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Title	Cytological Effects of N.N'.N ˢ-triethylenthiophosphoramide (TESPA) upon the PMCs of Paris verticillata M.v.BIEB
Author(s)	TANIFUJI, Shigeyuki
Citation	Journal of the Faculty of Science, Hokkaido University. Series 5, Botany, 8(2/3), 143-156
Issue Date	1962
Doc URL	https://hdl.handle.net/2115/26312
Type	departmental bulletin paper
File Information	8(2_3)_P143-156.pdf

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Cytological Effects of N. N'. N''-triethylenthiophosphoramide (TESPA) upon the PMCs of *Paris verticillata* M. v. BIEB.*

By

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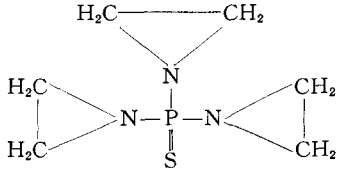
It is a well known fact that the alkylating agents produce the chromosome injuries of the radiomimetic types. In higher plants, 2:4:6: triethylenimino 1:3:5 triazine (TEM), which is one of ethylenimine compounds, was demonstrated by ARNASON & WAKONIG ('59) to be effective in causing chromosome breakage in roots of *Vicia* and *Allium*, and in microsporocytes of *Hordeum*, though not in microspores of *Tradescantia*.

In the present experiments, the effect of N. N'. N''-triethylenthiophosphoramide (TESPA), one of the alkylating agents, on the PMCs of higher plants was investigated cytologically. The structural formula for this compound is shown as follows:



The author's attention was also paid to the observations of the behaviour and structure of kinetochores, since the breakage within the kinetochore region of the chromosomes in roots of *Trillum* was observed rather frequently in the experiments with TEM** as a preliminary test.

Materials and Methods

TESPA was used at concentrations of 25 and 50 mg/l dissolved in tap water. The materials were taken from the PMCs of *Paris verticillata* M. v. BIEB. ($2n=10$, 5II) and, as a preliminary

* Aided in part by a grant defrayed from Mombusho Scientific Research Fund to Professor H. MATSUURA.

** This preparation was supplied through the courtesy of Dr. A. TONOMURA, National Institute of Genetics, Misima, Japan.

[Journal of the Faculty of Science, Hokkaido University, Ser. V, Vol. VIII, No. 2 & 3, 1962]

test, the PMCs of *Trillium kamtschaticum* PALL. ($2n=10$, 5II) were also utilized. The five chromosomes of each gametic set are represented as **A**, **B**, **C**, **D** and **E** respectively both in *Trillium* and *Paris* (HAGA '34).

In the first set of experiments with the PMCs of *Trillium*, young inflorescences with naked anthers after removal of their sepals and petals were immersed directly in TESPA solutions. On the other hand, in the case of *Paris* cut stems bearing young inflorescences were kept in the solutions for various hours. After treatment the solutions of TESPA were replaced by tap water and the PMCs were fixed and stained with aceto-carmine at various times. In order to clarify the structure of kinetochores in normal meiotic chromosomes of *Paris*, the Matsuura's pretreatment method ('44) was applied.

Results

1) Preliminary test with PMCs of *Trillium*.

The PMCs at pachytene or diplotene were treated with 25 or 50 mg/l aqueous solutions of TESPA for 2 or 3 hours. At 24 or 72 hours after the end of the treatment almost all of PMCs were at first anaphase and telophase. Although the frequency of the aberrations observed in these cells was not so high, such abnormalities as akinetic fragments, chromatid bridges with fragments, univalent laggards, and in addition, monopolar cells with or without cell wall, and tripolar telophase (Fig. 4) were found. Delayed metaphase I cells with over-contracted chromosomes and abnormal prophase cells, in which the nuclei became homogeneous pycnotic mass, were frequently observed among the normal cells at ana- or telophase I. Chromosomal breakage at metaphase I was also observed, but stickiness of chromosomes was not so remarkable. The frequency of abnormal cells at first metaphase, anaphase and telophase in one anther is shown in Table 1 as an example.

TABLE 1. Frequency of aberrant PMCs of *Trillium* treated with 50 mg/l solution of TESPA for 2 hours (24 hrs after the end of the treatment).

Metaphase I			Ana- and Telophase I				
Normal cells	Cells with chromosome breakages	Cells with over-contracted chromosomes	Normal cells	Cells with chromatid breakages	Cells with bridge and fragment	Cells with fragments	Monopolar cells
71	3	5	198	2	5	9	2

2) Inner structure of the kinetochore in *Paris*.

Many authors hold that the kinetochore is of a compound structure. LIMA-DE-FARIA ('58) distinguished the interior and median zones within the

kinetochore of *Tradescantia* at meiosis. He observed regularly the occurrence of the two chromomeres in the interior zone and two in the median. The compound structure with chromomeres was also revealed by him in the kinetochores of *Secale* and *Agapanthus*. Somewhat similar description was given in the somatic chromosomes of *Scilla liliohyacinthus* by GIMÉNEZ MARTÍN ('58) as the centromere having six equidistant chromomeres.

In the present material, *Paris*, the fixation techniques without pretreatment are unfavorable to the study of the fine structure of kinetochores. In the present study, the pretreatment with 0.28 mol solution of KCl, as stated above, was employed in order to reveal the inner structure.

As shown in Figs. 5 & 6, the double structure of the kinetochore is evident but the appearance of chromomeres is not clear.

MATSUURA ('41) illustrated the inner structure of the kinetochore in the meiotic chromosomes of *Trillium kamtschaticum*. He states that the kinetochore is a compound body consisting of the chromonematic thread (kinetonema) and of the matrix surrounding it. The present findings seem to correspond with those by MATSUURA.

3) Experiments with PMCs of *Paris*.

The treatment of *Paris* was carried out with the PMCs almost all of which were at the earlier stages, i.e. early prophase or leptotene (Fig. 1). The durations of treatments with TESPA were 8 and 16 hours for each of two concentrations, i.e. 25 and 50 mg/l. In addition, 3 hour-treatment with 50 mg/l solution was carried out.

The quantitative relationship between the frequencies of aberration and these different treatments was not so clear as expected.

§ *Abnormal nuclei at prophase.* Among the PMCs treated from early prophase on, there were more or less abnormal prophase cells showing the homogeneous pycnotic structure of nuclei, sometimes with the chromatic bodies in cytoplasm (Fig. 7). Since these aberrant cells with the pycnotic nuclei and the diplotene cells with the chromatic bodies showing a similar structure of abnormal prophase nuclei, are frequently observed in company with normal early diplotene cells, the cause of this aberration might not be attributed only to the extreme clotting of chromosomes at prometaphase or metaphase I. This aberration is not caused specifically by the treatment with TESPA, but recognized also in the cases of the treatment with dihydro-streptomycin sulfate (TANIFUJI '60) and with the water extracts of *Arisaema japonicum*, one of toxious plants (OHNO & TANIFUJI '60).

§ *Over-contraction of chromosomes.* Over-contraction of chromosomes at

metaphase I was recognized with a few frequencies among the abnormal prophase cells, and also among the proceeding normal or only slightly damaged cells, i.e. anaphase I, telophase I, interphase and so on.

§ *Stickiness and clotting of chromosomes.* Clotting or stickiness of chromosomes was rarely found at metaphase I or II (Figs 8, 9 & 10). Another study on the somatic chromosomes of *Trillium* (unpubl.) suggests that remarkable stickiness is not induced by TESPA. In the present experiments, however, the observation was made at 2 or 3 days after the treatment, and so conclusive decision may not be drawn. WAKONIG & ARNASON ('59) stated that no chromosome stickiness occurred in *Vicia* and *Allium* when treated with TEM.

§ *Lagging at telophase I.* Lagging of univalents at telophase I was met with, though rarely. Cases of non-disjunction of chromosomes at anaphase I were found in a few cells.

§ *Tripolar telophase.* Two days after treatment with 50 mg/l solution for 8 hours, tripolar telophase was observed in a few cells. In one of them three polar groups consisted of 4, 3 and 3 bivalents.

Although the frequency was extremely low, cases of monopolar telophase were also observed.

§ *C-meiotic configurations.* At 34 hours after treatment with 50 mg/l solution of TESPA for 16 hours, the c-meiotic figures appeared rather frequently; that is, all bivalents were scattered throughout the cell (Figs. 12 & 13). Moreover, in some of these cells the gradual loss of stainability with aceto-carmine and the alveolation of chromosomes were observed (Fig. 13).

In the present case, it is not conceivable that the functional evanescence of both poles resulted in such c-meiotic configurations, because the non-convergence of 5 univalents at one polar region is not recorded. Accordingly, it seems likely that the destruction or non-organization of the spindle or attractoplasm was caused by the TESPA treatment and this related to the occurrence of c-meiotic figures.

§ *Uncoiling and dispersal of chromonema.* Cells showing the uncoiling of the chromonemata were observed among the first metaphase, anaphase and telophase cells in cases of two days after treatment with 50 mg/l solution for 8 hours. In the configurations shown in Figs. 15 & 16, the two uncoiling threads run parallel with each other. Sometimes, partially affected chromosomes are found. One of these cells is shown in Fig. 14 (A), which is at anaphase I and accompanied with the extreme fragmentation of uncoiled chromonemata.

The treatment with TESPA causes also the dispersal of chromonematic threads. As shown in Figs. 17 & 18, the chromonematic threads disperse into

spherule-like substances which are only faintly stained with aceto-carmine. Quite similar abnormalities were reported by BAL & KAUFMANN ('59) in the PMCs of *Tradescantia* when treated with desoxyribonuclease after exposure to ribonuclease. They proposed that removal of nucleic acids facilitates uncoiling of the protein threads of chromosomes.

Although it remains obscure whether or not TESPA is able to efface nucleic acids from chromosomes or able to inhibit nucleic acid synthesis, the following possibility may be supposed that general metabolic disturbances by TESPA come to secondarily produce the aberration of chromosomes. Uncoiling of chromosomes was also observed in the PMCs of *Paris* when treated with streptomycin at earlier prophase stages (unpubl.). Furthermore, MATSUURA & IWABUCHI ('62) observed similar chromosomal aberrations in the same material after the administration of several chloride compounds.

As two chromatids of univalent are generally separated with each other except kinetochore regions at anaphase I, the two threads lying parallel may be uncoiled chromatids. For accurate decision of whether they are chromatids or not, the counting of numbers of these threads within the cell is necessary. This is a difficult task, since these threads are long and entangled with each other. However, roughly counted number of the threads in a cell is about 20, not 40 at best, suggesting the uncoiling of chromatid threads. On the other hand, as shown in Fig. 14, there is a structure as indicated by arrow which seems to be half-chromatid without coiling. In this partially affected cell at anaphase I, the chromosomes keeping their ordinal shape are **a**, **a**, **b**, **b**, **c**, **d** and **e** univalents (univalents of each chromosomes are represented by small letters), in addition to them there are the long arm of **d**, and one chromatid of **c** chromosome. Accordingly, **e** univalent, one short arm of **d**, and one chromatid of **c** would have been presumably converted to the rest unusual structure. However, it seems that the total amounts of these bodies are a little in excess of the chromosomes which are presumed to have deformed. The loss of distal part of some chromosomes might have been involved in this situation. Regardless of a few obscurities, it will be allowable to assume that the faintly stained structure which consists of two parallel threads indicated by arrow is a single chromatid of **c**, each of the two threads being a half-chromatid. It will not be improbable that half-chromatids of meiotic chromosomes become visible precociously under certain conditions. MATSUURA & HAGA ('40) found that meiotic chromosomes splitted into half-chromatids in *Trillium* after exposure to high temperature, and they called it "ultra-mitotic type" of division. Another possibility, however, is that the configuration at question represents a pair of chromatids which remain uncoiled, as in Figs. 15 & 16, although

such a partially affected cell as shown in Fig. 14 is of rare occurrence.

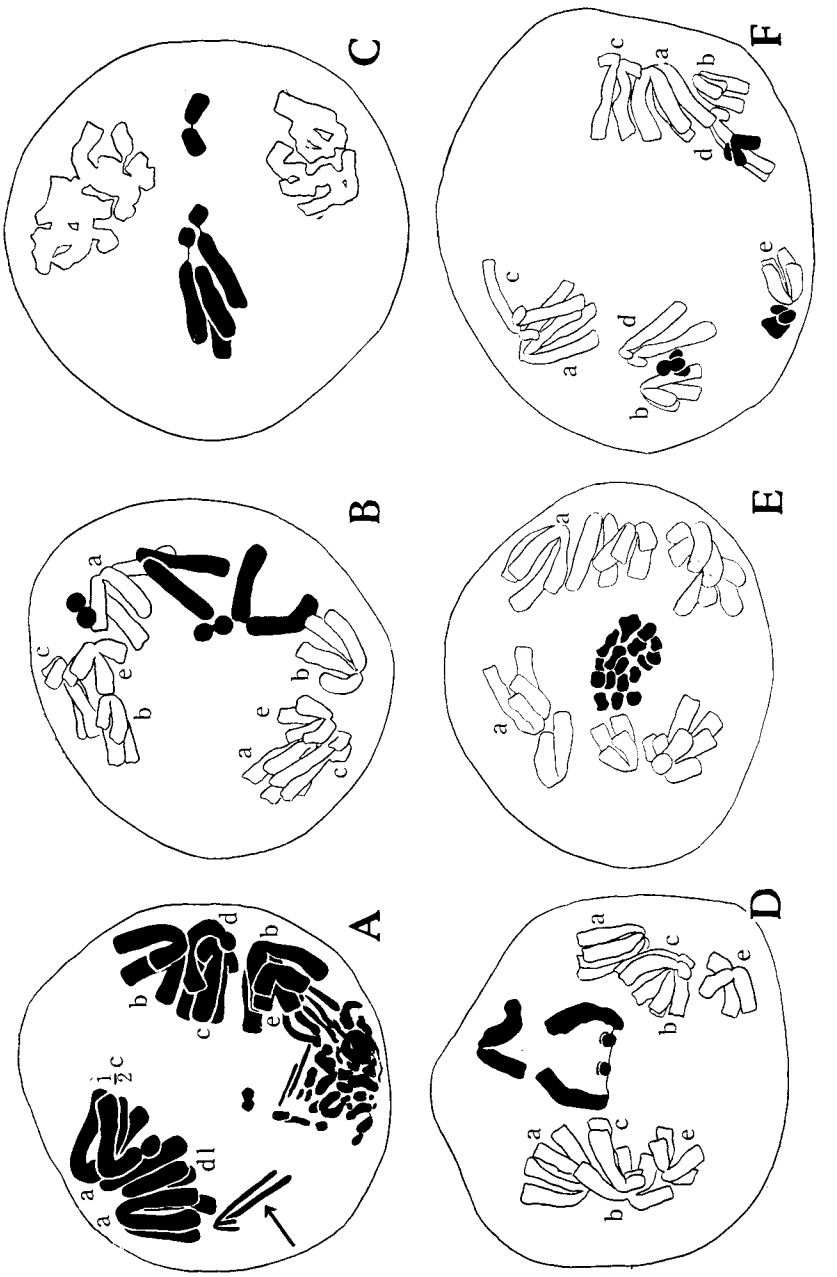
§ *Chromosome breakage.* In the earlier experiments with the PMCs of *Paris*, it was shown that the breaks induced by streptomycin consisted of both chromatid and chromosome types (TANIFUJI '60). Furthermore, a configuration was observed, which seemed to have derived from four isolocus chromatid breakages in a bivalent. Such a configuration might have resulted from "chiasma breakage" as proposed by MATSUURA ('50), that is, simultaneous breakages of the four chromatids of a bivalent at one chiasma. It has been reported that the chiasma breakage is mostly confined to hybrid plants in *Trillium* (MATSUURA '50, KURABAYASHI & SAHO '57). Therefore, it is assumed that in the diploid *Paris*, the frequency of chiasma breakage is also low. However, it may be possible that the occurrence of chiasma breakage is affected by some chemicals when the first metaphase chromosomes are exposed. Apart from this question, it is a subject of deeper interest to know how the chromosomes constituting bivalents at earlier stages of prophase response to the action of radiomimetic substance. Simultaneous breakage of the four chromatids at isoloci in a bivalent was frequently recognized at metaphase I. Such a breakage will be referred, for brevity, as bivalent breakage. Resultant configurations of bivalent breakage were observed at ana- or telophase I. When any reunion did not take place, four isomorphic akinetic fragments will be expected to appear at anaphase I (Fig. 21). Cases of isolocus breakage of two chromatids in a half bivalent were also observed. Consequently, cells with only one or two akinetic fragments were frequently recorded at ana- or telophase I. The frequency of these aberrant cells are represented in Table 2.

The sister reunion at each broken end of the four fragments produced by such a bivalent breakage, would result in the formation of two isomorphic akinetics. However, it seems likely that the reunion took place only rarely in the present material, since the bridge formation seldom occurred as compared with the frequency of akinetic fragments.

§ *Breakage within kinetochore region.* Breaks within the kinetochore region also appeared with considerably high frequency. Among them there are breaks of bivalent and those of one of two chromosomes of a bivalent. The former is shown in Figs. 20, 23 (B) and 24 (C), and the latter in Figs. 19, 22 and D. The configuration shown in Fig. 24 (C) could not be decided whether it is due to the imperfect bivalent breakage of **C** chromosome or to two simple chromosomal breakages of **C** and **D** within the kinetochore region. Laggards shown in Fig. D are clearly the result of simultaneous breakage and reunion in the kinetochore region of the bivalent of **D** chromosome. The configuration of Fig. 23 (B) is also an illustration of the bivalent breakage within the

TABLE 2. Frequency of aberrant PMCs of *Paris* after exposure to TESPA solution (Aberrations per 100 cells).

Duration of treatment, hours	Concentration	Days after the end of treatment	Metaphase I						
			Total cells observed	Normal	Contraction of chromosome	Clotting of chromosome	Bivalent breaks	4II+2I	Unclear chromosome breaks
8	50 mg/l	2	63	50.8	3.2	1.6	22.2	6.4	15.9
8	50 mg/l	3	24	41.7	16.7	0.0	25.0	12.5	4.2
16	25 mg/l	1	7	14.3	28.5	0.0	28.5	28.5	0.0
			Anaphase I						
			Total cells observed	Normal	One akinetic fragment	One bridge and one fragment	Extreme breaks	Monopolar	
8	50 mg/l	2	68	70.6	11.8	2.9	11.8	2.9	
8	50 mg/l	3	31	77.4	6.5	3.2	12.9	0.0	
16	25 mg/l	1	2	100.0	0.0	0.0	0.0	0.0	
			Telophase I						
			Total cells observed	Normal	One akinetic fragment	Two isomorphic akinetics	Four isomorphic akinetics	Extreme fragmentation	
8	50 mg/l	2	406	94.6	3.5	0.0	0.5	1.5	
8	50 mg/l	3	226	96.0	1.8	0.4	0.0	1.8	
16	25 mg/l	1	101	91.9	2.0	3.0	1.0	3.0	


A
B
C
D
E
F

kinetochore region of **D** chromosome. One of the short arms is located at the polar region, though it is not decided whether the newly organized kinetochore is still functional or not. Moreover, the isobrachial laggards (one arm of a univalent) at late telophase I are shown in Fig. 25 (presumably one arm of **B**), and Fig. 26.

It has been shown by DARLINGTON ('39), MCCLINTOCK ('32) and other authors that the misdivision of kinetochore leads to the formation of isochromosomes. On the basis of the recognition of centromeric chromomeres, MARKS ('57) and SANCHEZ-MONGE ('50) discussed the various types of misdivision.

In the cells shown in Figs. 25 and 26, the rest part of the isobrachial laggard must be incorporated into one of the daughter nuclei. It may have been incorporated in main nucleus by chance like sex chromosomes in animal cells which wander about throughout the cell during the division. However, if it is maintained in the nucleus through the successive cell divisions, the establishment of functional isochromosomes may not be impossible. Regarding the inner structure of kinetochores in *Paris* chromosomes, it must be rare that breaks within its region occur just at the middle point. Therefore the simultaneous formation of two or more functional isochromosomes from one univalent or bivalent may be of rare occurrence. Although the isochromosomes observed in the present experiments are not abundant, almost all of them remain as laggards, suggesting the rarity of establishment of functional isochromosomes.

§ *Extensive fragmentation.* In the case of two or three days after treatments with 25 and 50 mg/l solutions, the extensive fragmentation of chromosomes at ana- or telophase I was frequently recorded (Figs. 28 & 29). The sizes of these fragments are similar within a cell, though different among different cells. In almost all cases both chromatids of chromosomes are involved in the fragmentation. Fig. 29 represents a typical configuration like the bunches of grapes. Sometimes, the rosary-like structure of chromosomes was observed at telophase I, which was distinguished from the former by the linear arrangement and being the chromosomal type (Fig. 30). The configuration shown in Fig. 27 seems to represent the potential type of the extensive fragmentation of the first anaphase chromosomes, which was presumably induced after the separation of the chromosomes constituting a bivalent. In this case, it is considered that the granules remain in line because of the imperfect breakage. Such an extensive or excessive fragmentation has been reported by many authors. LOVELACE ('54) found that ultraviolet radiation produced similar aberrations, i. e. extensive fragmentation and bead-like structure, in the pollen tube chromosomes of *Tradescantia* and substituted the term "shattering" for the extensive fragmentation of the parts of chromosomes. According to LOVELACE's account,

the localized shattering of chromosomes and the different degrees of it were caused by the differential dosage of UV-rays within a nucleus. SWAMINATHAN and NATARAJAN ('59) observed the extensive fragmentation of the root chromosomes of wheat when treated with peanut, mustard, and caster oils. And the rosary-like structure of anaphase bridges was demonstrated by KATO ('57) in the onion roots treated with the water extract of spinach fruits. The extensive fragmentation of meiotic chromosomes was also found by MATSUURA and IWABUCHI (loc. cit.) in *Paris* treated with various salts such as NaCl, KCl and $CaCl_2$, and further by MATSUURA and TAKEHISA ('62) in the PMCs of *Trillium* treated with a chelating agent, EDTA.

In the case involving alkylating agents, ARNASON & WAKONIG ('59) also reported such an aberration in the meiotic chromosomes of barley after administration of TEM. Furthermore, KOLLER ('58) inferred that the metabolic disturbances by alkylating agents caused indirectly the excessive fragmentation. Even in the LOVELACE's experiments with UV irradiation, the possibility that the certain chemicals, produced by UV-rays, affected the induction of shattering, if not all, would also be postulated. BLOOM, ZIRKLE and URETZ reported that the spindle was destructed or diminished by the irradiation of UV microbeams, and these events also occurred by the bombardment of a small portion of the cytoplasm. Accordingly spindle "poison" which is formed by UV-rays from some cytoplasmic constituents was assumed by them to be responsible for these aberrations (ZIRKLE '59).

An explanation about the extensive fragmentation in meiotic chromosomes will be given through the "parallelization hypothesis", proposed by MATSUURA ('40, '49). According to his account, two paired chromatids constituting the relational spiral at early metaphase are converted to the parallel system through the isolocus breakages of chromatids at each half coil and subsequent reunion. When such reunions failed to occur after the breakages, it may result in many fragments with equal length, or with multiple length of a certain unit. Although the final conclusion should be awaited until further studies are accomplished, the case reported by MATSUURA and TAKEHISA (loc. cit.) seems to suggest this possibility.

Summary

In the present study, the effects of N. N'. N''-triethylenthiophosphoramide (TESPA) on the PMCs of *Paris verticillata* M. v. BIEB. and, as a preliminary test, on the PMCs of *Trillium kamtschaticum* PALL. were dealt with. Furthermore, the inner structure of kinetochores in meiotic chromosomes of *Paris* was

investigated by means of the pretreatment with KCl solution.

The main results obtained are as follows:

1) The TESPA treatment on the PMCs of *Trillium* produced the following abnormalities; akinetic fragments, chromatid bridges, univalent laggards, monopolar cells, tripolar cells, over-contraction of chromosomes at metaphase I, and abnormal prophase nuclei showing the homogeneous pycnotic mass, though the frequencies of these abnormities were not high.

2) In addition to these abnormalities, in the case of *Paris*, slight stickiness and clotting of chromosomes at metaphase I or II, c-meiotic figures, uncoiling and dispersal of chromonemata, and extensive fragmentation were recorded.

3) The simultaneous breakages of four chromatids and two chromatids at isoloci of one bivalent were sometimes found. Moreover, the breakage within the kinetochore region was also observed.

4) The pretreatment with KCl solution revealed the double structure of kinetochores in the normal bivalent chromosomes of *Paris*, but failed to demonstrate any chromomere-like structure.

Acknowledgment

The writer is deeply indebted to Professor Hajime MATSUURA, Hokkaido University, Sapporo, for his kind guidance and encouragement during the cource of this study.

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Explanation of Text-figures.

Fig. A, partially affected chromosomes at telophase I. The two parallel threads indicated by arrow are presumed to consist of half-chromatids.

Fig. B, resultant configuration from the breakage within the kinetochore of **D** bivalent. A pair of long arms and one short arm are seen as laggards.

Fig. C, configuration assumed to be resulted from either the breakage within the kinetochore region of **C** bivalent, or that of **D** and **C** chromosome (as univalent unit).

Fig. D, resultant configuration from the breakage within the kinetochore region of **D** bivalent. The affected chromosomes are also observed as laggards.

Fig. E, extensive fragmentation at telophase I. It is shown that almost all of the chromosomes are subjected to fragmentation.

Fig. F, cell at telophase I having three fragments which are obviously resulted from the fragmentation of only one chromosome of **E** (half-bivalent).

Explanation of Plates

Plate I

Figs. 1–3. Normal prophase cells of *Paris verticillata* M. v. Bieb..

1, two nuclei at early prophase. 2, a nucleus at middle prophase. 3, a nucleus at late prophase.

Fig. 4. Tripolar telophase of the PMCs of *Trillium kamtschaticum* Pall. treated with TESPA solution.

Figs. 5–6. Inner structure of the kinetochore of normal meiotic chromosome of *Paris verticillata*, stained with aceto-carmine after the pretreatment with 0.28 mol KCl solution.

Figs. 7–15. Abnormal PMCs of *Paris verticillata* treated with TESPA.

7, abnormal nucleus at prophase and chromatic bodies showing the homogenous structure of chromatin mass. 8, clotting of chromosomes at metaphase I. 9–10, stickiness and clotting of chromosomes at metaphase II. 11, tripolar telophase. 12–13, c-meiotic configurations. Note the alveolation of chromosomes in Figure 13. 14, a partially affected chromosomes at telophase I. Uncoiling of a few chromosomes and the extreme fragmentation of the threads (cf. Fig. A). 15, uncoiling of chromosomes.

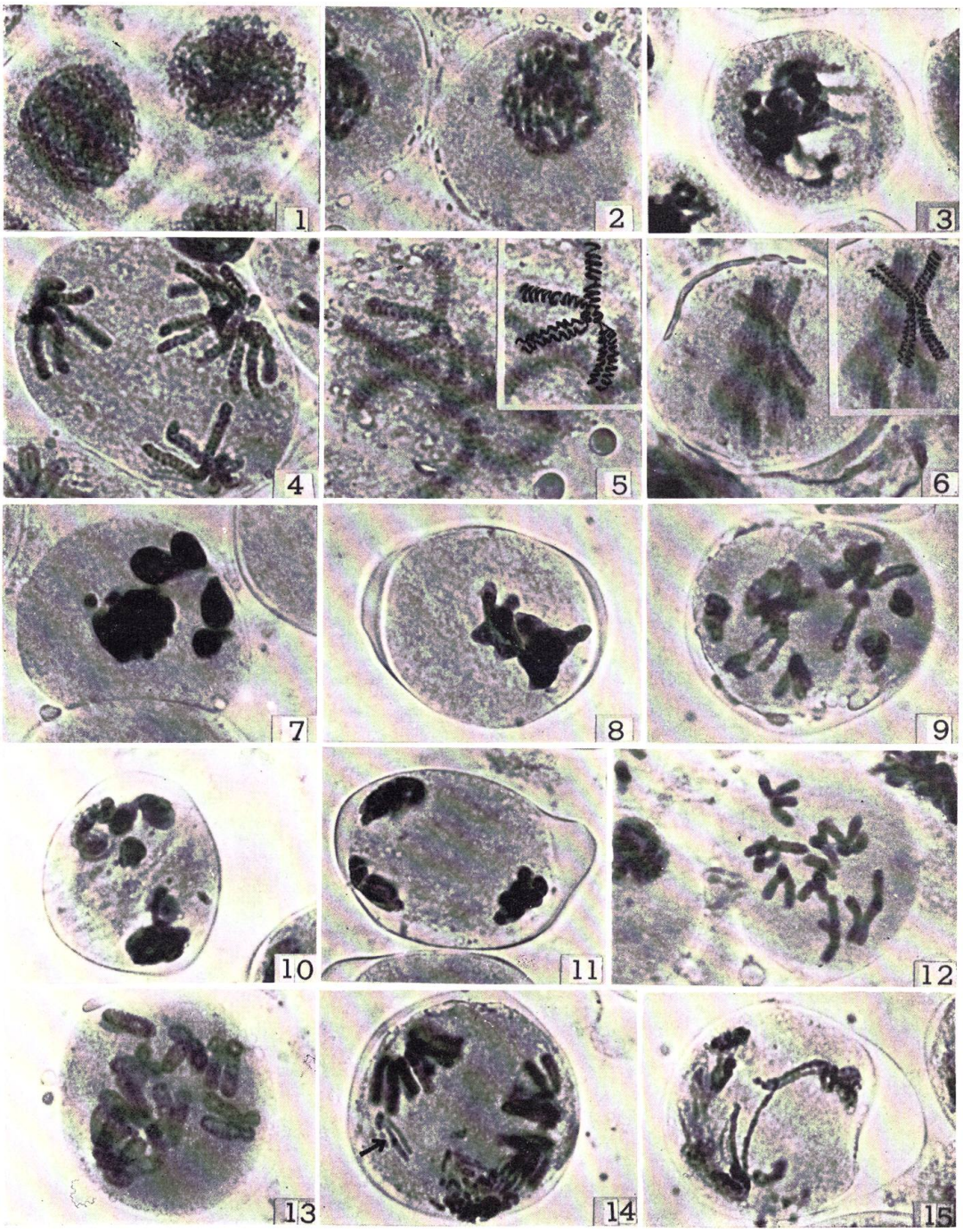
Plate II

Figs. 16–30. Abnormal PMCs of *Paris verticillata* treated with TESPA.

16, uncoiling of chromosomes. 17–18, dispersal of chromonemata. 19, simultaneous breakages of two chromatids (kinetonemata) within the kinetochore region of **A** chromosome at metaphase I. 20, simultaneous breakages of the four kinetonemata of the bivalent of **E** chromosome. 21, two and four isomorphic akinetics at telophase I, the latter suggesting the simultaneous breakages of four chromatids at isoloci of a bivalent.

Figs. 22–26. Resultant configurations at telophase from the breakage within the kinetochore region.

22, simultaneous breakages of the two kinetonemata of **d** univalent. 23, simultaneous breakages of the four kinetonemata of **D** bivalent (cf. Fig. B). 24, configuration assumed to be resulted from either simultaneous breakages of four kinetonemata in **C** bivalent, or independent breakages in two non-homologous chromosomes, **c** and **d** univalents (cf. Fig. C). 25–26, laggards of one whole arm of a univalent resulted from the breakage within the kinetochore region. Figs. 27–30. Extensive fragmentation of chromosomes at telophase I. 27, potential type of extensive fragmentation (presumably at telophase). 28–29, extensive fragmentation. It is clearly observed that both chromatids of chromosomes are involved in the fragmentation. 30, extensive fragmentation. Note the linear arrangement of fragments.



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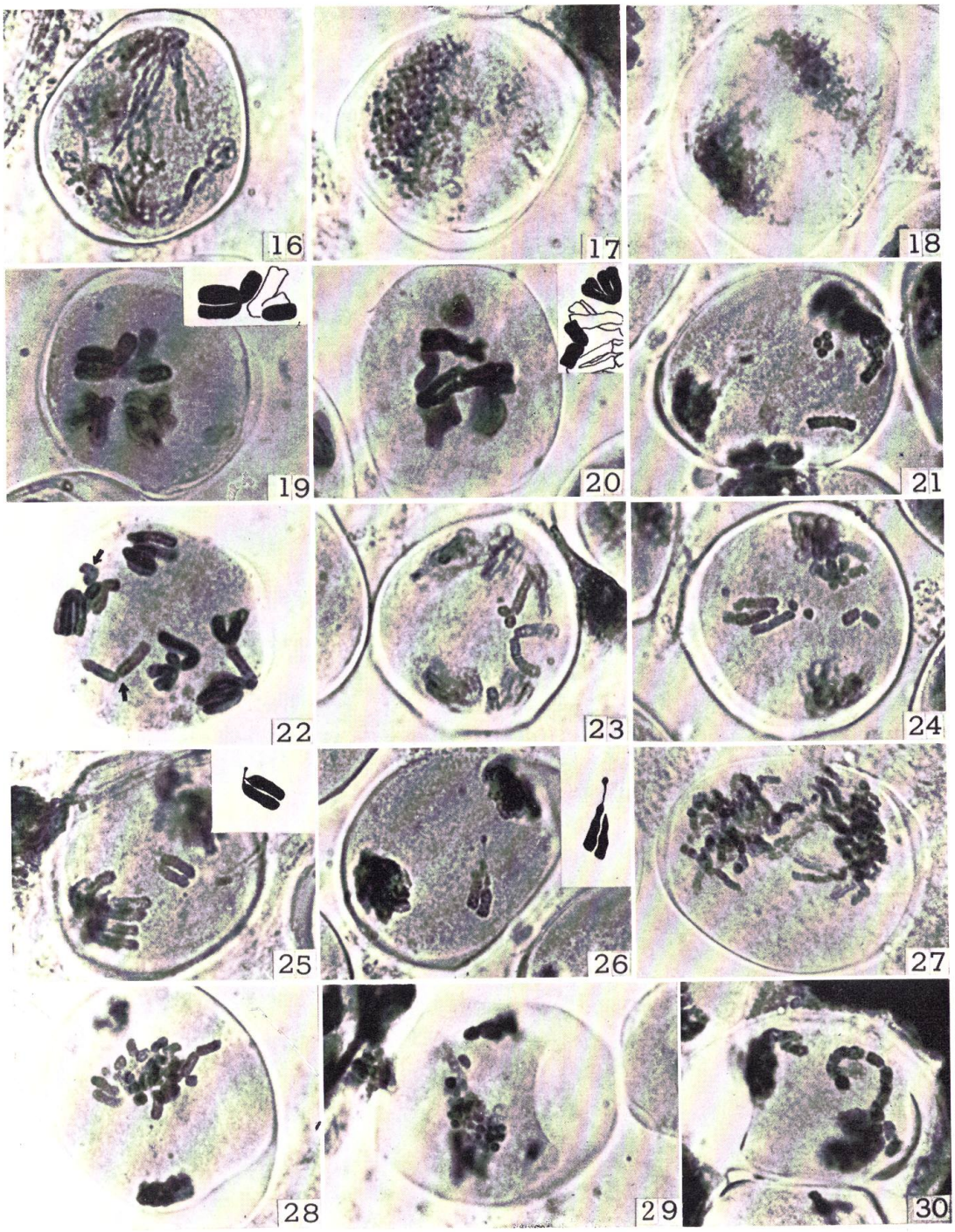

S. Tanifuji: Cytological effect of TESPA on Paris chromosomes.