X-RAY FRACTIONATION AND CHROMOSOME
BREAKAGE

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I. INTRODUCTION

THE study of dose fractionation in relation to the biological effect of ionising radiation, apart from its direct application to radiotherapy, has played a considerable part in the investigation of the mode of action of the radiation. It has played this part together with the study of dose effect, of intensity effect and of the relative effectiveness of different wave-lengths and types of radiation. Thus certain effects, such as the inactivation of viruses, the killing of bacteria and the production of gene mutation in *Drosophila* have been interpreted as being due to single ionisations within the region known as the "target", rather than to a cumulative effect of radiation. A full treatment of the data and arguments upon which this conclusion is based, has been given by Lea (1946). Briefly, the recognition of this type of action is based upon survival curves showing the effect to be proportional to dose (densely ionising radiations being less effective), and upon the effect for a given dose being independent of intensity and the manner in which the dose is fractionated so long as the sensitivity of the material to the radiation remains constant during the period over which the dose is spread.

Not unnaturally the same method of approach, based on study of the dependence of the effect on dose, type of radiation, intensity

and mode of fractionation, has been applied in the investigation of the mode of action of ionising radiation in causing chromosome breakage. But with chromosome breakage, investigation of the quantitative relationship between ionisation and effect is obviously far more complicated, and the full extent of its complexity has not been generally realised. The complexity of the problem arises from the fact that the chromosomes are part of a physiological system, the cell, which undergoes cyclic changes affecting their sensitivity to breakage by ionising radiations. This developmental cycle is affected by the environment which includes the radiation itself.

The study of chromosome breakage is, moreover, complicated by the fact that it can be detected and analysed only after a period of development of a few hours to many days according to the material. During this time restitution and various types of reunion have occurred, so that only a proportion of the original breaks can be detected either as breaks or more complex structural changes. Hence, in the study of chromosome breakage, by examination at a later period of development, a correct interpretation of the changes which have taken place between irradiation and observation, and a knowledge of the factors influencing these changes is essential for a correct estimate of the initial effects of the radiation.

The most extensively used materials for the quantitative study of radiation-induced chromosome breakage have been *Drosophila* sperm and *Tradescantia* pollen-grains. The methods used in the study of breakage in *Drosophila*, although they have the advantage that the material at the time of irradiation is in a relatively stable and uniform stage of development, have the limitation that many divisions occur between irradiation of the males and observation of the results in the salivary gland chromosomes of larvæ resulting from mating of the treated flies with normal females. Consequently many of the structural changes resulting from irradiation are inevitably lost, owing to inviability of the cells containing them. Only certain types of interchange, inversions and minute deletions unaccompanied by inviable changes can be observed, and anything approaching a complete analysis is impossible. It is therefore not surprising that data on the relation between dose, intensity, manner of fractionation and the resulting changes in *Drosophila* show a lack of consistency.

The method initiated by Sax (1938) and subsequently used by many other workers, for the quantitative study of breakage in developing pollen-grains of *Tradescantia* does not possess the same limitation. The pollen grains are examined at the metaphase and anaphase following irradiation so that all cells survive for examination. None-the-less the period of time elapsing between irradiation and observation necessitates a design of experiment and a method of analysis which take into consideration as fully and correctly as possible all that takes place during this period. Moreover, as the stage at which the pollen grains are irradiated can be deduced only from the

time elapsing between irradiation and fixation, great care must be exercised to ensure that the design of experiments allows for any factors, whether environmental (including the radiation itself) or internal, which may influence the rate of development. Differences in the rate of development are important because they may lead, particularly when development is rapid at the time of irradiation, to significant differences in the stage of development at which different anthers or pollen grains are irradiated, with the same time interval between irradiation and fixation. Moreover, there is considerable evidence that the rate of development itself affects the sensitivity to radiation.

The full significance of these complications, which arise from the use of developing biological material for quantitative experiments in radiation breakage, has not generally been recognised. This was first pointed out by Darlington and La Cour (1945) and subsequently Koller (1946) and Darlington and Koller (1947) have drawn attention to the danger of accepting, without due caution, theoretical deductions based on experiments in which the above complications have not been fully taken into account. Darlington and La Cour (1945) expressed the view that in quantitative experiments on X-ray breakage certain rules must be followed; briefly, that the cell cycle must be studied at close stages, that temperature and other conditions must be controlled, that doses must be low and analysis must be as complete as possible, breakage and reunion being recorded separately. These rules have particular significance for studies of the time factor, *i.e.* intensity and fractionation effect.

Study of breakage at stages throughout the mitotic cycle in *Tradescantia* pollen grains (Sax and Swanson, 1941; Darlington and La Cour, 1945; Catcheside and Lea, *see* Catcheside, 1948) shows that the breakage frequency changes sharply, peak frequencies occurring at certain periods, the time of the peaks in relation to metaphase varying according to the conditions of growth and the dose administered. Sax and Swanson, using uncontrolled temperatures during the period of development considered that the most sensitive period is shortly before mid-prophase; Darlington and La Cour, on the other hand, using controlled temperatures concluded that the peaks occur at the end of the resting stage. Whatever the stage in the mitotic cycle at which the peaks occur, the data of all authors show that the sensitivity is rapidly changing at short periods of 12-36 hours before fixation when chromatid breakage is prevalent. If a dose of radiation is spread over varying periods by altering the dosage rate or by fractionation, the dose may consequently cover different parts of the sloping sensitivity curve especially if the radiation is given at periods around 24 hours.

Koller (1946) in investigating the effects of dosage rate in *Tradescantia* pollen attached little value to data obtained from material fixed 24 hours after irradiation (with material kept at 23-24° C.).

The 24-hour samples showed excessive variation, which he attributed to relatively large effects of environmental variation at the period of chromosome division (when the cell is in an unstable state), and also to the effect of the radiation itself upon the time scale of development. Darlington and La Cour (1945) have, in fact, inferred that the duration of the development cycle (as shown by breakage frequency) is reduced when the dose is increased. Intensity also may have some effect although present data are insufficient (Catcheside 1948). Further, Darlington and La Cour (*loc. cit.*) produced evidence that, in *Allium* and *Trillium* roots, suppression of mitosis occurs depending on duration and amount of the dose; Koller (1946) also reported mitosis suppression with doses of 200 r. given at 50 r./min. and 5 r./min. but not at lower intensities. Clearly the effect of radiation on the rate of development of the pollen grains is not a simple one; it depends on the stage of development at which the radiation is given as well as on the dose and possibly the intensity.

From the above considerations it follows that experiments using relatively short time periods with *Tradescantia* pollen to investigate the effect of varying intensity and fractionation are of little value. In the experiments of Sax (1939, 1940, 1941) and Fabergé (1940), designed to investigate the effect of fractionation of X-ray dose with *Tradescantia* pollen, a reduction in the frequency of certain types of aberration with fractionation of the dose was demonstrated. Considerable weight was attached by Sax to observations of chromatid breakage (at periods of about 24 hours after irradiation). For example, the conclusion that the frequency of simple breaks is unaffected by fractionation was based entirely on observations of chromatid breakage.

Further, Sax made no mention of temperature control in the period between irradiation and fixation. Omission of temperature control during development particularly vitiates the results in the case of chromatid breakage because, for reasons stated above, any variation in the rate of development will have particularly far-reaching consequences when the time interval between irradiation and fixation is short.

Again, the methods of scoring and analysis used by both Sax and Fabergé in their fractionation experiments are open to the objection that they are incomplete and do not permit an analysis of breakage and reunion. Fabergé (*loc. cit.*) used relatively high doses of 1320, 2640 and 3960 r. and consequently anything approaching a complete analysis was impossible. His method of scoring was to count the total number of chromosome bodies in each cell. Sax, for chromosome breakage, scored only centric ring and dicentric chromosomes at metaphase and anaphase. All acentric fragments were ignored. Chromatid breakage was scored as "2-hit" or "1-hit" breaks, according to whether the aberration involved reunion or not.

The data from these early experiments still constitute the only source of our knowledge of the effect of fractionation of X-ray dose

on breakage in *Tradescantia*. Their interpretation, moreover, still forms an important part of the basis for certain theories concerning reunion, restitution and sister reunion, which have far-reaching implications for the interpretation of the observed chromosome changes in terms of the target theory of radiation action (see Gray, 1944 ; Lea, 1946 ; Catchside, 1945, 1948).

In view of the deficiencies in design and inference inherent in this earlier work, the desirability of further experiment in order to obtain more critical information is obvious. The experiments described below have been designed and carried out with this in view.

2. EXPERIMENTAL METHOD

(i) General Plan of the Experiments

The experiments, which cover three growing seasons (1947, 1948 and 1949), were planned to investigate quantitatively the effect on

TABLE 1

Code letters for doses and time intervals used. In all cases where the dose was divided into fractions the time interval between X-raying and fixation was measured from the first dose

Experiment	Date of Irradiation	Batch of Material	Dose and fractionation	Time between dose and fixation of anthers
" R "	19/6/47	A	100 r.—4 hrs.—100 r.	48 hrs.
	12/6/47	B	200 r.	48 hrs.
	19/6/47	C	100 r.	48 hrs.
" S "	27/5/48	A	180 r.—4 hrs.—180 r.	48 hrs.
	"	B	360 r.	48 hrs.
	"	AA	180 r.—4 hrs.—180 r.	72 hrs.
	"	BB	360 r.	72 hrs.
" T "	18/6/48	A	180 r.—4 hrs.—180 r.	48 hrs.
	"	B	360 r.	48 hrs.
	"	AA	180 r.—4 hrs.—180 r.	96 hrs.
	"	BB	360 r.	96 hrs.
	"	3B	360 r.	144 hrs.
" U "	15/6/49	B	360 r.	72 hrs.
	"	E	180 r.—1 hr.—180 r.	"
	"	D	180 r.—2 hrs.—180 r.	"
	"	A	180 r.—4 hrs.—180 r.	"
	"	F	180 r.—8 hrs.—180 r.	"
	"	C	180 r.	"
" W "	17/6/49	B	360 r.	"
	"	E	180 r.—1 hr.—180 r.	"
	"	G	180 r.—6 hrs.—180 r.	"

chromosome breakage in the developing pollen grains of *Tradescantia*, of dividing a given dose into two fractions separated by a period of a few hours. The experiments carried out in the first two years were

designed not only to obtain data on the effect of separating fractions by four hours, but in addition, to obtain information on the following important matters : (a) the appropriate dose which will give sufficient breakage and yet allow a complete analysis, (b) suitable conditions of growth between irradiation and fixation of the material, and methods of controlling these conditions, (c) the most suitable time period to allow between irradiation and fixation, bearing in mind the importance of avoiding irradiation at a time of rapid development and change of sensitivity. The final experiments (1949) were designed to explore the effect of varying the time interval between two fractions.

(ii) Material and Conditions of Growth

In all the experiments the material used was clone 20' of *Tradescantia bracteata* obtained from the John Innes Horticultural Institution. This material was selected because the pollen grains have only six chromosomes which are large and technically suitable for detailed cytological analysis and contain no centric fragments. The plants of the clone, which is self-sterile, can easily be distinguished from seedlings resulting from cross-pollination, by the light colour of the flowers. The main disadvantage of the clone is its lack of vigour in all but the most suitable conditions.

Young shoots with no mature flowers were cut on the day before irradiation and placed in a chamber with the temperature controlled at about 23° C., the actual temperature throughout the experiment being recorded. In 1947 the temperature in the growth chamber dropped at times from 23° C. to 20° C., but in the later experiments the method of control was improved, enabling temperature to be strictly controlled between 23° C. and 24° C.

Young shoots were always used because it was found that they retained their vigour as cut shoots more readily for several days, and were more likely than older shoots to possess buds at the required stage of development at the time of fixation. Five or six shoots were removed from the temperature control chamber for each X-raying and were replaced in temperature control between irradiation and fixation and, in the case of fractionated doses, between the fractions. The irradiation was carried out at room temperature which in no case varied more than 1° C. from 18° C. A short period without temperature control occurred immediately before and after irradiation while the material was conveyed from the growth chamber to the X-ray room. Tests were made for temperature variation during these periods and showed that no great fluctuation occurred.

It was found that conditions of illumination are an important factor in the survival in good condition of the pollen grains over a period of several days. After the third day in very weak illumination the anthers tended to dry up and the pollen grains to abort. In 1948 the material was illuminated in the growth chamber by an 80-watt fluorescent tube and for the later experiments a lighting unit was constructed with six 40-watt fluorescent tubes which could be arranged against the glass of the growth chamber. The heat given out by these tubes complicated temperature control, but the difficulty was overcome by using an electric fan cooling system. Under these conditions the shoots and pollen grains could be maintained in good condition for many days.

Owing to climatic variation during the three years, the plants from which material for the different experiments was taken were not grown under uniform conditions. This fact, and the variation in the controlled conditions from year to year, may account for the difference in breakage frequency observed in different years. As Koller (1946) has pointed out, a "standard breakage frequency" does not exist. Quantitative comparisons of irradiation effects produced in different years are thus of little value without adequate controls. In all cases variations in the controlled

conditions are recorded, and where comparisons are made between material irradiated on different days, with periods of time intervening in which the conditions of growth of the plants may have changed, controls were used with the same dose to ensure comparability. The only exception to this was the comparison made in 1947 between the results of two irradiations carried out a week apart.

(iii) *Cytological Technique*

At the time for fixation, buds were tested for stage of development by removing one anther and squashing in acetic lacmoid. If the frequency of metaphase stages was fairly high, the other five anthers were immediately smeared on one slide and fixed in one part acetic acid with three parts absolute alcohol, freshly mixed for each batch of three slides. After 20-30 minutes in the fixative the slides were transferred to 95 per cent. alcohol and left overnight, before staining by the Feulgen method. Slides were partially flattened before mounting, using the method of La Cour (1947).

(iv) *X-raying*

In all experiments the X-ray treatment was given at 135 kv. and 5 mA. with focal distance of 50 cm. (average) using an applicator of 10 cm. diameter circle. Filtration of 1.0 mm. aluminium gave a half-value layer for the radiation of 0.07 mm. copper. The dose was calculated but checked periodically with a dosimeter. The dosage rate was constant for each experiment, but varied from 25.5 to 29.5 r. per minute for the different experiments.

(v) *Method of analysis and scoring*

In sampling the slides, the first suitable anther smears encountered in an examination of the batch from each irradiation, were chosen for analysis, the criteria of suitability being a sufficient number of metaphase stages and a relatively early developmental stage as deduced from the proportion of binucleate cells. Anthers with a percentage of binucleate cells above 40 were rejected, and in most cases the percentage in those analysed was between 10 and 30. The importance of taking the developmental stage into consideration lies in the fact that the developmental rate and hence the sensitivity of the pollen grains as they approach mitosis is dependent upon the developmental stage of the anther, owing to convergent development within the anther (*see* Darlington and La Cour, 1945). Where possible, two anthers from separate slides were chosen for analysis from each irradiation and recorded separately as a check on variability between anthers and buds. As can be seen from the tables of breakage frequency, the variability between the two anthers is usually remarkably small.

In cell sampling, in order to avoid unconscious choice of metaphases for analysis, every metaphase in polar view, coming into the field as the smear was traversed under oil immersion objective, was analysed until the required number was reached.

The method of scoring is based on that elaborated by Darlington and Upcott (1941) and Darlington and La Cour (1945). This method enables the various events occurring from the time of irradiation to observation of the resulting chromosome configurations to be

separated, as far as possible, and is considered to be the most complete and consistent in observation and inference.

Metaphases only were included in the analysis, as the inclusion of anaphases introduces additional difficulties of interpretation. For example, the configuration illustrated in plate II, fig. 5, a dicentric ring at anaphase, could result from an earlier centric ring or a centric fragment with sister reunion at both ends (*see* Darlington and Upcott, 1941). Similarly, the odd dicentric in plate II, fig. 7 could result either from sister reunion following a single distal break, or from chromatid union following distal breaks in two separate chromosomes accompanied by reunion of acentrics. The terminology of chromosome structures and changes is that given in the appendix and glossary of Darlington and Mather (1949).

Certain difficulties of interpretation were encountered, some inherent and some due to circumstances such as the position of the chromosomes or their degree of contraction. For example, fig. 2 shows a configuration similar to fig. 18, 48 h. of Darlington and La Cour (1945), a ring surrounding another chromosome. Several such figures were observed; they were interpreted as a centric ring surrounding an unbroken chromosome when an undoubted acentric chromosome fragment of the appropriate size occurred in the same cell.

In a few of the anthers analysed, dwarf pollen grains of the type described by La Cour (1949) were observed in mitosis in small numbers. The proportion in metaphase or anaphase was always very low because the anthers were chosen with early developmental stage, mitosis in these dwarf pollen grains usually beginning at about the time it ceases in the normal grains. These anomalous pollen grains were not included in the analysis and were easily recognisable by the abnormal orientation of the spindle. Plate II, fig. 10 shows interlocked dicentric chromosomes at anaphase in a dwarf pollen grain; the longitudinal orientation of the spindle can be seen in contrast to the normal orientation in plate II, fig. 8, which shows the same configuration in a normal pollen grain.

Occasionally pollen grains were observed with abnormal chromosome numbers. The most common were grains with a diploid nucleus, two haploid nuclei and grains incompletely separated in sporogenesis. These were rejected in analysis and the anthers bearing them in any numbers were rejected. They are probably due to abnormal conditions such as drought or sudden temperature change during meiosis. Various types are shown in plate III, together with normal grains.

The most frequent type of radiation-induced chromosome abnormalities observed were (a) paired more or less spherical acentric fragments of various sizes (figs. 7, 8, etc., plate I, fig. 7), (b) dicentric chromosomes accompanied by acentric fragments (fig. 7 and plate I, fig. 3), (c) centric rings accompanied by acentric fragments (figs. 4, 9, 11, etc.). Sometimes a single cell contained more than one dicentric (fig. 5), more than one centric ring (fig. 2), a ring and a dicentric

FIGS. 1-8.—Pollen grains of *Tradescantia bracteata* in metaphase after X-raying. Dose, time after X-raying, time between fractions, symbols of inferred structural change and scoring of breakage and reunion are given for each figure :—

- FIG. 1.—360 r., 72 hrs., 1 hr., $C_1r + C_0$, ($B'' \cdot R''$).
 FIG. 2.—360 r., 72 hrs., 4 hrs., $2C_1r + C_2 + 3C_0$, ($3B'' \cdot 3R''$).
 FIG. 3.—360 r., 72 hrs., 1 hr., $C_1r + C_2 + 2C_0 + 2m$, ($2B'' \cdot 2R'' \cdot 2mB''$).
 FIG. 4.—360 r., 72 hrs., 4 hrs., $C_1r + C_0$, ($B'' \cdot R''$).
 FIG. 5.—360 r., 72 hrs., — $3C_2 + 3C_0 + m$, ($3B'' \cdot 3R'' \cdot mB''$).
 FIG. 6.—360 r., 48 hrs., 4 hrs., $C_2 + C_0 + m$, ($B'' \cdot R'' \cdot mB''$).
 FIG. 7.—360 r., 72 hrs., — $C_2 + C_0 + m$, ($B'' \cdot R'' \cdot mB''$).
 FIG. 8.—360 r., 72 hrs., — $2m$, ($2mB''$).

(fig. 3) or a tricentric chromosome (fig. 10 and plate I, fig. 4). Usually only one acentric accompanied each dicentric or centric ring chromosome, indicating that reunion of centrics is usually accompanied by reunion of acentrics. Where the number of acentrics in a cell exceeded one per dicentric chromosome or centric ring, those in excess of this number were usually small and spherical. Thus in fig. 6 and plate I, fig. 2, a dicentric is accompanied by one rod-shaped acentric chromosome fragment and one pair of spherical fragments.

Minutes. The interpretation of these small acentric fragments is one of the main difficulties in analysis of chromosome breakage and has been discussed in relation to *Tradescantia* by Rick (1940) and Darlington and La Cour (1945). The difficulty is that cytological examination cannot distinguish whether these small fragments are terminal deficiencies resulting from single breaks or whether they are intercalary deletions resulting from two breaks close together on the chromosome, followed by reunion of the terminal portions of the chromosome to form apparently unbroken chromosomes, and reunion of the ends of the deletion to form small acentric rings. Giles (1943) has pointed out that in some previous work deletions had not been distinguished from deficiencies and that this accounted for some of the discrepancies between different quantitative estimations of breakage in *Tradescantia*. Thoday (1942) scored approximately the same frequency of simple breaks as of dicentrics and rings five days after an X-ray dose of 100 r. and 200 r., whereas Sax (1939) ignored simple deficiencies because of their rarity at five days after irradiation. Considerable discrepancies have appeared between subsequent estimations of the proportion of acentrics considered to be simple deficiencies at three days after X-raying (*cf.* Darlington and La Cour, 1945 and Koller, 1946). It is possible that these discrepancies are due to different interpretation of the small acentrics.

Consideration of the distribution of breaks in the chromosome arms and of the proportion of small acentric fragments bearing satellites and of their frequency curves in relation to dose, led Rick (1940) to the conclusion that in *Tradescantia* such fragments are almost entirely of ring structure and of intercalary origin. Newcombe (1942) came to this conclusion on the grounds that they showed little, if any, sister reunion. From theoretical considerations, the same interpretation had previously been given to minute deletions in *Drosophila* by Muller (1940) and to very small acentrics in *Tradescantia* pollen by Sax (1938), the assumption being that the two breaks result from a single ionisation. Darlington and La Cour (1945) include under the term "minute" spherical acentric fragments less than the diameter of a chromatid and often visibly double. They deduce, on the basis of a statistical comparison with breakage and reunion involving major fragments, that the majority of minutes are the result of reunion following two independent breaks close together in the chromosome, and that they depend for their survival on reunion (to give rings)

FIGS. 9-16.—Pollen grains of *Tradescantia bracteata* in metaphase after X-raying. Dose, time after X-raying, time between fractions, symbols of inferred structural change and scoring of breakage and reunion are given for each figure :—

FIG. 9.—360 r., 72 hrs., — $C_1r + C_0 + m$, ($B'' . R'' . mB''$).

FIG. 10.—360 r., 72 hrs., 8 hrs., $C_3 + 2C_0$, ($2B'' . 2R''$).

FIG. 11.—180 r., 72 hrs., — $C_1r + C_0$, ($B'' . R''$).

FIG. 12.—360 r., 48 hrs., constriction considered as restitution.

FIG. 13.—360 r., 72 hrs., 1 hr., $C_0' + m$, ($B' . mB''$).

FIG. 14.—360 r., 72 hrs., — C_2 (triradial) + C_0 , ($2B'' . R'' . R' . C_0SR$).

FIG. 15.—360 r., 72 hrs., 4 hrs., C_0' , (B').

FIG. 16.—360 r., 72 hrs., 1 hr., $C_1r + C_0 + C_0'$, ($B'' . R'' . B'$).

in competition with restitution. This conclusion was arrived at earlier by Darlington and Upcott (1941) in regard to small acentrics arising by spontaneous breakage in *Tulipa*, on a number of grounds including comparisons of frequencies of acentrics of different sizes and the lack of sister reunion in them.

In the present experiments a considerable number of small acentric fragments were found, of about the diameter of the chromatid or sometimes slightly larger and usually visibly double (fig. 8). They have the appearance of "large minutes" but their size and contraction preclude the possibility of distinguishing by cytological examination whether they are simple terminal deficiencies or intercalary deletions of the ring type. In a few cases they are clearly the product of reunion of two small terminal acentrics, *i.e.* when a single small fragment accompanies an exceptionally long dicentric chromosome or a large centric ring (figs. 4, 7, Plate II, fig. 6). With regard to the rest, a small proportion of them may be short terminal deficiencies caused by breaks in the distal portions of chromosomes, but they are scored as minutes on the following grounds. Undoubted rod-shaped acentrics are very rare in the absence of dicentrics or centric rings in the same cell, and when they do occur, they and the corresponding centric fragment often show sister reunion (plate I, fig. 5, and plate II, fig. 9), whereas the small acentrics are almost always visibly double and sister reunion cannot have occurred. The occurrence of what can only be interpreted as small centric rings accompanied by acentrics not appreciably shorter than the total length of the chromosome (fig. 11 and plate I, fig. 8) shows that reunion of the minute type (to give fragments of about the size of the diameter of the chromosome) does occur; in fact small centric rings are much more frequent than large rings of the type shown in figs. 2 and 4. Moreover, it also demonstrates, as does the occurrence of considerable numbers of dicentrics with short segments between the centromeres (fig. 6 and plate I, fig. 6), that breakage is frequent in the proximal portion of the chromosomes. This, coupled with the rarity of the survival of simple proximal breaks to leave rod-shaped acentrics, suggests that simple deletion as a whole is rare and that breaks depend largely on reunion for their survival at fixation time of 48 hours or more after irradiation.

Additional evidence of the ring structure of the round fragments is obtained from study of prophase nuclei where obvious ring chromosomes of various sizes are common (plate II, figs. 1 and 2). The smaller ones are more common, two occurring occasionally in a single nucleus. Most of them are probably acentric rings giving rise to the commonly occurring round metaphase acentrics. Clear ring chromosomes of larger size are, however, much more common than at metaphase and it is assumed that most of them lose their obvious ring structure owing to the greater contraction at metaphase (figs. 9 and 16), only the largest showing clear ring structure as in figs. 2

and 4. It is concluded, therefore, that the vast majority of small paired spherical acentric fragments, of whatever size, are intercalary deletions surviving by virtue of reunion to form rings. They are recorded separately as minutes and scored as single *mB*".

Breakage survival. Another difficulty in the complete analysis of chromosome breakage as distinct from chromatid breakage is the fact that breaks in chromosomes as distinct from breaks in chromatids can disappear before metaphase, not only by restitution but by reunion. Thus reciprocal interchanges formed by symmetrical reunion of centric with acentric fragments can be detected only if the interchange is so unequal that it gives rise to chromosomes with appreciably abnormal short or long arms (fig. 3 and plate I, fig. 6). Such interchanges must consequently be neglected in direct scoring. Similarly, symmetrical chromosome reunion within chromosomes giving rise to inversions can never be detected at metaphase as can chromatid inversions. Reunion of two acentric fragments to form one, cannot be detected directly by cytological observation, though it can often be inferred from a complete analysis of the cell in which it occurs, and can be suspected in the absence of sister reunion.

Darlington and La Cour (1945) recognised this difficulty and dealt with it by the use of a correction. The number of chromosome breaks (*B*"') is in the first place estimated by counting acentrics and for certain purposes the correction $2R''C_1$ (twice the number of chromosome reunions in centrics) may be added to this estimate. Whereas this undoubtedly gives a truer statistical analysis of breakage, it does not solve the difficulty of classification of individual cells by number of breaks. The need for the above correction makes classification of cells by number of breaks of doubtful value (see Darlington and La Cour, 1945), as the correction, which is statistical, cannot be applied to individual cells.

A small number of apparent chromatid breaks were observed distributed throughout the samples. In some cases they appeared merely as very small unstained gaps in one of the chromatids (fig. 13), but occasionally as typical chromatid breaks with a considerable gap between the chromatid ends (fig. 15). In addition one or two triradial configurations were observed (fig. 14). These configurations are considered to be due to delayed restitution of one of the chromatids with or without reunion of the other, in both cases following chromosome breaks (see Darlington and Upcott, 1941; Darlington and La Cour, 1945, and Darlington and Koller, 1947). The occasional occurrence of a slight constriction in the unbroken chromatid, at the same locus as the break in the sister chromatid, confirms this interpretation (fig. 16). No other explanation would appear to account satisfactorily for their occurrence at 48 and 72 hours after irradiation at this temperature.

Occasionally, short unstained gaps, sometimes traversed by thin stained threads, were observed in both chromatids of a chromosome

(fig. 12) but they were not scored as B" unless the break was distinct and the fragments clearly separated and out of alignment (fig. 5). In the few cases where the two fragments remain aligned, but separated by a gap, they were scored as B", although it is probable that they are in reality cases of restitution.

3. EXPERIMENTAL RESULTS

First Series.—The results from the experimental work carried out in 1947 are summarised in table 2. They show that at two days after an X-ray dose of 200 r. the breakage frequency is considerably reduced when the dose is split up into two fractions separated by four hours. The frequency of acentric fragments (C_0) excluding

TABLE
*Analysis of breakage and reunion in Tradescantia bracteata
ture between X-ray and fixation approximately*

Code for batch and slide	Dose and time	Number of cells	B" (C_0'')	mB"	R" $C_1r + C_2$
R C 1, 6	100 r., 48 hrs. (continuous dose)	160	22	6	10
R A 6, 8	100 r., 48 hrs. } 100 r., 44 hrs. } (fractionated dose)	160	37	8	22
R B 1, 5	200 r., 48 hrs. (continuous dose)	160	60	18	38

"minutes" is reduced by approximately 40 per cent., of minute acentrics (m), by over 50 per cent., and of reunion of centrics ($R''C_1$) by 42 per cent. Total breakage, as measured by adding the correction $2R''C_1$ to the number of acentrics (*see* Darlington and La Cour, 1945) is reduced by 42.5 per cent. A very small number of chromatid breaks (B') were observed but are not recorded and are omitted in the estimation of breakage frequency.

Thus the general conclusion of Sax (1939, etc.) and Fabergé (1940) that continuous radiation is more effective than intermittent radiation in producing permanent breakage, is supported by this experiment. The question as to how far the further widely accepted conclusions regarding the cause of the different effectiveness (namely, the occurrence of healing or restitution (Sax) or "repair" (Fabergé) in the interval between fractions) are supported or refuted by the present experiments is discussed below in relation to the experiments as a whole.

Breakage frequency from the single half dose of 100 r. is approximately one-third of that from the full continuous dose. On the general assumption that breakage is initially proportional to dose, a more than linear relationship between surviving breakage and dose is to be expected if a proportion of breaks survive (to be seen) by virtue of reunion. As the number of initial breaks increases with dose, the greater number produced in each cell will have a disproportionately greater chance of survival in competition with restitution. The chance of two breaks occurring in close enough proximity for reunion will be greater. Reunion will compete more successfully with restitution. The empirical coefficient of chromosome reunion (ECR") (*see* Darlington and La Cour, 1945), obtained by dividing the number of reunions in major centric fragments (*i.e.* in dicentrics, polycentrics

2

*pollen grains after continuous and fractionated doses. Tempera-
23° C. June 1947. For Code letters see table I*

Breakage per cell				ECR" $R''/B''(C_0'')$
$B''(C_0'')$	$B''(C_0'' + 2R''C_1)$	mB''	Total B'' $C_0'' + 2R''C_1 + m$	
0.14	0.26	0.04	0.30	0.45
0.23	0.50	0.05	0.55	0.60
0.38	0.85	0.11	0.96	0.63

and centric rings) by the number of breaks as estimated from major acentrics (C_0), gives a measure of the proportion of recognisable breaks which have undergone recognisable reunion.* The data show that there is no significant difference between the ECR with continuous and fractionated dose, though that from the single half dose is lower.

The frequency of reunion of centrics (*i.e.* in dicentrics and centric rings) per cell is reduced in the samples from the fractionated dose to about twice that from the single half dose (table 2, *cf.* Sax, 1939 and 1941). However, the data show that the frequency of acentrics,

* The comparatively low figure of 0.45-0.63 may, however, give a false impression of the proportion of breaks which survive by virtue of reunion, firstly, because reunion of centrics to form dicentrics or centric rings may leave the acentrics unreunited and secondly, because many of the acentrics classed in this analysis as $B''(C_0)$ are probably of the "minute" type (and have been classed so in the later experiments). Thus the ECR is in this case of doubtful value as an indicator of the proportion of breaks which have survived by reunion, but it is of value in comparisons between the different irradiations, as the criterion for scoring minutes is constant throughout the experiment.

whether major fragments or minutes, and the total corrected breakage frequency are both reduced to considerably less than twice that from the half dose.

For a number of reasons the results of the above experiment cannot in themselves be considered as a reliable basis for conclusions as to

TABLE

*Analysis of breakage and reunion in Tradescantia bracteata pollen grains after 360
between X-ray and fixation 24° C. May*

Code for batch and slide	Time between fractions (hours)	Time of fixation	Number of cells		B"(C ₀ ")	mB"	R"			B'
			Total	Damaged			C ₃	C ₂	C ₁ '	
T A 2 S A 6 }	4	2 days {	40	21	13	16	...	8	3	3
			40	20	12	13	...	6	6	2
		Total	80	41	25	29	...	14	9	5
T B 2 S B 5 }	0	2 days {	40	22	19	27	...	9	5	3
			40	23	20	18	2	12	3	...
		Total	80	45	39	45	2	21	8	3
S AA 6 S AA 6 }	4	3 days {	40	21	16	8	...	8	4	3
			40	21	12	19	...	11	2	2
		Total	80	42	28	27	...	19	6	5
S BB 4 S BB 4 }	0	3 days {	40	28	18	24	...	12	5	2
			40	25	20	24	...	10	4	1
		Total	80	53	38	48	...	22	9	3
T AA 2 T AA 4 }	4	4 days {	40	26	16	16	...	9	4	1
			40	21	16	6	...	8	4	...
		Total	80	47	32	22	...	17	8	1
T BB 4 T BB 4 }	0	4 days {	40	28	20	29	2	13	4	...
			40	30	21	27	...	9	8	1
		Total	80	58	41	56	2	22	12	1
T 3 B	0	6 days	40	37	38	47	...	20	9	2

the cause of the reduction in breakage with fractionation. The conditions during the experiments were considered to be unsatisfactory in the following respects. The temperature was insufficiently rigidly controlled; the lack of illumination of the cut shoots during the experiment led to deterioration in the condition of the pollen grains,

making analysis difficult, and caused complete failure in development of pollen in those shoots which remained in the growth chamber for a day longer than those used for analysis. The number of breaks scored was considered to be too low to insure that the results were of statistical significance, particularly in regard to the samples from

a continuous dose, and dose separated into two fractions of 180 r. Temperature June 1948. For Code letters see table I

Breakage per cell					ECR" $R''/B''(C_0'')$	Per cent. binucleate cells
$B''(C_0'')$	$B''(C_0''+2R''C_1)$	$B''(C_0'')+m$	Total B'' $C_0''+2R''C_1+m$	mB''		
...	...	...	...	...	...	14
...	...	...	...	...	...	27
0.31	0.89	0.68	1.25	0.36	0.92	...
...	...	...	...	...	...	20
...	...	...	...	...	...	16
0.49	1.26	1.05	1.82	0.56	0.80	...
...	...	...	...	...	...	12
0.35	0.98	0.69	1.32	0.34	0.89	...
...	...	...	...	...	...	22
0.48	1.25	1.08	1.85	0.60	0.82	...
...	...	...	...	...	...	15
...	...	...	...	...	...	17
0.40	1.03	0.68	1.31	0.28	0.78	...
...	...	...	...	...	...	29
0.51	1.41	1.20	2.11	0.70	0.88	...
0.95	2.40	2.13	3.58	1.18	0.77	38

the half dose. For this and for other reasons stated below, it was considered that a higher dose of 360 r. would be more satisfactory. The most serious limitation of the experiment is that the developmental changes of the pollen grains during the varying periods of irradiation, which as we have seen may be an important factor

influencing the breakage yield, was not taken into account. Only one time period after irradiation, namely 48 hours, was used; consequently any change in yield due to fractionation might be due, at least in part, to the irradiation having been given at a time when sensitivity was falling, and hence the second dose in the fractionated samples might have been given at a time of lower radio-sensitivity. Slight differences in the rate of development due to external conditions and to the different doses of radiation might also in these circumstances lead to differences in yields of breaks. The seriousness of this limitation is shown by the data of Darlington and La Cour (1945) which indicate that at two days after a dose of 180 r. at 24° C. the frequency of B" might be expected to alter with changes of a few hours in the timing of the dose and that the time scale of development of the pollen grains may be materially affected by altering the dose.

Second series.—Table 3 summarises the results of experiments carried out in 1948. More confidence can be placed in these results for the following reasons. The temperature was strictly controlled and the illumination of the shoots during the period of development between irradiation and fixation kept the anthers and pollen grains in good condition for several days, enabling a more reliable analysis to be made and allowing fixation to be carried out at several intervals after irradiation. The choice of the higher dose of 360 r. enabled the experiments of Darlington and La Cour (1945) to be used as a basis for choice of the time periods between irradiation and fixation. Their data show that under the experimental conditions prevailing, little variation in frequency of B" occurs between anthers fixed at two, three and four days after a dose of 360 r. (table 10B and graph 7A), whereas with doses of 180 r. the stable period begins at three days after irradiation, the temperature in all cases being 24° C. (appendix II, table 1 and graph 1). In the 1948 experiments therefore, fixation was carried out two days, three days and four days after a dose of 360 r. given continuously and separated into two equal fractions by a four-hour interval. A few shoots, which remained after taking the four-day samples, were examined for buds showing the appropriate stage of development, at six days after irradiation. A bud suitable for analysis was found only in the material which had been given the continuous dose.

The results show that breakage yield varies comparatively little between samples fixed after two, three and four days at 24° C. (table 3 and graph 1), thus confirming the results of Darlington and La Cour. They show, moreover, a considerable reduction in breakage frequency for all three periods when the dose is given in two equal fractions separated by four hours. This indicates, firstly, that the reduction of breakage frequency is not due directly to the difference in time period before fixation, at which the second fraction was given and, secondly, that it is not due to alterations in the time scale of development of the pollen grains. The actual reduction in total corrected

breakage frequency is approximately 30 per cent. at two days, 27 per cent. at three days and 38 per cent. at four days. As in the 1947 results, there is no significant difference between the empirical coefficient of reunion (ECR'') after continuous and fractionated doses at any of the time periods, nor did the time period itself significantly

GRAPH 1.—Breakage frequency estimated in different ways in relation to the time after X-ray dose of 360 r. continuous and separated into two equal fractions by four hours. (from Table 3).

affect the coefficient. It appears to be much higher than in 1947 but this is at least in part due to the difference in criteria used for scoring small fragments. Breakage as measured by the frequency of acentrics is somewhat lower than the comparable figures of Darlington and La Cour (1945) and it varies less between the three time periods.

TABLE 4

Time	$(B''(C_0)+m)$ per cell	
	D. and LaC. 1945	1948 data
2 days . . .	1.94	1.05
3 days . . .	1.23	1.08
4 days . . .	1.47	1.21

The breakage frequency ($B''C_0+mB''$) at 6 days was considerably higher than at earlier periods, being as high as 2.13. As this figure is based on a single sample of 40 cells, it should perhaps be treated with caution.

TABLE

Analysis of breakage and reunion in Tradescantia bracteata 72 hours after the beginning also after single dose of 180 r. Temperature between X-ray

Code for batch and slide	Dose and time between fractions (hours)	Number of cells		$B''(C_0'')$	mB''	R''		B'
		Total	Damaged			$C_2 + C_3$	$C_1 r$	
U B 4	360 r./0	80	66	57	56	36	14	2
W B 3 * ,, 5 *	,, 0	80	57	55	61	29	17	1
	,, 0	80	57	54	47	36	13	4
	Total	160	114	109	108	65	30	5
W E 2 ,, 5	360 r./1	80	56	46	38	29	11	7
	,, 1	80	57	38	45	26	9	5
	Total	160	113	84	83	55	20	12
U E 3 * ,, 5 *	360 r./1	80	52	40	41	27	8	5
	,, 1	80	54	46	34	25	15	8
	Total	160	106	86	75	52	23	13
U D 1 ,, 3	360 r./2	80	45	28	33	19	6	4
	,, 2	80	47	35	39	21	10	4
	Total	160	92	63	72	40	16	8
U A 1 ,, 7	360 r./4	80	42	23	27	12	8	4
	,, 4	80	45	30	38	23	4	4
	Total	160	87	53	65	35	12	8
W G 3 ,, 6	360 r./6	80	53	35	37	23	7	4
	,, 6	80	43	29	35	20	7	4
	Total	160	96	64	72	43	14	8
U F 6 ,, 7	360 r./8	80	56	44	43	31	7	2
	,, 8	80	57	53	42	39	9	1
	Total	160	113	97	85	70	16	3
U C 2 ,, 5	180 r./0	160	26	34	36	23	9	1
	,, 0	160	25	39	25	25	7	2
	Total	320	51	73	61	48	16	3

* Replicates

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of treatment, with either a 360 r. continuous dose or separated into two equal fractions, and fixation 24 °C. June 1949. For Code letters see table I

Breakage per cell					$R''C_1$ per cell	ECR'' $R''/B''(C_0'')$
$B''(C_0'')$	$B''(C_0''+2R''C_1)$	$B''(C_0'')+m$	mB''	Total B'' $(C_0''+2R''C_1+m)$		
0.71	1.96	1.42	0.70	2.66	0.63	0.88
...	...	...	...	...	...	...
0.68	1.87	1.36	0.68	2.55	0.60	0.87
...	...	...	...	...	...	...
0.52	1.47	1.04	0.52	2.00	0.47	0.89
...	...	...	...	...	...	...
0.54	1.47	1.01	0.47	1.94	0.47	0.88
...	...	...	...	...	...	...
0.39	1.09	0.84	0.45	1.54	0.35	0.89
...	...	...	...	...	...	...
0.33	0.92	0.74	0.41	1.33	0.29	0.89
...	...	...	...	...	...	...
0.40	1.10	0.85	0.45	1.55	0.36	0.89
...	...	...	...	...	...	...
0.61	1.69	1.14	0.53	2.22	0.54	0.89
...	...	...	...	...	...	...
0.23	0.63	0.42	0.19	0.82	0.20	0.88

see table I.

Third series.—The design of the 1949 experiments was different from the previous experiments in that the time interval between the fractions was varied. The same dose of 360 r. was used as in the previous year, full continuous doses and a single half dose being given

GRAPH 2.

GRAPH 3.

GRAPH 4.

GRAPH 5.

GRAPHS 2-5.—Frequency of breakage and reunion 72 hours after X-ray dose of 360 r. in relation to the time interval between two equal fractions. Abscissa axis: time interval in hours. Frequency from single half dose shown for comparison. "Half dose x 2" in Graph 5 corresponds to the "base line" of Sax's curves. (from Table 5.)

for comparison with the fractionated doses. The fractions were separated by intervals of 1, 2, 4, 6 and 8 hours. In this way it was hoped that more information could be obtained on the cause of the reduction following fractionation.

On the basis of the 1948 experiments, 72 hours was considered

to be the most suitable fixation time counted from the beginning of irradiation. This period is short enough to ensure that anthers and pollen grains remain in the best condition for analysis, and it is fairly safe to assume that no significant changes in yield would result from the shorter time period for the second fraction, even if the time between fractions is extended. The half dose would come within the stable resting stage period, even with a more extended development cycle following the lower dose. The shoots were illuminated during the period between irradiation and fixation as in the experiments of the previous year, and the anthers remained in good condition throughout the experiment.

The results are summarised in table 5 and in graphs 2-5. The breakage frequency from the full continuous dose is consistent between samples and is greater than in the experiments of the previous year, for the same dose, time interval and temperature. This is probably partly due to the difference in illumination and emphasises the necessity for strict control of the growth conditions during the interval between irradiation and fixation. The variation from year to year may, however, be partly due to differences in the condition of the plants from which the material was taken. In the present experiments a repeat control was given the same dose even when an interval of only two days elapsed between irradiation of shoots with different doses. It was considered that only in this way could the validity of comparisons be ensured with any certainty.

The breakage frequency from the four-hour fractionated dose is almost equal to that in the previous year, the reduction following fractionation being approximately 50 per cent. This reduction is the maximum recorded. From the single half dose the breakage frequency is a little less than one-third of that from the full continuous dose, a result comparable with that obtained in 1947. The frequency both of reunion of centrics and of breakage drops considerably lower than twice that from the single half dose (*see graphs*). The frequency of breakage in whichever way it is measured, (*see graphs*) drops progressively as the time interval between fraction is extended up to 4 hours, the drop being most rapid over the first 2 hours. A recovery in the frequency is apparent at 6 hours, becoming more rapid from 6 to 8 hours, the longest time interval given between fractions. At 8 hours' separation the reduction in total breakage frequency is only 15 per cent. And the curve points to a further recovery—which I shall test with further experiments.

With the additional information given by this series of experiments, we are now in a position to approach the problem of interpretation and to re-examine the bearing of fractionation experiments on theories of X-ray breakage and reunion.

4. DISCUSSION

On the basis of the early quantitative experiments of Sax on X-ray breakage in *Tradescantia* pollen, the conclusion was reached that the relation between irradiation and chromosome breakage is fundamentally similar to that between irradiation and mutation (Sax, 1938), a conclusion which had already been arrived at by many other workers using different experimental materials. The main basis for this conclusion was the study of dose curves ; certain aberrations, assumed to be "2-hit aberrations" were observed to increase geometrically with dose, single breaks or "1-hit aberrations" being more or less proportional to dose. Subsequently many other experiments have been carried out to investigate the relation between dose and chromosome breakage using X-rays, neutrons and other ionising radiations. These investigations have been widely interpreted as supporting Sax's main conclusions.

Fallacies of method and inference, some of which have already been referred to, render much of the data of doubtful value. Nevertheless, certain broad conclusions concerning the action of radiation on the chromosomes appear to be widely accepted, namely, that breakage is immediate or occurs within a short time of the passage of an ionising particle through, or in the vicinity of, a chromosome and that the broken ends may subsequently rejoin, enter into new reunions or remain as broken ends. Thus breakage is considered as, in a sense, a direct effect on the chromosomes rather than a cumulative effect on the cell as a whole. However, it is now usually recognised that chemical processes must intervene between ionisation and break, but little is as yet known regarding their extent and nature. The study of chemical breakage suggests that radiation breakage may not be as "direct" as has usually been supposed (Darlington and Koller, 1947).

The early work on the relation of dose to breakage frequency in *Tradescantia* was followed by investigation of the effect of separation of breaks in time (assuming that breakage is immediate) by alteration of the intensity of irradiation and by fractionation (Sax, 1939, 1940, 1941). It is in the conclusions drawn from these and subsequent investigations of the time factor in relation to breakage frequency that we find fundamental contradictions in interpretation which are difficult to resolve. Sax's data on intensity variation indicated that spreading of the dose over periods up to a few hours progressively reduced the yields of so-called "2-hit aberrations" (dicentric and centric rings). His interpretation of this effect was that if the radiation is given slowly a break may heal (rejoin) before a second break occurs with which it might have entered into reunion. As the drop in yield of breaks was apparent even when the dose was spread over short periods of under an hour, it followed that rejoining or reunion occurs mainly within a comparatively short time after breakage.

Acting on the assumption that there is a critical time period during which reunion or restitution occurs, Sax (1939) set out to determine by fractionation experiments the "maximum time a break can remain 'open' in an unstable condition"; and this maximum period was estimated to be of the order of one hour.

The use of the ambiguous term "unstable condition" and of the term "healing" together with "restitution" (apparently as synonyms) has led to considerable confusion (Darlington and Upcott, 1941; Lea, 1946). This confusion has arisen particularly over the question as to how far healing on the one hand or restitution on the other hand have been held to account for the effect of intensity and fractionation on chromosome breakage. Marinelli *et al.* (1942) for example, supported Sax's hypothesis and used both the terms "restitution" and "healing" in their interpretation of their data in the light of the theory of recovery of Swann and Del Rosario, but it is not clear whether healing is used as a synonym for restitution or implies healing as such. Although it appears that Sax did not use the term "healing" in the usual sense (*i.e.* healing against rejoining of ends), he refers to "a proportion of breaks producing broken ends which appear to have lost their capacity for fusion" (Sax, 1940). Moreover, although he admitted that the assumption that all fusions are completed within a short period of time (about an hour) after irradiation did "not necessarily mean that a broken end can remain in an unstable condition for only an hour" (Sax, 1940, p. 62-3), the final assertion (Sax, 1941, p. 101) that breaks can remain open for only 26-60 minutes clearly supposes, not only rapid reunion or restitution, but a healing process in those ends which do not reconstitute or reunite within that period. The supposed lack of sister reunion in terminal chromosome acentric fragments was held to support this conclusion (Sax, 1940).

Lea and Catcheside (1942) accepted the theory of a limited period during which the majority of primary breaks reconstitute or enter into reunion, and the conclusion that a lack of sister reunion in chromosome breaks surviving until chromosome division (with irradiation five days before fixation), indicates healing at some time between breakage and chromosome division. From a mathematical treatment of certain of Sax's data they concluded that, although a few breaks "remain open" for a much longer period and may subsequently form reunions, the average time elapsing between breakage and reunion for most of the breaks is as low as 3 or 4 minutes. Catcheside, Lea and Thoday (1946) made similar calculations from their own data, arriving at a similar result, but again were "forced to the conclusion" that this short period does not fit all the facts when doses are spread over a period of more than 30 minutes, and that reunion may occur after a much longer period measured in hours, not minutes.

Darlington and Upcott had, on the other hand, as early as 1941, expressed the view that there is no evidence for healing against non-

sister reunion, and they pointed to the existing evidence that broken ends retain permanently the ability to rejoin. Sister reunion, being interpreted as a reproductive error appearing as a reunion of chromatids when the chromosomes have divided, was considered to be in quite a different category as regards the conditions of reunion. They further pointed out that the assumption of a healing process against reunion is in any case unnecessary in order to explain the intensity effect, because the facts could be explained by the competition between restitution and reunion. These arguments were further substantiated by study of the relation between radiation breakage and reunion and the nucleic acid cycle (Darlington and La Cour, 1945), and study of chemical breakage (Darlington and Koller, 1947).

Koller (1946), in his study of the effect on breakage of reducing the dosage rate in *Tradescantia*, accepted the above explanation (*i.e.* the competition of restitution with reunion) for the reduction observed in breakage at 48 hours and 72 hours, when the dose is spread over 6 hours and more. This interpretation was supported by the observation of a smaller proportion of breaks involved in reunion (*i.e.* a lower ECR) as the dosage rate is decreased. He concluded, however, that other factors play a part in the reduction, for example, the length of the cell cycle relative to the duration of exposure to the radiation, and changes in the time scale of development which might account for reduction in the frequency of simple breaks at low intensities. For reasons stated above he rejected data obtained from fixation at short intervals after irradiation. The data gave no support, however, to Catcheside and Lea's conception of very rapid reunion or restitution.

Newcombe (1942) rejected the interpretation of the intensity effect as due to competition of restitution with reunion, on the grounds that the frequency of simple breaks showed in his data just as much intensity effect as that of reunions, and he suggested inhibition of reunion by the radiation as the explanation. However, it is probable that many of the acentrics which were observed at 3, 5 and 6 days after X-raying and were interpreted as "fractures" were in fact intercalary deletions involving reunion—especially as their frequency showed an exponential relation to dose.

Different methods of experiment and interpretation of radiation breakage in *Tradescantia* pollen have thus given rise to two distinct and conflicting conceptions of the sequence of events following breakage and giving rise to the observed aberrations at metaphase and anaphase :—

(1) that reunion and restitution are rapid, being almost completed within an hour and mostly occurring within a few minutes, and that healing against restitution and reunion occurs at some time between breakage and division of the chromosomes,

(2) That reunion and restitution are delayed and cumulative, that healing against non-sister reunion does not normally take place

and that sister reunion is delayed and simultaneous with the division of the chromosome into chromatids.

These two conceptions have far-reaching implications for the interpretation of the observed aberrations. Thus the conception of isochromatid breaks (breaks in no way distinguishable from chromosome breaks except that they occur together with chromatid breaks in the same cell) has arisen from the deduction that reunion and restitution follow rapidly after breakage, thus precluding the possibility of chromatid reunion or restitution following chromosome breakage, to give rise to apparent chromatid breaks. If, on the other hand, reunion is delayed, the isochromatid break can be interpreted as a chromosome break, from which it is in any case cytologically indistinguishable, and the accompanying chromatid break can be interpreted as a chromosome break which has undergone delayed chromatid restitution or reunion.

The evidence for the first conception is drawn from the results of three types of experiment :—

(1) observation of a decrease in frequency of reunions (“2-hit aberrations”) when the same X-ray dose is given at lower intensity ;

(2) observation of a smaller increase in frequency of reunions when the dose is raised at constant intensity, than when it is increased keeping the time constant ;

(3) observation of a reduction in frequency of reunion when the same dose is divided into fractions separated by time intervals.

The conception of a time limit for reunion and restitution originated from the third type of experiment. As it is with this type of experiment that we are particularly concerned, the data and interpretations from earlier experiments of this type with *Tradescantia* pollen grains must be critically examined in relation to the results of the present experiments.

Fabergé (1940), as we have seen, used high doses which necessitated a very crude scoring system, namely counting of the total number of chromosome bodies per cell. A reduction in the frequency of gross breakage (*i.e.* breakage minus reunion) was detected when doses were divided into 6 fractions separated by about 16 minutes, the total spread of the dose being 3 hours, but no definite conclusions as to the cause of the reduction could be drawn from the data.

In the experiments of Sax (1939, 1940, 1941) designed with a view to estimating the maximum time a broken end can remain “capable of fusion with other broken chromosomes,” relatively low doses were used and the doses, numbers of fractions and periods between fractions were varied, breakage being examined at 24 hours and 5 days after irradiation. The design and interpretation of these experiments was based on the assumption that any reduction in breakage frequency observed was due to a reduction in reunion and that consequently only the frequency of so-called “2-hit breaks” would be affected. This assumption was based on the general interpretation

of the results of intensity experiments and the results of two experiments with 24-hour fixation which indicated that fractionation did not affect the frequency of simple chromatid breaks. In the experiments on chromosome breakage at 5 days, only reunions (centric rings and dicentrics) were scored and a progressive drop in their frequency was recorded as the time interval between fractions was increased. The time interval (about an hour) at which the frequency dropped to the same as that from a single fraction, multiplied by the number of fractions (the "base line" of the curves) was considered to be the maximum time a break can remain open. It was argued that with this and longer periods between the fractions, restitution is completed during the intervals, reunion occurring only between breaks resulting from the same fraction of the dose. If, on the other hand, no restitution occurs in the intervals between fractions, the values obtained would be equal to that from the full continuous dose.

The results of the present experiments confirm the reduction in the number of reunions per cell when the interval between fractions is increased up to 4 hours. They show, however, that the frequency rises again if the interval is increased beyond 4 hours up to 8 hours and that at 4 hours the frequency of reunions drops considerably below that from twice the half dose. This reduction and recovery affects not only reunion but also the estimates of breakage (graphs 2-5). The accepted explanation of the reduction cannot account either for the magnitude of the reduction or for the later increase or recovery.

The recovery is not apparent in Sax's experiments. The reason is evident. The maximum time interval left between consecutive fractions was 4 hours; and in all but one of the experiments the dose was separated into more than two fractions. In two experiments the dose was separated into 4 fractions by intervals of 4 hours, the total spread of the dose being 12 hours, and in one experiment the dose was spread into 4 fractions over a period of 6 hours, but no significant recovery was observed, though in two of these cases the frequency showed some recovery as the interval was increased. Sax noticed that in some of the experiments the frequency of centric rings and dicentrics from the fractionated dose dropped to below the multiplied value from the single fraction (though not to the extent shown in the present experiments). The possibility of "a physiological disturbance caused by the radiation" was tentatively considered as an explanation (Sax, 1939).

The almost complete recovery shown in breakage and reunion frequency in the present experiments, when two fractions of the dose are separated beyond 4 hours, at once suggests that the reduction itself is due to some temporary effect of the first fraction reaching a maximum at about 4 hours and then wearing off. Such an explanation would obviously be in direct contradiction to the general assumption, made in quantitative comparisons of chromosome breakage with varying dose, intensity and mode of fractionation, that the sensitivity

to irradiation remains constant. As we have seen, this assumption is frequently unjustified owing to the lack of attention paid to possible variations in the timing of irradiation in relation to the development of the material. But we can now see that, even if these factors are fully taken into consideration, the possibility remains that the irradiation itself may affect not only the rate of development but the subsequent sensitivity of the chromosomes to breakage.

Sax recognised this possibility, and carried out experiments (Sax, 1942) designed specifically to test the effect of irradiation on subsequent breakage. The main object of the experiments was to test whether the action of X-rays in chromosome breakage was a "direct hit mechanism" or whether cumulative indirect physiological effects played a role in breakage, previous irradiation increasing subsequent sensitivity. Two doses were given; the second one (24 hours before fixation) produced chromatid breaks which could be distinguished from the chromosome breaks produced by the earlier dose. The experiments indicated that the first dose did not increase the sensitivity to the second dose. On the contrary, a slight decrease in chromatid breakage was observed after previous irradiation. This appeared to be due to a reduced sensitivity of acentric fragments, an effect which has been attributed to a higher charge of nucleic acid on the acentrics (Darlington and La Cour, 1945).

These results are widely held to give support to the theory of direct action of X-rays in breakage rather than a cumulative effect on the cell as a whole. But they do not demonstrate the absence of any physiological effect on breakage. Disregarding any effect on the result due to possible change in the rate of development caused by the first dose, they merely show that such an effect does not last 24 hours or 3 days, for these were the periods of separation of the two doses.

The question naturally arises: how might irradiation be expected to affect subsequent sensitivity to breakage, in view of what is known of the effects of X-rays on the cell metabolism and the factors affecting sensitivity to radiation breakage? There is ample evidence, some of which we have mentioned, that radiation may produce temporary effects on the cell metabolism, but these effects have usually been ignored in quantitative experiments on breakage or have been considered as minor factors which tend to upset the results and cause deviations from statistical expectation, for it is generally assumed that they are independent of the "direct" effect of breakage. Darlington and La Cour (1945), on the other hand, drew attention to the difficulty of separating nuclear from cytoplasmic effect of X-rays, and to the general assumption that they are naturally independent of each other. They furthermore pointed out that these effects which they succeeded in separating to a certain extent in experiment, were in fact connected in nature by way of the nucleic acid cycle. There is now considerable evidence that radiation causes

disturbances in the nucleic acid metabolism (Darlington and La Cour, *loc. cit.*; Koller, 1947; Darlington, 1947). These effects are usually temporary after small doses, their duration and degree depending upon the dose; for example, stickiness of chromosomes (interpreted as being due to an excess of non-polymerised nucleic acid on the chromosomes, Darlington, 1942) appears at 2 hours, reaches a maximum at 5 hours and disappears at 12 hours after a dose of 360 r. at 20° C.; after 90 r. it reaches a maximum at 7 hours (Koller, 1947). The timing of this effect is thus very similar to that of the reduction and recovery in breakage and reunion frequency observed in the present experiments.

From comparative studies of chromosome breakage throughout the mitotic cycle and in euchromatin and heterochromatin, and from the observation that in nuclei with a resting phase of short duration (and lower nucleic acid charge) the chromosomes are more easily broken (Darlington and La Cour, 1945), it has been shown that breakability has an inverse relation to the nucleic acid charge on the chromosomes. A most likely explanation, therefore, of the temporary effect on breakage is that it is due to a temporary accumulation of nucleic acid on the resting phase chromosomes reaching a maximum, in the conditions of the experiments, at about 4 hours after the first dose. Such an accumulation would be expected also to increase temporarily the rejoinability of broken ends (Darlington and La Cour, *loc. cit.*). This cannot be directly detected by a comparison of the empirical coefficient of reunion (R''/B'') after the continuous and fractionated dose, because the frequency of reunion is so high with long time intervals between irradiation and fixation that breaks survive almost entirely by virtue of reunion.

We may now consider the question of how far the above interpretation of the fractionation effect can be reconciled with the results of previous fractionation experiments with *Tradescantia* pollen. The results of Sax's experiments at five-day intervals after irradiation, in which only reunions were scored, clearly offer no obstacle to the present interpretation. The number of fractions used, coupled with the fact that the intervals between fractions were not sufficiently protracted, accounts for the failure to observe a significant recovery in yield of aberrations. On the other hand, much significance has been attached to certain experimental results which indicate that so-called "2-hit breaks" (*i.e.* breaks involved in reunion) show a significant reduction in frequency when the dose is fractionated, whilst simple breaks do not (Sax, 1939, 1941), and that with neutrons neither type of aberration shows any dependence upon fractionation of the dose (Giles, 1943). These observations have been considered as an important part of the evidence in support of the theory that the reduction in yield of aberrations with fractionation is due to the prevention of reunion by the rapid occurrence of restitution between fractions.

In assessing the significance of these experiments, the following points should be noted :—

(i) The evidence for lack of dependence upon fractionation with both X-rays and neutrons is based on a very few observations taken at periods of approximately 24 hours after irradiation. For reasons which have been given in an earlier section, observation at such short time intervals after irradiation introduces a number of variable factors, which may significantly affect the breakage yields and render reliable interpretation of the results impossible.

(ii) Even if these results are taken at their face value it must not be forgotten that any factor which causes a reduction in initial X-ray breakage will affect disproportionately the frequency of breaks surviving by virtue of reunion. If we interpret the reduction in yield of surviving breaks following fractionation of X-ray dose as being due to a reduction in initial breakage in the later fractions, we should expect the yield of reunions to be disproportionately affected, as it is when the dose is reduced. With neutrons the disproportion would not be expected on the generally held assumption that, with neutrons, reunions usually occur between breaks caused by a single ionising particle (Thoday, 1942 ; Giles, 1943). Simple breaks observed at metaphase, on the other hand, constitute the remainder (perhaps a small one) which survive after restitution and reunion, and they may also depend for their survival on sister reunion. Consequently the relation of their frequency to that of initial breaks is in all probability not a simple one. (This was recognised by Lea (1946) and Catcheside (1948) as a weakness in the deductions on the mechanism of induction of breaks, made from curves relating dose and intensity to frequency of surviving breaks).

(iii) Examination of the few experiments, which have been interpreted as demonstrating the absence of a fractionation effect, reveals that the maximum intervals between fractions were relatively short. In the two experiments with X-rays (Sax, 1939, 1941) the maximum periods were in one case 27 minutes between each of three fractions and in the other 80 minutes between four fractions. The latter experiment showed a reduction, with fractionation of the dose, of nearly 20 per cent. in the frequency of simple breaks as compared with a maximum reduction of nearly 50 per cent. in the case of reunions. In the single experiment recorded by Giles (1943) using fractionated doses of neutrons, the maximum period between fractions was 32 minutes. Thus the shortness of the period between fractions in these experiments must also be taken into consideration when estimating the significance which may be attributed to their results.

I conclude that observations of chromatid breakage at one day after irradiation, giving results which have been held to indicate that only independently produced breaks involved in reunion are affected by fractionation and that consequently the effect is primarily one on reunion, do not in fact provide critical evidence on the matter.

A further point arises in relating the present results to previous experiments with fractionated doses. The absence of fractionation and intensity effect on structural change in *Drosophila* is generally contrasted with its existence in *Tradescantia*. The experiments on *Drosophila* (e.g. Muller, 1940; Dempster, 1941; Kaufmann, 1941) are difficult to relate to those on *Tradescantia* because (a) the doses used are far higher; (b) in fractionation the intervals between fractions are much longer (in days not hours). In experiments on intensity variation, Muller (*loc. cit.*) showed that there was no reduction in frequency of structural changes when the dosage rate was very low, a result which is in contrast to that of Koller (1946) using low dosage rate with *Tradescantia*. The absence of intensity effect in *Drosophila* is explained by assuming that no reunion takes place until fertilisation. The fractionation experiments owing to their design could not, however, reveal any temporary radiation-induced effect on sensitivity, consequently no fractionation effect such as appears in the *Tradescantia* experiments would be expected.

5. CONCLUSIONS

The reduction in breakage yield observed in the present experiments is mainly, if not entirely, due to a temporary physiological effect of the first part of the dose on breakage by the later irradiation. In view of what is known concerning the effects of radiation on the nucleic acid metabolism of the cell, this effect may well depend on a temporary accumulation of nucleic acid on the chromosomes. The results of previous experiments carried out to investigate the effect on chromosome breakage, of dividing a dose of ionising radiation into separate fractions, present no serious obstacle to this view.

This interpretation leads to certain general conclusions. In the first place, radiation-induced changes in the sensitivity to breakage must clearly be taken into consideration in the interpretation of all quantitative experiments, in which the doses last long enough for the temporary effect to come into action. Hence the interpretation of all experiments, in which the duration of the dose is varied, needs to be radically reviewed. Many earlier workers have ignored these complications. But they are obvious from the reaction of the temporary effect with the breakage frequency. The present fractionation experiments and the investigations of Darlington and La Cour (1945) and Koller (1946, 1947) into radiation-induced stickiness and sister reunion frequencies may be considered as first steps in the study of the temporary effect of radiation on the cell.

It follows that not only the fractionation effect but the intensity effect on frequency of reunion can no longer be regarded as evidence that reunion and restitution occur rapidly after breakage. Indeed, we now see that little restitution can have taken place during the first eight hours after breakage; thus positive support is given

to the already existing evidence that reunion and restitution are delayed.

Two further conclusions follow as a consequence of the rejection of the theory of rapid reunion : 1. The competition between reunion and restitution need no longer be regarded as a process occurring rapidly after breakage. It may be envisaged as a much slower process, effective only in the elimination of reunion as shown by experiments with very low dosage-rates (Koller, 1946). 2. The crucial argument for the postulation of isochromatid breaks loses its validity. It has been argued that reunion cannot be delayed because its frequency is influenced by intensity and fractionation. In consequence, apparent chromatid breaks occurring in the same cell with chromosome breaks, could not generally result from delayed chromatid reunion or restitution. Now we see that the overlapping of apparent chromosome and chromatid breaks which is so characteristic of the metaphase of X-rayed *Tradescantia* pollen grains is the result of delayed reunion of broken ends. This result is inevitable if reunion can occur at any time between breakage and the end of the resting stage.

Finally, the results of the present experiments point to the necessity of studying radiation breakage, not in isolation as a mechanical effect on the chromosomes, but in relation to the physiological processes and development of the cell and to radiation effects other than breakage. This necessity was demonstrated by the experiments of Darlington and La Cour, relating breakage to the nucleic acid cycle ; they showed that it affects not only the interpretation but the design of experiments.

The results, moreover, indicate the importance of further study of radiation effects other than breakage and particularly those which manifest themselves as changes in sensitivity to radiation breakage and in rate of development. These effects, though more elusive and less amenable to quantitative study than breakage of chromosomes, should not be neglected either in their relation to the quantitative study of breakage or in the general study of the biological effects of ionizing radiations and their application in radiotherapy.

6. SUMMARY

1. Experiments with *Tradescantia* pollen show that the effect of separating an X-ray dose of 360 r. into two equal fractions is as follows :—

- (a) Breakage frequency decreases as the period between fractions is increased up to four hours.
- (b) Beyond four hours' separation the breakage frequency begins to recover and at eight hours' separation it is little below that from the continuous dose.
- (c) The frequency of breakage and reunion is reduced at four hours' separation to considerably below twice that from the half-dose.

Plate I.—Pollen grains of *Tradescantia bracteata* (clone 21') in metaphase after X-raying. Dose 360 r., except fig. 5, dose 100 r. Time in hours after X-raying and time in hours between fractions, if any, are given in the legend applying to each figure, followed by the symbols for the structural changes and the scored breaks and reunions. $\times 1750$.

FIG. 1.—72 hrs., C_1r+C_0 , $(B''.R'')$.

FIG. 2.—72 hrs., 1 hr., C_2+C_0+m , $(B''.R''.mB'')$.

FIG. 3.—48 hrs., C_2+C_0 , $(B''.R'')$.

FIG. 4.—72 hrs., 1 hr., C_3+2C_0 , $(2B''.2R'')$.

FIG. 5.—48 hrs., C_1+C_0 , $(B''.C_1SR.C_0SR)$.

FIG. 6.—72 hrs., 4 hrs., C_2+C_0 , $(B''.R'')$. Short and long chromosomes indicate reciprocal interchange (not scored).

FIG. 7.—72 hrs., m , (mB'') . Note wide separation of minute chromatids.

FIG. 8.—72 hrs., C_1r+C_0 , $(B''.R'')$. Note small size of centric ring.

Plate II.—Pollen grains of *Tradescantia bracteata* (clone 21') after X-raying, dose 360 r. Figs. 1-4 in prophase, 5-10 in anaphase. Time in hours after X-raying and time in hours between fractions, if any, are given in the legend applying to each figure. $\times 1750$.

PROPHASES

FIG. 1.—72 hrs., 1 hr. Large ring chromo- some enclosing fragment. FIG. 2.—72 hrs. Small ring fragment.

FIG. 3.—72 hrs., 1 hr. Long dicentric with very small acentric.

FIG. 4.—48 hrs. Two very small minutes, one with wide separation.

ANAPHASES

FIG. 5.—48 hrs. Dicentric chromatid ring and acentric. Probably results from centric ring chromosome (see text p. 8).

FIG. 6.—72 hrs., 1 hr. Dicentric and acentric in early anaphase showing zig-zag arrangement.

FIG. 7.—48 hrs. Flattened anaphase. Pair of short dicentrics with corresponding pair of acentrics. Single long dicentric with short acentric. probably results of SR in centric and acentric fragments following B" (see text p. 8).

FIG. 8.—72 hrs., 4 hrs. Interlocking dicentrics with acentric fragment (*cf.* fig. 10).

FIG. 9.—72 hrs., 2 hrs. Dwarf grain, dicentric resulting from sister reunion. Accompanying acentric with SR not visible in photo.

FIG. 10.—72 hrs. Interlocking dicentrics accompanied by acentric fragment in dwarf pollen grain. Note longitudinal orientation.

Plate III.—Various types of diploid and double pollen grains, together with normal grains, observed after irradiation. These abnormalities are probably due to unfavourable conditions such as drought or sudden temperature change during meiosis. $\times 1400$.

FIG. 2.—Double pollen grain showing generative nuclei joined by bridge.

FIG. 1.—Right, double pollen grain arising by failure of cell separation at second meiotic division. Left, haploid grain. Below, unreduced pollen grain at metaphase.

FIG. 4.—Unreduced pollen grain at metaphase showing centric chromosome fragment overlying acentric (B''), and chromatid interchange ($2B' \ 2R'$) involving two chromosomes. Below, unreduced pollen grain and normal haploid grain.

FIG. 3.—Double pollen grain showing unreduced nucleus in metaphase.

2. These results show that the reduction is not in the main due to an irreversible change such as restitution occurring in the interval between fractions; reunion and restitution must therefore occur usually more than eight hours after breakage.

3. The reduction and later recovery in breakage frequency from the second half-dose, with increasing intervals between fractions, is intelligible on the assumptions (i) that radiation has a depressing effect on sensitivity to breakage, and (ii) that this effect is temporary. Such an effect might result from an accumulation of nucleic acid on the resting-phase chromosomes which is lost after an interval of time. Within a certain range of intervals between fractions, the accumulation induced by the first half-dose would then reduce the sensitivity to breakage by the second half-dose.

4. The results have an important bearing on the interpretation not only of fractionation experiments, but of all quantitative dose and dosage-rate experiments in which the doses last long enough for the effect on sensitivity to breakage to come into action. They also explain the wide overlap between apparent chromosome and chromatid breaks in time-scaled studies of resting-stage breakage.

5. Previous experiments can no longer serve as a basis for the rejection of the evidence that restitution and reunion are delayed. With the rejection of the theory of rapid reunion, the crucial argument for the postulation of isochromatid breaks is invalidated.

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