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**Some Observations of Abnormal Divisions in Grasshopper
Germ Cells after Treatment with Hypotonic
and Hypertonic Solutions¹⁾**
(Studies on Abnormal Nuclear Divisions, 3)

By

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(With 2 Plates)

Thanks to the extensive studies carried out by Kuwada (1937) and his followers (Shinke 1937, '39, '41, Shigenaga 1937, '44, '45, '49), our knowledge on the mechanism of the nuclear and cell divisions has been promptly advanced in these years. Kuwada (1937) pointed out that various processes having taken place in the nucleus through mitotic as well as meiotic divisions, especially those relating to chromosomes, may be regarded as a series of phenomena relative to changes in the water-relation in the cell. Then, experiments have repeatedly been made by Shinke (1937, '39, '41), Shigenaga (1937, '44, '45, '49) and some others along his views, by subjecting cells to abnormally high or low temperatures, by treating them with various narcotics, chemicals and hypotonic or hypertonic solutions or by exposing them to X-rays. The remarkable abnormalities thus artificially induced are the pseudo-amitosis, the coalescence of chromosomes by stickiness leading to the formation of bridges in division, the formation of giant di-diploid nuclei and multi-nucleate cells due to the failure of the telophase reconstruction and the abnormal vacuolization of the cytoplasm. In connection with the question how these abnormalities are brought about under these artificial conditions, the results of their investigations cited above are highly suggestive of the supposition that the most favorable interpretation for the causes of these abnormalities is largely concerned with the hydration and dehydration phenomena of the cell.

By reference to the literature it is evident that most of the experiments in this field have been carried out with plant material. In the present investigation

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the effects of hypotonic and hypertonic solutions were observed by taking the germ cells of the grasshopper, *Podisma sapporensis* (Acrididae), as material, in order to learn what is the cause of rendering the division abnormal. The present study may be important, on the one hand, in having an intimate connection with the elucidation of abnormal divisions in tumor cells, since just like abnormal features as those experimentally induced are common in the latter.

Before going further, I wish to acknowledge my sincere indebtedness to Professor Sajiro Makino for his kind guidance and suggestion given during the course of this work. Further my thanks are extended to Dr. Kyôjiro Shimakura of the Obihiro Zootechnical College, for his kind advice especially concerning the technical side of the present experiment.

Material and Method

The present work was carried out with male germ cells of *Podisma sapporensis*. Recently Shimakura (1950) has showed that in testis immersed in Ringer-Lock-Barta's solution¹⁾, the germ cells of the same species proceed in their meiotic divisions without showing any remarkable sign of injurious effects for a considerable length of time. Based on his results, therefore, Ringer-Lock-Barta's solution was in this case regarded as isotonic. This solution was used as the standard solution for the preparation of test media in the following experiments. In the subsequent description, 1R denotes the original solution prepared after the regular formula of Ringer-Lock-Barta's solution, 10R denotes a solution ten times as concentrated as the standard of 1R solution, and 0.5R that of half standard concentration, and so on. For preparation di-distilled water was used. The solutions were buffered with NaHCO_3 for maintaining their pH at about 8.

The data with which the present paper is concerned consist of those obtained by the following two methods of experiment :

i) One of them is to observe the spermatocytes of *Podisma* in the living condition by the hanging-drop method, applying various kinds of solutions as test medium. A small part of a testis follicle was placed on a clean coverglass and the surrounding fatty and other adhering tissues were taken out. By cutting, the follicle germ cells are obtained on the coverglass. To the germ cells thus prepared, a drop of one of the test solutions above mentioned was added. Then the coverglass was placed being inverted on a hollow-glass which was filled with the same kind of solution. The edge of the coverglass thus prepared was sealed with liquid paraffin, and the preparation was then brought under microscope.

ii) The other method consists of the following procedures: the testis-follicles, after removing the adhering tissues, were submerged, without squashing, in one of the test media filled in a clean glass vessel of proper size. After being left for a

1) This consists of 10 c.c. of 9 percent NaCl, 2 c.c. of 1 percent KCl, 2 c.c. of 1 percent CaCl_2 , 2 c.c. of 10 percent glucose, 0.02 grams of NaHCO_3 and 100 c.c. of di-dist. water.

necessary length of time, the follicles were taken out of the medium, placed on a slideglass, and smeared with aceto-carmin. With the preparation thus made the microscopical examination was performed.

The experiments to be described here were carried out from June 23 to July 13 in 1949, and from June 2 to July 21 in 1950, at a room temperature ranging from 20°C to 26°C.

Observations

(1) *Preliminary experiments*: In order to learn whether the meiotic divisions proceed regularly in the material immersed in standard solution (1R) or not, some preliminary experiments were carried out after the methods described above. The spermatocytes taken out of the testis-follicle were immersed into a 1R solution for about ten or more hours. Some testis-follicles were treated also with a 1R solution for 48 hours. In both experiments it was found that the meiotic divisions of the spermatocytes normally proceeded to a considerable extent (Figs. 1-7 and 46). In the observations under the living state of cells, the behavior being in process from separation of chromosomes at anaphase to formation of daughter nuclei, was found to be quite regular through both the first and second meiotic divisions.

(2) *Effects of hypotonic solutions*: The abnormalities of the cell and nuclear divisions caused by different lengths of treatment and by different concentrations of the solution used were the subjects mainly observed here, taking the first spermatocytes as material. Di-distilled water and solutions from 0.2R to 0.9R were used as test-media. The results obtained here are summarized in Table 1. The notable abnormalities chiefly observed are (a) unravelling of chromonemata and (b) formation of bi-nucleate cells.

The unravelling of chromonemata was one of the very common abnormalities occurring when the cells were placed in a hydrated condition. The unravelling of chromonemata was remarkable in di-distilled water, and it also appeared in various degrees by treatment with 0.4R and 0.5R solutions. When the cells were treated with di-distilled water, they showed swelling and then became homogeneous. In smear preparation of the testis-follicle after having been treated for 5 minutes, swelling of the chromosome had not yet taken place, but the cells seemed to show the maximum state of swelling. In a prolonged treatment the chromosomes showed a sign of swelling and the chromonemata became visible within them to a considerable extent. The whole outline of chromosomes was visible until 8 minutes of treatment, while by longer treatment their outline was lost from sight (Fig. 47). And then, the unravelling of chromonemata took place showing an extremely deformed outline (Figs. 48 and 49). A still longer immersion resulted in the drawing out of chromonemata flowing outside the cell body together with the cytoplasmic substance. By a treatment longer than 60 minutes the cells began as a whole to show a poor affinity to the dye, and the spiral structure of chromosomes became also indistinct.

Table 1. Summary of the results produced due to the treatment with hyptonic solutions.

Abnormal type Conc.	Meiotic division		Chromosomes
	Regular division	Formation of bi-nucleate cells	Uncoiling of chromonemata
di-dist.	—		+
0.2R	—		+
0.4R	—		+
0.5R	±	+	±
0.6R	±	+	±
0.7R	±	+	—
0.8R	+		—
0.9R	+		—

Table 2. Summary of the results produced by the treatment with hypertonic solutions.

Abnormal type Conc.	Meiotic division						Chromosomes
	Regular division	Amoeboid behavior	Di-nucleate cell	Di-diploid cell	Chromosome bridge	Bending of spindle	Fusion
2R	+	+				+	
3R	±	+	+	+	+	+	±
5R	±	+	+	+	+	+	+
6R	±	+	(+)	(+)		+	+
8R	—		(+)	(+)		+	+
10R	—		(+)	(+)		+	+

(+) is a sign indicating occurrence after replacement into 1R solution.

By treatment with 0.4R and 0.5R solutions, the swelling of the cell was not so remarkable as by di-distilled water, but the meiotic division was found inhibited. This resulted in the uncoiling of chromonemata to some extent.

The formation of bi-nucleate cells was remarkably observed when cells were treated with solutions of higher concentrations, such as 0.6R and 0.7R solutions. After treatment, the swelling of the chromosomes was induced and their general appearance became indistinct. But the outline of the cell and the mitochondrial bodies included were evident in appearance. The meiotic divisions took place for a certain length of time, proceeding from metaphase to anaphase. But it was found that some of these cells failed to separate into two daughter cells, in spite of the considerable elongation of the cell body. Due to a longer immersion they recovered into the oval form again. In the cells fixed at this stage with 1% acetic acid, occurred the formation of bi-nucleate cells, containing two independent nuclei (Figs. 8-11). In the treatment with 0.8R and 0.9R solutions, the chromosomes became obscure

in outline, after swelling. But, the meiotic divisions proceeded as regularly as had occurred in a 1R or standard solution, and no remarkable abnormality was found. Thus, the abnormalities obtained as a result of hydration of cells are unravelling of chromonemata, inhibition of the meiotic division and formation of bi-nucleate cells. For general indications of the data from various experiments, one can refer to Table 1 which was prepared by way of summary.

(3) *Effects of hypertonic solutions*: When the cells were treated with hypertonic solutions, phenomena completely reverse to those observed in hypotonic solutions were found. The cells showed a strong shrinkage and an increase in refractivity as a result of dehydration. In the case with a solution of higher concentration they took the appearance of a highly compact mass with no visible structure. In 2R solution it was found that some cells continued their meiotic divisions in a rather regular manner, while in some cases the division was inhibited at first metaphase. In the latter case the chromosomes exhibited morphologically no visible abnormality, except that they were smaller in volume and more distinct in outline than with 1R solution.

In those of higher concentrations such as 3R, 5R and 10R solutions, the cells were mostly disturbed at metaphase, showing various abnormal figures of chromosomes. On the other hand, in the anaphase cells the inhibition of division hardly took place in lower concentrations than 5R. The abnormalities induced are as follows:

(a) Amoeboid behavior of cytoplasm during from anaphase to telophase was frequently observed when the cells in process of division were placed in a dehydrated condition. At anaphase the cell stretched its cytoplasmic process in a like manner as that usually observed in amoeba. This behavior has continued up to the completion of separation into two daughter cells. As a result, there were often produced two cells unequal in volume of cytoplasm and having two nuclei nearly equal size (Figs. 12-16).

(b) The bi-nucleate cells were produced due to failure of formation of the cell boundary at anaphase (Figs. 17-23 and 50). As a whole, the bi-nucleate cells produced by the treatment of a rather short time, showed two daughter nuclei placed at a considerable distance, while in those produced by a longer treatment two nuclei were found lying closely together.

(c) Formation of a di-diploid nucleus: After separation at anaphase, the divided chromosomes without migrating to the poles, are enclosed together by a common nuclear membrane, thus resulting in the production of a di-diploid nucleus (Figs. 24-25). Both the formation of di-diploid cells and of bi-nucleate cells took place when treated with a solution of rather low concentration such as a 3R.

(d) Bending of the spindle body was remarkably found in highly dehydrated cells. After examination in the living state, it was recognized that the spindle body became first irregular in both shape and structure, and then its bending followed

(Figs. 26-29, 51 and 52.).

(e) Formation of micro-nuclei: A few chromosomes extruded out of the spindle due to irregular behavior of chromosomes at anaphase resulted in the production of a certain number of micro-nuclei (Fig. 53).

(f) Chromosome bridges: In both the first and second meiotic divisions chromosome bridges made their frequent appearance at anaphase (Figs. 30-33 and 54). Sometimes, the chromosomes forming bridges failed to separate at telophase. Two connecting nuclei thus formed came to approach each other and finally conjugate into a single body. The formation of a di-diploid cell with an anuclear cytoplasmic mass thus took place through the process as described above (Figs. 34-41).

(g) Fusion or coalescence of chromosomes: Fusion often occurred between two or sometimes more chromosomes (Fig. 44). In an extreme case, the chromosomes were found grown together into some irregular masses, due probably to their stickiness (Figs. 42-43 and 45). Such phenomena were rather remarkable in hypertonic solutions of higher concentration.

(h) Deformation of chromosomes: An unusually prolonged treatment with hypertonic solutions made the cells irreversible, leading to their degeneration. In the highly damaged cells the chromosomes were found in the form of a short rod or even with a ball-like outline (Fig. 55). It appears that such a deformation of chromosomes may be the result of a break-out of chromonema within chromosomes, and not due to the fragmentation of chromosomes.

The general indications of the results obtained by treatment with hypertonic solutions, are summarized in Table 3. The abnormalities herein concerned contain (i) the abnormalities concerned with the cytoplasm, (ii) those relating to the spindle substance, and (iii) those of the chromosomes. A series of abnormalities given in (a) in the above description is no other than the cytoplasmic abnormalities. Those described from (b) to (e) seem to belong to the abnormalities having occurred in the spindle substance, and finally those described from (f) to (h) are concerned with the chromosomes.

Remarks

By the present experiments, the effects of hypotonic solutions upon the cells were shown as irregular arrangement of chromosomes, unravelling of chromonemata, and production of bi-nucleate cells, as a result of hydration.

The demonstration of the inner structure of chromosomes involving spiral chromonemata, has been reported heretofore by several workers with application of various methods in plant cells (Sakamura 1927, Kuwada & Nakamura 1933, Oura 1936, Shinke 1937, Kuwada, Shinke & Oura 1938, Matsuura 1938, Shigenaga 1940). It has been suggested from these studies that the spiral structure of chromosomes is caused either by swelling of the matrical substance or by dissolution of the latter (Sakamura 1927, Kuwada 1937). Very recently Makino & Momma (1950) have

advised a simple new technique for the demonstration of chromonemata. It involves the treatment of cells with cold or hot water previous to smearing. The interpretation for the method advocated by Matsuura (1938) and Makino & Momma (1950) seems to lie in a swelling of the matrical substance due to hydration in cells. Thus it appears that a swelling of the chromosome matrix caused by hydration makes the inner spirals of chromosomes visible.

It may be noticeable that the bi-nucleate cells were produced due to hydration of cells, since no such a case has been reported heretofore. It seems to the author that this phenomenon was caused by hydration of cytoplasmic material taking place during anaphase. Shinke (1939) described that the mitotic abnormalities, such as chromosome scattering or irregular chromosome distribution at anaphase, and formation of micro-nuclei, are those occurring as a result of hydration.

Experiments of dehydration, on the other hand, have been carried out rather extensively producing pronounced results in plants. Shinke (1939) described that the mitotic abnormalities which are regarded as connected with dehydration are chromosome bridges, chromosome coalescence, pseudo-amitosis, di-diploid nucleus, failure of the telophasic reconstruction, etc.. Frequent productions of bi-nucleate cell and also di-diploid nucleus were shown by Shigenaga (1937, 1944, 1949). Shigenaga (1937) observed them by means of replacing cells into water after dehydration with hypertonic solutions. The results of the present experiments are fairly compatible with the view expressed by Shinke (1939), so far as the formation of the bi-nucleate cell and the di-diploid nucleus is concerned.

The mechanism of coalescence and pycnosis of chromosomes, associated with structural changes taking place in chromosomes are a matter wholly unknown. The observations made by Shimakura (1937) seem to give a suggestive evidence on this point. He treated the chromosomes of pollen mother cells of *Tradescantia* and reported that the chromosomes showed shrinking in semipermeable condition of the plasm-membrane, while they swelled when the semipermeable condition was lost. And then, they stuck together with each other in more or less degree when the cells were treated again with hypotonic medium. In this respect he pointed out that hypertonic solutions did not act as osmotic operation upon the chromosomes any longer, but served as harmful agency of the molecules or ion concentration of the salts used.

In the present experiments with hypertonic solutions some abnormalities having occurred in the spindle are also interesting. Shinke (1939) regarded abnormal dehydration of the cellular components, especially that of the spindle, as the primary cause of mitotic abnormalities caused by plasmolysis. Bělář (1929) reported the evidence of abnormality of the spindle in the case of treatment by hypertonic solutions in the spermatocytes of *Chorthippus*. In a recent study, Ris (1949) demonstrated in grasshopper germ cells that after the exposure to X-ray and also to high temperatures abnormal lateral expansion of the spindle was brought about due to the

stickiness of chromosomes taking place at anaphase, and then there were formed a cell with the diploid number of chromosomes and an anucleate cytoplasmic bud. Working with the mechanism of mitosis, Wada (1936, 1949) expressed the view that the spindle substance showed a reversible change of structure from globular protein molecules into unfolded polypeptid chains in the living cell, and that the fibrous polypeptid molecule, more unstable than the globular protein molecule was disturbed in its hydrated state on account of higher dehydration, and then the change of the molecule-structure of the spindle substance followed.

Generally speaking, we may say that the abnormalities obtained by influence of hypotonic and hypertonic media in the present experiments involve most of the abnormal figures described by Shinke (1939) as hydration and dehydration figures of the cell.

Summary

(1) Abnormal meiotic figures were observed, using the spermatocytes of the grasshopper, *Podisma sapporensis*, as material, after treatment with hypotonic and hypertonic solutions.

(2) The solutions of various concentrations employed as test media were prepared using Ringer-Lock-Barta's solution as standard solution. The observations were carried out with a hanging-drop method on living cells by application of test media and by immersing the testis-follicles in various test solutions.

(3) Under the influence of hypotonic solutions the cells induced irregular arrangement of chromosomes, unravelling of chromonemata and formation of bi-nucleate cells.

(4) The abnormalities regarded as the effects of hypertonic solutions are amoeboid behavior of the cytoplasm, formation of bi-nucleate cell and di-diploid nucleus, irregular bending of the spindle body, formation of micro-nuclei, chromosome bridges, coalescence of chromosomes and deformation of chromosomes.

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Explanation of Plate V

All figures, except Figs. 29, 44 and 45, are camera-lucida drawings made from living spermatocytes, observed with the hanging-drop method. Figs. 29, 44 and 45 were made from aceto-carmine smear preparations. Magnification: ca. $\times 1200$. In reproduction, they were reduced to 1/3 in printing.

In explanation, 20-M-T, for instance, denotes the treatment of 20 minutes with the solution indicated.

Figs. 1-7. Successive stages of regular meiotic division of a first spermatocyte after treatment with 1R solution; a control series. 1); 20-M-T. 2); 32-M-T. 3); 55-M-T. 4); 73-M-T. 5); 95-M-T. 6); 130-M-T. 7); 160-M-T. Figs. 1-7 are successive stages observed in one and the same cell.

Figs. 8-11. Successive stages in formation of bi-nucleate cell, observed in one and the same spermatocyte, after treatment with 0.6R solution. 8); 20-M-T. 9); 55-M-T. 10); 90-M-T. 11); 115-M-T.

Figs. 12-16. Successive stages of asymmetrical cell division taken place due to amoeboid behavior of cytoplasm during ana-telophase, observed in one and the same spermatocyte, after treatment with 3R solution. 12); 40-M-T. 13); 160-M-T. 14); 240-M-T. 15); 255-M-T. 16); 350-M-T.

Figs. 17-21. Successive stages in production of bi-nucleate cell, observed in one and the same spermatocyte, after treatment with 3R solution. 17); 30-M-T. 18); 65-M-T. 19); 105-M-T. 20); 140-M-T. 21); 255-M-T.

Figs. 22-23. The same, after treatment with 5R solution, without formation of cell boundary. Successive stages observed in one and the same spermatocyte. 22); 45-M-T. 23); 65-M-T.

Figs. 24-25. Successive stages in production of di-diploid nucleus, by replacing the material in 1R solution after treatment with 8R solution. 24); 57-M-T. 25); 105-M-T.

Figs. 26-29. Bending of spindle body observed in four different spermatocytes.

Fig. 26. First spermatocyte after treatment with 3R solution for 120 minutes.

Fig. 27. Second spermatocyte after treatment with 6R solution for 55 minutes.

Fig. 28. Second spermatocyte after treatment with 5R solution for 70 minutes.

Fig. 29. First spermatocyte after treatment with 10R solution for 100 minutes, showing a change of cell into spherical form.

Figs. 30-33. Successive stages in formation of chromosome bridge, observed in one and the same spermatocyte, after treatment with 5R solution. 30); 50-M-T. 31); 85-M-T. 32); 150-M-T. 33); 220-M-T.

Figs. 34-41. Successive stages in formation of di-diploid cell and anucleate cytoplasmic mass, observed in one and the same spermatocyte, after treatment with 6R solution. 34); 33-M-T. 35); 53-M-T. 36); 58-M-T. 37); 68-M-T. 38); 78-M-T. 39); 98-M-T. 40); 111-M-T. 41); 142-M-T.

Figs. 42-45. Fusion of chromosomes, observed in different cells.

Fig. 42-43. Two successive stages. After treatment with 10R solution. 42); 40-M-T. 43); 120-M-T.

Fig. 44. After treatment with 5R solution for 15 minutes. Showing chromosome coalescence at first metaphase, low degree.

Fig. 45. Chromosome coalescence after treatment with 8R solution for 60 minutes, high degree.

Explanation of Plate VI

All figures are photomicrographs taken with the aid of 'MAKAM', from acetocarmine smear preparations, by the courtesy of Prof. Makino.

Fig. 46. First spermatocyte metaphase after treatment with 1R solution for 120 minutes, showing the normal state of chromosomes. $\times 400$.

Figs. 47-49. Hydration figures representing drawing-out of chromonemata, after treatment with di-dist. water. $\times 600$.

47, after treatment for 8 minutes.

48, after treatment for 30 minutes.

49, after treatment for 50 minutes.

Figs. 50-55. Dehydration figures including the various meiotic abnormalities.

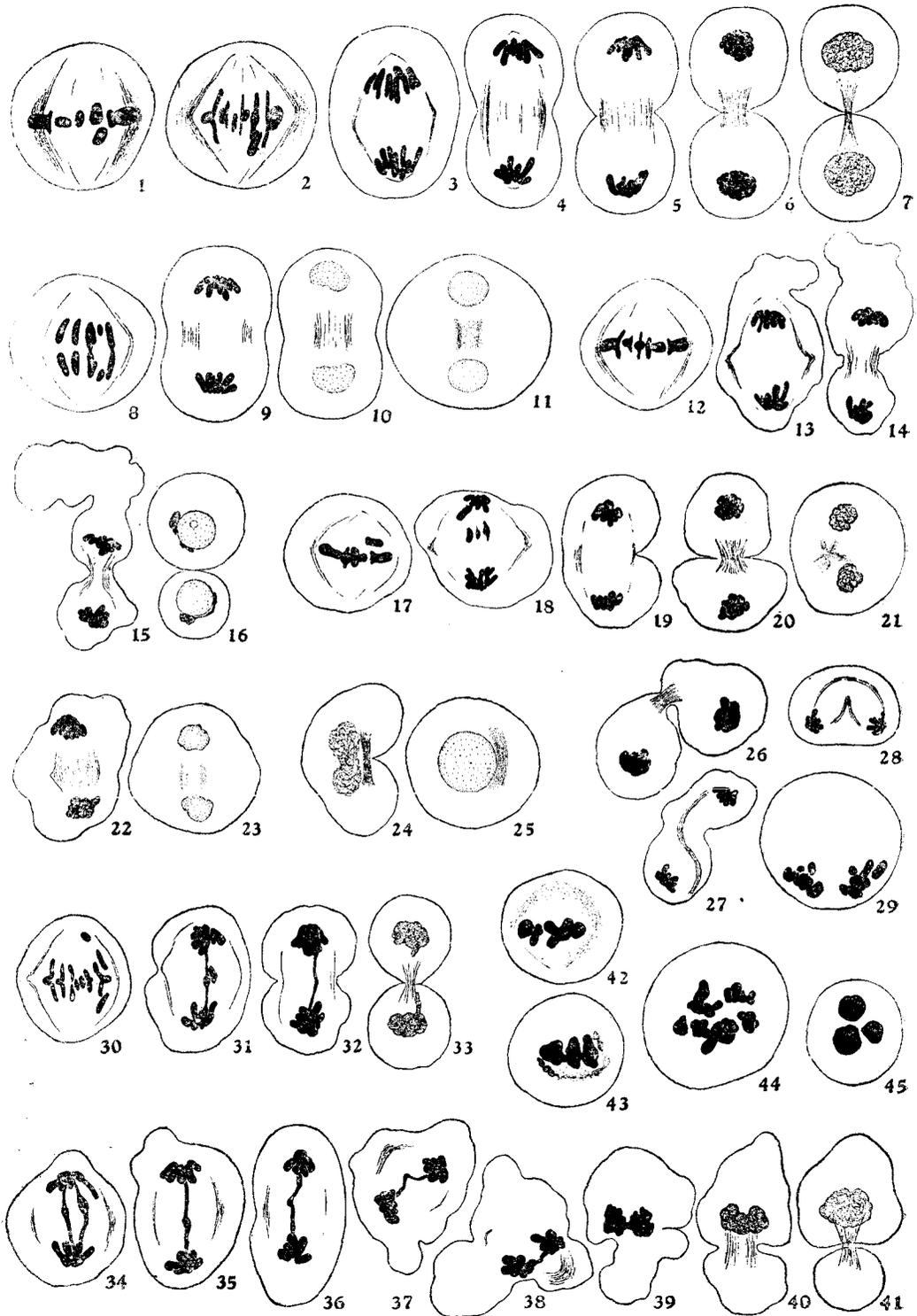
50, formation of bi-nucleate cell, after treatment with 5R solution for 80 minutes. $\times 600$.

51-52, two spermatocytes showing bending of the spindle body, Fig. 51 after treatment with 5R solution for 15 minutes and Fig. 52 after treatment with 10R solution for 120 minutes. $\times 400$.

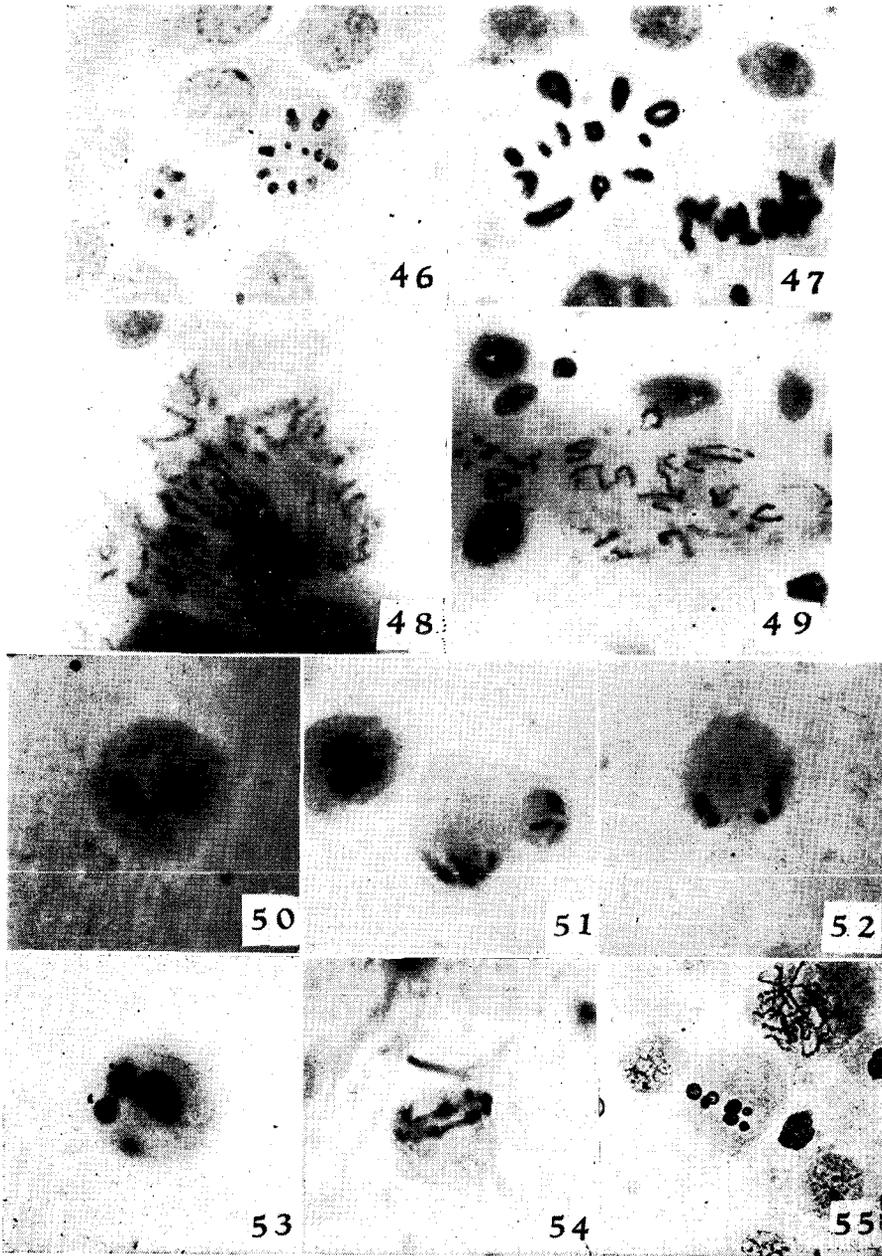
53, production of micronuclei after treatment with 10R solution for 120 minutes. $\times 600$.

54, two chromosome bridges in a single cell, after treatment with 5R solution for 60 minutes. $\times 400$.

55, deformation of chromosomes showing ball-like feature, after treatment with 5R solution for 60 minutes. $\times 400$.



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