THE CONSEQUENCES OF LOCALISED CHROMOSOME BREAKAGE

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1. INTRODUCTION

CHROMOSOMES can be broken by various chemical reagents in specific regions and at specific times during the mitotic cycle. With 8-ethoxy-caffeine (EOC) and maleic hydrazide (MH) breaks and re-arrangements are induced in the resting nucleus and are first observed at the ensuing mitosis (Kihlman and Levan, 1951 ; McLeish, 1952 ; see plate, figs. 1 and 2). The consequences of these limited and regular effects in succeeding cell generations have now been studied in an attempt to answer the following questions :

(i) After breakage at different positions, can the acentric fragments and the micronuclei that they form be classified into recognisable groups ?

(ii) Does the loss or gain of specific parts of nuclei tell us anything about their structure and the elements necessary for their survival ?

(iii) To what extent can the centric and acentric products of primary breakage be used as markers for timing new breakage and reunion in later resting nuclei ?

2. MATERIALS AND METHODS

Seedlings of *Vicia faba* var. Claudia Aquadulce were used in the present experiments. I have already described the methods used in their growth and treatment (1952).

MH treatment.—A dose was selected which, while giving a high breakage frequency, subsequently allowed growth and mitosis to continue so that later cell generations could be studied :

$$0 \cdot 5 \text{ mM}/2 \text{ hrs.}/20° \text{ C.}$$

EOC treatment.*—A dose similar to that employed by Kihlman and Levan (1951) was found suitable in these respects :

$$8 \text{ mM}/8 \text{ hrs.}/7° \text{ C.}$$

In this case, the plants were kept at 7° C. for 2 days prior to treatment and afterwards were allowed to recover at 20° C.

Preparations.—For metaphase :—

(i) Pre-treatment : Normally, 2 hours in 0·05 per cent. colchicine. In the EOC experiments, breaks in the nucleolar constriction were more easily detected if the chromosomes were excessively contracted so that the acentric fragments became free. Pre-treatment was therefore extended to 4 hours.

(ii) Fixation : 2 BD overnight.

(iii) Post-fixation : The roots were washed in distilled water and then softened and bleached for 10 mins. in a mixture of ammonium oxalate (sat. aq. soln.) and hydrogen peroxide (20 vol.) 1 : 1 (Dr C. E. Ford, private communication).

(iv) Staining : Feulgen squash method.

For anaphase :—This same technique was used but pre-treatment was omitted. For studying the positions of acentric fragments and micronuclei :—

(i) Fixation : 2 BD overnight.

(ii) Embedding : 52° C. M.P. paraffin wax.

(iii) Sectioning : at 20 μ.

(iv) Staining : iodine-crystal violet method.

For nucleoli :—A. Sections.

(i) Fixation : 1 : 3 acetic alcohol.

(ii) Embedding : 52° C. M.P. paraffin wax.

(iii) Sectioning : at 6 μ.

(iv) Staining : methyl green-pyronin (Kurnick, 1952).

                B. Squashes.

(i) Fixation : formalin-chromic acid overnight (Lewitsky, 1931).

(ii) Staining : Feulgen-light green (Semmens and Bhaduri, 1941).

Measurements.—Outline camera-lucida drawings of nuclei and nucleoli were made at a magnification of ×2520. Their areas were then computed from counts made on a superimposed grid of 1/16 in. squares for nuclei and, to reduce error, 1/32 in. squares for the smaller nucleoli.

Scoring and mapping of breaks.—The symbols of Darlington and La Cour (1945) were used in scoring structural changes. The positions of breaks were mapped by camera-lucida drawings and checked, where possible, by comparisons with the lengths of undamaged homologues.

Detection of later cell generations (X_2-X_n).—The progeny of damaged cells were recognised by the occurrence of any one of the following :—

(i) micronuclei ;

(ii) deficiency or translocation of chromosome parts ;

(iii) reproduced acentrics ;

(iv) chromosome reunion (R'') giving dicentric chromosomes—a reunion which very rarely occurs at X_1 (McLeish, 1952).

3. CLASSIFICATION OF ACENTRICS AND MICRONUCLEI

The action of EOC and MH in *Vicia* is to induce limited and localised breakage, EOC chiefly in the nucleolar constriction, MH in the heterochromatin. Its distribution is shown in graphs 1 and 5. Characteristic acentric fragments and micronuclei are thus produced especially by the breakage of the M chromosome.

Acentrics.—Because of the positions of breakage, only four main types of acentric fragment need concern us. They are classified according to the way in which euchromatin, heterochromatin and nucleolar organisers are combined or separated (fig. 1). At X_1 telophase they may be included in main nuclei or lost. If included, all types retain their individuality throughout the ensuing resting stage and reproduce along with the centric chromosomes at X_2 (plate, fig. 7). If lost, they constitute micronuclei of characteristic form.
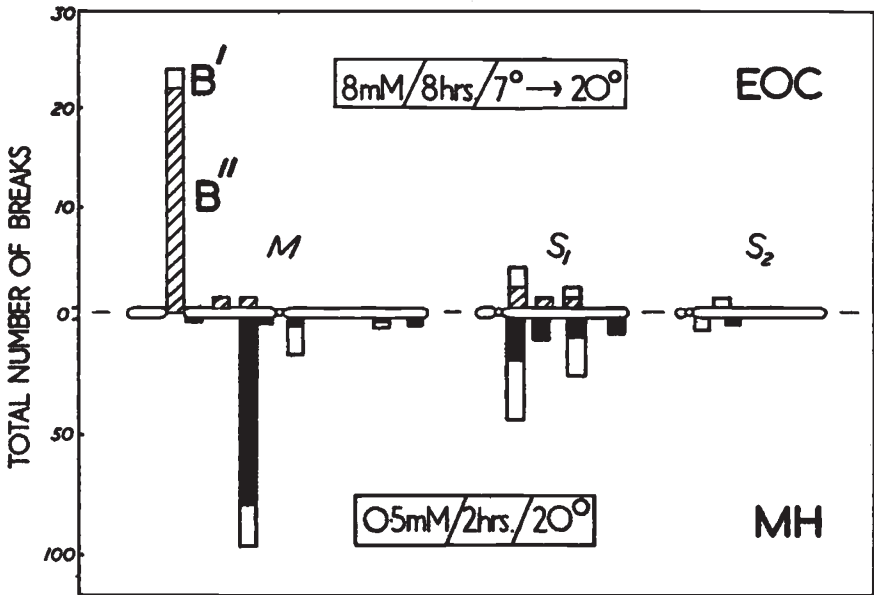
GRAPH 1.—Comparison of the distribution of breakage segments, in X_1 cells, in the 3 chromosome types M, S_1 and S_2; one day after treatment with EOC (above) and up to and including 5 days after treatment with MH (below). Tabular data not given for EOC. MH data from McLeish (1952), table 4.
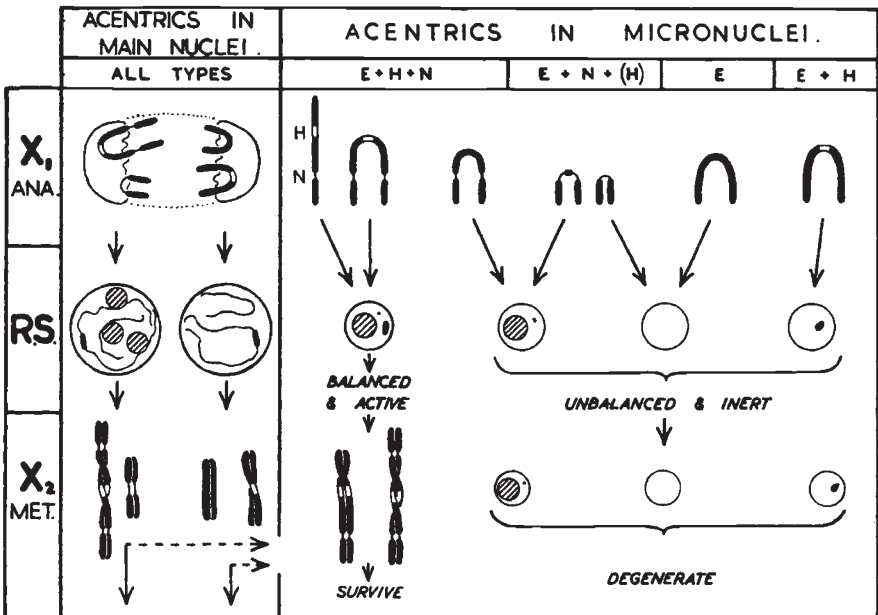

FIG. 1.—Diagram showing how the 4 main groups of acentrics, with various combinations of euchromatin (E), heterochromatin (H), and nucleolar organisers (N), behave when included in main nuclei or micronuclei. The inferred small heterochromatic segment near the nucleolar organiser is not represented. R.S.—resting stage.

Nucleolar organisers are marked by secondary constrictions at mitosis and are thus detectable in acentrics. They organise nucleoli in micronuclei. Heterochromatic segments are, of course, indistinguishable from the euchromatin at mitosis under normal temperature conditions. But they betray their presence in micronuclei by their typical allocyclic behaviour. They are then visible as Feulgen-positive chromocentres (fig. 4, *a-d*; see also Darlington and La Cour, 1940, 1941).

Nearly all acentrics in *Vicia* arise in one of two ways : from B″ SR or B′ R′ (fig. 2). Those arising from B″ SR are *iso-acentrics*. They are double since the arms on each side of the point of reunion are derived from sister chromatids. As we shall see, the extent of such duplication as well as of translocation (B′ R′) in acentrics, influences both the structure and subsequent behaviour of their micronuclei.
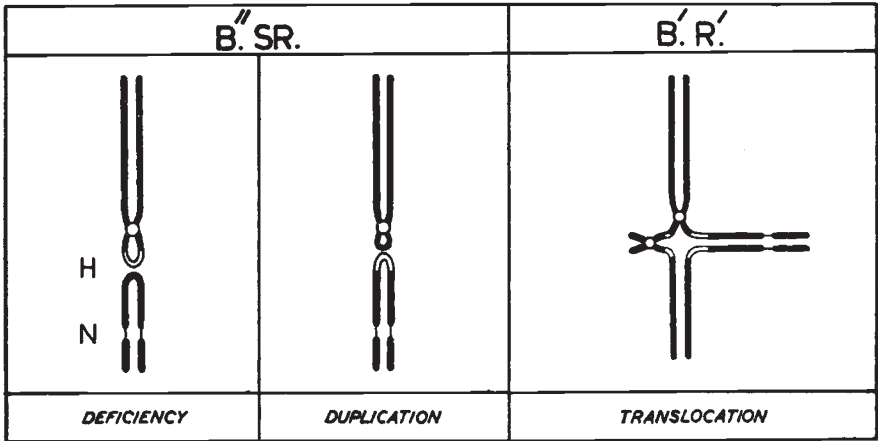

FIG. 2.—Diagram showing how the amount of heterochromatin (H), carried by acentrics with nucleolar organisers (N), can be determined by both the position and the type of breakage and reunion.

Micronuclei.—The contents of micronuclei formed after random X-ray breakage must remain largely unknown unless displayed during a further mitosis. On the other hand, localised chemical breakage can provide a method of segregating known parts of the nucleus as standard acentrics in micronuclei. These, and their complementary but now deficient main nuclei can be examined in some detail.

I have accordingly made measurements of main nuclei, micronuclei and their nucleoli in selected cells of the type shown in fig $4a$ which occur 24 hours after MH treatment. They are daughter cells derived from an X_1 mitosis in which most of the nucleolar arm of one M chromosome had been lost from the nucleus but included in the cell. Their contents can be inferred from the observed breakage

occurring up to 24 hours. This is of low frequency with high localisation in segment 2 (graph 5 ; see also McLeish, 1952, tables 2 and 4). Moreover 94 per cent. of these breaks are B″-s followed by SR to give nucleolar iso-acentrics (fig. 2). Thus the majority of micronuclei selected for measurement contain two sister nucleolar organisers and their main nuclei only one. Nucleoli are organised in both.

At the time of fixation (*i.e.* 24 hours) most micronuclei are less than 12 hours old. This is because, as I previously showed, most breakage appears in mitosis more than 12 hours after treatment. Their size and structure are then accurate indicators of their constitution since any variability due to their survival or degeneration is not yet likely to be apparent.

For comparison, I measured those cells (45·9 per cent.) in untreated roots where the products of the two organisers had fused to give a single nucleolus.

The results of all these measurements are given in graph 2 and are discussed in the next section.

4. THE ORGANISATION OF NUCLEOLI IN *VICIA*

A study of the positions and products of localised breakage can give us an idea of the position of origin of nucleoli and the factors controlling their size.

Position of origin.—The evidence concerning nucleolar origin in *Vicia* is derived from the two types of localised breakage (with EOC and MH) and from observations on X_2 cells.

The positions of breakage with EOC are shown in graph 1. 88·2 per cent. of the resulting acentrics carry varying lengths of the nucleolar constriction (plate, fig. 1) yet only 13·5 per cent. of the total micronuclei have nucleoli (table 1). These micronuclei are approximately the same size as those seen in MH-treated cells (fig. 4*a* ; plate, fig. 3). They are probably formed by acentrics derived from the 6·5 per cent. of breaks occurring between the organiser and the centromere. The production of a nucleolus would then depend not on the constriction region as a whole but on some locus at its proximal end.

The constriction or " SAT zone," observed at mitosis, has often been supposed to be the result of nucleolar growth during the preceding telophase and resting stage. This was suggested by the observations of McClintock (1934) in *Zea Mays* and Navashin (1934) in *Crepis*. The suggestion can be tested in *Vicia* by observing the behaviour of nucleolar acentrics in micronuclei. As we shall see, some of these remain viable ; they reproduce and reappear at X_2 (fig. 6 ; plate, figs. 2 to 5). We then see that the lengths of their nucleolar constrictions are not significantly diminished despite the fact that much smaller nucleoli were formed at the preceding telophase. Possibly the difference in nucleolar size is insufficient to give detectable

TABLE 1

Frequencies of nucleolar and non-nucleolar acentrics and micronuclei at different times after EOC treatment. Scored at X_1 metaphase and anaphase, during the resting stage (R.S.) and at X_n prophase. Nucleolar acentrics are those with nucleolar constrictions

X_1

Time	Cells	Per cent. with acentrics	Total nucleolar	Total non-nucleolar
1 D	Met. 60 Ana. 100	*40·0* *44·0*	25 48	2 8
2 D	Ana. 25	*8·0*	2	...
		Total . . Per cent. .	75 *88·2*	10 *11·8*

R.S.

Time	Resting cells	Per cent. with micronuclei	Total nucleolar	Total non-nucleolar
2 D	737	*5·4*	5	36
3 D	651	*2·6*	3	16
4 D	635	*2·2*	2	12
		Total . . Per cent. .	10 *13·5*	64 *86·5*

X_n

Time	Prophase cells	Per cent. with micronuclei	Total nucleolar		Total non-nucleolar	
			Active	Inert	Active	Inert
2 D	712	*5·6*	1	3	...	38
3 D	983	*0·61*	...	...	...	6
4 D	947	*1·1*	...	1	...	10
		Total . . Per cent. .	1 *1·7*	4 *6·9*	...	54 *91·4*

differences in constriction length at the ensuing mitosis. On the other hand, the nucleolar constriction of *Vicia* may be a segment of constant length, which, in conjunction with an adjoining proximal locus, forms a compound organiser gene. The constriction could be regarded as a part of the chromosome which is necessary for the proper functioning of the organiser and around which the substances of the nucleolus are secreted (fig. 3).
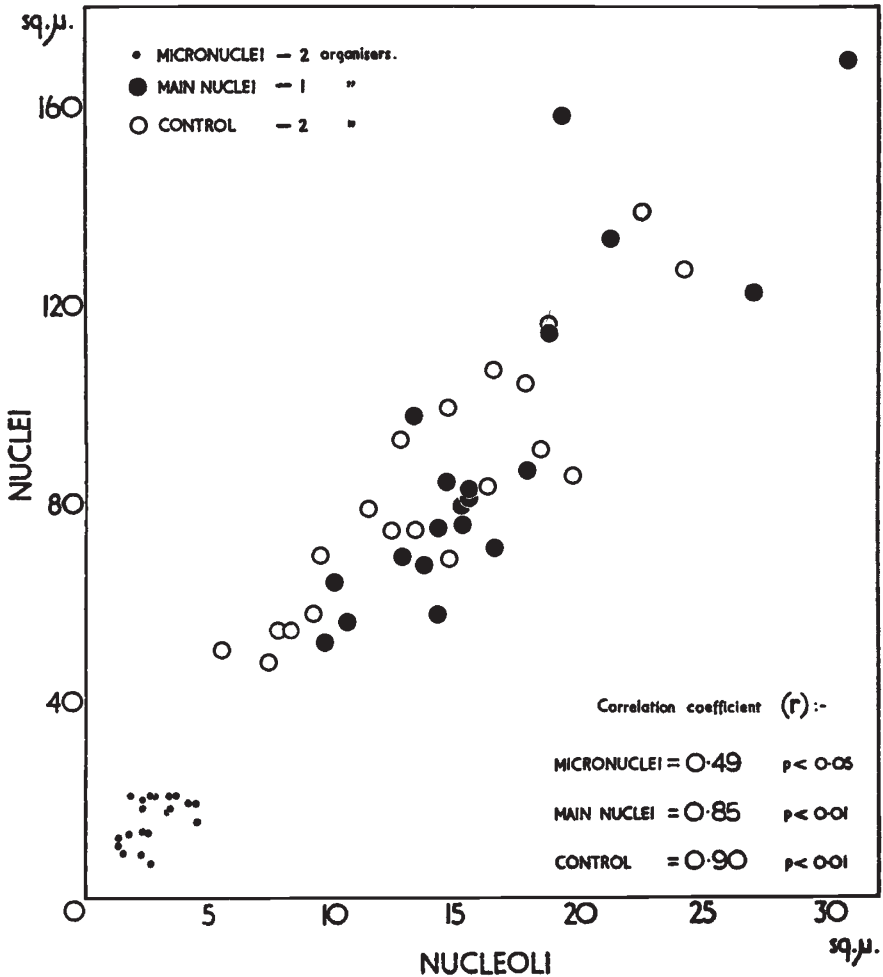
GRAPH 2.—Showing the linear correlations between nuclear and nucleolar size in the 3 selected classes. Tabular date not given.

Occasionally, in MH-treated diploid cells of *Vicia*, more than two nucleoli appear (fig. 4c). Most of these cases probably result from the inclusion in daughter cells at X_1 of extra nucleolar organisers carried by acentrics. Others, as we shall see, appearing 2 days or more after treatment, can be the result of reproduction of such acentrics.

As Heitz (1931) originally suggested, other chromosomes may

have the ability to organise nucleoli. Darlington and Haque (1954) have obtained additional evidence of this from the structure of microcytes at the second meiotic division of *Allium*. In the present experiments with *Vicia* I think such behaviour must be rare for two reasons. First, more than two nucleoli never appear in normal *Vicia* cells ; and secondly, in MH-treated cells, the relative frequency of nucleolar and non-nucleolar acentrics at mitosis 24 hours after treatment agrees, approximately, with the relative frequency of their corresponding micronuclei at this time (table 2).

Size.—The following results were obtained from measurements made in the types of selected cells described in the previous section :

> (i) There is a significant linear correlation between nuclear and nucleolar size in each of the three classes considered (graph 2 ; *cf.* Fernandes and Serra, 1944). The scatter may be due to the nuclei being in different physiological stages of interphase since a similar correlation has been observed by Lesher (1951) during increases of chromosome size in salivary gland nuclei of *Drosophila*.

FIG. 3.—Diagram showing the way in which the euchromatin (E), heterochromatin (H) and nucleolar organiser (NO) may be distributed in the nucleolar arm of the M chromosome of *Vicia faba*. C—centromere ; NC—nucleolar constriction. The large heterochromatic segment can be revealed by cold treatment. The positions of the two smaller ones are inferred.

> (ii) The single nucleolus of the micronucleus is the product of two organisers yet is always smaller than that of its accompanying main nucleus which is the product of one. These latter nucleoli fall into the same range of size as those of controls which are the fusion products of two organisers (graph 2).

> (iii) Only 6·6 per cent. of the total micronuclei occurring 24 hours after treatment contain two separate nucleoli in contrast to the 54·1 per cent. of controls. The separate nucleoli are then further reduced in size (fig. 4*b*). Nucleolar fusion here must be due partly to the frequent proximity of sister organisers in iso-acentrics derived from B″ SR (fig. 2), and partly to the restricted internal volume of the micronuclei.

Together, I think these data confirm the general conclusions of McClintock (1934) in showing the size of nucleoli to be influenced by three factors : by competition between their organisers, by the amount of chromosome material which surrounds the organisers, and by the existing physiological condition of their nuclei.

TABLE 2

Frequencies of cells with nucleolar and non-nucleolar acentrics and micronuclei at different times after MH treatment. Scored at X_1 metaphase, during the resting stage (R.S.) and at X_n prophase. Nucleolar acentrics are those with nucleolar constrictions. Reproduced in graph 3.

X_1

Time	Meta-phase cells	Per cent. with acentrics	Nucleo-lar	Per cent.	Non-nucleo-lar	Per cent.	Both types	Per cent.
1 D	50	*18·0*	5	*55·6*	4	*44·4*	...	...
2 D	200	*31·0*	32	*51·6*	20	*32·3*	10	*16·1*
3 D	200	*23·0*	26	*56·5*	15	*32·6*	5	*10·9*

R.S.

Time	Resting cells	Per cent. with micro-nuclei	Nucleo-lar	Per cent.	Non-nucleo-lar	Per cent.	Both types	Per cent.
1 D	5673	*0·8*	24	*53·3*	20	*44·4*	1	*2·2*
2 D	2025	*10·9*	101	*45·9*	110	*50·0*	9	*4·1*
3 D	2487	*16·7*	180	*43·4*	219	*52·8*	16	*3·8*
4 D	2482	*14·5*	111	*30·7*	238	*65·9*	12	*3·3*
5 D	2231	*8·4*	45	*23·9*	138	*73·4*	5	*2·7*
6 D	1839	*2·8*	21	*40·4*	29	*55·8*	2	*3·8*
7 D	1178	*2·3*	15	*55·5*	11	*40·7*	1	*3·8*

X_n

| Time	Prophase cells	Per cent. with micronuclei	Total nucleolar		Total non-nucleolar	
			Active	Inert	Active	Inert
3 D	557	*6·1*	12*	7	...	15
4 D	794	*4·9*	20†	8	...	11
5 D	1023	*1·3*	4‡	1	...	8
		Total	36	16	...	34
		Per cent.	*41·9*	*18·6*	...	*39·5*

* One in advance of main nucleus.
† One retarded slightly.
‡ One cell contained two.

5. THE BEHAVIOUR OF ACENTRICS IN MICRONUCLEI

We have already seen how acentrics can be classified into recognisable groups, each forming characteristic micronuclei. A study of the frequency and behaviour of these micronuclei at various times after their formation shows that they are mostly lost. Of those
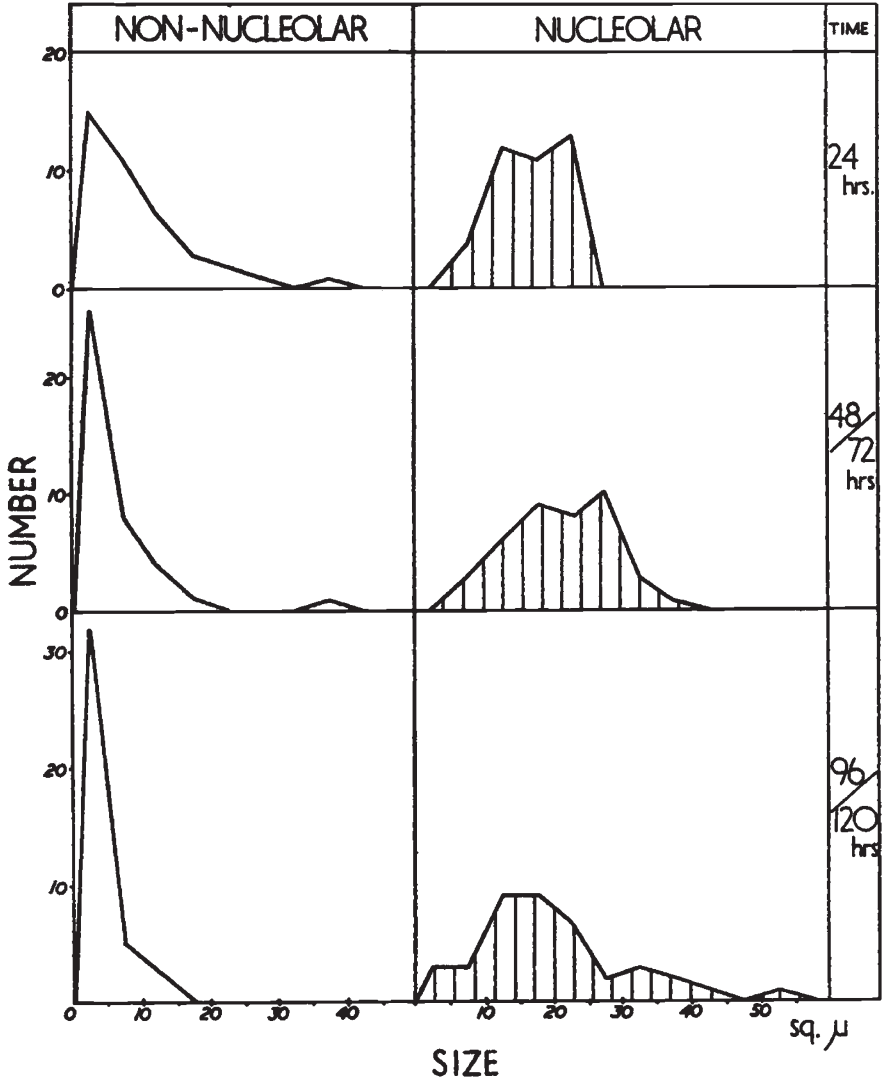

DAYS AFTER TREATMENT
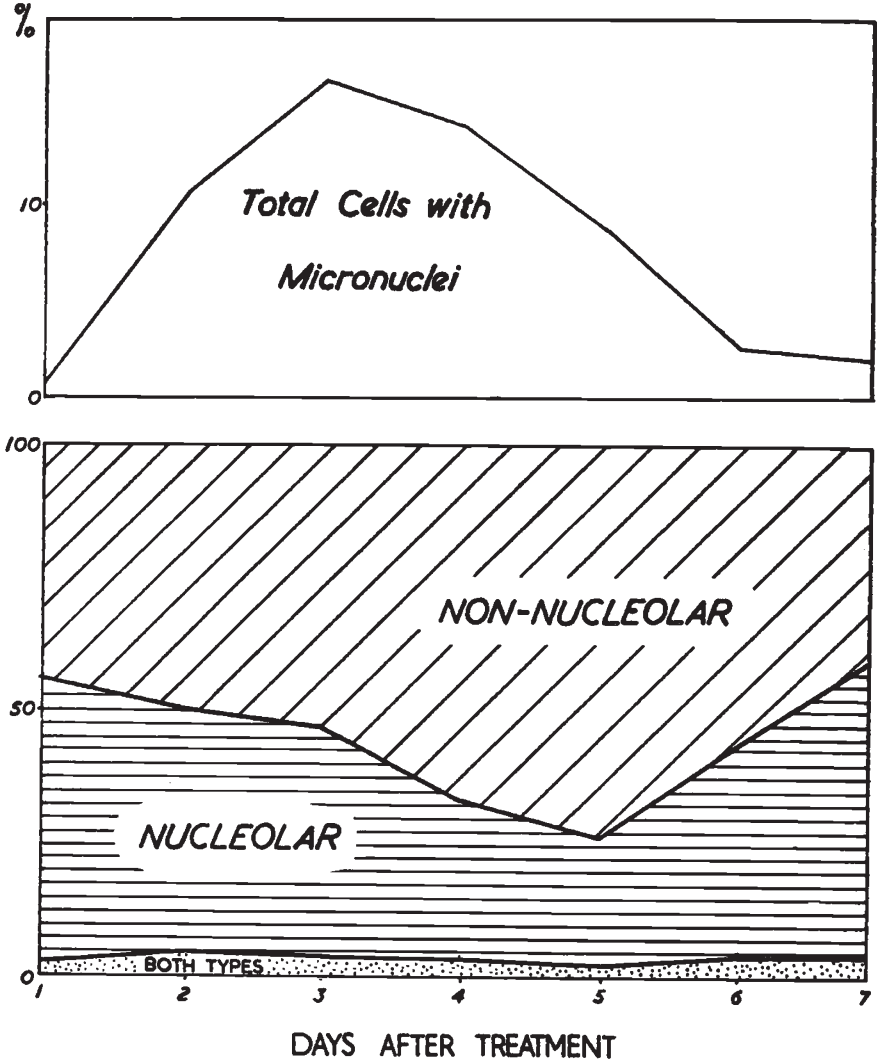
GRAPH 3.—Frequencies of resting cells containing micronuclei at different times after MH treatment. From table 3.

that survive, some appear to be able to play an active part in cell metabolism.

Elimination of micronuclei.—Two main factors operate in eliminating micronuclei from the root meristem. Some degenerate and are absorbed into the cytoplasm or into the main nucleus, while others

are carried away from the apex into differentiated tissue by growth and mitosis. After MH treatment a few new micronuclei continue to arise as a result of deferred breakage. But these are insufficient to interfere with the quantitative estimates of those of primary origin. A small proportion of micronuclei are presumably retained for variable

GRAPH 4.—Comparison of the sizes of nucleolar and non-nucleolar micronuclei at different times after MH treatment. 40 micronuclei of each type were measured for each of the 3 comparisons (the 48 and 72 hrs. and the 96 and 120 hrs. data showed no significant difference and were lumped). Tabular data not given.

times in the meristem by being carried forward in mitosis. The survivors of this proportion must be mostly nucleolar since in contrast to non-nucleolar types, their frequency rises again after 5 days (table 2, graph 3). This conclusion is confirmed in graph 4. Here,

the distinction between the two types is expressed as a size difference. For, while the non-nucleolar micronuclei continually decrease in size with time, the nucleolar types exhibit a greater variability. A spread occurs, representing, as we shall see from other evidence, degeneration in one direction, and in the other, active survival. The degenerating nucleolar micronuclei become pycnotic and hence indistinguishable from non-nucleolar types. This must be the reason for their diverging frequency after 2 days (table 2, graph 3). It also explains the discrepancy between the relative frequency of the two types of acentric and that of their corresponding micronuclei at this time.

One further discrepancy is shown in table 2. After MH treatment, X_1 cells containing both types of acentric are more frequent than resulting daughter cells with both types of micronuclei. This may be due partly to the segregation of the two types into separate daughter cells at X_1 mitosis and partly to the formation of single micronuclei by both types.

The physiology of survival in micronuclei.—During the resting stage following X_1 mitosis (after MH), some excluded nucleolar acentrics evidently remain viable since 69·2 per cent. of their micronuclei become actively synchronised with the main nucleus at subsequent prophases (fig. 4e and f; plate, fig. 4, table 2).* The remaining 30·8 per cent. may possibly play a minor role in cell metabolism but are evidently incapable of mobilising their proteins, nucleic acids and other components in order to become effective working units. There is consequently no further visible reproduction of their constituent acentrics and they eventually die (fig. 4d).

We must now examine the three components which may control the balance between survival and death :—

(i) EUCHROMATIN.—This is present in varying amount in all acentrics, micronuclei and main nuclei. Unlike the nucleolar organisers and heterochromatin it cannot be eliminated from any one of them by localised breakage and hence its relative functions are uncertain. It can, of course, be segregated alone in some micronuclei but these are inert and soon degenerate.

(ii) NUCLEOLAR ORGANISERS.—The active survival of micronuclei is dependant on their containing a nucleolar organiser. La Cour (1952) found this to be true in *Hyacinthus*. In *Vicia* we can determine the relative parts played by nucleolar organisers and heterochromatin since they are to some extent separable by breakage.

(iii) HETEROCHROMATIN.—The amount of heterochromatin which is carried into the micronucleus is known to be variable. It depends directly on the position of breakage and on the type of reunion (figs. 1 and 2).

All nucleolar micronuclei are formed by acentrics derived entirely or in part from breakage in the nucleolar arm of the M chromosome. The combined evidence from cold treatment and breakage with

* Acentrics derived from EOC breakage are rarely viable (see table 1).

MH has shown that this arm carries one of the largest blocks of heterochromatin in the *Vicia* nucleus—corresponding with breakage segment *2*—and possibly two smaller blocks corresponding with breakage segments *1* and *3* (fig. 3). There may be additional smaller blocks which have so far eluded detection since numerous tiny chromocentres can occasionally be resolved in the micronuclei (fig. 4*d*).
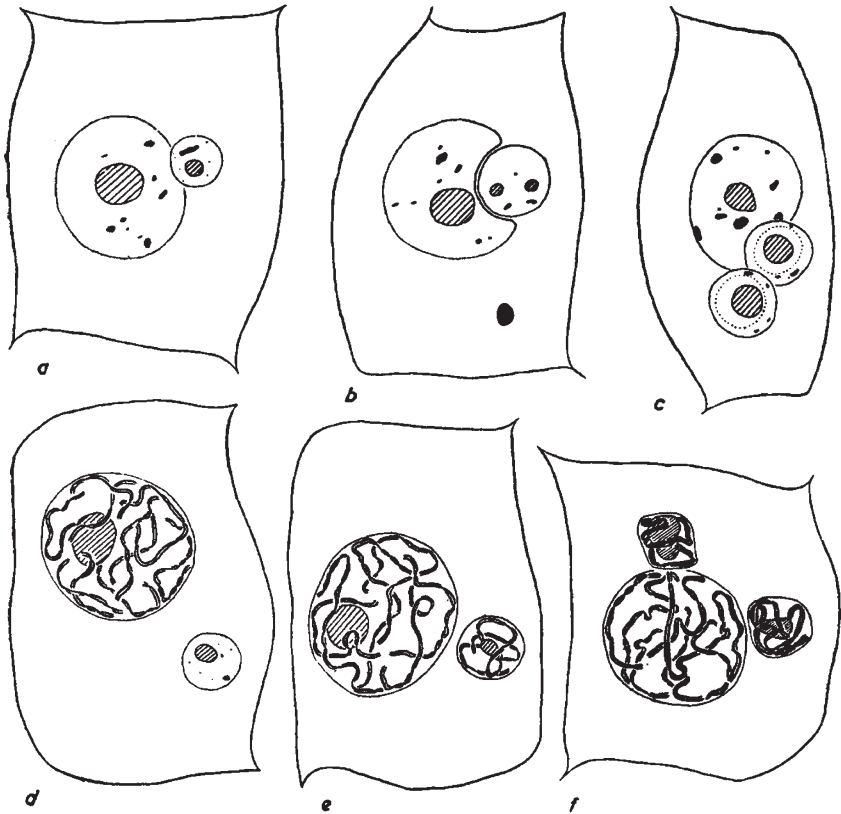

FIG. 4.—(*a*) to (*f*). Root cells of *Vicia faba* containing micronuclei (m.n.) at different times after MH treatment.

(*a*) 24 hrs. : main nucleus and m.n. containing heterochromatin (black) and nucleoli (hatched).

(*b*) 48 hrs. : nucleolar m.n. partially enclosed by main nucleus. Small pycnotic m.n. degenerating in cytoplasm.

(*c*) 72 hrs. : 2 nucleolar m.n. A third nucleolus in the main nucleus.

(*d*) 72 hrs. : Active main nucleus at prophase. Inert nucleolar m.n. deficient in heterochromatin.

(*e*) 72 hrs. : Main nucleus and nucleolar m.n. synchronised at prophase.

(*f*) 96 hrs. : Main nucleus (deficient in nucleolar organisers) synchronised with 2 nucleolar m.n. × 1300.

Even allowing for the possibility of fusion, the largest block must form one of the most prominent chromocentres in the resting nucleus. Yet, during resting stage 24 hours after treatment, only 44·4 per cent. of nucleolar micronuclei contained chromocentres which appeared to be of comparable size.

This may represent the proportion which remains viable. For, later on, of the 30·8 per cent. which were inert at X_n prophase, only 2 contained such large chromocentres; 10 had small to medium-sized chromocentres and 4 had none at all. Evidently, heterochromatin was the balancing factor for viability.

However, one inert micronucleus contained a nucleolus, 3 large and 3 medium-sized chromocentres. It apparently had all the requirements for activity—adequate heterochromatin and at least one nucleolar organiser—and yet was inert. Possibly, a non-nucleolar acentric, carrying the extra heterochromatin, had been included along with the nucleolar acentric. If this was the case, it would then appear that for activity, the nucleolar acentric must carry its own requirements of heterochromatin. The presence of a small amount near the nucleolar organiser which I previously inferred from breakage

TABLE 3

Localisation of the total breakage and reunion occurring up to 28 days after MH treatment in cell generations after X_1. High R′ values are due to the reunion of broken ends from X_1 with deferred B′-s (see fig. 8). As at X_1, unreunited ends are rare. Reproduced in graph 5

| Breakage segments (centrics)	B″	SR		B′	R′
		C_1	C_0		
2	5	*5*	*5*	2	4
7	...	...	...	2	2
8	1	*1*	*1*	2	2
10	1	*1*	*1*	4	6
11	...	...	...	1	2
14	1	*1*	*1*	1	...
Acentrics : all nucleolar					
Median . . .	9	8	8	2	4
Sub-median . .	1	1	1	...	...

in segment *1* is apparently not enough. Although short acentrics derived from breakage in this segment can occasionally reproduce, I think they do so only after inclusion in the main nuclei.

Supporting evidence that heterochromatin is the decisive factor for the survival of nucleolar micronuclei, comes from the localisation and frequency of deferred breakage. This, as we shall see, occurs at X_2 and X_3 following MH treatment. Breakage in these cells is more frequent in the middle of the nucleolar iso-acentric than in segment *2* of the remaining normal M chromosomes (table 3, graph 5, fig. 5*b*). Breakage frequency seems to be roughly proportional to the size of heterochromatic segments as it is at X_1 (McLeish, 1952). Thus it follows that the segment in these nucleolar iso-acentrics must be as large, if not larger, than the segment in the M chromosomes.

It may be lengthened as a result of duplication by SR (fig. 2). This would also account for the long rod-like chromocentres which can sometimes be seen in micronuclei (fig. 4a).

These cells are, of course, a selected sample. The acentrics can reproduce because of their large content of heterochromatin, yet it is through this heterochromatin that they are more susceptible to breakage.

From their study of the B chromosomes in *Sorghum*, Darlington and Thomas (1941) have suggested that the effect of heterochromatin is to stimulate nuclear and hence cell division. Further, Caspersson *et al.* (see Caspersson, 1950) have shown that heterochromatin and
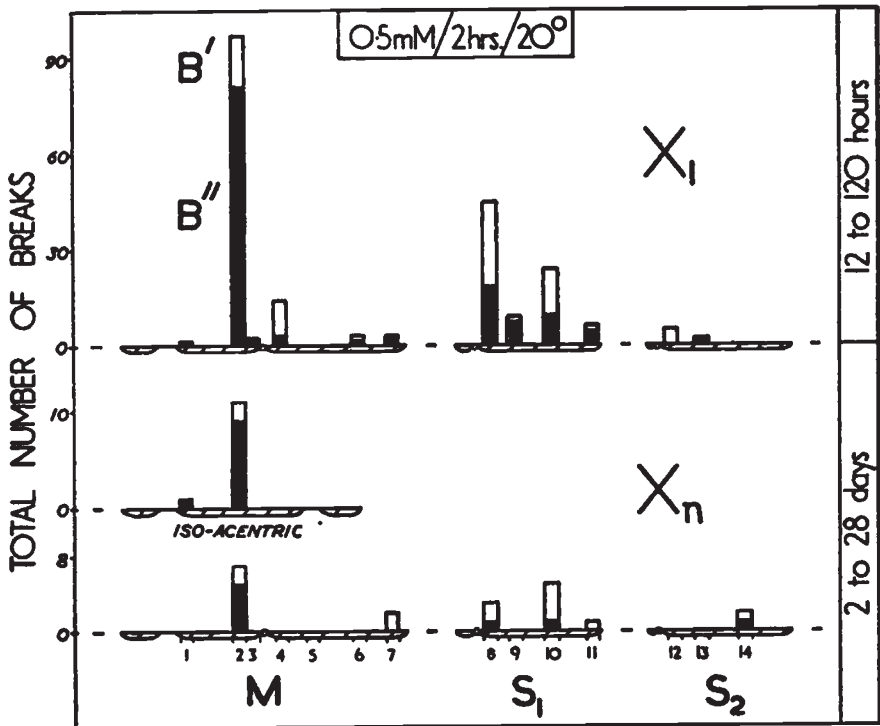
GRAPH 5.—Distribution of the segments (numbered *1* to *14* in the 3 chromosome types M, S_1 and S_2) in which primary breaks (at X_1) and deferred breaks (at X_n) occur after MH treatment. The change in the relative breakage frequencies in nucleolar and non-nucleolar chromosome arms in X_n cells is not significant. From table 3 and McLeish (1952), table 4.

nucleoli are chemically inter-related ; they appear to share similar functions in the protein and nucleic acid metabolism of the cell. The present results confirm the importance of their physiological relationship for chromosome reproduction and the working existence of the nucleus.

Nuclear co-operation.—The studies of Barber (1941) in *Uvularia* have shown how complementary and physiologically active resting nuclei can co-operate to make a balanced system in the cell.

In *Vicia* the close association of a main nucleus with a nucleolar micronucleus often gives us an indication of their co-operation and of their impending synchronisation in mitosis. Non-nucleolar types seem never to be associated or synchronised with main nuclei in this way.

In 14·5 per cent. of cases, the nucleolar micronucleus is partially enveloped by the main nucleus (fig. 4b). Yet the two remain distinct with a clear dividing zone and do not fuse. This must be due to the fact that nuclei are bounded by real membranes and not merely

FIG. 5 (*a*) to (*d*).—Metaphase cells derived from the X_1 generation following MH treatment. Showing fragment chromosomes (black) and their homologues (stippled where recognisable).

    (*a*) Cell deficient in the nucleolar arm of one M chromosome. The other M and two S_1 chromosomes show partial breakage.

    (*b*) X_2 cell with reproduced nucleolar iso-centric with deferred B″+SR.

    (*c*) Deferred breakage and reunion, involving an M chromosome and a nucleolar acentric, giving a triradial.

    (*d*) An $S_1 : S_2$ triradial. No fragment present. × 1250.

surfaces (Callan and Tomlin, 1950; Bovey—see Wilkins, 1951). Dowrick (1954) has seen a similar situation in pollen grains of the diploid pear, Beurré Bedford. They contain groups of nuclei which remain closely associated but never fuse until mitosis when the membranes have gone.

In *Vicia* the close nature of the relationship between main nucleus and micronucleus is shown by the way they react to the loss or gain of nucleolar organisers. Evidently, the loss of one organiser from the nucleus and the cell does not impede mitosis since I have observed

29 metaphases deficient in the nucleolar arm of one M chromosome (fig. 5a). But if both organisers are lost, it seems that no further mitosis can occur. If, however, both organisers are lost from the nucleus but included in the cell in micronuclei, a balanced system can result. Then the main nucleus is helped along by its two accessory nuclei and all three co-operate during the resting stage to achieve synchronisation at mitosis (fig. 4f).

This raises a further question. Why is it that a balanced micronucleus can help a deficient main nucleus and yet a balanced main nucleus cannot help a deficient micronucleus? The answer may lie in the fact that these micronuclei differ from main nuclei in one constant respect : they never contain centromeres.

We know that apart from its well-known function on the mitotic spindle, the centromere can be distinguished by its earlier behaviour inside the nucleus. It is known to influence the localisation of X-ray and ultra-violet radiation breakage as well as the frequency of sister reunion (Sax and Mather, 1939 ; Swanson, 1942 ; Darlington and Upcott, 1941 ; Darlington and La Cour, 1945). In *Scilla*, Rees (1952) has shown how it interferes with X-ray-induced errors of chromosome separation at anaphase.

In the absence of nucleolar organisers and detectable heterochromatin, centric chromosomes can form viable micronuclei in *Uvularia* (Barber, loc. cit.). Here, as in the case just described in *Vicia*, nucleolar organisers were present in the accompanying nucleus.

Thus the absence of heterochromatin or nucleolar organisers or both reveals that the centromere can play a part in maintaining the viability of a nucleus. This nucleus is then capable of co-operating in development with one or more complementary nuclei.

6. REPRODUCING ACENTRICS AS CYTOLOGICAL MARKERS

After MH treatment, some acentric fragments survive and reproduce through successive mitoses in viable micronuclei as can all types by inclusion in main nuclei. They all provide markers for timing the deferred breakage and reunion which continues to arise from the persistent effects of initial treatment.

Nucleolar acentrics were originally reported as reproducing in *Vicia* by Levan and Lotfy (1951). They occur frequently in the present experiments and provide the most reliable markers (plate, fig. 6). Occasionally they can be studied in X_2 and X_3 prophase micronuclei. They appear to be able to reproduce through two successive mitoses ; an original acentric chromatid gives four chromatids in two pairs (fig. 6). The paired chromatids often show relational coiling. At X_2 anaphase they rarely fall far apart and are both included in one of the daughter cells. Thus a further reproduction is an accurate indication of X_3.

I have no evidence of their ability to undergo a further reproduction

at X_4. By this time, the cells may have become unbalanced. Many will also have passed into differentiated tissue. In either case, they will not undergo mitosis again.

7. DEFERRED BREAKAGE

Initial treatment with MH causes breakage at X_1 and in succeeding cell generations. It occurs at X_2 and X_3 with similar type and distribution as at X_1 but with diminishing frequency (tables 3 and 4 ; graph 5, fig. 6). It occurs in hitherto undamaged chromosomes, in centric fragment chromosomes and in acentric chromosomes which have survived either by inclusion in main nuclei or through viable

TABLE 4

Frequencies of cells in secondary lateral roots of Vicia faba, *showing primary and deferred breaks at different times after MH treatment. Some cells can be timed in relation to the initial breakage (see text). The history of normal cells cannot be determined since they are unmarked*

Time	Cells	Normal	X_1	Broken					
				X_2		X_3		Untimed X_n	
				Total	Rebroken	Total	Rebroken	Total	Rebroken
2 D	200	125	72	3	...	...	...	...	...
3 D	315	219	61	15	*3*	...	...	20	*1*
4 D	266	164	35	34	7	4	*2*	29	*4*
5 D	63	46	4	8	*1*	...	...	5	*2*
6 D	65	50	2	5	*1*	2	*1*	6	*2*
10 D	28	19	1	3	*1*	...	...	5	*1*
19 D	18	12	...	3	*2*	1	...	2	*1*
28 D	30	24	2	2	...	...	...	2	...

micronuclei (figs. 5 and 6). Furthermore, X_1 and X_2 breakage reappears in newly formed tertiary roots 28 days after treatment.

Deferred breakage may be the later expression of the partial breakage seen at X_1. Particularly if, as Darlington (1950) suggests, the chromosome has a multiple fibre structure. For breakage occurring in a few of these sub-units would not become effective in the whole chromosome until they had been sorted out in successive mitoses.

An alternative and more likely explanation is that the reagent persists in the cells for a long period, where it continues to induce its characteristic action in the chromosomes. In view of its molecular structure, which has been discussed by Kihlman (1951), Loveless (1952) and others, it could conceivably be incorporated in the cell in some way to form a substitution nucleic acid precursor.

A further indication of its continued presence is its permanent effect on the size of the meristematic zone. This is gradually reduced

in length during the first 3 days after treatment and often remains so for at least 28 days. Within this zone, however, mitosis continues at an approximately normal level. The effect on mitosis is probably,
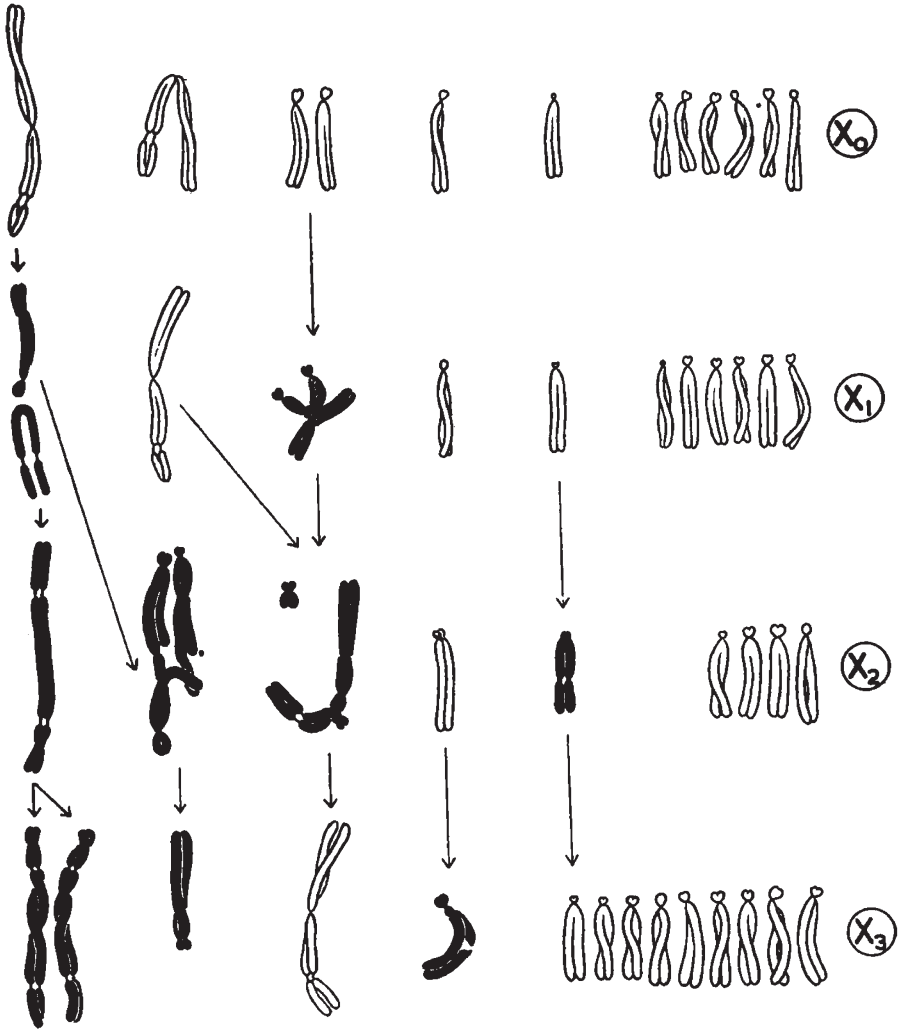

FIG. 6.—Metaphase chromosomes observed in a normal cell (X_0) and in 3 consecutive cell generations (X_1 to X_3) after MH treatment. Illustrating some of the possible ways in which configurations formed at one mitosis can influence those formed at the next. X_2 and X_3 are timed in relation to X_1 by the number of reproductions of the nucleolar iso-acentric (left).

X_1, 48 hrs.: B″ SR in segment 2 giving a nucleolar iso-acentric. $S_1 : S_1$ interchange forming a C'_2.

X_2, 96 hrs.: fragment chromosomes from X_1 reunited with deferred breaks. Partial break in an S_2 chromosome.

X_3, 96 hrs.: Deferred B′ in an S_1 chromosome. × 1250.

as I pointed out previously, something different from the chromosome breaking effect. It could possibly be correlated with the findings of Leopold and Klein (1951) that MH is an anti-auxin.

8. SECONDARY REUNION

The chief consequences of X_1 anaphase separation, after MH treatment, are shown in fig. 8. Dicentric chromatid bridges resulting from SR or R' break at random. The two daughter nuclei then receive equal or unequal fragment chromosomes whose broken ends can behave in one of three ways :

(i) They can undergo R'' with other broken ends to give dicentrics (fig. 7b). This occurs in a restricted period of movement during telophase which has been defined by La Cour (1952).

(ii) If R'' does not occur before the initiation of the X_2 mitosis, the broken ends then have the opportunity to undergo secondary or delayed SR (fig. 7a) as in the breakage-fusion-bridge cycle of McClintock (1941). In *Vicia* after MH treatment, as in *Hyacinthus* after X-raying (La Cour, 1952), this is rare.

a   *b*
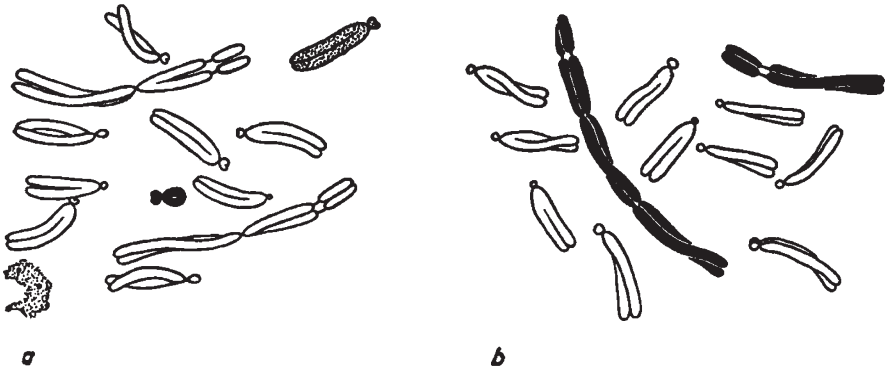
FIG. 7 (a) and (b).—Secondary reunion in X_2 metaphase cells of *Vicia* 96 hrs. after MH treatment.

(a) SR in an S_1 centric fragment ; remainder of chromosome present as a micro-nucleus. Possible undamaged S_1 homologue stippled.

(b) C_2'' derived from non-homologous R'' between the broken ends of two M fragment chromosomes carried through from X_1. Nucleolar Co'' formed by reproduction of an original Co'. × 1250.

(iii) Finally, the residue of broken ends failing to undergo either R'' or SR, remain open for a sufficient time to reunite with centric and acentric fragments which have suffered deferred breakage. Such reunion of new and old ends accounts for the common occurrence, at X_2, of triradials (fig. 5c and d).

This sequence of events shows SR to be a different type of reunion from either R'' or R'. For, although the separate sister chromatid ends remain capable of reunion after the split, they do not seem to reunite with one another. Thus, it is easier to look upon SR as resulting earlier from failure of chromosome reproduction rather than later from a real reunion of sister chromatids.

An accurate quantitative assessment of these various types of secondary reunion is impossible for the following reasons :

(i) Dicentric chromosomes seen at X_2 need not be the result of

R". They can also arise by the subsequent duplication of complete dicentric *chromatids* which passed complete to one pole at X_1 (fig. 8). (ii) The absence of SR in fragment chromosomes at X_2 is not necessarily an indication of its failure. Such small chromosomes can arise from chromatid translocation at X_1. Their breakage and reunion points are interstitial and consequently hidden and sealed against further reunion. (iii) In a somewhat similar way, the high frequency of
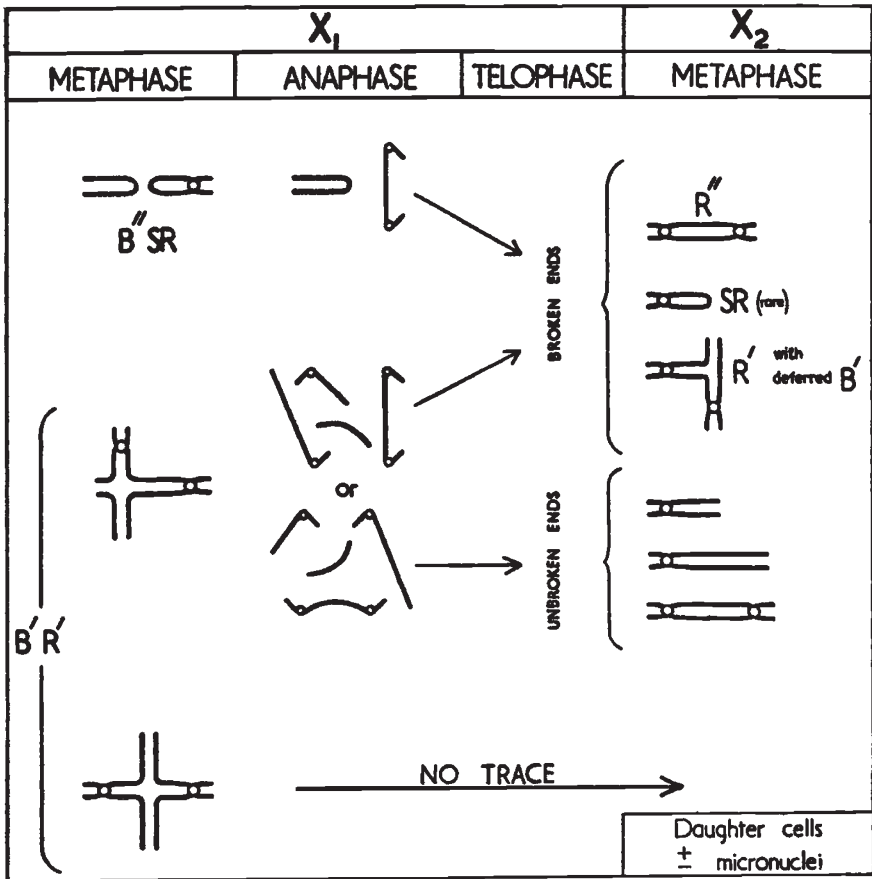

Fig. 8.—Typical X_1 metaphase configurations seen after MH treatment and the chief consequences of their separations at anaphase. Derived X_2 daughter cells may or may not contain micronuclei.

reunion in acentrics at X_1 leaves few with broken ends (McLeish, 1952). Thus even if they are included in main nuclei, they are seldom involved in secondary reunion unless re-broken.

9. SUMMARY

1. Treatments with maleic hydrazide (MH) and 8-ethoxycaffeine (EOC) lead to specific and different patterns of chromosome breakage and reunion in *Vicia faba*. Standard types of centric and acentric fragments therefore enter into the main nuclei and the micronuclei.

2. Acentrics and micronuclei can thus be classified into four types according to their combinations of euchromatin, heterochromatin and nucleolar organisers.

3. Nucleoli are organised in some micronuclei. As in the main nuclei, they appear to be formed on a specific chromosome segment, by an organiser at the *proximal* end of the secondary constriction. Their size is influenced by competition between their organisers and by the size of the nuclei.

4. Viable micronuclei have to carry both nucleolar organisers and heterochromatin. They co-operate with main nuclei during interphase and become synchronised with them during prophase. The nuclei, which have definite membranes, are often closely associated, but they rarely, if ever, fuse.

5. When included in main nuclei, all types of acentric can reproduce in successive mitoses (X_2, X_3). Nucleolar types can also do so in micronuclei. They thus mark the number of the mitosis and show when deferred breakage and secondary reunion are taking place.

6. The regular pattern of breakage seen at X_1 after MH treatment can thus be shown to be repeated in X_2 and X_3 cells for at least 28 days.

7. Broken ends in centrics and acentrics are carried through from X_1 and X_2. They can then reunite with contemporary, as well as with deferred, breaks. Moreover the reunions are of new kinds : at X_1-SR but rare R″ ; at X_2-R″, but rare SR.

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FIG. 1.—X_1 anaphase after EOC. Breakage and reunion (probably B″SR) has occurred in the nucleolar constriction, part of which is now visible in the centre of the C_2' bridge and part in the centre of the Co′.

FIGS. 2 to 5.—Reproduction of a balanced acentric after MH breakage.

    2. X_1 anaphase. Breakage and sister reunion (B″SR) in segment *2* has given a nucleolar iso-acentric and a C_2'. The undamaged sister M chromosomes have passed to the poles.

    3. Daughter cell arising from a mitosis as shown in fig. 2. Nucleoli organised by the undamaged M chromosome in the main nucleus and by the nucleolar iso-acentric in the micronucleus.

    4. Synchronisation of main nucleus and micronucleus at X_2 prophase.

    5. Early anaphase of X_2. The original Co′ is now a Co″.

FIGS. 6 and 7.—X_2 metaphases after MH.

FIG. 6.—B″SR probably occurred in segment *2* of both M chromosomes at X_1. Both the resulting nucleolar iso-acentrics have reproduced (at 3 and 5 o'clock) ; one shows a median deferred break. The centric fragments have undergone R″ to give a C_2''.

FIG. 7.—A non-nucleolar acentric (9 o'clock) derived from breakage in an S_1 chromosome at X_1 has reproduced by inclusion in a main nucleus. The centric fragment is lying in the centre of the cell.

All figures : × 1800.

1.

2.

3.

4.

5.

6.

7.
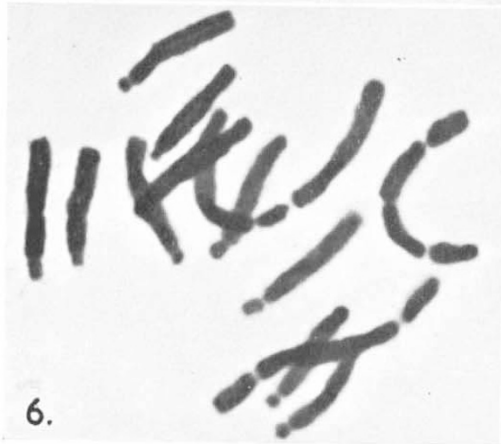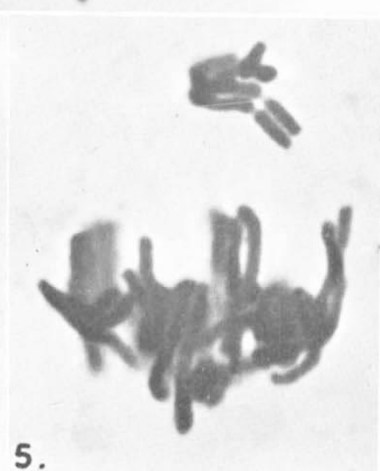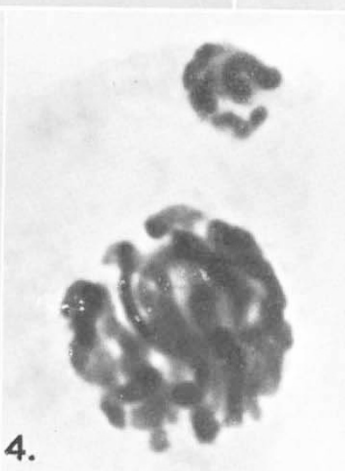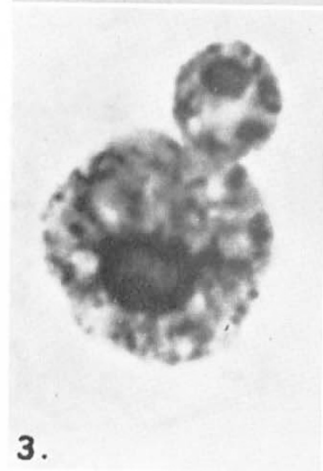
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