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Effect of Inorganic Salts on Cell Division II. Production of Meiotic Abnormalities in PMCs of *Paris* by NaCl, KCl and $CaCl_2$*

By

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Many authors have reported that a number of mitotic abnormalities are induced by various inorganic salts (YAMAHA, '27; SHIGENAGA, '44; LEVAN, '45; VON ROSEN, '53; MAKINO & TANAKA, '53; KAUFMANN & MCDONALD, '56; HINDMARSH, '59; MATSUURA & IWABUCHI, '62). VON ROSEN ('54a, b) divided almost all fundamental elements of the periodical system into three groups according to their activity on chromosome substance and the degree of poising effect on cell plasm, that is, (1) very strongly active metals, (2) weakly active metals and (3) inactive metals. He postulated that all sorts of active metals react with proteins in cell plasm, cell enzymes and directly with constituents of chromosome body, thus, inducing directly or indirectly various types of abnormalities in chromosomes. However, it was found that Mg and Na metals, which are included in the inactive group according to VON ROSEN's classification, produced the mitotic abnormalities such as chromosome breakage, stickiness of chromosome, spindle impairment and so on when used as the chloride compounds (KAUFMANN & MCDONALD, l.c.; MATSUURA & IWABUCHI, l.c.).

In the previous paper, the writers assumed that the meiotic abnormalities in PMCs of *Tradescantia* induced by NaCl treatment are due to the ionic unbalance within cells, resulting from predominant absorption of Cl-ions over Na-ions. The present paper is concerned with the influence of some neutral slats, KCl, NaCl and $CaCl_2$ upon the meiotic division of PMCs in *Paris verticillata*. It is expected that the treatments with these salts enhance too the induction of cytological abnormalities at meiosis, being caused by the alteration of ionic environment in cells.

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Materials and Methods

Flower buds of *Paris verticillata* M. BIEB. (=*Paris hexaphylla* CHAM.; $2n=10$, 5II) which were collected from a natural population at the foot of Mt. Teine near Sapporo City were used throughout the present experiment. The solution of sodium chloride (NaCl), potassium chloride (KCl) and calcium chloride ($CaCl_2$) were prepared with deionized water. Furthermore, half and half mixture of NaCl and $CaCl_2$ solutions, each of which being isotonic, was used in order to reduce the permiability to protoplasm membrane by an antagonistic effect of Ca-ion with Na-ion, the latter being known to increase the permiability. The concentrations of the salt solutions employed are listed in Table 1. NaCl, KCl, $CaCl_2$ and the mixture of NaCl and $CaCl_2$ will be expressed in abbreviation as N, K, C and M, respectively. Since van't Hoff coefficient* of KCl is equal to that of NaCl, the solutions of the same concentrations as NaCl were prepared for KCl. Each concentration of the $CaCl_2$ solutions and the mixture solutions of NaCl and $CaCl_2$ was made to be isotonic to each concentration of the NaCl solutions. These salt solutions were not buffered as in the previous experiment, for the purpose of the present studies is to investigate the specific effect of each ion dissociated from the salts on cell division, particularly on chromosomes.

The stems of the material plants were cut at about 10 cm. below the flower buds and the cut stems were immersed about 7 to 8 cm. in 500 ml. of the active solutions at room temperature (13°±2°C) for 46 to 60 hours until fixation. Since in *Paris verticillata*, as in *Trillium*, the meiotic stages of PMCs in a flower bud are almost synchronized, the meiotic stages of PMCs at the beginning of treatment was examined with one of eight anthers in a flower bud and only the plants whose PMCs are at early leptotene to early zygotene were used. With or without delay of several hours after treatments, preparations were made by the acetocarmine

TABLE 1. Salts and concentrations adopted in the present experiment.

Salt	NaCl	KCl	$CaCl_2$	$NaCl+CaCl_2$
Isotonic scale**	Abbreviation			
	N	K	C	M
1	5×10^{-3}M	5×10^{-3}M	3.5×10^{-3}M	4.2×10^{-3}M
2	1×10^{-2}M	1×10^{-2}M	7×10^{-3}M	9×10^{-3}M
3	5×10^{-2}M	5×10^{-2}M	3.5×10^{-2}M	4.2×10^{-2}M
4	1×10^{-1}M	1×10^{-1}M	7×10^{-2}M	9×10^{-2}M
5	5×10^{-1}M	5×10^{-1}M	3.5×10^{-1}M	4.2×10^{-1}M

** The numbers indicated as isotonic scale in this Table abbreviatedly represent salt concentrations. For instance, N-3 indicates 5×10^{-2}M NaCl solution and C-3 does 3.5×10^{-2}M $CaCl_2$ solution, both being isotonic to each other based on the van't Hoff coefficients of NaCl and $CaCl_2$.

* The van't Hoff coefficients, 1.84 for NaCl and KCl, and 2.59 for $CaCl_2$, were employed as isotonic coefficients in preparing these salt solutions.

smear method. As a control, the material plants were treated with tap water instead of the experimental solutions. In addition, deionized water (abbreviatedly, D.W) treatment was carried out to examine whether it influences the meiotic division or not.

Both the stems and leaves of the plants treated with the highest concentration of each salt (N-5, K-5, C-5, M-5) wilted slightly in several hours and still more in twenty hours after the beginning of treatments. These plants could apparently be recovered from the wilting condition within several hours by the replacement of the active solution with tap water, while extreme wilting of the plant body which was brought about by continuous 40 hrs treatment with the highest concentration of salts could not recover even when transferred in tap water. On the other hand, neither wilting of the stems nor that of leaves occurred when treated with the other concentrations of these salts, evidently indicating that only the highest concentration of each salt exerts hypertonically to cell fluid of the plants. Therefore, the observations of meiosis were carried out excluding the materials treated with such solutions. However, the meiotic division of the recovered plants from the wilting condition, in some cases, were also observed.

The designation of the chromosome complements by Haga ('34) was adopted here; that is, each of the five chromosomes of a complement was represented as A, B, C, D and E according to their length order. The precise observation of cell division was carried out on cells at various stages of meiosis except the first prophase. The statistical data were collected at the stages from mid-anaphase to telophase of the first and second divisions.

Observations and Results

The various types of abnormalities of meiosis were recorded in the salt treated materials at the various stages from first metaphase to tetrad as in the case of the NaCl-treated *Tradescantia* reported in the previous paper. It could not be found, however, that a specific type of abnormality was brought about by any salts used in the present experiments. The abnormalities of meiosis frequently obtained in the materials which were transferred into tap water after 20 hrs treatment with hypertonic solution of each salt were mostly those resulting from extreme stickiness of chromatin substance; clotting chromosomes at metaphase (Fig. 11) and heavily sticky bridges at first ana- or telophase, which were frequently seen in the cells where all the anapahse chromosomes were segregating losing their conventional figure as adhesive mass of chromatin substance, as seen in Fig. 12. In N-5 and M-5 groups, a decrease in stainability of telophase chromosomes with carmine dye and the failure of cell wall formation at late telophase were observed only rarely. GILES ('39) investigated the effect of dehydration on microsporogenesis in *Tradescantia* and found that the cytokinesis by cell wall formation is inhibited by dehydration. Similar result with respect to the dehydration effect on somatic cell division has been reported by SHIGENAGA ('44). He frequently observed bi-nucleate cells and

di-diploid nuclei when the living cells of *Tradescantia reflexa*, young leaf epidermis and petal cells, were palced in hypertonic solution of several neutral salts or of some kinds of heavy metal salts. As a consequence, he has attained the conclusion that the dehydrating action due to the treatment with the hypertonic solution of these salts would produce the denaturalization of spindle and phragmoplast to result in these aberrations in mitotic cells. It may be surely concluded therefore that the meiotic abnormalities resulted from the treatment with the highest concentration of each salt used here are caused by the dehydration of cell fluid rather than by the effect of the ions dissociated from the salts. Total aberration frequency in the materials treated with the hypertonic solution was not always high and showed some variation among the individuals and among the different salts.

The aberrations scored at first and second ana- or telophase were classified into the following three categories in order to analyse the degree of the effect of these salts.

I) The first category includes the aberrations that are commonly accepted as "structural change" of chromosomes such as chromosome breakage;

(1) chromosome fragment,

(2) chromatid bridge resulted from the rejoining of two broken ends of chromosomes, termed "true bridge".

II) The second category includes the following aberrations, usually called as "non-structural change" of chromosomes;

(3) chromatin bridge ascribed to anomalous stickiness of anaphase chromosomes, termed "sticky bridge",

(4) the aberration derived from impairment of mitotic apparatus, leading to non-disjunction, lagging of chromosomes or disturbance of bipolarity giving mono-, tri- or multipolar nuclei at telophase.

III) The aberrations indistinguishable whether they are structural or non-structural were dealt with in the third category. For instance, there were some cases where it was difficult to distinguish at first ana- or telophase whether chromatid laggards are acentric fragments or precociously segregated half-univalent laggards, as pointed out by TANIFUJI ('60) who observed the various meiotic abnormalities in *Paris hexaphylla* treated with streptomycin. Such laggards, therefore, are included in this category.

In addition to the aberrations mentioned above, the following types of abnormalities were relatively often observed in the salt treated materials, though the statistical observation was not made;

(5) abnormalities of chromonema coiling,

(6) clotting of first metaphase chromosomes,

(7) complete or partial suppression of cell wall formation to result in the bi-nucleate cells.

The statistical data of aberrations at the first and second divisions of meiosis are summarised in Tables 2 and 3, respectively, giving the average

TABLE 2. Frequency of aberrant PMCs at the stage from AI to TI in *Paris verticillata* treated with different concentrations of NaCl, KCl and $CaCl_2$.

Treatment	Total cells observed	Aberrations* (%)			
		Structural	Non-Structural	Indistin-guishable	Total** s
Control	1,466	1.0	1.2	1.2	3.3± 0.7
Deionized water (D.W.)	1,212	2.1	3.1	0.8	6.0± 1.7
NaCl					
N-1	1,373	5.8	3.9	2.8	12.5± 4.4
N-2	1,704	10.0	5.9	3.2	19.5± 3.8
N-3	1,515	10.7	8.8	4.7	24.3±11.8
N-4	1,035	8.5	7.5	1.4	17.5± 3.5
KCl					
K-1	1,668	9.5	4.3	1.4	15.2± 9.4
K-2	2,143	13.3	3.9	1.8	18.9± 4.8
K-3	798	13.5	5.7	3.4	22.7±13.8
K-4	2,146	5.4	3.9	2.4	11.7± 3.4
K-5†	1,585	7.2	8.8	1.5	17.5± 3.9
$CaCl_2$					
C-1	3,129	4.9	1.7	0.8	6.5± 1.9
C-2	4,288	3.7	1.8	1.3	6.8± 3.9
C-3	1,140	10.1	8.7	5.3	24.1±14.7
C-4	2,020	3.7	2.2	1.3	7.2± 1.3
C-5†	2,183	6.2	3.6	2.4	12.2± 2.4
NaCl+$CaCl_2$ (1:1 Mixture)					
M-1	1,417	5.4	2.3	1.4	9.2± 2.1
M-2	2,094	5.7	2.6	0.9	9.2± 2.1
M-3	3,798	4.7	3.2	2.1	10.0± 3.2
M-4	3,387	6.4	4.0	2.0	12.5± 2.3

† The groups replaced in tap water 42 hours after 20 hrs treatment with active solution.
* For details, see text.
** "Total" representing the average value of four to five plants.

TABLE 3. Frequency of aberrant PMCs at the stage from AII to TII in *Paris verticillata* treated with different concentrations of NaCl, KCl and $CaCl_2$.

Treatment	Total cells observed	Aberrations* (%)			
		Structural	Non-Structural	Indistin-guishable	Total** s
Control	1,534	0.3	1.2	0.3	1.8±0.6
Deionized water (D.W.)	3,313	1.0	2.3	0.6	4.0±1.1
NaCl					
N-1	1,579	1.5	3.5	1.2	6.2±2.7
N-2	1,337	3.0	4.6	3.1	10.7±4.6
N-3	2,161	6.5	5.7	2.2	14.8±3.9
N-4	467	0.4	9.2	1.7	11.3±3.2
KCl					
K-1	755	0.7	1.8	0.1	2.6±0.7
K-2	345	1.8	1.7	0.6	4.1***
K-3	534	0.9	3.4	0.3	4.6±1.6
K-4	1,449	0.8	1.3	0.8	2.8±0.9
$CaCl_2$					
C-1	2,621	0.9	0.7	0.3	1.9±0.7
C-2	1,810	0.9	2.2	0.7	3.8±0.6
C-3	2,900	1.3	1.6	0.8	3.7±1.9
C-4	392	1.3	4.6	0.5	6.4±0.1
C-5†	627	0.6	2.4	0	3.0±0.1
$NaCl+CaCl_2$ (1:1 Mixture)					
M-1	1,142	1.1	2.0	0.8	3.9±1.0
M-2	1,324	1.2	1.7	1.0	3.9±1.3
M-3	1,991	1.7	1.7	1.3	4.6±0.6

† The group replaced in tap water 47 hours after 20 hrs. treatment with active solution.
* For details, see text.
** "Total" representing the average value of two to four plants.
*** The value from one plant.

values of four to five plants in each treatment group. However, relatively large fluctuation of aberration frequency is found among the individuals in certain groups; that is, N-3, K-1, K-3 and C-3 groups. These fluctuations may be attributable to the difference of practical concentrations of the chemicals achieved in PMCs and not to the difference in frequencies of spontaneous

aberrations, because the fluctuation among five control plants was within 1.5% in the frequency of aberrant PMCs. From the comparison of the frequency of the aberrant PMCs in each salt treatment group with that in the control, it will be certainly concluded that these salt solutions induce the abnormalities of meiotic division. The aberration frequency was higher in the first division than in the second one and was found not to be proportional to the concentrations of the salts, except the M-treatment in which there was a proportional relationship between them. In N-, K-, and C-treatment groups there was found a tendency that the aberration yields generally reach a maximum at N-3, K-3 and C-3, respectively, these concentrations being isotonic to each other. The relationship seen in the M-treatment groups is supposed to have arisen from the ionic antagonism between Na^{+} and Ca^{++}.

In addition, comparing the result of D.W. group with that of the control, it was revealed that the frequency of the abnormal cells in the former is approximately twice that in the latter. It seems likely that deionized water brings about a modification of the physiological environment of cells so as to result in the aberrations.

In both the control and the materials treated with deionized water there exists a relationship indicating that the frequency of the structural aberrations of chromosomes at first ana- or telophase is lower than that of the non-structural ones, but in the salt treated materials this relationship was reversed in spite of the increase of the non-structural aberration yields. This clearly suggests that the salts used here are capable of inducing the structural changes of chromosomes besides the injury of the mitotic apparatus.

Fragmentation of chromosomes

At first metaphase in the salt treatment groups the isolocus chromatid breakages in half-bivalent chromosomes were very often observed, and the isolocus breakages of the four chromatids in a bivalent chromosome were met with only twice. As pointed out in *Trillium* by MATSUURA ('50) and in *Paris* by TANIFUJI ('62,) it is rersonable to suppose that the latter abnormal configuration might result from "chiasma breakage" as proposed by MATSUURA (l.c.). Four isomorphic chromatid fragments which were rarely found at first ana- or telophase, as seen in Figs. 2, 3, may be produced due to the above-mentioned breakage event, if sister-reunion of the broken ends does not occur.

At first and second ana- or telophase, chromatid fragments were frequently seen in the salt treated groups, mostly ranging from one to four in number per PMC. In addition, the extensive fragmentation of chromosomes was also induced by the application of the salts (Figs. 4-9), but occurred very rarely in

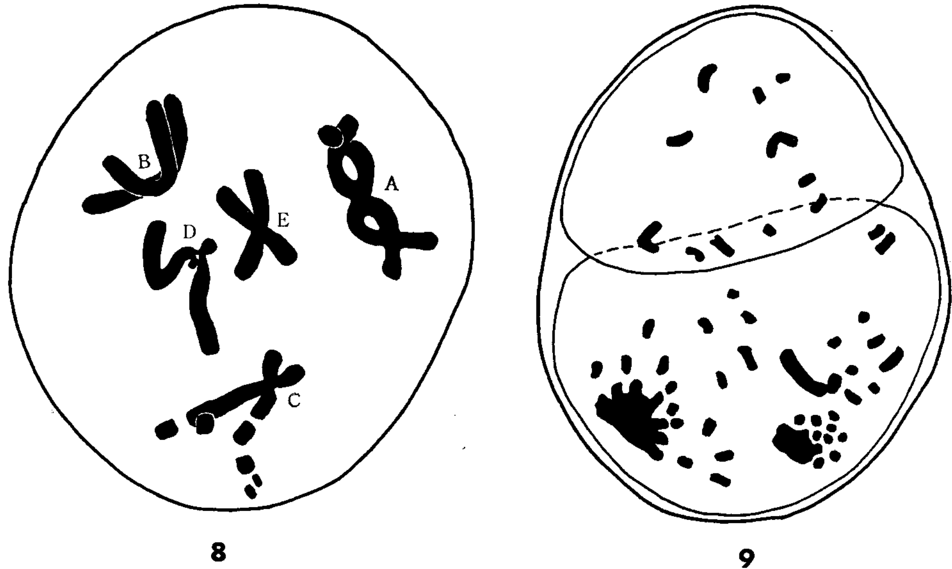


Fig. 8. Extensive fragmentation seen on C-chromosome at first metaphase.
Fig. 9. A case of the extensive fragmentation of chromosomes.

the D.W. group and in the control. In some cases, it was recognized that the fragments due to the extensive chromosome breakages in a cell have nearly equal size (Fig. 7), but in the majority of cases the size of them was not similar to each other (Figs. 4, 5). In almost all of the cells where the extensive fragmentation of chromosomes took place, only some chromosomes of the complement were subject to breakage. It is worthy to note that this type of chromosome aberration was hardly found at first metaphase but frquently met with at first anaphase. It is supposed, therefore, that abundance of matrix in metaphase chromosomes will serve to maintain the broken chromosomes not to be crushed into fragments and many fragments are ready to be released at anaphase when paired chromatids become free from each other.

The extensive chromosome fragmentation was reported to be induced by UV-irradiation, by alkylating agents or by other substances (LOVELACE, '54; SWAMINATHAN & NATARAJAN, '59; TANIFUJI, '62). SWAMINATHAN et al. (l.c.) have observed this type of chromosome aberration in the meristematic cells of root tips of *Triticum* treated with mustard oil or caster oil. It has been found in our laboratory that similar chromosome aberrations are induced by TESPA (an alkylating agent) in the PMCs of *Paris* (TANIFUJI, '62) and by EDTA (a chelating agent) in those of *Trillium* (MATSUURA & TAKEHISA, '62). A discussion will be later given of the cause of this aberration.

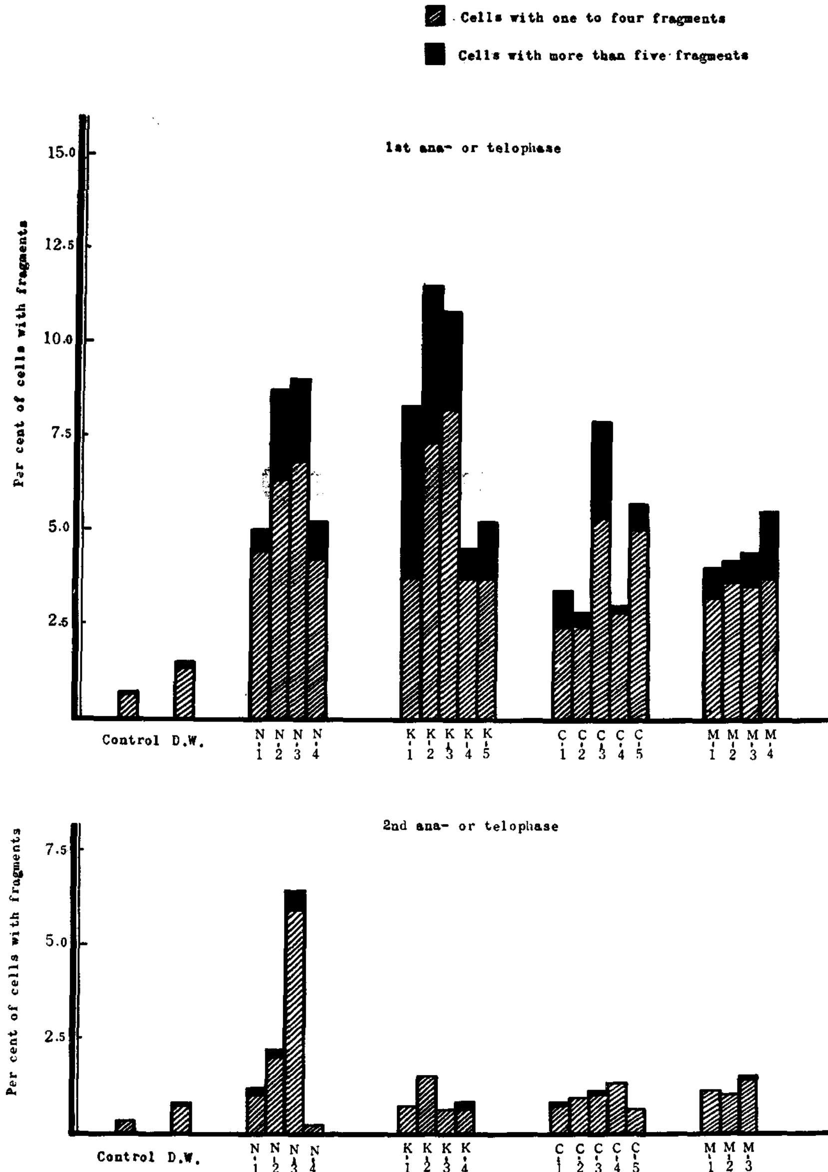
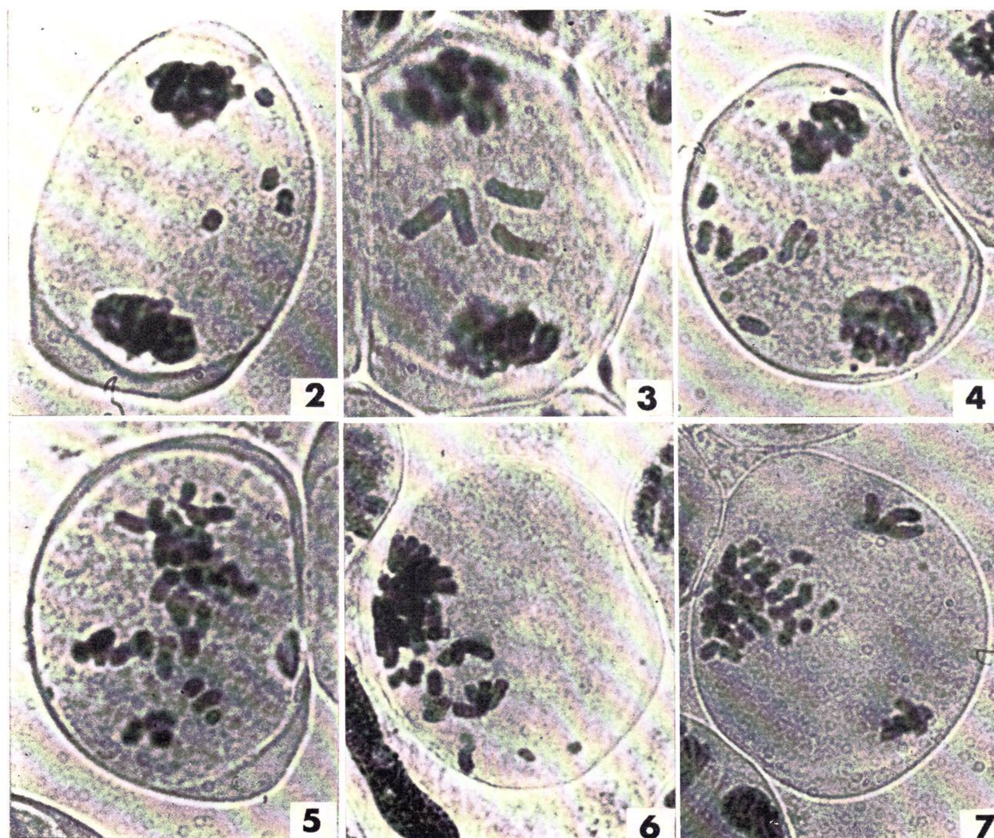


Fig. 1. Frequency of cells with chromosome fragments in the meiotic division of *Paris verticillata* after treatment with NaCl, KCl and $CaCl_2$.



Figs. 2, 3. Four isomorphic acentric fragments at first telophase.
Figs. 4-7. Numerous fragments due to the extensive fragmentation of chromosomes.

The statistical data of chromosome fragments scored at first and second ana- or telophase are shown in Fig. 1. The frequency of the cells with fragments are distinctly higher in the salt treatment groups than in the control, indicating that all the salts adopted in the present experiment have the ability to induce chromosome breakage at meiosis. The occurrence of chromosome fragmentation by the salt treatments was more striking in the first meiotic division than in the second one. In some cells having numerous fragments (more than five), the aberrations such as impairment of spindle or bipolarity, which will be described later, were also observed (Figs. 4-6).

Chromosome bridges

As mentioned above, there were two types of the chromosome bridges in the present investigation; bridges accompanied with acentric fragments (=true bridges) and those without them (=sticky bridges) which are mainly attributable to anomalous stickiness of chromosome matrix. It is without doubt assumed for the former to have derived from the structural changes of chromosomal thread (i.e., breakage and reunion), and for the latter to have arisen from the increase in viscosity of chromosome matrix due to the phy-

TABLE 4. Frequency of true and sticky bridges in meiotic division of *Paris verticillata* treated with NaCl, KCl and $CaCl_2$.

—per 100 cells—

Treatment	True bridge		Sticky bridges	
	1st division	2nd division	1st division	2nd division
Control	0.2	0.1	0.1	0.4
D.W.	0.7	0.3	0.5	0.8
NaCl				
N-1	0.9	0.4	0.5	0.5
N-2	1.7	0.8	0.1	1.5
N-3	1.8	0.5	0.7	1.4
N-4	3.4	0.2	1.9	1.9
KCl				
K-1	1.2	0	0	0.5
K-2	0.9	0	0.1	0.3
K-3	2.8	0.3	0	0.6
K-4	0.9	0	0	0.4
K-5	2.1		0.2	
$CaCl_2$				
C-1	1.5	0.2	0	0.1
C-2	0.9	0	0.1	0.3
C-3	2.2	0.2	0	0.5
C-4	0.8	0	0.1	0.5
C-5	0.6	0	0.1	1.3
$NaCl+CaCl_2$				
M-1	1.1	0	0	0.8
M-2	1.5	0.2	0.1	0.2
M-3	0.8	0.2	0.1	0.1
M-4	1.0		0.1	

siological alteration of cells. DARLINGTON & KOLLER ('47) and Haque ('53) have reported sister-reunion bridges without any accompanying fragments which are postulated as due to the reunion of the two unbroken ends of sister chromatids. Similar bridges without any fragments were rarely seen in the present investigation. However, in many cases, it was difficult to decide strictly whether the bridges in question are due to chromatid union of the unbroken ends or stickiness of chromatin substance. It, therefore, must be kept in mind here that the bridges without acentric fragments were, for convenience' sake, included in the category of the sticky bridges.

With respect to the occurrence of both types of the chromosome bridges, neither specific differences in frequency were obtained by the different kinds of salts, nor the frequencies of both types of bridges was proportional to the salt concentrations. (Table 4). According to the observation at first anaphase, true bridges were usually formed between the homologous chrosmosomes and rarely between the non-homologous ones. In the salt treated materials, stickiness of the chromosomes forming the sticky bridges was prominent but considerably variable in its degree. In many cases, one or two sticky bridges were found in a cell (Figs. 13, 14) and heavily sticky bridges were observed in some cells, in which the normal configuration of anaphase chromosomes was lost (Fig 12). Bundle-like bridges were also found in only a few cells of the salt treated materials, which seem owing to the serious stickiness of chromatin substance. It must be noticed here that simialr bundle-like bridges are clearly shown in Fig. 15 of the previous paper which represents an aberrant PMC of *Tradescantia* induced by NaCl. As seen in Table 4, the true bridges are predominantly scored over the sticky ones in the first meiotic division, but this relationship is reversed in the second one. Although both the bridge yields in some salt treated groups are lower than those in the control, there is a general indication that the production of both types of chromosome bridges is enhanced by the salt treatments.

Lagging of chromosomes

In the present section, only the laggards that could be identified in regard to the chromosome morphology (A, B, C, D and E) was dealt with. In the first meiotic division were observed univalent laggards and half-univalent (chromatid) laggards derived from the precocious division of univalent chromosomes, while in the second division almost all of the lagging chromosomes were of chromatid type and univalent laggards were very rarely scored in some of the salt treated materials. The frequencies of both types of the laggards in each division of meiosis are given in Table 5. The frequency of the univalent

TABLE 5. Frequency of lagging chromosomes in meiotic division of *Paris verticillata* treated with NaCl, KCl and $CaCl_2$.

—per 100 cells—

Treatment	Univalent laggards		Chromatid laggards	
	1st division	2nd division	1st division	2nd division
Control	0.3	0	0.6	0.6
D.W.	0.5	0.1	0.7	1.0
NaCl				
N-1	0.3	0.2	2.0	2.5
N-2	0.9	0	2.6	1.5
N-3	1.5	0.2	3.1	2.3
N-4	0.5	0	1.9	0.4
KCl				
K-1	0.3	0.3	1.6	0.4
K-2	0.5	0	0.7	1.5
K-3	0.6	0.3	1.4	1.5
K-4	0.2	0.2	1.8	0.4
K-5	0.3		1.8	
$CaCl_2$				
C-1	0.3	0	1.1	0.4
C-2	0.2	0.1	0.7	1.2
C-3	2.2	0.1	3.0	0.5
C-4	0.3	0	1.1	3.3
C-5	0.4	0	2.0	0.8
$NaCl+CaCl_2$				
M-1	0.6	0	0.4	0.5
M-2	0.2	0	0.6	1.4
M-3	0.5	0.1	1.9	0.9
M-4	0.5		1.5	

laggards at the first division did not differ between each of the salt treated groups and the control, with the exception that it is higher four to five times in N-3 and C-3 groups than in the control. On the contrary, the frequencies of the chromatid laggards in each division of meiosis are generally increased by the treatment with the salts though the degree of increase was not always proportional to the salt concentrations. It may be supposed that occurrence of the chromatid laggards in the first division was mainly attributable to the injury of the function of their kinetochores rather than to the impairment of

TABLE 6. Relation of the frequency of lagging in each chromosome to ratio of univalent formation of chromosome pairs.

Chromosome type	Relative length of chromosome (%*)	Ratio of univalent formation (%) (spontaneous)*	AI ~ TI				AII ~ TII	
			Control		Salt treatment		Control	Salt treatment
			Univalent laggard (%)	Chromatid laggard (%)	Univalent laggard (%)	Chromatid laggard (%)	Chromatid laggard (%)	Chromatid laggard (%)
A	27.6	7.4	8.3	7.6	17.1	12.5	16.6	20.9
B	20.7	16.1	12.3	15.0	15.3	18.0	22.2	22.9
C	19.0	21.4	18.3	10.6	17.6	10.0	13.9	16.4
D	17.6	22.6	20.6	21.2	19.8	16.4	27.8	9.5
E	15.1	32.5	40.5	45.6	23.2	34.1	19.5	30.3
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Total chromosomes analyzed		917	48	66	129	438	36	201

* Cited from Haga. (1944).

spindle mechanism.

HAGA ('44) indicated in *Paris verticillata* that the shorter the length of chromosomes, relatively the more frequent in univalent formation. Furthermore, a report has been made by TANIFUJI ('60) in the meiosis of the same material after administration of streptomycin that the frequency of univalent laggards in the individual chromosome type is roughly parallel to that of the spontaneous univalents at first metaphase. A similar result concerning to the frequency of lagging of each chromosome, as represented in Table 6, is also obtained at the first meiotic division of the control material in the present study—namely, the frequencies of the univalent and chromatid laggards in each chromosome type at first ana- or telophase have a parallel relation to the frequency of the spontaneons univalent formation at first metaphase scored by HAGA (l.c.). Since little differences were recognized in frequency and type of lagging chromosomes among the different salts or among the different concentrations, the data were summed up in a group of "salt treatment" (see Table 6). It becomes clear that the parallel relationship of the frequency of lagging chromosomes to the degree of univalent formation at first metaphase which was found in the control tends to be lost in the case of the salt treatment. As a consequence, it may be surmised that the salt solutions promote and modify the induction of lagging of chromosomes.

Abnormality of chromonema coiling

Some modifications in coiling of chromonemata were relatively often recorded in the salt treated materials. The meiotic stage of some cells appeared to be at first prophase judging from the state of chromonema coiling, though almost all of the PMCs in the same anther were at first ana- or telophase or interphase. In some cells where chromonema coiling delayed or failed to occur and remained as prophasic state, a part of the chromonema was broken in the fragments with one or two loose gyres as indicated by arrows in Fig. 20. In most of these cells which have assumed to have remained in prophasic state, the nuclear membrane has disappeared. This implicates that the meiotic division of these cells has proceeded at least until late first prophase, since the disappearance of the nuclear membrane in meiosis usually marks the end of diakinesis. On the other hand, in only some chromosomes of a complement whose chromonemata were in the normal state of spiralization the extensive fragmentation of chromosomes mentioned above was observed (Fig. 21). More attractive configurations of chromonema coiling were brought about by the salt treatments (Figs. 25–28). The anaphase chromosomes of the left-sided cell in Fig. 28 seem to be a little longer and thinner

than the normal anaphase chromosomes of the right-sided cell in the same photograph. The extremely long and thin anaphase chromosomes are shown in Fig. 27, which were relatively often found. In Figs. 25 and 26, it is obvious that the major spirals of the chromosomes are completely uncoiled, and furthermore the uncoiled chromosomes tend to segregate toward both poles so that many chromonema bridges are formed due to the entanglement of them. Thus, such segregation of the uncoiled chromosomes will have a potentiality to result in abnormal cells in the successive cell division. Partial failure of spiralization in a chromosome was met with only rarely (Fig. 23.) It must be emphasized here again that the meiotic stages of almost all the PMCs in an anther in which the above-mentioned types of aberrant cells were present were synchronized to be at first ana- or telophase or interphase, and that such aberrations were rarely scored in the D.W. treated group or in the control.

The minute and loosely coiled fragments, as already shown in Fig. 20, seem to be due to the failure of coiling in the broken chromonema rather than to the despiralization of the major coils in the small chromosome fragments, because no despiralization was found in the small fragments resulting from the extensive chromosome fragmentation. However, in most cases it is impossible to state definitely whether the aberrations of chromonema coiling observed here are attributable to the despiralization of the major coil of metaphase chromosomes or to the loose spiralization of chromonemata at prophase.

Previously some reports concerning the abnormal despiralization of chromosomes induced by means of chemical and physical treatments have been published by several authors. DARLINGTON et al. ('53) have found the sporadic failure of spiralization of chromosomes at first meiotic anaphase of *Uvularia* when irradiated at first prophase with X-rays. VON ROSEN (1953) has observed in *Pisum* rootlets treated with the metals, Au and Ag, some prophase cells in which the chromosomes are prevented from contraction and, thus, from entering a normal metaphase-anaphase cycle. He gave it the name "the despiralization type" of chromosomes, and ascribed it as due to a direct action of the metal ions. Moreover, similar types of the abnormalities have been reported to be induced by temperature (DOWRICK, '57; JAIN, '57), by DNase (BAL & KAUFMANN, '60) and by an alkylating agent (TANIFUJI, '62). Regarding the causes of such abnormalities of chromonema spiralization, therefore, it may be said that the normal processes of chromonema coiling and uncoiling are in some relation to the physiological alteration of cells,

Other aberrations

In the materials treated with salts non-disjunction of anaphase chromosomes, unequal segregation such as 4-6 or 3-7 segregation (Fig. 16) and precocious chromatid division to both poles at first anaphase (Fig. 10) was relatively often observed. There is no doubt of that these aberrations are derived from the disturbance of the spindle mechanism and the kinetochore function. In addition, the abnormalities which were thought to result from the disturbance of bipolarity, e.g., tripolar segregation of chromosomes (Fig. 17) or apolar segregation of them (Fig. 14), were brought about by the salt treatment.

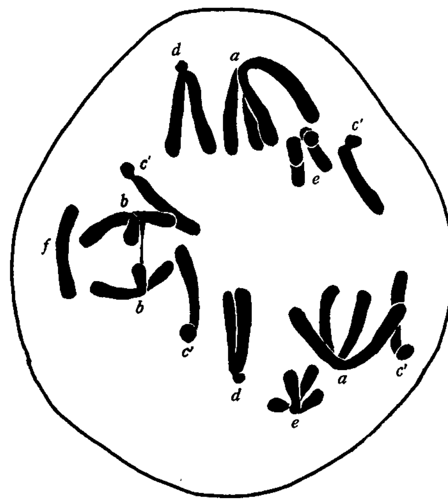


Fig. 10. A true bridge and precocious division of c-chromosomes into their sister chromatids at first anaphase.

Clotting of the first metaphase chromosomes was frequently found in the salt treated materials (Fig. 11). It is clear that this abnormality is due to the extreme stickiness of chromatin substnace. Such aberrations will, of course, have a potentiality to result in the complicated abnormalities in the successive cell division, as seen in Fig. 12.

In some of the materials placed in 0.01 mole NaCl, the stainability of chromatin substance with carmine dye was found to be decreased in the second telophase chromosomes (Fig. 19). Similar abnormality was produced

in the tetrad nuclei of PMCs in *Tradescantia* after application of NaCl (MATSUURA & IWABUCHI, '62). However, it is not apparent here whether this abnormality was induced by only NaCl, and in this respect further investigations are needed.

Discussion

As introduced at the beginning of this paper, VON ROSEN ('54a, b) classified the fundamental elements of the periodical system into three groups on the basis of their activity on cell division. He has mentioned that the elements of the halogenic series have an ability to produce chromosome brekage though Cl-ion is completely ineffective when it is bound in a salt, KCl. According to his result, Na and K metals belong to the inactive group and Ca metal to the weakely active group. However, it became obvious in the present study that the several types of meiotic abnormalities including chromosome breakage can be induced by the treatment with NaCl, KCl and $CaCl_2$ solutions.

Then, what action of the salt solutions should be considered as the cause of the abnormalities? Two interpretations may at least be possible on this question. One of them is based on the assumption that the general ionic environment in cells is modified by the unequal absorption of the cations and the anions dissociated from the salts. The unequal absorption of Na^+ and Cl^- into plant cells was already demonstrated with wheat root cells by LUNDEGÅRDH ('40). Such changes of the ionic environment in cells will be expected to occur likewise in the cases of the KCl and $CaCl_2$ treatments, because the protoplasm membrane of plant cells carries an ability to make only some kinds of metal ions pass selectively into the cells. Therefore, the meiotic abnormalities induced by KCl and $CaCl_2$ may possibly be ascribed to the alteration of the ionic environment in cells as in the cases of NaCl. MATSUURA & IWABUCHI (l.c.) considered that NaCl-induced aberrations may presumably be attributed to the alteration of the ionic environment in cells due to the predominant absorption of Cl^- over Na^+.

The evidence that the ionic alteration of cells exerts an effect on cell division has been obtained by several investigators in the experiment employing chelating agents. McDONALD & KAUFMANN ('57) observed the various types of mitotic abnormalities in the cells of the root-tip meristem of *Allium cepa* treated with ethylenediaminetetraacetic acid (EDTA) which chelates divalent or trivalent cation. They have inclined to conclude that this chemicals directly changes the ionic environment of cells, indirectly resulting in the chromosome

aberrations (KAUFMANN & McDONALD, '59). Their conclusion has been supported in part by TAKEHISA ('61) who studied in *Vicia* the effect of EDTA on the mitotic chromosomes. It is therefore conclusive that the alteration of the general ionic environment of cells impaires the normal progress of the cell division.

Another interpretation is based on the possibility, as proposed by VON ROSEN ('54a), that metal ions are able to react with the different proteins in the cell plasm or directly with the constituents of chromosomes, resulting in the structural changes of chromosomes or in the abnormal cell division.

Regarding the causal relation of the alteration of ionic environment in cells to the induction of the structural aberrations of chromosomes, a plausible assumption that the structural integrity of chromosomes is affected due to the disturbance of ionic environment in cells has been suggested in the previous paper. The structural aberrations such as the chromosome breakages induced by the salt treatments in the present experiment will also easily be explainable on that assumption.

Up to date, the chemical composition of the chromosomes in the higher organisms has been studied chemically, histochemically and by the UV-absorption technique. The components of the chromosomes have, in view of the above result so far achieved, been generally believed to be DNA, RNA, basic protein such as histone or protamine, and non-basic protein, though the relative proportions of these constituents vary in the chromosomes from different organisms. However, it has unfortunately not been clear how these macromolecular constituents build up chromosomes, and various chromosome models, in which the relationship among these constituents is supposed, have been proposed by several authors (TAYLOR, '57 ; FREESE, '59 ; DOUNCE, '59 ; SCHWARTZ, '58, '60 ; CAVALIERI & ROSENBERG, '61).

On the other hand, recently the numerous studies focusing attention on the possible role of divalent or trivalent cations in maintaining chromosome integrity have been carried out by some workers (MAZIA, '54 ; STEFFENSEN, '55, '57, '59 ; KIHLMAN, '57, '59). A hypothesis that Ca and Mg metals from chelate bonds with terminal phosphate groups between different DNA species along the chromosomes has been proposed by STEFFENSEN ('57, '60) on the basis of the results from the serial studies with respect to the effect of metal deficiency in cell on chromosome breakage. In this connection, KIHLMAN ('57) has suggested that Fe metals may also form similar bonds or they may combine the guanine bases of the nucleic acids. Furthermore, KIRBY ('56) reported that DNA-protein complex in cell nuclei of mammalian tissues is built up by the metallic bonds (Fe, Co, Cu, Mn and Zn metals) between the carboxyl

groups of the proteins and the phosphate groups of the DNA.

Thus, it is quite likely that the matallic bonds are concerned in the connection between the macromolecular constituents in chromosomes. Therefore, if the alteration of ionic environment induced by the salt treatments can make the chelate bonds unstable which have relatively low energy when compared with the covalent bonds, it will be inferred that the structural changes of chromosomes are more feasible to be produced by the cellular ionic alteration.

It must be noticed here that a few undamaged chromosomes were so often found in the cells in which only some chromosomes in the complement were extensively broken. What is the cause of such a concentrated breakage? Since it is neither conceivable that the ionic alteration of cells caused by the salt treatments occurs only on some places in the cells, nor that each chromosome reacts differentially to the chemicals, other possibilities must be considered.

When at least one break is produced in a chromosome, the damaged chromosome is weakened in the ability to maintain the integrity of the chromosome so as to become more breakable, that is, the initial breakage at a certain point in a chromosome can enhance the potentiality to induce the subsequent breaks in the same chromosome. If the above assumption is admitted, it will be explicable why the extensive fragmentation of chromosomes occurs and why it concentrates only on some chromosomes of the complement.

An alternative explanation for such extensive fragmentation of meiotic chromosomes will be offered by the MATSUURA's "parallelization hypothesis" proposed on the basis of behaviour of the first metaphase chromonemata in meiosis of *Trillium kamtschaticum* PALL. (MATSUURA, '40, '49). According to this hypothesis, the two paired chromatids of each meiotic chromosome arm forming the relational spiral system at early metaphase are transformed into the parallel one through the breaks and reunions between the two chromatids at twisting points existing in the spiral system, which are forced to break at late metaphase by the longitudinal cleavage of the matrix of the chromosomes probably initiating from both the proximal and distal ends without permitting any rotation of free ends. Therefore, if the normal process of the subsequent reunion is inhibited by some causes, it will result in many fragments of nearly equal length or its multiple length. This assumption is very attractive, but at present the definite conclusion may not be safely drawn until more critical evidence is obtained.

It was apparent in the present experiment that the production of the chromosome aberrations due to the stickiness of chromatin substance, *viz.*,

the clumping at metaphase and sticky bridges at ana- or telophase, were enhanced by the salt treatments. The abnormalities of this kind must be thought to have a direct connection with the viscosity of the chemical constituents of chromosomes. The DNA, RNA and protein have the physico-chemical property of polyelectrolyte in solution. The viscosity of such polyelectrolytes has been confirmed to be altered by the changes in ionic strength, salt concentration and temperature in the solution (GULLAND & JORDAN, '47 ; DOTY, '59). Consequently, a possibility could be supposed that the chromosome aberrations due to the stickiness of chromatin substance were also mainly attributable to the modification of the ionic environment in cells which was induced by the salt treatments.

As one of the characteristic abnormalities observed in the present investigation the aberrations of coiling spiralization of chromonemata may be pointed out. As introduced in the foregoing section, many authors have reported that the despiralization of chromonemata is brought about by the chemical and physical treatments. Then a question may arise what action of the salts was operative for the induction of the abnormalities in chromonema coiling. The question can not competely be dissolved only by the result obtained in the present investigation, but it is certain that the solution of this problem may give us a clue to the mechanism of chromonema coiling throughout the cycle of cell division. In respect to the mechanism involved in the process of chromonema coiling, many interpretations have been advanced. DARLINGTON ('35) postulated that a molecular coil sets up an internal twist in the chromosome thread, which determines the microscopically visible pattern (DARLINGTON & MATHER, '49). On the other hand, it was assumed by KUWADA ('39) that the contraction of the matrix of chromosomes plays a role on chromonema coiling. In addition, MATSUURA ('41) has suggested that the spiralization of chromonemata is closely connected with the strength of the internal repulsion force due to the electrical charges of the string, the elasticity of chromonema and the external limitation by chromosome matrix, and hence the pitch of a regular spiral can be determined by the equilibrium among these three factors. These hypotheses, however, involve several problems which have remained unsettled.

On deliberating the mechanism of chromonema coiling, it is important to take the physico-chemical properties of the constituents in chromosomes, nucleic acids and proteins into consideration. The following evidence suggests the validity of this idea. The behaviour of cell nuclei or of chromatin substance at different ionic strengths or at various salt concentrations shows striking parallel events in number of their physico-chemical properties which the DNA molecules exhibit in solution of the same condition (reviewed by

ANDERSON, '56). ANDERSON (l.c.), assuming that the chromosome condensation is essentially same to that of coiling up of the DNA molecules in chromosomes, has proposed a probable mechanism that the condensation is produced by the polyvalent cations, namely, histone, protamine or similar basic substance, which constrain the DNA in the nuclei. It is postulated there that the negatively charged phosphate groups of the DNA tend to cross-link with the positively charged groups of the adjacent histone, so that the chromosomes result in condensation. He showed that a similar result can be obtained by the changes of the ionic character of environment, or by addition of polycation. The above-mentioned view will be furthermore supported by a recent work that the polyamines such as putrescine, cadaverine and spermine are capable of upsetting the normal process of chromonema coiling and uncoiling (DAVIDSON & ANDERSON, '60). Accordingly, it seems probable that the aberrations of chromonema coiling frequently seen in the present experiments are mainly due to modification of ionic environment in cells induced by the salt treatments. This indicates probably that the normal process of the chromonema coiling and uncoiling has a connection with the shift of ionic environment in cells.

Recontly, the intramolecular changes of the polymeric chains such as polynucleotides or polypeptides, *viz.*, helix-coil transition, have been found to be brought about by the change of pH value or of temperature in solution (reviewed by DOTY, '59). In addition, the intermolecular changes seen in actin protein of muscle, G–F transition, have been reported to occur in the presence of Ca or Mg ions (reviewed by OOSAWA, '61). FELSENFELD & RICH ('57) found the interesting evidence that a three-stranded complex, (Poly A + Poly U) Poly U, is made from two strands of polyuridylic acids and one of polyadenylic acid by addition of a small number or divalent cations, and it was shown that the ionic composition of the solution is effective upon the tendency to form two-stranded complex or three-stranded one.

Thus, based on the fact that pH, temperature and small ions are able to modify the secondary structure of the macromolecules in living system, it will be postulated that the coiling of chromonemata may be directly or indirectly caused by the changes of the secondary or high-order structure of the macromolecules constituting chromosomes.

Summary

1) The present paper dealt with the effect of the neutral salts, NaCl, KCl and $CaCl_2$ on the meiotic division in PMCs of *Paris verticillata* M. BIEB. ($2n=10$). The PMCs at early leptotene to early zygotene were treated

at room temperature by immersing the cut stems of material plants in the salt solutions for 46 to 60 hours until fixation. The statistical observations of the meiotic aberrations were carried out at the stage from mid-anaphase to telophase of the first and second divisions.

2) In the salt treated materials various types of meiotic abnormalities were frequently observed; chromosome fragments, true and sticky bridges, lagging chromosomes and the abnormalities of chromonema coiling. Clotting of metaphase chromosomes, non-disjunction and tripolar segregation were also recorded.

3) The PMCs with one to four acentric fragments were very often seen and their frequency was higher at the first division than at the second one. The true bridges were recorded more frequently at the first division and the sticky bridges were more at the second one. The occurrence of chromatid laggards was more conspicuous than that of the univalent laggards.

4) In order to know the nature and degree of the effect of these salts on meiosis, the abnormalities were classified into three categories according to the property of them; the structural chromosome aberrations (i.e., the aberrations due to the breakage of chromosomes), the non-structural chromosome aberrations (i.e., the aberrations ascribed to the stickiness of chromatin substance or to the impairment of mitotic apparatus), and the aberrations indistinguishable whether structural or non-structural. The yield of the three types of aberrations was much higher in the salt treated groups than in the control.

5) In NaCl, KCl and $CaCl_2$ treatments, the frequencies of the PMCs with these aberrations did not rise in parallel with the increase of the salt concentrations, but generally reached a maximum at the isotonic scale 3, while in the treatment with the mixed solution of NaCl and $CaCl_2$ there was a proportional relationship between the aberration frequency and the salt concentration. However, it was not found that only a certain type of aberrations was specifically induced by any salt.

6) In both the control and the deionized water treated group, there existed a relationship indicating that the non-structural aberrations occur somewhat frequently than the structural ones, but in the salt treated groups the above relationship was completely reversed. This implies that the salts adopted here may bring about the structural changes of chromosomes as well as the impairment of mitotic apparatus.

7) It is presumed then that the salts primarily induce the alteration of the ionic environment in cells, indirectly resulting in the various meiotic abnormalities including the structural modification of chromosomes.

8) Certain striking configurations indicating the extensive fragmentation

of chromosomes and those of abnormal spiralization of chromonemata were recorded rather frequently in these salt-treated materials. Discussion was made as to the causality of these phenomena.

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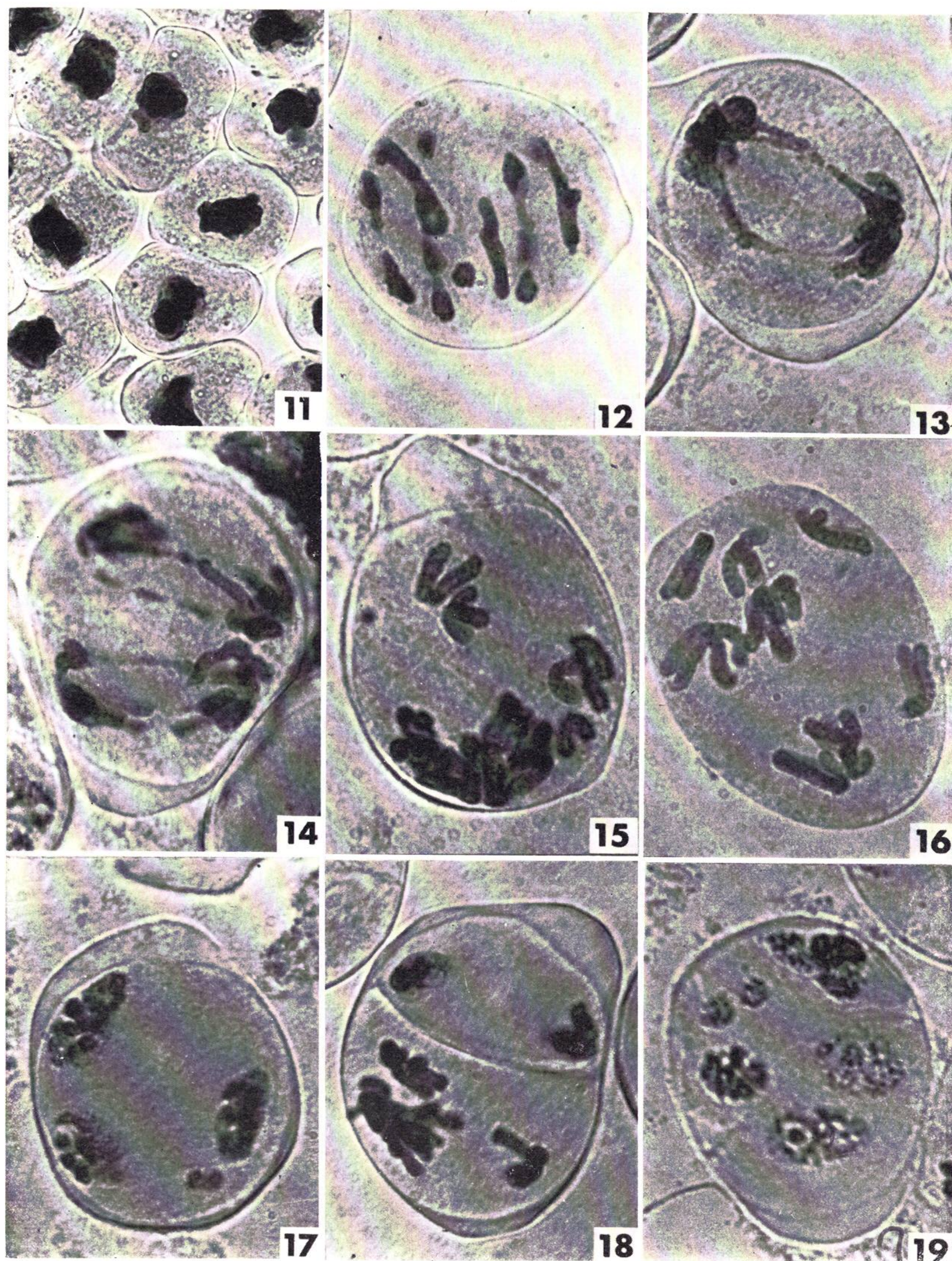
Explanation of Plates

Plate I

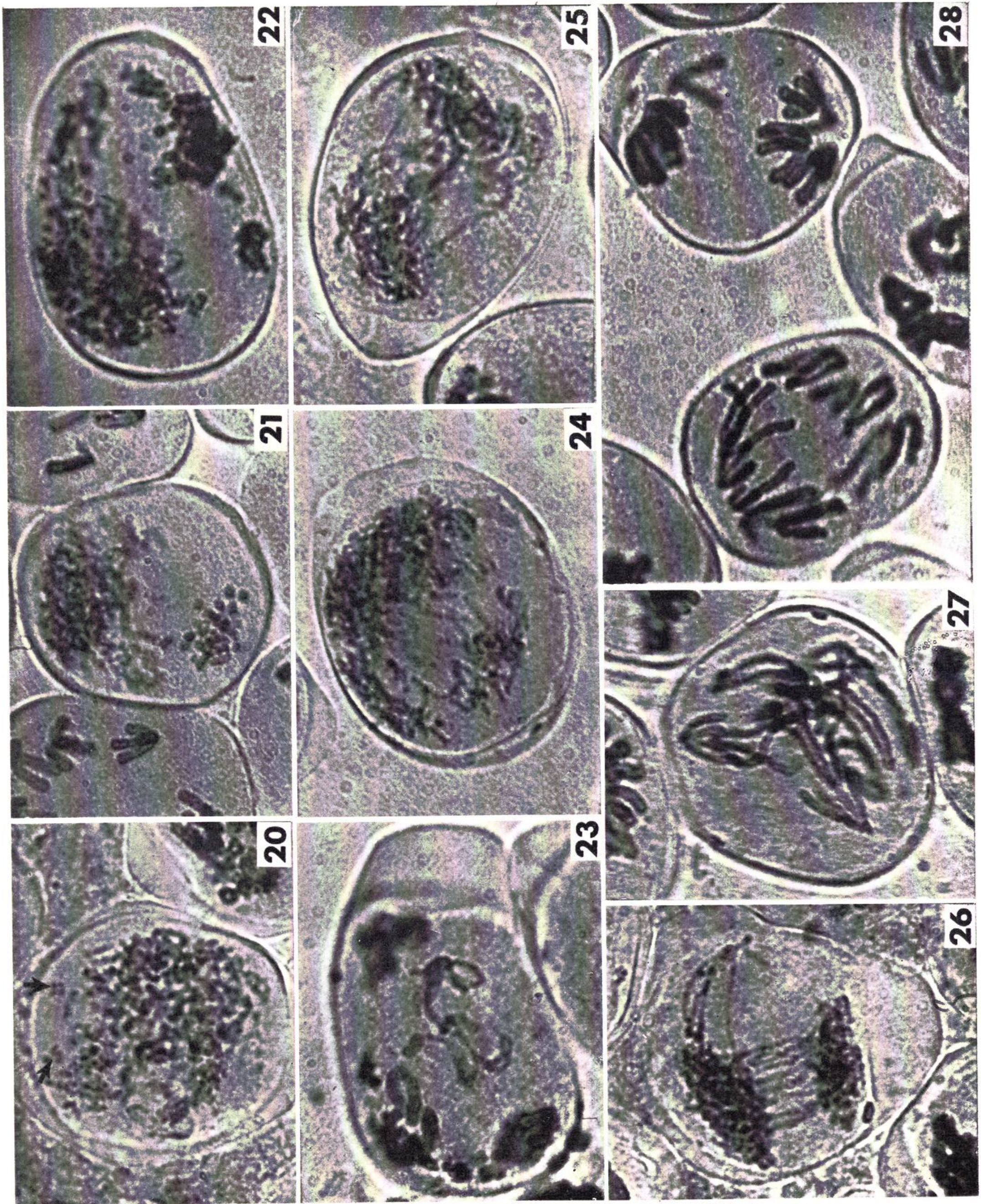
Fig. 11. Clotting of the first metaphase chromosomes.
Fig. 12. Extreme stickiness of chromatin substance resulting in heavily sticky bridges.
Figs. 13, 14, Sticky bridges at the first and second divisions.
Fig. 15. Complete impairment of the mitotic apparatus.
Fig. 16. Unequal segregation, 6-4 segregation, at first anaphase.
Fig. 17. Tripolar segregation at the first division.
Fig. 18. Abnormal second division resulting from the abnormality at the first division.
Fig. 19. Decrease of the stainability of chromatin substance with carmine dye at second telophase.

Plate II

Figs. 20-28. Representing the various types of abnormalities of chromonema coiling induced by the salt treatments. For explanation, see text.



H. Matsuura and M. Iwabuchi: Effect of inorganic salts on cell division II.



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