidine is applied before or immediately after X-irradiation;

c) changes observed, in L cells, in the relation between stage in cell development and proportion of aberrations occurring at L chromosome zones.

The interpretation of the evidence summarised above is by no means straightforward, and there are a number of factors which must be considered before the relative importance of these various observations can be weighed.

1. We have stressed the fact that the presence of B' aberrations in L cells where H^3 label was applied after x-irradiation need not imply a doubled state of the chromosome prior to DNA incorporation. This of course follows from the fact that a nucleus takes, on an average, about 7.5 hours to synthesize all its DNA, the rate of synthesis varying in different chromosome zones. We have attempted to overcome the indecisiveness of relating aberration type to presence or absence of label in the whole nucleus by examining the labelling status of the chromatid zones actually involved in aberration formation. However, we must ask ourselves whether this approach may itself be open to misinterpretation.

The presence of label was determined in relation to a 1-μ length of metaphase chromatid, an aberration being scored as labelled if a minimum of two silver grains were found within about 0.5 μ of either side of the point of breakage or exchange. With our cytological techniques the sum total of the lengths of the 24 chromatids of the metaphase complement of *Vicia faba* is about 211 μ, and DNA duplication of the whole complement takes about 450 minutes. However, not all the DNA in any given 1-μ region is synthesized in a few minutes, for DNA incorporation at some chromosome zones may occur over a period of hours. From this it follows that it should be possible for a B' aberration to be induced in a chromatid zone in which the majority, but not all, of the molecules in the zone had undergone DNA replication prior to x-irradiation. A B' aberration could therefore be induced in a duplicated region which, because of relatively low resolving power, is scored as a region which incorporates DNA after x-irradiation. That some of the labelled B' aberrations observed must fall into this category is suggested from the following considerations on the number of H^3 atoms required to be incorporated into a given locus in order to give significant labelling at that locus.

If 200 disintegrations from tritium are required to activate one silver grain in the emulsion overlying the labelled locus (36), then to obtain an average of two grains per micron of chromatid in 21 days' exposure requires the presence of 1.2×10^6 H^3 atoms, the half-life of tritium being 12.26 years. Only the hydrogen in the 5-methyl position of the thymine ring was tritiated, and since at the time of use its specific activity was 4.7 c/mmole, then it follows that only 16 per cent of the total thymidine molecules in the H^3-thymidine solution contained tritium. In other words, 7.5×10^6 thymine bases derived from the H^3-thymidine applied to the roots must be incorporated into 1 μ of chromatid in order to yield two silver grains in that micron. Since the 4-c DNA content of the diploid *Vicia* nucleus is around 3×10^{-11} gm (references in (1), p. 256), then the "new" DNA synthesized into 1 μ of metaphase chromatid is about 7.1×11^{-14} gm. This amount of "new" DNA represents about 7.3×10^7 base pairs, so that if the adenine + thymine to guanine + cytosine ratio is about 1.5:1 (37), then there must be around 4.38×10^7 molecules of thymine built into each micron of chromatid. Thus in order to obtain two silver grains over a 1-μ region of chromatid, one-sixth of the new thymine in that region must be built up from the H^3-thymidine solution applied to the roots.

The above calculations thus show that when H^3-thymidine is applied after x-irradiation, some of the B' aberrations which are classed as L types may well have been induced in chromosome zones which had already incorporated DNA prior to x-irradiation. This must always cause a complication in any analysis simply because breakage or exchange may involve very few DNA base pairs, whereas a considerable number of tritium-labelled thymine bases must be present in order to give detectable labelling.

2. The difficulty of determining whether a given

zone of chromatid in an early S nucleus had not incorporated any DNA at the time of irradiation is by no means the only complication in studying the transition phase between the response of the chromosome from a single to a double structure. The interpretation of our results must inevitably be influenced by any assumptions which are made about the mechanism of aberration formation. The processes involved in the formation of an aberration may be separated into two: breakage, or some other change in chromatid structure (38), followed by exchange between the damaged zones or broken chromatid ends. Breakage is generally believed to occur immediately or shortly after exposure to the radiation, whereas the actual exchange of damaged parts may take place over periods of up to 1 hour later. However, if breakage is delayed then it would be possible that breakage in a chromosome damaged whilst in an unduplicated G_1 state could be deferred until after duplication; in this way B' aberrations might result following irradiation in late G_1 for example (8). It may well be that a certain amount of delayed breakage may occur in either the pre- or the postduplication phase, but we reject the possibility that such a delay would account for our finding that B' aberrations can be induced in late G_1 cells, since it is difficult to conceive of a damaged zone's undergoing duplication and then yielding latent breakage in only one of the sister chromatids, especially as new DNA is built into both chromatids (39). We must also at this point make it clear that the spectrum of B' aberration types observed in those cells irradiated in late G_1 is similar to that found in irradiated G_2 cells. These include chromatid interchanges and intrachanges together with the rare chromatid breaks and the very frequent isochromatid breaks showing sister reunion. Furthermore, although a few of these late G_1 cells may contain both B' and B'' aberrations, almost all the cells contain only one aberration type.

3. From a consideration of the effects of certain base analogues on the amount of chromosome damage induced by x-rays, Taylor *et al.* (40) have suggested that the rejoining of radiation-induced chromosome breaks requires the replication of the whole or parts of the damaged "DNA replicating units." On this hypothesis, DNA incorporation at damaged sites showing rejoining should occur in nuclei at all stages of development throughout interphase. Thus if the "replicating unit" were sufficiently large, then the presence of B' aberrations in a labelled chromosome zone in experiment 3, where x-rays were given before the exposure to H^3-thymidine, might be explicable on the above hypothesis. Our results, however, rule out this possibility, as all the aberrations in the first damaged cells to arrive at metaphase, in experiments where treatments with H^3-thymidine are given after x-irradiation, are always unlabelled, as is also indicated by the results for the early fixation times in experiment 3 (Table III). The presence of label in a damaged chromosome zone under these conditions has thus nothing to do with the process of chromosome rejoining. These observations also indicate that if DNA incorporation is necessary for chromosome rejoining, then the amount of DNA incorporated is probably very much smaller than the equivalent amount of new DNA found in 0.5 μ of a metaphase chromatid.

4. One factor which requires mention is the possibility that some of the aberrations observed in the experiments were induced not by the x-irradiation, but rather by the internal β emission from tritium. Aberrations are indeed induced by the isotope, but under the conditions used in the present experiments their frequency is extremely low and their contribution to the total aberration yield is negligible.

Bearing in mind the difficulties discussed above, we believe that the experimental evidence taken as a whole strongly points to the conclusion that B' may be induced in chromosome regions which are *preparing* to incorporate DNA. This conclusion is of course based principally on the labelling patterns observed in cells carrying both B'' and B' aberrations and on the changes in the relation between the presence of label and the positions of aberrations, particularly in cells in the late G_1 to early S period at the time of irradiation. These results also indicate that a chromosome zone in a G_1 nucleus may react as double to x-rays for a period of probably less than 1 hour before DNA incorporation into that zone, and that this doubled response to x-rays is maintained throughout the S and G_2 periods. Independent work by Wolff and Luippold (41), using the same material, has led to a similar conclusion. In their experiments, H^3-thymidine was administered either immediately before, or at various times after, x-irradiation. They conclude from their observations that the change from B'' to B' occurs either in late G_1 or in

very early S before much DNA has been duplicated.

Our results also suggest the possibility that shortly before and at the very commencement of DNA synthesis a considerable proportion of the total chromosome length in the nucleus may react as double to x-rays. This is indicated by the fact that in experiment 3 (Table III) the mean aberration yield in damaged cells which were irradiated whilst in late S (10 to 14 hours' fixation) is only slightly higher, 3.0 per cell, than the mean yield of 2.2 aberrations per cell obtained in cells irradiated whilst in late G_1 to early S (36 to 40 hours' fixation). It must be remembered that these are aberration yields in damaged cells, and more precise information on the difference in aberration yields between cells irradiated in early and late S would be obtained by scoring both normal and damaged cells at these fixation times. However, these results and their implications suggest an explanation for the somewhat puzzling and well known finding that cells containing both B' and B'' aberration types are rare. Their rarity does not appear to be a simple consequence of the fact that the frequency of B'' relative to B' aberrations is low, for even with the relatively long duration of the S phase and the high aberration yields obtained in the present experiments, few cells containing both aberration types were observed. It would seem then that the low frequency of cells of this type may be due, at least in part, to a fairly rapid transition from an effectively single to an effectively double structure in a large proportion of the nucleoprotein in cells in late G_1. That this transition to a double state is not effected in all the chromatin in late G_1 to early S cells is evidenced by the fact that cells containing both B' and B'' aberrations are found in populations which were in the mid S phase at the time of irradiation.

Finally, we must consider the implications of the above conclusions in relation to the structure of the irradiated interphase chromatids. We have continually referred to the resolution by x-rays of a single or doubled state of the chromosome, or more precisely of an interphase structure which must be directly related to the chromatids seen at mitosis. However, our results indicate that this doubleness is not coincident with the completion of chromosome duplication, but may perhaps be associated with a particular phase in the process of chromosome duplication. If we consider the process of chromosome duplication from the standpoint of a single replicating unit, then duplication may be separable into a number of sequential phases, *viz.* (*a*) *template organisation* (this may involve the removal of RNA protein and possibly the unwinding of the DNA double helix) resulting in physical separation of the replicating units, whether these are half DNA helices or complete double helices (42); (*b*) *replication* of DNA, proteins, and RNA; and (*c*) *integration* of the templates and their replicated products into the complete duplicated unit or chromatids. Thus the earliest transition from a single to a doubled response of the chromosome to x-rays might occur at the stage of template organisation or at the stage of replication, particularly if the protein component of the chromosome duplicates before the DNA. In fact there is considerable evidence indicating that nuclear histone replication is closely associated in time with DNA synthesis (43–45), the synthesis of chromosomal protein being coincident with or occurring very shortly after the incorporation of DNA (46). From this it would seem that the doubleness of the chromosome to x-rays prior to DNA incorporation is unlikely to be a consequence of an early replication of chromosomal protein, but rather might reflect a physical separation of the replicating units prior to the actual processes of replication.

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