Chromosome Studies on *Trillium kamtschaticum* PALL. and Its Allies. XXVII. Effect of EDTA on the structure of meiotic chromosomes*

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Recently STEFFENSEN (1953, 1955, 1957, 1959) asserted that Ca and Mg cations are responsible for the structural integrity of chromosomes, holding together macromolecular units of chromosomal nucleoprotein in the form of end-to-end linkage. This hypothesis favours the explanation of crossing-over mechanism at the molecular level of chromosome structure, in the sense that the crossing-over, i. e., breakage and reunion at the chromosomal level, must comprise breakage and rebonding of chemical bonds in nucleoprotein complex. It seems likely that at the time of crossing-over chromosome reunion takes place instantaneously after breakage, and there is a fact that the reunion of chromosome breakage needs energy supply (WOLFF & LUIPPOLD, 1955; BEATTY & BEATTY, 1959, 1960; MATSUURA et al, 1962). From these two points, it is assumed that points of break-reunion in chromosomes at the time of crossing-over are of low-energy-requiring chemical bond. Such bridges of Ca and Mg cations in chromosome organization as presumed by Steffensen fit well with this type of chemical bond. On the other hand, the hypothesis which assumes the participation of Ca and Mg cations in the structural integrity of chromosomes has been objected by KAUFMANN & his co-workers (1956, 1957a, 1957b, 1957c), LEVINE & his co-workers (1956, 1958), and many others.

Decades ago Matsuura observed the change of spiral system of metaphase I chromosomes in meiosis of *Trillium*, i. e., the two relationally coiled chromatids of each arm in early metaphase I are transformed into two parallely coiled strands in late metaphase I, and thus he advocated the spiral theory of crossing-over that this transformation of spiral system represents the crossing-

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over itself and is due to the breakage and reunion in each half-coil of spiral (MATSUURA, 1940, 1950).

We conjecture then that these broken and rejoined points at every half-coil might be Ca- and Mg-bridging points. If our conjecture be right, the treatment with EDTA which deprives Ca and Mg cations, when acted at the critical period of spiral-parallelization, ought to induce chromosome fragmentation which indicates breakage at half-coil unit distance.

The present paper deals with the result of experiments with an aim to ascertain the above conjecture.

Material and method

Anthers of *Trillium kamtschaticum* PALL. collected from Nakagoya population in the plains of Ishikari were used as material. At first one of six anthers in a flower was excised and its meiotic division stage was examined by usual aceto-carmine smear method. When almost meiocytes (PMCs) in the examined anther were at the stage of meiotic late prophase or metaphase I, three of remaining five anthers were excised and floated on the 0.01 M aqueous solution of EDTA (ethylenediaminetetraacetic acid) adjusted to pH 6.5–7.0 with 1N NaOH, and the rest on deionized water as control for 24 hours. Preliminary works indicated that the concentration of EDTA is suitable at 0.01 M for a purpose of the present experiment.

The PMCs were fixed and stained by usual aceto-carmine smear technique with water-pretreatment introduced by MATSUURA (1938) in order to demonstrate the spiral configuration of chromosomes. The temperature before and during the treatment was maintained at 7°–11°C. The PMCs observed in the present experiment were taken from the upper part of each anther.

Results

Usually the PMCs at late prophase or metaphase I of *Trillium kamtschaticum* PALL. comes up to telophase I or interkinesis in 24 hours (OHNO & TAKEHISA, 1962). When anthers just passing through the stage of late prophase or metaphase I are excised and floated for 24 hours either on EDTA solution or on deionized water, the meiotic division in the majority of these excised anthers proceeds further and reach telophase I, although this does not imply that EDTA was not taken into PMCs through tapetum.

In cases of treatment with EDTA which started from late prophase, it was found in some anthers that meiosis did not proceed remaining at late prophase (cf. Table 4). As a whole it is also evident that the treatment with EDTA slows down the division speed when compared with that of control (cf. Table 3). The average number of cells with aberration per 100 telophase I cells is, in the case of treatment from metaphase I, 9.76 cells in EDTA treat-

TABLE 1. Frequency of aberrant PMCs observed at telophase I treated with EDTA from metaphase I on. (per 100 cells)

Anther No.*	No. cells with aberration										
	Structural aberration								Non-structural aberration		
	chromo. frag.	chromat. frag.	isochrt. frag.	macro frag.	mirco frag.	minute frag.	b+f**		sticky bridge	others***	
EDTA treatment series.											
35-S	1	·	4	·	4	4	5		·	1	
35-S	·	6	·	·	4	2	2		·	·	
35	·	1	1	1	·	1	·		4	·	
34	·	2	·	6	·	1	·		1	·	
34	·	·	·	2	·	6	·		·	·	
31	·	·	·	·	3	2	·		1	·	
31	·	1	·	·	1	·	·		4	·	
31-2	1	·	·	2	4	6	·		3	·	
31-2	1	·	·	·	2	2	·		·	·	
2-23****	·	1	1	·	·	1	1		3	1	
2-23	·	1	·	·	2	·	·		2	·	
2-23	·	·	1	·	2	1	·		13	·	
2-23	·	·	·	1	·	8	·		1	·	
2-23	·	·	·	·	2	·	1		4	1	
24-F	·	·	·	·	5	·	4		12	4	
24-FA	·	·	·	·	·	·	1		·	·	
24-FA	·	·	·	·	·	·	·		·	·	
mean	0.18	0.71	0.41	0.71	1.71	2.00	0.82	total (6.54)	2.82	0.40	total (3.22)

Control series											
35-S	·	3	·	·	·	2	2		1	1	
34	·	·	·	2	·	1	·		·	·	
31	·	·	·	·	1	·	1		1	·	
31-2	·	2	1	·	·	·	1		4	1	
31-2	·	·	·	·	·	1	1		·	·	
2-23	·	·	·	·	2	·	1		4	·	
2-23	·	·	·	·	·	·	1		4	·	
2-23	·	·	·	·	·	·	2		1	·	
24-F	·	·	·	·	·	·	·		·	·	
24-FA	·	·	·	·	·	·	·		·	·	
mean	0.00	0.50	0.10	0.20	0.30	0.40	0.90	total (2.40)	1.50	0.20	total (1.70)

* The anthers with a common Anther No. were derived from one flower.

** Bridge and fragment.

*** Mainly chromosome lagging.

**** Anthers with Anther No. 2-23 were derived from three individuals.

TABLE 2. Frequency of aberrant PMCs observed at telophase I treated with EDTA from late prophase on. (per 100 cells)

Anther No.	No. of cells with aberration									
	Structural aberration							Non-structural aberration		
	chromo. frag.	chromat. frag.	macro frag.	micro frag.	minute frag.	b+f		sticky bridge	others	
EDTA treatment series.										
EL-221*	1	1	·	2	·	·		6	1	
EL-221	·	1	·	·	1	·		6	6	
EL-221	·	1	1	·	·	·		4	·	
mean	0.33	1.00	0.33	0.67	0.33	0.00	total (2.66)	5.33	2.33	total (7.66)
Control series.										
EL-221	·	1	·	1	1	1		·	·	
EL-221	·	·	1	·	·	·		·	1	
EL-221	·	1	·	·	·	·		·	2	
EL-221	·	1	·	·	·	2		1	·	
EL-221	·	·	·	1	·	·		1	1	
mean	0.00	0.60	0.20	0.40	0.20	0.60	total (2.00)	0.40	0.80	total (1.20)

* Anthers with Anther No. EL-221 were taken from five individuals.

ment, 4.10 cells in control, and in the case of treatment from late prophase 10.32 in EDTA treatment, 3.20 in control (Tables 1 & 2). These results indicate obviously that EDTA was taken into PMCs.

The treatment with EDTA causes various types of chromosomal aberration. The treatment from metaphase I produces mainly chromosome fragments of various sizes at anaphase I or telophase I, which are due to the disruption of linear continuity of chromosomes (Table 1). The smallest fragments are as large as approximately the half-coil of spiral of metaphase I chromosomes (Fig. 1). These were termed minute fragments. In the present work, the chromosome fragments were divided into six classes in order of their size and shape, i.e., (1) minute fragments, (2) micro fragments which consist of nearly two or three half-coils of spiral (Fig. 2), (3) macro fragments which are similar in length but larger in breadth twice or more than micro fragments (Fig. 3), (4) iso-chromatid fragments which are supposed to originate from isolocus chromatid breaks, regardless of their length (Fig. 4), (5) chromatid fragments which are of more than half-coil units and larger than micro or macro fragments and

TABLE 3. Correlation of the frequency of structural and non-structural aberrations with the stage distribution of PMCs within each anther. (PMCs treated with EDTA from metaphase I on).

Anther No.		Percentage of cells with			Stage distribution of PMCs at the time of fixation within upper part of each anther.				
		Structural aberration	Non-structural aberration	Total	Meta. I	Ana. I	Telo. I	Meta. II	Ana. II
35-S	(EDTA)	18	1	19	+++	++	+++		
	(EDTA)	14	0	14	+++	+++	±		
	(Cont)*	7	2	9	±	+	+++		
35	(EDTA)	4	4	8		++	+++		
34	(EDTA)	9	1	10			+++		
	(EDTA)	8	0	8			+++		
	(Cont)	3	0	3			+++	++	
31	(EDTA)	5	1	6			+++	++	
	(EDTA)	2	4	6			+++		
	(Cont)	2	1	3			+++	+	±
31-2	(EDTA)	13	3	16	+++	+++	++		
	(EDTA)	5	0	5	+++	+++	+		
	(Cont)	4	5	9	+	+++	++		
	(Cont)	2	0	2	++	+++	++		
2-23	(EDTA)	4	4	8			+++		
	(EDTA)	3	2	5	±	+	+++		
	(EDTA)	4	13	17	+++	+	++		
	(EDTA)	9	1	10	++	+	+++		
	(EDTA)	3	5	8	+		+++		
	(Cont)	3	4	7	++	++	++		
	(Cont)	1	4	5			+++		
	(Cont)	2	1	3			+++		
24-F	(EDTA)	9	16	25			+++		++
	(Cont)	0	0	0			+++		
24FA	(EDTA)	1	0	1			+++		
	(EDTA)	0	0	0			+++		
	(Cont)	0	0	0			+++		

* Control.

TABLE 4. Correlation of the frequency of structural and non-structural aberrations with the stage distribution of PMCs within each anther. (PMCs treated with EDTA from late prophase on).

Anther No.		Percentage of cells with			Stage distribution of PMCs at the time of fixation within upper part of each anther.				
		Structural aberration	Non-structural aberration	Total	Pro.	Meta. I	Ana. I	Telo. I	Meta. II
EL-221	(EDTA)	4	7	11		+++		+	
	(EDTA)	2	12	14		+++	±	+	
	(EDTA)	2	4	6		+	++	+++	
	(EDTA)	—	—	—	++++				
	(EDTA)	—	—	—	+++	+			
	(EDTA)	—	—	—		++++			
	(EDTA)	—	—	—		++++			
EL-221	(Cont)	4	0	4	+	+++	+++	++	
	(Cont)	1	1	2		+++	++	+	
	(Cont)	1	2	3		++	++	++	
	(Cont)	3	1	4				+++	
	(Cont)	1	2	3		+	+	+++	
	(Cont)	—	—	—	+++	+			

show spiral diameter of chromatid unit (Figs. 6, 7c), (6) chromosome fragments which are the same in their length as chromatid fragments but show spiral diameter of chromosome unit (Fig. 5). Since usually fragments of various classes are met with within a cell, the cell was classified in scoring into types such as the type of cell with minute fragments, the type of cell with macro fragments and so forth according to the smallest fragments in it. In this way of classification the frequencies of the cells with aberration at telophase I were summarized in Tables 1 & 2. Cells showing extreme fragmentation of chromosomes (Figs. 8, 9) were not included in Tables 1 & 2, for it is sometimes difficult to determine their division stage in meiosis.

The treatment from late prophase on gave results different from the treatment from metaphase I on in the main type of induced aberrations at anaphase I or telophase I. The treatment from late prophase on brought about mainly the "non-structural" chromosome aberrations such as sticky bridges which seem to be due to certain physiological change of chromosomes (Table 2, Figs. 13, 14). Such an alteration of the main types of EDTA-induced aberration between these two treatments is also ascertained by the comparison of the ratio of structural aberrations such as chromosome fragments to non-structural aberrations in each treatment. Namely the ratios "structural" versus "non-struc-

tural" in the case of the treatment with EDTA from metaphase I on and that from late prophase on are 6.54/3.22=2.00, 2.66/7.66=0.34, and in the case of the contral 2.40/1.70=1.41, 2.00/1.20=1.67 respectively. This suggests that the EDTA-sensitive period of chromosome structure which brings about chromosome fragmentation may lie in the metaphase I stage.

In this connection, it is worthy to note that in some cases any difference between the aberration frequencies of EDTA treatment from metaphase I on and that of control was not recognized (for example, Anther No. 31, 2-23, 24-FA in Table 3). It is hard to consider that failure of PMCs in absorbing EDTA is responsible for this. The distribution of PMCs in each anther with respect to meiotic stages are shown in the right column of Table 3 or 4. From this column of Table 3, one may become aware of that nearly all anthers which have a similar high frequency of EDTA-induced chromosome fragmentation in the treatment from metaphase I on are similar in the mode of stage distribution of PMCs. Namely, the frequency of EDTA-induced structural aberration is high in anthers where the ratio of cells at metaphase I, anaphase I and telophase I is nearly 1 : 1 : 1 (Anther No. 35-S, 31-2). On the contrary there is only a few or no structural aberration in anthers where PMCs were mostly at telophase I (Anther No. 24-FA). Such difference in the mode of stage distribution of PMCs at the time of fixation indicates that there was already certain difference in the anthers at the time of initiation of treatment, some anthers being at an earlier stage within subdivided stages of metaphase I and some at later stage within metaphase I, although they were regarded to be at the same division stage. It is difficult to detect the difference in the mode of subdivided stage distribution in each anther at the time when the treatment initiated, because such a difference may exist among anthers within a flower and also between the upper part and the lower one of an anther (Table 5). After all, the fact that the frequency of EDTA-induced chromo-

TABLE 5. The distribution of PMCs with respect to meiotic stages within an anther.

Anther No.	Part of anther	Number of cells					
		Pro-phase	Meta. I	Ana. I	Telo. I	Meta. II	Total
35-S*	upper half of anther	0	431	299	473	0	1203
	lower half of anther	0	ca. 1000	0	0	0	ca. 1000
35-S*	upper half of anther	0	466	480	63	0	1009
	lower half of anther	0	458	41	11	0	550

* These anthers were treated with EDTA.

TABLE 6. Re-arrangement of Table 3 and 4.

Treatment	Representative stages of anthers at the time of observation.*		Anther No.	Frequency of EDTA-induced telophase I aberrations.		
				Percentage of cells with		
	Group	Stage		structural aberration	non-structural aberration	total
EDTA	A	late metaphase I	EL-221	4	7	11
			EL-221	2	12	14
			mean	3.0	9.5	12.5
	B	mid-anaphase I	35-S	18	1	19
			35-S	14	0	14
			31-2	13	3	16
			31-2	5	0	5
			2-23	4	13	17
			mean	10.8	3.4	14.2
	C	late anaphase I	35	4	4	8
			2-23	3	2	5
			2-23	9	1	10
			EL-221	2	4	6
			mean	4.5	2.8	7.3
	D	mid-telophase I	34	9	1	10
			34	8	0	8
			2-23	4	4	8
			2-23	3	5	8
			24-FA	1	0	1
			24-FA	0	0	0
			mean	4.1	1.7	5.8
	E	late telophase I and thereafter	31	5	1	6
			31	2	4	6
			24-F	9	16	25
			mean	5.3	7.0	12.3
Control	B	mid-anaphase I	EL-221	4	0	4
			EL-221	1	1	2
			mean	2.5	0.5	3.0

(to be continued)

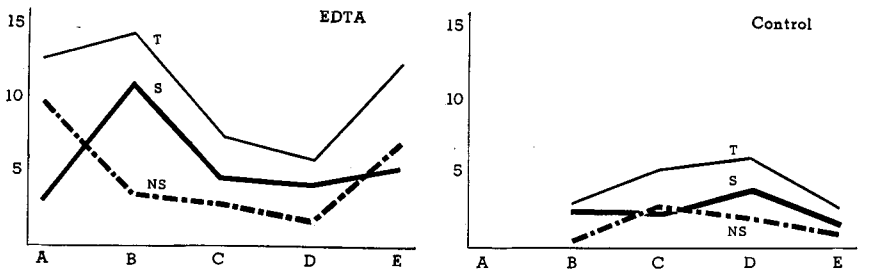
C	late anaphase I	2-23	3	4	7
		EL-221	1	2	3
		31-2	4	5	9
		31-2	2	0	2
		mean	2.5	2.8	5.3
D	mid-telophase I	EL-221	1	2	3
		35-S	7	2	9
		mean	4.0	2.0	6.0
E	late telophase I and thereafter	24-FA	0	0	0
		EL-221	3	1	4
		34	3	0	3
		31	2	1	3
		2-23	1	4	5
		2-23	2	1	3
		24-FA	0	0	0
		mean	1.6	1.0	2.6

* As judged from the distribution of meiotic stages of their PMCs.

some fragmentation depends on whether the majority of PMCs are at earlier subdivided stage within metaphase I or at later stage, can not be explained without assuming the existence of EDTA-sensitive period within metaphase I. Namely, in the case of Anther No. 24-FA, it is presumed that the majority of PMCs have passed through the EDTA-sensitive period when EDTA came up to the effective concentration in PMCs to induce chromosome fragmentation.

This assumption is also ascertained by the fact that there was certain tendency of a group of aberrant cells with chromosomal fragments to localize in one preparation, and that there was also within one preparation a difference of the division stage between the group of aberrant cells and that of normal cells (Figs. 15, 16, 17). The existence of EDTA-sensitive period is further emphasized in Table 6 and Graph 1 where the anthers shown in Table 3 and 4 were grouped by degrees of resemblance in the mode of stage distribution of PMCs at the time of fixation, and where the fluctuation of the frequency of EDTA-induced aberration through these anther-groups was demonstrated.

As the other types of aberration which was brought about by the treat-



Graph 1. Graphic representation of Table 6.

Vertical line indicates the percentage of cells with aberration and horizontal line the groups in Table 6. Curve S, N and T represent the structural, non-structural and total aberrations respectively. The stage when EDTA-chelation acted effectively is presumed in the group A to be at very early metaphase I, in B, C, D and E at gradually later stages, the last one (E) being at the stage of ending of metaphase I or rather that of initiation of anaphase I.

ment with EDTA from metaphase I on, there were the chromatin bleb which is strangulated from the structure of metaphase I chromosomes, and the chromatin protrusion that the chromatin material is protruded to the outside of cells (Figs. 23, 24). In addition to these aberrations, it was often noted that at anaphase I minute fragments seem to be pulled to the pole, being held by chromosomal matrix (Figs. 18, 19, 20, 21, 22).

Discussion

The results described above indicate that when EDTA acts on the chromosomes which are passing through the EDTA-sensitive period, it causes the chromosome fragmentation. This EDTA-sensitive period which lies in a certain subdivided stage of metaphase I may represent a period when chromosomes themselves become unstable structurally. This instability of chromosomes may be brought about at the time, as postulated by MATSUURA (1940, 1950), when the spiral system of metaphase I chromosomes is transformed from relational spiral system into parallel spiral one by means of the break and reunion at every half-coil. This assertion is confirmed by the occurrence of the minute fragments which are supposed to have originated from the breakage of half-coil unit. Since the breaks at the moment of spiral-parallelization are, in normal condition, to be followed by instantaneous reunions, chromosome fragmentation can not manifest itself. It is presumed that chromosome fragmentation, in the present experiment, results from inhibition of reunion by EDTA. This is well

explained by assuming that Ca and Mg cations are participated in the structural integrity of chromosomes and that these cations must be supplied for the breaks to reunite. Thus, from the results presented in this paper, it will be possible to draw the following conclusions, that is, (1) as Steffensen has asserted, the bridge of Ca or Mg cation must lie in the chromosome structure, (2) the spontaneous break and reunion at every half-coil, which was reported by Matsuura, must occur at the site of Ca or Mg chelate bond of chromosome structure, and (3) the crossing-over is supposed to be the break and reunion at the site of Ca or Mg chelate bond.

Contrary to the present experiment, some workers studying the effect of EDTA on the mitotic chromosomes observed no chromosome fragmentation, e. g. HYDE in *Vicia* (1956), MCDONALD & KAUFMANN in *Allium cepa* (1957), Sarkar in grasshopper embryos (1957), HYDE & PALIWAL in *Vicia faba* (1958), DAVIDSON in *Vicia* (1958), and WAKONIG & ARNASON in *Vicia* (1958). Such results might be attributed, in part, to the long duration of EDTA treatment in their experiments. Namely, the EDTA treatment of long duration brings about some physiologically abnormal conditions in cells, enough to deprive the majority of Ca and Mg cations which acts as activators of cellular enzymes, and this supposedly acts against the occurrence of EDTA-induced chromosome fragmentation. An indication of such physiologically abnormal conditions was recognized in *Vicia* root-tips even when the EDTA treatment of short duration was applied (TAKEHISA, 1961). In the present experiment meiosis proceeded almost normally in spite of the EDTA treatment of long duration. This represents that the actual concentration of EDTA in PMCs was still low at the time of fixation, i. e., the degree of damages in cellular metabolic processes due to the deprivation of enzyme activator Ca and Mg by EDTA was not so severe that the chromosome fragmentation effect of EDTA was masked.

Summary

Anthers of *Trillium kamtschaticum* PALL., in which PMCs were either at the stage of late prophase or metaphase I, were excised and floated for 24 hours at 7°–11°C on the 0.01 M aqueous solution of EDTA adjusted to pH 6.5–7.0 with 1N NaOH.

The EDTA treatment from late prophase on produced mainly the aberration due to chromosome stickiness, while the treatment from metaphase I on produced mainly the chromosome fragmentation, indicating the existence of a specific period within metaphase I when chromosomes are particularly sensitive to chelating action of EDTA so as to result in chromosome fragmentation. It

is supposed that this EDTA-sensitive period may correspond to the time when the spiral system of metaphase I chromosome is transformed from relational spiral system into parallel one through the breakage and reunion of chromatids at every half-coil, as postulated by MATSUURA (1940, 1950). The fact that the size of the "minute" fragment observed considerably frequently is approximately equal to the size of half-coil enables the interpretation that deprivation of Ca and Mg cation by EDTA may prevent the broken ends of chromatids from reunion at the time of spiral-parallelization.

Thus, the results obtained here confirm that, (1) as Steffensen has asserted, the bridges of Ca or Mg cation must take part in the maintenance of linear continuity of chromosome structure, and (2) the spontaneous break and reunion at every half-coil, which has been asserted by Matsuura, may occur at the site of Ca or Mg chelate bond of chromosome structure.

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Explanation of Plates

Plate I

Fig. 1–7. PMCs with various types of aberration induced by the EDTA treatment from metaphase I on. ca. × 1000.

1, a cell with minute fragments. 2, a cell with micro fragments. 3, a cell with macro fragments. 4, a cell with isochromatid fragments. 5, a cell with a chromosome fragment. 6, a cell with one bridge and a chromatid fragment. 7a, a cell having double bridges and two macro fragments. 7b, a cell having fragments of various sizes. 7c, a cell having four chromatid fragments.

Plate II

Fig. 8–12. PMCs with various degrees of chromosome fragmentation induced by the EDTA treatment from metaphase I on. The left cell in Fig. 9 is normal. Fig. 13–14. PMCs with aberration of chromosome stickiness induced by the EDTA treatment from late prophase on. ca. × 1000.

Plate III

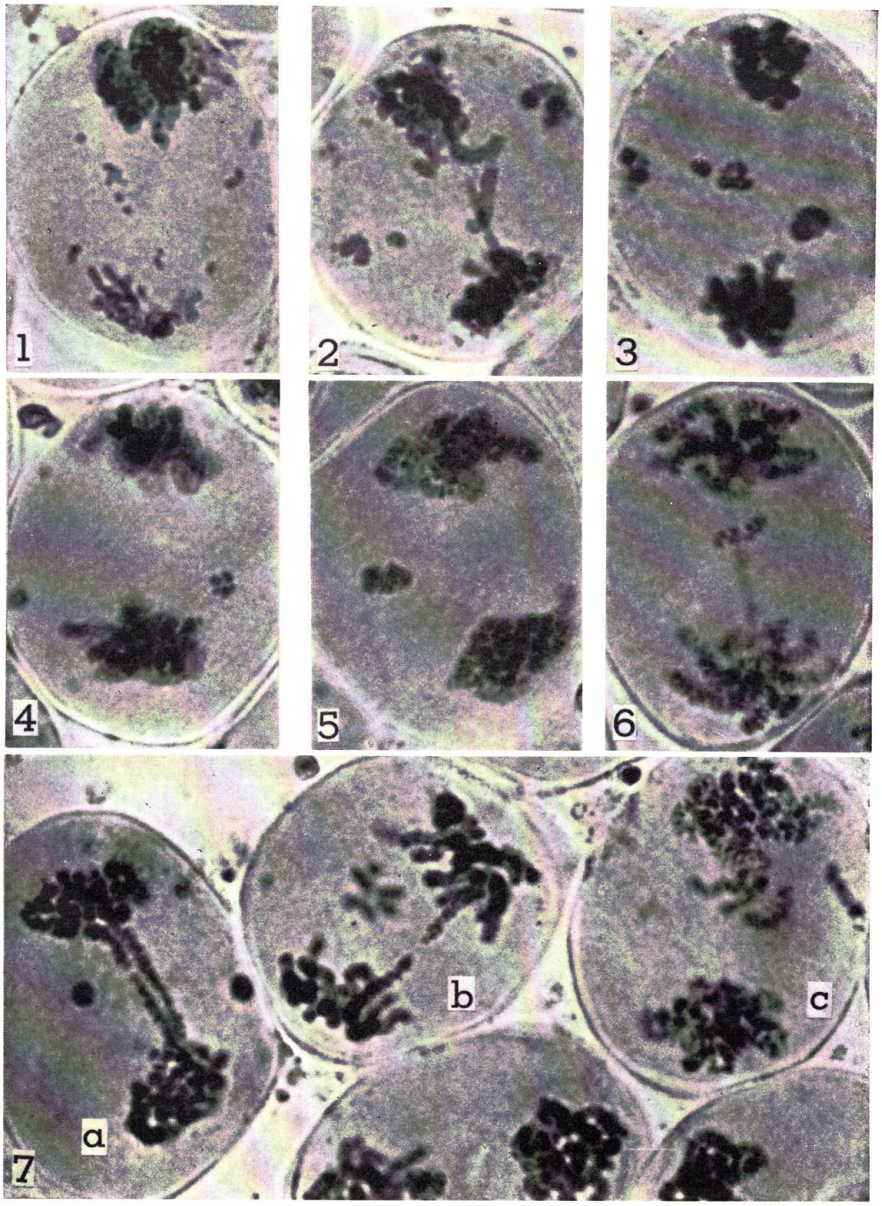
Fig. 15–17. Demonstration of stage difference of PMCs within an anther. ca. × 440.

15, a part of preparation where PMCs are uniformly at early interkinesis. 16, a part of preparation where PMCs are uniformly at late anaphase I. Note that most of PMCs at this stage are provided with aberrations. 17, a part of preparation where PMCs are uniformly at mid-anaphase I. The PMCs at this stage are rarely provided with aberrations.

Plate IV

Fig. 18–24. PMCs treated with EDTA from metaphase I. ca. × 1000.

18, 19, 20, 21, 22, PMCs in which chromosome fragments were pulled to the pole being held by chromosome matrix. Camera-lucida drawings of the portions indicated by arrows are shown in the lower right part of the Plate IV. 23, a cell having chromatin protrusion. 24, a cell with chromatin blebs.



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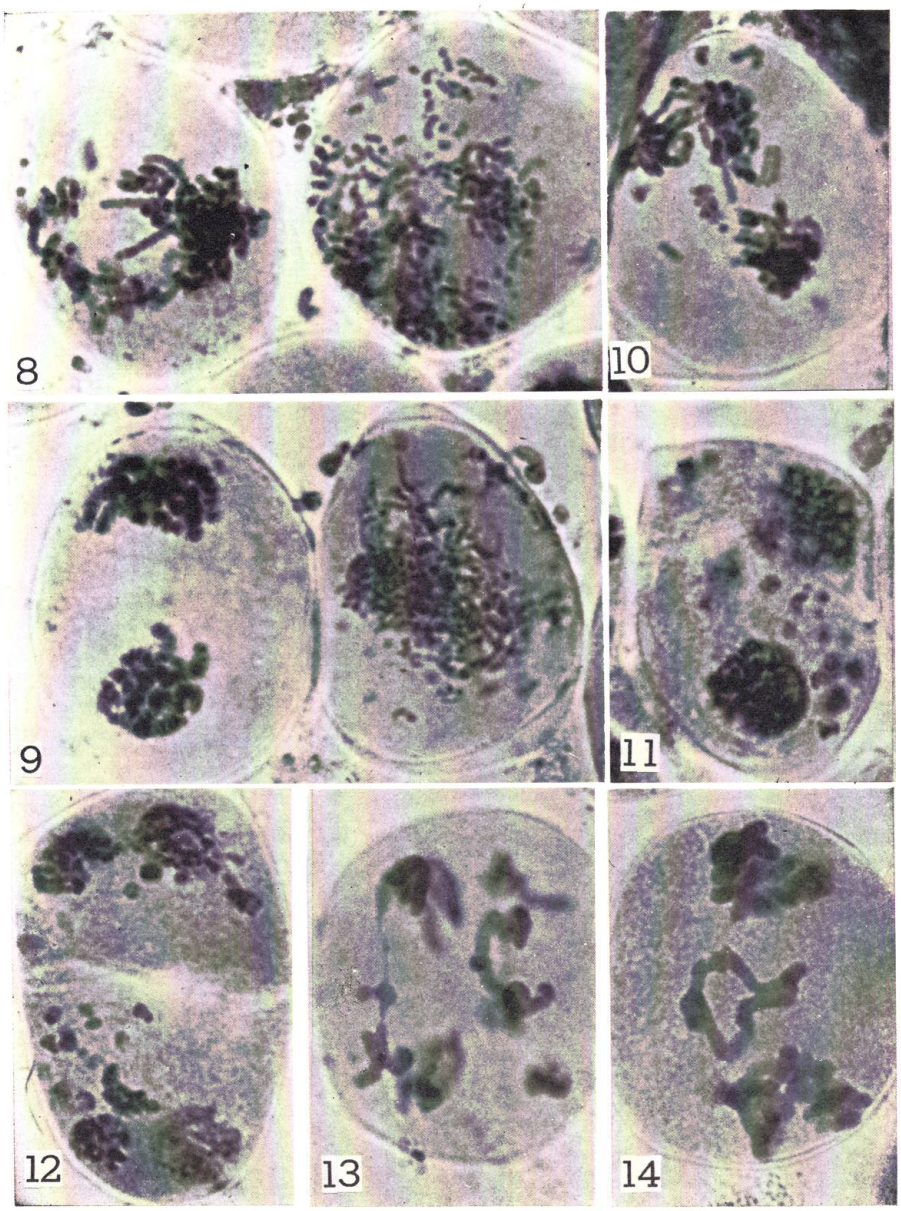

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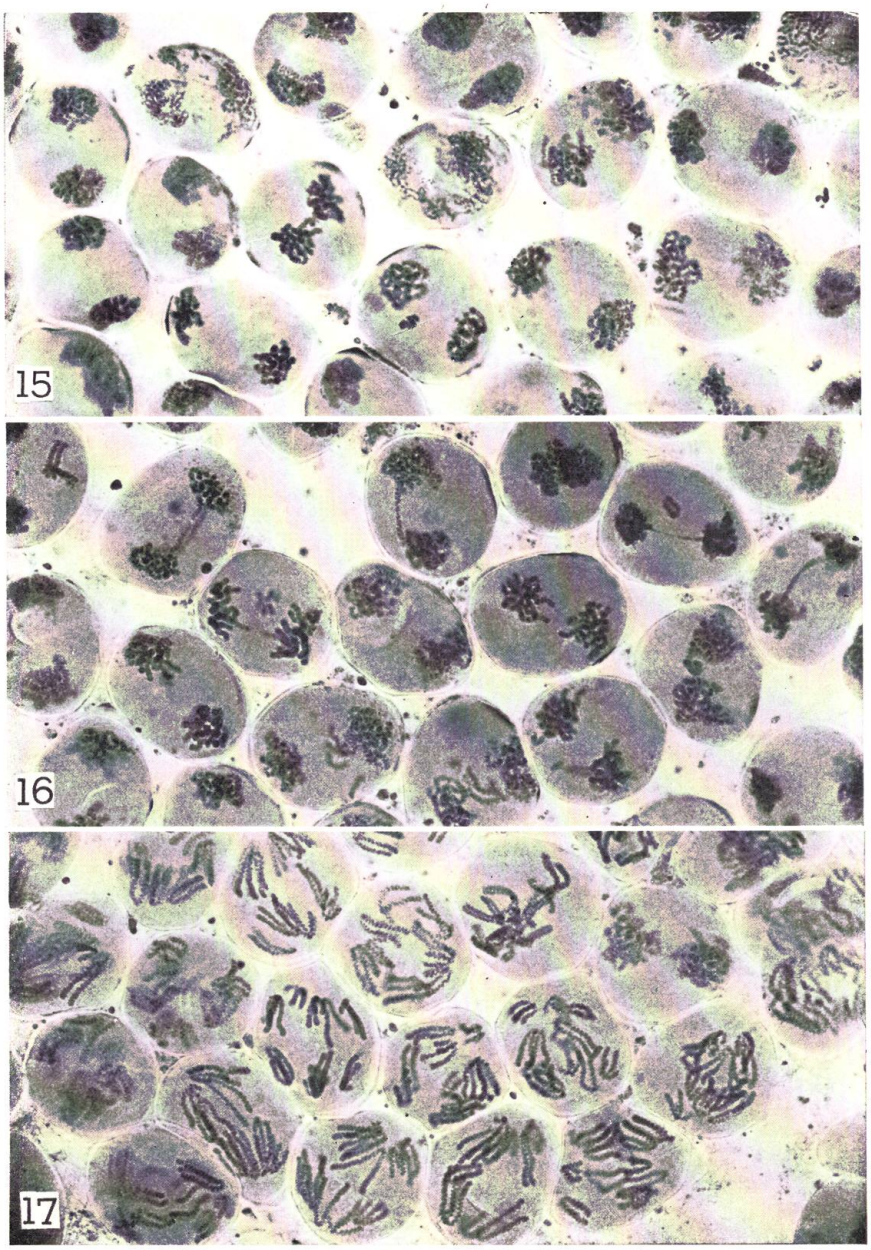

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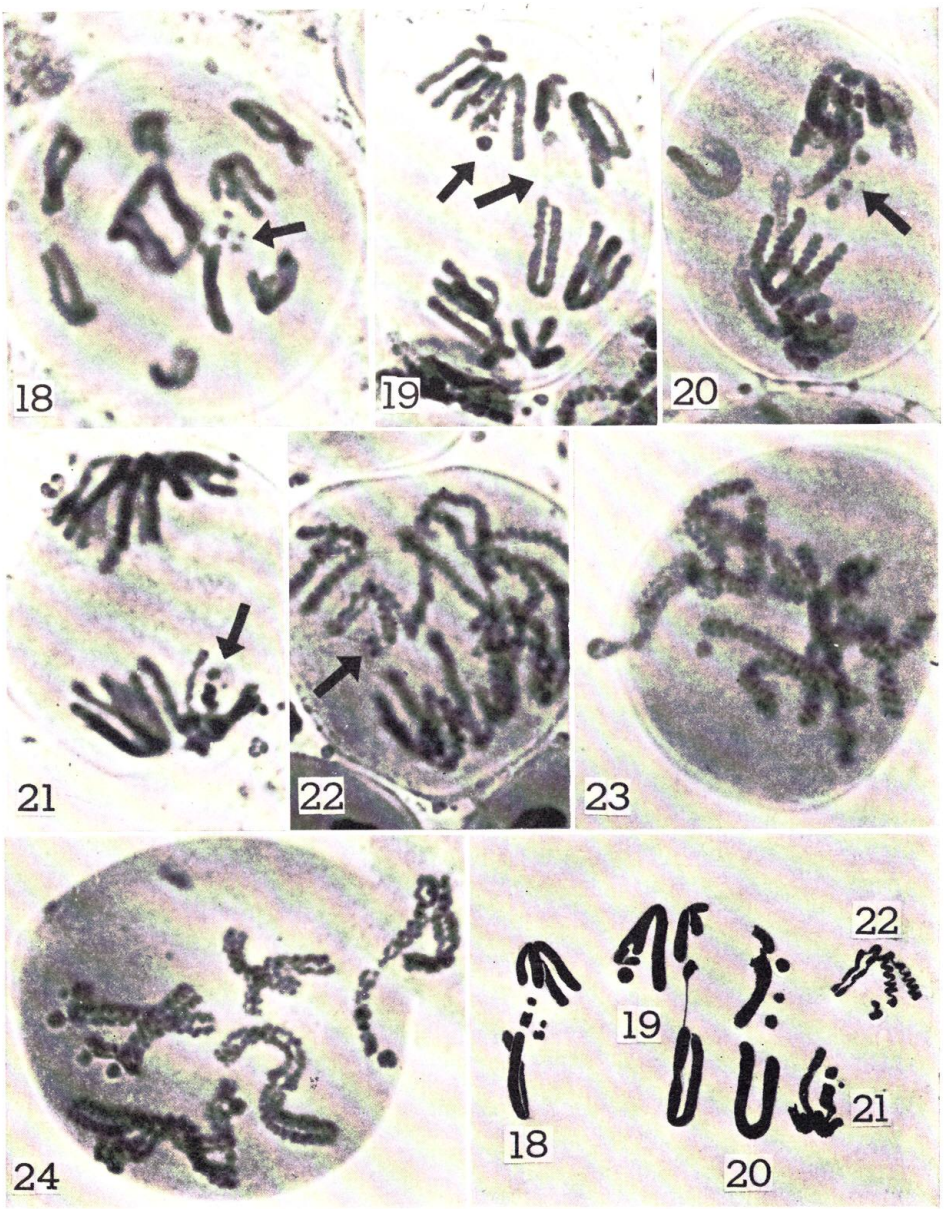

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