Chromosome Studies on *Trillium kamtschaticum* PALL. and Its Allies. XXVI. The effects of mitomycin C on the frequency of X-ray-induced chromosome aberrations*

By

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Mechanism of rejoining of radiation induced chromosome breaks has been studied by many investigators utilizing various chemical and physical agents which modify the type and frequency of aberrations. Especially the studies with the inhibitors of oxidative metabolism have brought about important informations on chromosome rejoining. From the results of experiments utilizing KCN, 2-4-dinitrophenol, CO, $Na_2S_2O_4$ and ATP etc., WOLFF & LUIPPOLD ('55) and BEATTY & BEATTY ('59, '60a, & '60b) illustrated that energy supply is necessary for the rejoining of broken ends. Recently WOLFF ('60) showed that protein synthesis is also necessary for the rejoining of broken ends as a conclusion from the experiment employing mainly chloramphenicol, and the author tends to deny the involvement of DNA and RNA synthesis in rejoining from the fact that chloramphenicol did not inhibit the incorporation of P^{32} into the DNA of germinated *Vicia* seeds. However, in our previous experiments it was found that mitomycin C, which is known to inhibit specifically the synthesis of DNA in bacteria, inhibits the sister reunion at X-ray induced broken ends, and remarkably increases relative frequency of heterochromatic breaks as compared with euchromatic ones (MATSUURA, TANIFUJI & IWABUCHI '62).

The present experiments were carried out in order to obtain more precise information on the mechanism of chromosome rejoining.

Material and Methods

Ovular tissue cells of *Trillium kamtschaticum* were used in the present experiments. Cut stems with flower buds were placed in 20 μg/ml solution of mitomycin C for 14 hours (8 hrs

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pre-, and 6 hrs post-irradiative treatment). The mitomycin solution was prepared with tap water and its initial pH was 6.8–7.0. A total dose of 60 r X-rays administered at 8.1 r/min. was given (180 kV, 6 mA, 0.5 mm Cu+1.0 mm Al filters, H.V.L.=0.95 mm Cu) 8 hours after the initiation of chemical treatment. The temperatures during irradiation and chemical treatment were 20°C and 15°C respectively. Immediately after the chemical treatment, the materials were transferred into beakers of tap water. The ovaries were fixed with LA COUR 2BE 48 hours after irradiation, hydrolysed with 1N HCl for 18 minutes at 60°C, and stained with leuco-basic fuchsin. Cytological observations were made on metaphase cells. As supplemental tests, the chromosome aberrations in the cells that elapsed 24 and 72 hours after irradiation were examined.

Results

When the present materials were treated with 0.2, 2 and 20 μg/ml of mitomycin for 8, 16 and 24 hours, by stem cut method, chromosome breakage was hardly observed 32 and 42 hours after the end of the treatment.

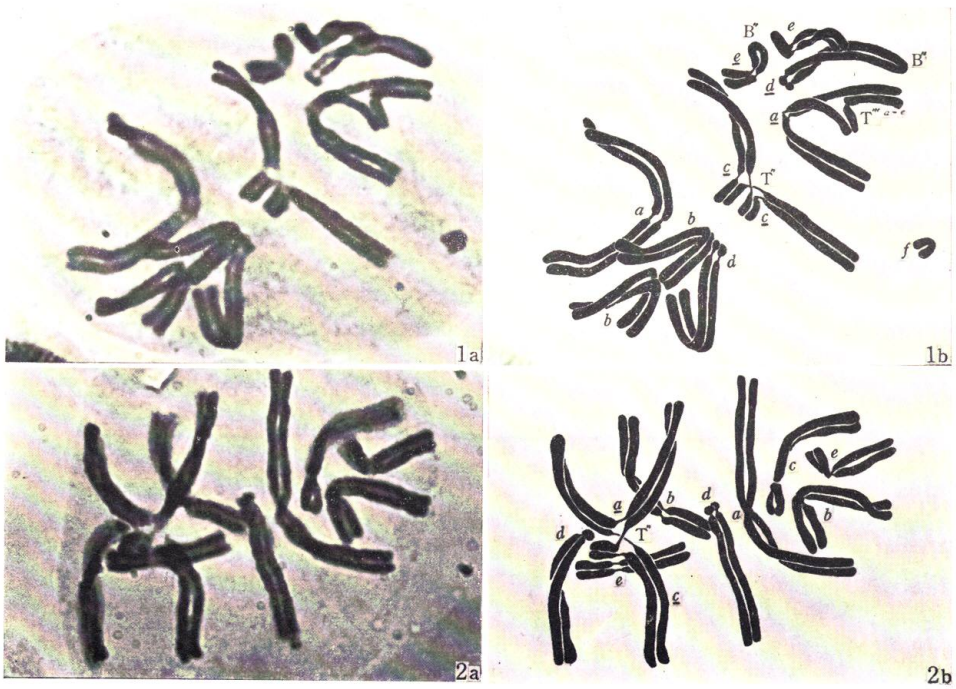
Whether X-ray induced chromosome aberrations are of chromatid type or of iso-chromatid type depends on the time elapsed after irradiation. In the present material 24 hours after irradiation the breaks of chromatid type are predominant over ones of iso-chromatid type, while 72 hours later the relationship is reversed. It is about 48 hours later that the frequency of both types become comparable to each other. However, it must be noted here that the relationship between chromatid breaks and iso-chromatid breaks in the mitomycin-X-ray treatment somewhat differs from that in the X-ray control. In the former the ratio of breaks of chromatid type to ones of iso-chromatid type is 121/80, while in the latter the ratio is 65/73. The ratio is a little higher in the combination treatment. This fact seems to indicate that mitotic cycle is slightly delayed by mitomycin treatment (Table 1).

TABLE 1. Frequencies of chromosome aberrations in *Trillium kamtschaticum* scored at metaphase 24, 48, and 72 hours after irradiation.

Elapsed hours after X-ray	Combined treatment	Total cells observed (plants)	Aberrations							B′	B″
			sB′	sB″	T″	T_0''	T_1''	T‴	T′		
24 hrs	——	69 (4)	45	36	13	0	1	0	1	73	37
48	——	81 (5)	42	67	9	4	0	1	1	65	73
48	Mito. 14hrs.	100 (5)	45	66	35	5	1	4	0	121	80
72	——	61 (3)	3	39	6	0	1	0	0	16	40

B′ = chromatid type breaks, B″ = iso-chromatid type breaks.

Both types of chromatid and iso-chromatid breaks could be classified into "one-hit" and "two-hit" aberrations respectively. The two-hit aberrations include chromatid-chromatid interchange (T″) (Figs. 1 & 2), chromosome-chromo-



Figs. 1 and **2.** X-ray-induced chromosome aberrations in ovular tissue cells of *Trillium kamtschaticm* Pall.

Fig. 1a and b, a chromosome-chromatid interchange (T'_0'') between **a**- and **e**-chromosomes, and a chromatid-chromatid interchange (T″) between two homologous chromosomes: a fragment (f) originated from an iso-chromatid breakage (B″) in **d**-chromosome.
Fig. 2a and b, a T″ near the kinetochore region between **a**- and **c**-chromosomes.

some interchange (T‴), and chromatid-chromosome interchange (T‴), which are subdivided into two types according as whether an akinetic fragment (Fig. 1) or a kinetic fragment following iso-chromatid breakage is involved (T'_0'' or T'_1'' respectively). The configurations showing the union of unaffected distal ends of chromosomes with broken ends of a chromatid break were sometimes met with (T′). One-hit aberrations of chromatid and iso-chromatid types produce the terminal deletions of chromatid (sB′) and of iso-chromatid type (sB″) (Fig. 1) respectively. Sometimes in the latter sister reunion (SR) of the broken ends is observed.

1) *Increase in the frequency of detectable aberrations.*

Frequencies of simple breaks and of rejoined breaks in metaphase cells were scored in each case of the X-ray control (5 plants, 81 cells) and the

mitomycin-X-rays treatment (5 plants, 100 cells). The data are represented in Table 2 as average frequency per 100 cells. From the table it is apparent that detectable aberrations are remarkably increased by mitomycin treatment.

According to the estimation by LEA ('47), 95% of the initial or potential breaks induced by X-rays restitute and become undetectable. Although there is a suspecion that mitomycin affects the production of initial breaks and operate on the acceleration of primary damage, it seems more likely that restitution is inhibited by mitomycin, because the rejoining process of the broken ends finally involved in interchange formation is also affected, rather delayed, by this treatment as will be mentioned in the next section.

TABLE 2. Frequencies of simple and rejoined breaks in the X-ray control and mitomycin-X-ray treatment. -per 100 cells-

	X-ray control (81 cells)	Mitomycin-X-rays (100 cells)
sB′	51.9	45.0
sB″	82.7	66.0
rB′	28.4	81.0
rB″	7.4	12.0
sB (B′+B″)	134.6	111.0
rB (B′+B″)	35.8	93.0
Total breaks	170.4	204.0
Two-hit aberration yield	18.5	48.0

sB = simple breaks, rB = rejoined breaks.

2) *Increase in the frequency of two-hit aberrations.*

The frequency per 100 cells of two-hit aberrations was 18.5 in the X-ray control and 48.0 in the mitomycin-X-rays. The frequencies of the rejoined breaks involved in interchange formation were 35.8 and 93.0 in the X-ray control and in the mitomycin-X-ray treatment respectively (Table 2).

The cause of the increase of two-hit aberration yield by mitomycin is considered as the increment of the chance of interaction between breaks. SAX ('43) and WOLFF & VON BORSTEL ('54) reported that centrifugation during the period of X-ray exposure enhances the frequency of chromosome exchanges. However, the rise of chromosome movement seems not to be the main factor

for the increment at least so far as the present case is concerned. When the centrifugation promotes chromosome movement and enhances the frequency of interchanges, the frequency of terminal deletions of iso-chromatid must be also increased as shown by SAX (loc. cit.) and CONGER ('48). However, in the present experiments the frequency of iso-chromatid deletions is rather decreased by mitomycin. Accordingly the fact of the increase in two-hit aberration yield by mitomycin must be explained on the basis of another assumption.

It was reported by many authors that induced broken ends stay open for relatively long period of time and the period is modified by many agents, such as the dose of irradiation, low temperature, 2-3-dimercaptopropanol (BAL), chloramphenicol and so on. The alteration of the length of the period during which broken ends remain open or/and that of the joinable period following after the open period may be more important factors.

3) *Distribution of breaks.*

The heterochromatic regions of somatic chromosomes of *Trillium* are revealed as Feulgen negative parts by low temperature (DARLINGTON & LA COUR '40 : HAGA & KURABAYASHI '53, '54). In *Trillium kamtschaticum* these regions in each chromosome are located at the proximal parts to kinetochores. It was difficult to decide precisely whether any break is of heterochromatic or euchromatic in the present experiment. Then the following method was adopted for convenience' sake. Each chromosome arm was divided into appropriate number of sections whose length is nearly equal with the length of section of other chromosome arms. The number of sections in the long arm of **D** and both arms of **A** chromosome are 7 and 6 respectively. The number of long arm of **C** is also 6. The long arms of **B** and **E** are divided into 5 and 4 sections and the short arms of **B**, **E**, and **C** into 3, 2 and 2 ones, respectively. The short arm of **D** chromosome was decided as one unit. The proximal one segment in each arm is heterochromatic region, although some euchromatin is included in them. For convenience' sake in the present article the first sections from kinetochores are called H-sections and the other ones called E-sections. Kinetochore breaks are included in heterochromatic breaks. The breaks within the H-sections are in the true heterochromatin or near the heterochromatin, while almost all of the breaks within the remaining sections are truely euchromatic breaks. The sum of the length of H-sections in all the five chromosomes is nearly 22.3% of the total length of all the chromosomes when calculated from the data of 12 metaphase cells. According to these categories, each break of simple and rejoined types is classified in two groups as to both types of chromatid and iso-chromatid breaks (Table 3). Total rejoined breaks and

TABLE 3. Frequencies of simple and rejoined breaks in heterochromatin and euchromatin regions of *Trillium kamtschaticum* in the simple X-ray treatment and the combined treatment with X-ray and mitomycin C (48 hrs after irradiation). -per 100 cells-

		X-ray control		Mito.-X-rays	
		Breaks in H-sections	Breaks in E-sections	Breaks in H-sections	Breaks in E-sections
sB′		3.7	48.1	6.0	39.0
sB″		16.0	66.7	11.0	55.0
rB′		13.6	14.8	47.0	34.0
rB″		1.2	6.2	3.0	9.0
sB (B′+B″)	obs.	19.8	114.8	17.0	94.0
	exp.	30.0	104.6	24.8	86.2
rB (B′+B″)	obs.	14.8	21.0	50.0	43.0
	exp.	8.0	27.8	20.7	94.0
Total breaks	obs.	34.6	135.8	67.0	137.0
	exp.	38.0	132.4	45.5	158.5

Expected numbers calculated on the basis of random distribution.
sB = simple breaks, rB = rejoined breaks.

total simple breaks of chromatid and iso-chromatid types and sum of both breaks are compared with the expected values based on the random distribution, which are obtained by dividing the total number of breaks in the proportion of 22.3 to 77.7.

From the comparison between the observed and the expected values, it appears that the distribution of total detectable breaks in the X-ray control is at random within both heterochromatic regions and euchromatic regions. However, the frequency of the breaks involved in interchanges was significantly higher in the heterochromatic regions. In the mitomycin-X-ray treatment, it is recognized that the rejoined breaks in H-sections are more than twice the expected value. This fact shows that the heterochromatic breaks become more favourable to the formation of interchange by the combined mitomycin treatment.

The comparison of the frequencies of total detectable breaks in H- and E-groups with the expected values would suggest also the consequence of restitution. From the results in Table 3, it is known that, in the mitomycin-X-ray treatment, the rejoining to restitute is prevented severely in heterochromatic regions. On the other hand, in the X-ray control restitution takes place equally in both heterochromatin and euchromatin regions.

The distribution of the breaks of each type over the five chromosomes of the complement in both cases of X-ray control and the combination treatment is summarized in Table 4.

The expected values as even distribution are calculated from the relative lengths of the five chromosomes and are shown within parenthesis. Although the number of breaks scored is not sufficient, the increase of rejoined breaks by mitomycin is recognized in **D** and **C** chromosomes. These two chromosomes have large heterochromatic segments.

TABLE 4. Distribution of induced breaks in each chromosome of *Trillium kamtschaticum* in the X-ray control and the mitomycin-X-ray treatment.

Treatment	Breaks	Chromosome					χ^2 values
		A	**B**	**C**	**D**	**E**	
X-ray control	sB (B′+B″)	44 (32.2)	18 (19.8)	17 (18.4)	17 (19.8)	8 (13.8)	7.428
	rB (B′+B″)	9 (8.1)	4 (4.9)	5 (4.6)	5 (4.9)	3 (3.5)	
	Total	53 (40.3)	22 (24.7)	22 (23.0)	22 (24.7)	11 (17.3)	6.930
Mito.-X-ray	sB (B′+B″)	29 (34.4)	21 (21.1)	24* (19.6)	26* (21.1)	11 (14.8)	3.949
	rB (B′+B″)	23 (23.9)	8 (14.6)	19* (13.6)	18* (14.6)	9 (10.2)	6.064
	Total	52 (58.3)	29 (35.7)	43* (33.3)	44* (35.7)	20 (25.0)	7.693
Relative length of chromosome (%)		31.0	19.0	17.7	19.0	13.3	

Numbers within parenthesis representing the expected values based on the random distribution.
Asterisk indicating observed number higher than expected value.
sB = simple breaks. rB = rejoined breaks.

4) *Sister reunion* (SR).

In the metaphase cells that elapsed 48 hours after irradiation, the sister reunions at iso-chromatid breaks are frequently observed (Table 5). The higher frequencies on the akinetic side than on the kinetic—in both cases of X-ray control and mitomycin-X-ray treatment—were obtained, agreeing with the general rule accepted by many investigators.

The frequency of SR, sum of SR's on akinetic and kinetic sides, in the mitomycin-X-ray treatment does not differ from that in the X-ray control. The fact that mitomycin did not change the frequency of SR in the present case

TABLE 5. Frequency of sister reunion (SR) on akinetic and kinetic sides of iso-chromatid breaks.

Treatments	Akinetic side		Kinetic side		Total	
	SRC_0	$NSRC_0$	SRC_1	$NSRC_1$	SR	NSR
X-ray control	21 (58.3)	15 (41.7)	9 (25.0)	27 (75.0)	30 (41.7)	42 (58.3)
Mito.-X-rays	24 (51.1)	23 (48.9)	15 (32.6)	31 (67.4)	39 (41.9)	54 (58.1)

Numbers within parenthesis representing percentage values.
SR = sister reunion. NSR = failure of sister reunion.

of 6 hour posttreatment suggests that after the removal of mitomycin effect broken ends recover their rejoinability and iso-chromatid breaks can rejoin to form SR to the same extent as in the control.

Discussion

In the present experiments it was suggested that restitution is reduced by mitomycin and further a conspicuous increase of two-hit aberration yield by mitomycin was recognized. As both restitution and interchange formations result from the rejoining of broken ends, they seem to be competitive process determining the final yield of aberration, if the number of breaks is scanty within a cell. In the present experiment series, however, the competition would not be effective. This will be shown by the results from the other experiments utilizing ATP, illustrating that the frequency of rejoined breaks involved in interchange formation is only slightly reduced by ATP, although the total frequency of detectable breaks is remarkably decreased.

The next step is to consider how the following phenomena are interpreted, that is, the increase of the rejoining to form interchange and the decrease of the rejoining to restitute by mitomycin treatment. When the duration of the treatment with the inhibitors of rejoining is of long period, the induced broken ends may remain open and this unrejoinable, potentially joinable, period seems to be considerably long. The breaks in the state during unjoinable period would undergo neither restitution nor interchange formation. As stated earlier, in the previous experiment, the results that SR at the broken ends was inhibited by mitomycin, which was inferred from the data of anaphase and telophase aberrations 42 hours after irradiation, are understood by supposing that the chromosomes in those ana- and telophase cells had undergone anaphase separation before the recovery of rejoinability.

With respect to SR, DARLINGTON & LA COUR ('45) and DARLINGTON &

KOLLER ('48) postulated the view that all isolocus breaks are of pre-splitting and the impairment of chromosome reproduction at broken ends caused by X-rays results in SR (Cf. THODAY '53). It seems true in the present materials that almost all of isolocus breaks are of pre-splitting, since the clear-cut shifting of breaks from chromatid type to chromosome type was observed according to the elapsed hours after irradiation. However, the idea that SR results from the impairment of the reproduction seems to be denied in the light of the evidence that mitomycin, which prevents the rejoining of broken ends, could not increase the frequency of SR.

According as the action of the inhibitors becomes ineffective, the broken ends may gradually recover their rejoinability. When mitomycin is administered, the duration of the rejoinable period will be prolonged. And further it might be concerned, in part, that the recovery begins simultaneously. Therefore the chance of the formation of two-hit aberrations increases. The longer the duration of the unrejoinable period, the less the chance of restitution, since the distance between two broken ends following a breakage become large. Furthermore these effects by mitomycin must be operative especially remarkably in heterochromatin. Why is the heterochromatic breaks extremely affected by mitomycin? From the discrepancy of stainability, heterochromatin is distinguished from euchromatin, and chemically the difference between both chromatin is considered to be either quantitative or qualitative, but rather quantitative in DNA synthesis. Recently LIMA-DE-FARIA ('59) reported that the heterochromatin of *Secale* chromosomes synthesizes DNA later than does the euchromatin as indicated by the incorporation studies of tritiated thymidine into the chromosomes. The rejoining of induced broken ends, either restitution or recombination, means the reconstruction of the part of chromosome. For this synthesis energy supply may be necessary, but in addition the synthesis of chromosomal protein and DNA must be essential events. Here is a fact that mitomycin inhibits specifically the DNA synthesis of *Escherichia coli* at certain concentration (SHIBA et al. '58, SEKIGUCHI & TAKAGI '60). Although it remains obscure whether or not the concentration of mitomycin in the ovular tissue cells of the present material was effective specifically to prevent the DNA synthesis, the modification effect of mitomycin on rejoining, especially that in heterochromatin, suggests strongly the involvement of DNA synthesis in the process. The fact that in the control group the interchanges are more frequent in H-segments suggests that biochemical process synthesizing nucleic acids, which may promote the rejoining of breaks, proceeds somewhat slowly in heterochromatin than in euchromatin. It is probable, therefore, that the DNA synthesis for rejoining is prevented by mitomycin more severely in heterochro-

matin. And the rejoining of broken ends in heterochromatin may be more inhibited by mitomycin and both the open period, during which the broken ends stay open, and the following joinable period may be prolonged more remarkably in heterochromatin.

A further available method in order to obtain full informations on the phenomenon is to supply exogenous DNA or the constituent elements of DNA into the cells irradiated. Along this line of research, another experiment was carried out with the same material of *Trillium* under the same conditions except the administration of chemical substance. The results of the study illustrate clearly the involvement of DNA to the rejoining of induced broken ends. The detailed description will be presented in another article.

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Summary

The combined radiation experiments with mitomycin C were carried out with the ovular tissue cells of *Trillium kamtschaticum* Pall.

It is ascertained that mitomycin C is effective in increasing the final yield of chromosome aberrations detectable at metaphase 48 hours after irradiation. The increase of two-hit aberrations was remarkable especially in the heterochromatic regions.

These results may well be explained by assuming that the rapid rejoining of induced broken ends is inhibited and the rejoinable period after the recovery of rejoinability is considerably prolonged by the mitomycin treatment, and that these alterations are more effective at or near heterochromatic regions.

Literature cited

BEATTY, A. V. and J. W. BEATTY. 1959. Metabolic inhibitors and chromosome rejoining. Amer. Jour. Bot. **46**: 317–323.

BEATTY, A. V. and J. W. BEATTY. 1960 a. Postirradiative effects on chromosomal aberrations in *Tradescantia* microspores. Genetics **45**: 331–344.

BEATTY, A. V. and J. W. BEATTY. 1960 b. Potassium gluconate and ATP effects on chromosome aberration yield. Proc. Natl. Acad. Sci. **46**: 1488–1492.

CONGER, A. D. 1948. The cytogenetic effect of sonic energy applied simultaneously with X-rays. Proc. Natl. Acad. Sci. **34**: 470–474.

DARLINGTON, C. D. and L. LA COUR. 1940. Nucleic acid starvation of chromosomes in *Trillium*. Jour. Genetics **40**: 185–213.

DARLINGTON, C. D. and L. LA COUR. 1941. The detection of inert genes. Jour. Hered. **32**: 115–121.

DARLINGTON, C. D. and L. LA COUR. 1945. Chromosome breakage and the nucleic acid cycle. Jour. Genetics **46**: 180–267.

HAGA, T. and M. KURABAYASHI. 1953. Genom and polyploidy in the genus *Trillum.* IV. Genom analysis by means of differential reaction of chromosome segments to low temperature. Cytologia **18**: 13–28.

HAGA, T. and M. KURABAYASHI. 1954. do. V. Chromosomal variation in natural populations of *Trillium kamschaticum* PALL. Mem. Fac. Sci. Kyushu Univ. Ser. E (Biology) Vol **4**: 159–185.

LEA, D. E. 1947. Actions of radiations on living cells. The University Press, Cambridge.

LIMA-DE-FARIA, A. 1959. Differential uptake of tritiated thymidine into hetero- and euchromatin in *Melanoplus* and *Secale.* Jour. Biophys. and Biochem. Cytol. **6**: 457–466.

MATSUURA, H., S. TANIFUJI, and M. IWABUCHI. 1962. Chromosome studies on *Trillium kamtschaticum* PALL. and its allies. XXV. The effects of some chemicals on the frequency of X-ray-induced chromosome aberrations. Jour. Fac. Sci., Hokkaido Univ., Ser V. Vol. **8**: 57–74.

SAX, K. 1943. The effect of centrifugation upon the production of X-ray induced chromosomal aberrations. Proc. Natl. Acad. Sci. **29**: 18–21.

SEKIGUCHI, M. and Y. TAKAGI. 1960. Effects of mitomycin C on the synthesis of bacterial and viral deoxyribonucleic acid. Bioch. Biophys. Acta **41**: 434–443.

SHIBA, S., A. TERAWAKI, T. TAGUCHI, and J. KAWAMATA. 1958. Studies on the effect of mitomycin C on nucleic acid metabolism in *Escherichia coli* strain B. Biken's Journal **1**: 179–193.

THODAY, J. M. 1953. Sister-union isolocus breaks in irradiated *Vicia faba*: the target theory and physiological variation. Heredity **6**, Suppl.: 299–309.

WOLFF, S. 1960. Radiation studies on the nature of chromosome breakage. Amer. Nat. **94**: 85–93.

WOLFF, S. and R. C. VON BORSTEL. 1954. The effects of pre- and postirradiation centrifugation on the chromosomes of *Tradescantia* and *Vicia.* Proc. Natl. Acad. Sci. **40**: 1138–1141.

WOLFF, S. and H. E. LUIPPOLD 1955. Metabolism and chromosome-break rejoining. Science **122**: 231–232.