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Studies on Amphibian Chromosomes
II. On the Chromosomes of *Bufo bufo japonicus Schlegelii*

By

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(With Plates VI & VII)

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Contents

INTRODUCTION

The phenomenon of sex-reversal is commonly known in amphibians, and a good many examples of this have already been recorded by various authors. Of the whole group the toad shows the strongest

inclination for amphisexuality, and works have been carried out on this problem by such authors as PONSE (1924), and WELTI (1925 a, 1925 b) mainly from the standpoint of the experimental biology. Our present knowledge of chromosomes of the toad remains still rather meagre, even though those of the European forms have been studied by KING (1907), BECCARI (1926), STOHLER (1926—28), and those of the Japanese form by KADOTA (1923). STOHLER, above all, has contributed much to our knowledge of the chromosomes, not only of the testis but also of the functional and potential ovaries. He employed, however, the classical method of preservation for his work, and it is but natural that the figures he obtained are not clear enough to enable us to make any crucial examination of the individual chromosomes. The new fixing method recommended by MINOUCHI (1927) for mammalian chromosomes, gives satisfactory results for anuran chromosomes also; the chromosomes are always well preserved with this method, without losing their characteristic features especially in the metaphase. Here we are to express our heartiest thank to Professor Taku KOMAI for his kind advice and criticism.

In pursuing the present study we have been aided by a grant from the Imperial Academy of Japan.

MATERIAL AND METHODS

The toad used in the present study is *Bufo bufo japonicus Schlegelii*, which was captured in August at Kagoshima in the southern part of Kyushu. The testes were taken out as quickly as possible from the animal, cut into small pieces, not exceeding 4—5 millimeters in diameter, and were dropped into the following mixture :

3% OsO_4	4 parts
1.5% CrO_3	8 parts
4.5% $K_2Cr_2O_7$	8 parts

After 24 hours of fixation the material was washed with water during the same length of hours or more. Dehydration was carried on with various grades of alcohol beginning with 50%; absolute alcohol was replaced by creosote-toluol, which was successively replaced by toluol and toluol-paraffin, and then the pieces were imbedded in paraffin. Sections were cut into 8, 10 or 15 micra in thickness. After bleaching with H_2O_2, they were put into CHURA's picro-acetic mixture for 24 or 48 hours and stained with HEIDENHAIN's iron-haematoxylin. The fixative is especially recommended for the metaphase of various kinds of the germ cells, but not for the prophase of the first meiotic

division. The chromosomes in this stage are hardly stainable though they are well preserved.

OBSERVATIONS

Chromosomes in the Spermatogonium

The primary spermatogonium of this toad shows a spherical form and is enclosed by follicle cells as reported in other forms of amphibians by many previous investigators (*Fig. 1*). It is the largest of all male germ cells and found solitarily in the follicle. The prophase shows no difference from that of the secondary spermatogonium to be described later. The polar view of the metaphase is given in *Fig. 2* which shows 22 chromosomes.

The secondary spermatogonia are spherical in form; invariably two or more of them are found together in a common follicle membrane. In the early generation each of them contains a polymorphic nucleus smaller than that of the primary spermatogonium. This nucleus becomes gradually smaller by successive divisions losing its polymorphic nature, and finally assumes a spherical form. In the prophase the chromatin appears as an apparently continuous spireme which breaks up into many segments in the later stage (*Fig. 3*).

Each chromosome is divided into two daughter halves by a longitudinal split (*Fig. 4*). The polar view of two equatorial plates of the secondary spermatogonia are given in *Figs. 5* and *6*, each showing 22 chromosomes. The one in *Fig. 6* is smaller than that in *Fig. 5*. As much smaller equatorial plates than those shown in these figures may be found in the testis, the nucleus of the ultimate spermatogonium is evidently smaller than that shown in *Fig. 6*. Crucial observations have revealed that, except the difference in size, there is no marked difference in structure between the nuclei of the primary and secondary spermatogonium. Each chromosome of the equatorial plate of the primary spermatogonium is larger than the corresponding one of the secondary spermatogonium.

After examinations of a large number of well preserved equatorial plates of both primary and secondary spermatogonia, it has become clear that they contain 22 chromosomes without exception. The chromosomes can be sorted out into two groups according to their sizes, namely, 12 longer and 10 shorter chromosomes. The longer chromosomes are V-shaped in both the primary and secondary spermatogonia. The angle between the arms of the V is variable, but no case is found where the V stretches out so as to become straitgh.

The shorter chromosomes in the primary spermatogonium are also clearly V-shaped, while it is sometimes difficult to determine in the later secondary spermatogonium whether they are V- or rod-shaped, because they become shorter than those in the primary or the earlier secondary spermatogonium after repeated divisions. The polar granule is found at the point of flexure of either long and short chromosome. This fact seems to indicate that all the spermatogonial chromosomes are V- shaped in principle. The long ones are situated always in the periphery of the equatorial plate, directing the vertex towards the center, while the short ones are distributed irregularly in the central space.

Chromosomes in the Primary Spermatocyte

a. The Prophase

In the leptotene stage the chromosome appears in the form of many granular, much convoluted threads. Several nucleoli of various sizes stained deeply with HEIDENHAIN's iron-haematoxylin are present in the nucleus of this stage (*Fig. 7*). In the zygotene stage the homologous chromosomes begin to arrange themselves in twos near the cell pole, the nucleus thus showing a bouquet-like appearance (*Fig. 8*). The pachytene stage appears as soon as the pairing of the homologous threads is completed (*Fig. 9*). The bivalent spiremes become shorter and thicker by condensation during these stages ; they show, however, no dual appearance. The nucleoli are still visible in the pachytene stage. The diplotene stage and diakinesis follow in succession, none of these nucleoli being now visible. *Fig. 10* shows the strepsitene stage and *Fig. 11* the stage immediately before the diakinesis. The process of development from strepsitene threads to the ring tetrads could not be followed. The long chromosomes form ring tetrads as shown in the other forms of anurans by KADOTA (1923), WITSCHI (1924), STOHLER (1928) and IRIKI (1929), while it is not clear as to whether such large V-shaped tetrads as found by IRIKI (1929) in *Hyla* is present or not in the stage of *Bufo*. All of the small chromosomes are dumbbell-shaped. In the later prophase the spiremes are hardly stainable with HEIDENHAIN's iron-haematoxylin, so that the detailed observation of the stage are left for a later study.

b. The Metaphase

The equatorial plate of the first division is composed of six large and five small tetrads. There is a marked contrast between these

two groups of tetrads. The large tetrads lie always at the periphery of the equatorial plate and the small ones in the central space under well-preserved condition. The typical arrangement of the chromosomes in the first division is therefore similar to that in the spermatogonial metaphase, each tetrad of the former representing a pair of homologous partner of the latter. Irregularities may however often be seen with regard to the chromosome arrangement, such as, that some of the large chromosomes are situated in the central space, and the small ones lie at the periphery, or that all of the large ones are found on one half of the plate and all of the small ones on the other.

A vertical V-shaped tetrad which belongs to the large chromosome group is often found. It lies at the periphery of the equatorial plate as shown in *Figs. 14* to *19*. The polar view of this chromosome is given in *Figs. 14* to *17* and its side view in *Figs. 18* and *19*. This chromosome is probably homologous with that found in *Hyla* which is characterized by a special behavior as reported by IRIKI (1930).

c. The Anaphase

Fig. 20 shows the side view of two daughter-sets of chromosomes in the anaphase, while *Figs. 21* and *22* show the polar view of the same stage found in the same section. All of these contain 6 large and 5 small double V's.

Chromosomes in the Secondary Spermatocyte

The chromosomes hold their individuality in the interkinesis, though they become more or less diffuse in appearance (*Fig. 23*). 11 chromosomes can be clearly recognized in the nucleus. The polar granules appear as deeply staining dots in the lightly stained nucleus. The number of the granules is larger than that of the chromosome vesicles. It is also often seen that the two polar granules lie closely associated in a chromosome vesicle as shown in *Fig. 24*. Judging from these facts, it is highly probable that the duality of the chromosomes which appear in the anaphase of the first division is also held in this stage.

In well preserved condition the chromosomes appear always 11 in number in the metaphase of the second division, of which large 6 lie at the periphery of the equatorial plate and small 5 in the central space (*Fig. 25—27*). This arrangement is therefore similar in principle to that found in the spermatogonium and primary spermatocyte.

The chromosomes are all horizontal double V's. The polar granules lie at the point of flexure of V's. Each horizontal double V-chromosomes divide into two daughter halves of single V's in the anaphase. As the result each daughter group of the chromosomes receives 6 large and 5 small monads of the V's. *Fig. 29* shows the side view, while *Fig. 30* and *31* show the polar view of the two daughter groups in the anaphase of a secondary spermatocyte which is found in two successive sections.

DISCUSSION

Chromosomes in the Spermatogonium

According to the works of MEVES (1891), MCGREGOR (1899) and others, the spermatogonia of urodeles multiply by the amitotic division and develop into the functional spermatozoa. But KING (1907) has found no amitotic division in the germ cells of *Bufo lentiginosus*. LEVY (1915) and MORITA (1928) have reached the same result in *Rana esculenta* and *Rana nigromaculata* respectively. As far as we have examined on the primary and secondary spermatogonia of *Bufo bufo japonicus* as well as of *Hyla arborea japonica*, no amitotic division was found in the spermatogonia. Even if amitosis occurs in the spermatogonia of anurans at all, it would be exceedingly rare and the cells produced in this manner would never develop into mature spermatozoa.

It has already been observed by such investigators as MEVES (1891), CHAMPY (1913), and others that the spermatogonium of amphibians has a polymorphic nucleus in the resting stage of its early generation. There are however only a few who have attempted to give any explanation of this peculiarity. SWINGLE (1921) has observed in *Rana catesbiana* that the spermatogonial nucleus is nothing more than an aggregate of chromosomal vesicles or a fusion of such vesicles. This is probably the case with that in *Bufo bufo japonicus*, though our observation is not crucial enough on this point.

It is not very difficult to count the diploid number of chromosomes in this toad because of their large size and relatively small number in comparison with those of higher vertebrates. According to KING (1907), chromatin forms an apparently continuous spireme in the prophase of the primary spermatogonia of *Bufo lentiginosus*, which breaks into 24 segments in the later stage, of which 4 are noticeably longer than the rest, other 4 are very short and the remaining

16 are of intermediate length. In the secondary spermatogonia the chromatin spireme breaks also into 24 segments of different lengths. Each of these segments condenses into a V-shaped chromosome which is somewhat smaller than that found in the primary spermatogonia. BECCARI (1926) has reported in *Bufo viridis* that 22 chromosomes are present in the cell of the tadpole and 21 in the germ cell of the adult male. STOHLER (1926—1928) has found 22 chromosomes in the germ cells of young *Bufo viridis*, after or a little before metamorphosis, as well as in those of the adult. He classifies these chromosomes into 12 "Schleifen" and 10 "Stäbchen"; the former are long and take always a form of bead, while the latter are variable in form, being straight, a little curved or strongly bent.

In the present study on *Bufo bufo japonicus* we also have found 22 chromosomes in the spermatogonia which consist of 12 long and 10 short chromosomes as in other forms of *Bufo*. It may be concluded from these facts that the basal diploid number of the chromosomes of *Bufo* is 22 in both male and female, contrary to the opinions of KING and BECCARI. The spermatogonial chromosomes are all V-shaped in well-preserved preparations and each of them has a polar granule at the apex of the V. All of the small chromosomes are, therefore, centromitic, V-shaped, and the variability in form reported by STOHLER seems to be due to an artifact.

Chromosomes in the Primary Spermatocyte

KING (1907) has given in the male of *Bufo lentiginosus* one equatorial plate of the first division, showing 6 large and 6 small chromosomes. BECCARI (1926) has found 11 chromosomes in the first spermatocyte of *Bufo viridis* and sorted them into 9 large and 5 small ones. STOHLER (1926) has observed 11 chromosomes in the equatorial plate of the first spermatocyte of *Bufo viridis* and classified them into 4 large, 2 intermediate and 5 small ones, while in a later study (1927—1928) he has classified the chromosomes into two groups, 6 large and 5 small, because of the difficulty of making distinction between the smallest of the large ones and the largest of the intermediate ones. Two groups of tetrads, 6 large and 5 small, have also been observed by STOHLER (1928) in *Bufo calamita* and in *Bufo vulgaris*. In the present study on *Bufo bufo japonicus*, 11 chromosomes, 6 large and 5 small, are found in the equatorial plate of the primary spermatocyte as mentioned by BECCARI and

STOHLER. It is, therefore, clear that *Bufo* has 11 as the basal haploid number of chromosomes, though a doubtful example has been given by KING. Of these 11 chromosomes, large 6 are usually situated at the periphery of the equatorial plate and the small 5 in the central space. This arrangement of the chromosomes seems to be fundamental in *Bufo*. Such irregularities as noted by the previous authors is probably be due to an artefact.

Previous authors have hardly succeeded in obtaining any complete figure of the anaphase of the first division. Fortunately we obtained beautiful figures of the anaphase in which most of the chromosomes reveal a dyad nature, taking the form of double-V except only a few in which this nature is not so apparent. Careful studies of many polar and side views of the anaphase have made it clear that all of the chromosomes are double V's, the spindle-fibers being attached always at the apices of the double V's.

Chromosomes in the Second Spermatocyte

KING (1907) has given an equatorial plate of the second division in which 9 dumbbell-shaped chromosomes of different sizes are shown. STOHLER (1926—1928) has found in the second division 11 dot-like chromosomes connected one another by chromatin strands. It is, however, well known that there is a certain fixed rule on the behavior of the chromosomes in the development of the germ cells. The V-chromosomes in the spermatogonia change into double V's in the anaphase of the first division, horizontal double V's in the metaphase, and then into single V's in the anaphase of the second division. From these facts it can safely be concluded that such dumbbell-shaped or dot-like chromosomes of the second division as shown by KING (1907) and STOHLER (1926—28) are not preserved satisfactorily enough to determine their number and morphology. Generally speaking, the equatorial plate of the second division is more difficult to preserve than any other stage of germ cells, so that in our opinion few workers on vertebrate chromosomes by means of the ordinary fixing methods has succeeded in getting accurate figures of the equatorial plate. The metaphase chromosomes in the second division in the present study are all V shaped, not dumbbell-shaped as those in the works of KING and STOHLER. In the metaphase they lie horizontally in the equatorial plate, and in the anaphase they separate into two daughter V's thus recovering the form found in the spermatogonium. These facts are similar, in principle, to those found in the spermatogenesis of various

orthoptera by several authors, as well as the same of mammals by MINOUCHI (1928) and of reptiles by NAKAMURA (1928).

To determine fundamental chromosome number of any species, the following points must be made clear: (1) The number of spermatogonial chromosomes, (2) that of the first spermatocyte chromosomes, (3) that of the second spermatocyte chromosomes, (4) the forms of the meta- and anaphase chromosomes in these three kinds of cells. In the present study all of these points are made clear, so that there is no doubt that the diploid number of chromosomes is 22 and the haploid 11 in *Bufo bufo japonicus*.

Sex Chromosomes

Very little is known about the sex chromosomes of amphibians. WITSCHI (1924) has stated on the chromosomes of the first division of *Rana temporaria* that "Das Chromosom vierter Grösse beginnt nähmlich jetzt ein Sonderverhalten zu zeigen, durch das es sich schliesslich als Geschlechtschromosom zu erkennen geben wird." and "bei polarer Ansicht solcher Spindeln findet man dann natürlich die Teilstücke eines über dem anderen liegend." He has also reported in the second division that "In der Regel—vielleicht immer— teilt sich das Geschlechtschromosom später als die Autosomen. Es zeigt wiederum die von der ersten Reifeteilung her bekannte Zusammensetzung aus zwei ungleich grossen Segmenten, die wir nun als X- und Y-chromosomen erkennen." According to his opinion, therefore, the first division is equational and the second reductional as to the sex chromosomes. STOHLER (1928), who has contributed a great deal on the problem of sex determination of European toads from the cytological standpoint, has observed 22 chromosomes in the diploplate of both male and female and 11 in the haploplate of the male, and stated with regard to the sex chromosomes that, "bei allen von mir untersuchten Krötenarten war ein Geschlechtschromosom als solches nicht nachweisbar, weder in der einen noch in der anderen Ausprägung." In the male germ cells of *Bufo bufo japonicus*, so far as our examination has gone, no trace of heteropycnosis of the sex-chromosomes is recognized, though several nucleoli are present which disappear toward the end of the prophase. In the metaphase of the first division there is a large V-chromosome lying always in the periphery of the equatorial plate and taking a form of vertical V as reported by IRIKI (1930) in *Hyla*, while the other large chromosomes show always a form of vertical or tangential ring. This vertical V-chromosome is composed of

two atelomitic, probably centromitic, homologous univalents as in the case of *Hyla*. It can therefore be concluded that this chromosome which shows a peculiar behavior is probably the sex-chromosome of a XX-type, corresponding to that of *Hyla*.

MOHR (1915) maintains that the heteropycnosis of a monosome is due to the lack of the homologous partner. But many examples which contradict this assumption can be given from various forms of plants and animals. For example, NAKAMURA (1928) has reported that the heteropycnosis occurs in the sex-chromosome of male germ cells of *Natrix* and other snakes and lizards* where the male is homogametic (XX). DE WINIWARTER and SAIMONT (1908) have observed the same phenomenon in the oocyte of the cat, where the female is homogametic (XX). Generally speaking, in Amniota the heteropycnosis seems to occur always in the growth period of the first maturation division of the germ cell where the sex-chromosomes are homogametic or not.

STOHLER has carried on cytological studies on the spermatogenesis and ovogenesis in the functional ovary of the female and in the potential ovaries of the male and female. From the results thus obtained and also from other experimental evidences obtained by other works as PONSE and WELTI, he has concluded that " auch das Weibchen wie das Männchen der Kröten genetisch bisexell ist." It can, therefore, be safely assumed that the phenomenon of sex-reversal may occur not only in *Bufo* but also in various forms of amphibians, and that the sex-chromosomes remain in undifferentiated state showing no trace of heteropycnosis.

It is therefore probably safe in vertebrates to conclude that the heteropycnosis of sex-chromosomes is due to the high sex-differentiation of organisms, but not due to the lack of a homologous partner of the chromosomes.

SUMMARY

1. The spermatogonia can be classified into the primary and secondary, although no marked morphological difference except the size is found between the nuclei of both the spermatogonia.

2. In the equatorial plate of both primary and secondary spermatogonia, there are always 22 chromosomes, 12 large and 10 small. They are all essentially V-shaped. The long ones are situated

* The papers will be published shortly.

at the periphery of the equatorial plate and the short ones in the central space.

3. The equatorial plate of the first division is composed of 6 large and 5 small tetrads. Generally the large ones lie at the periphery of the plate and the small ones in the central space. One of the large tetrads is vertical-V-shaped, which lies always at the periphery of the plate.

4. The tetrads divide each into two equal parts in the first division, so that each of the daughter sets of chromosomes receives identical halves.

5. In the metaphase of the second division the chromosomes are always 11 in number, of which the larger 9 lie at the periphery of the equatorial plate and the rest in the central space. All of the chromosomes appear as horizontal double V's.

6. In the anaphase of the second division each horizontal double V-chromosome divides into two single V's, so that each daughter set consists of 6 large and 5 small V-shaped chromosomes.

7. The Japanese toad is amphisexual like the European toad as reported by STOHLER.

8. No trace of hetoropycnosis of the sex chromosomes is recognized in the growth period of the first spermatocyte, though several nucleoli are present which disappear at the end of prophase.

9. The vertical V-shaped tetrad which is present in the metaphase of the first division is composed of two homologous univalents. This is probably the sex chromosome, as in the case of *Hyla* reported by IRIKI.

10. In vertebrates the phenomenon of heteropycnosis is a sign of high sex-differentiation and not due to the lack of homologous partner of the chromosomes.

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EXPLANATION OF PLATES VI—VII

All the figures except Fig. 1 have been drawn with the acid of Abbé's Zeichenapparat, Zeiss Obj. Ap. 1.5 mm.; Oc. K 18; tube length 160 mm.; paper about 34 mm. below the stage, except Fig. 1 which has been drawn Oc. K 12 instead of Oc. K 18; others likewise.

Fig. 1. Primary spermatogonium surrounded by follicle cells, containing a polymorphic nucleus.

Fig. 2. A primary spermatogonium in the metaphase, showing 22 chromosomes, 12 long and 10 short. Black spots at the apices of the chromosomes (V's) are polar granules.

Fig. 3. Prophase of the secondary spermatogonium.

Fig. 4. Secondary spermatogonium in the metaphase, showing the longitudinal split through which each chromosome divides into two daughter halves.

Fig. 5 and 6. Secondary spermatogonia of different sizes in the metaphase, each containing 22 chromosomes, 12 long and 10 short.

Fig. 7. The primary spermatocyte nucleus in the leptotene stage.

Fig. 8. The same in the zygotene stage.

Fig. 9. The same in the pachytene stage.

Fig. 10. The same in the strepsitene stage.

Fig. 11. The same immediately before the diakinesis.

Figs. 12—17. Equatorial plates of the primary spermatocyte; 11 tetrads, 6 larg and 5 small. Vertical V-chromosomes are shown in Figs. 14—17.

Fig. 18 and 19. Side views of the primary spermatocyte; vertical V-chromosomes are blackened. Black spot on each arm of vertical V's is the polar granule (Fig. 19).

Fig. 20. A side view of the primary spermatocyte in the anaphase.

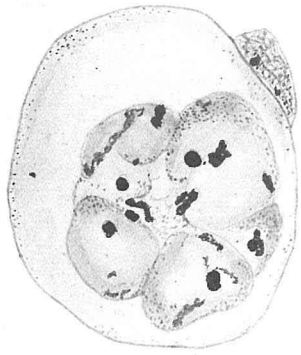
Fig. 21 and 22. A polar view of the daughter sets of the primary spermatocyte in the anaphase.

Figs. 23 and 24. The interkinesis.

Figs. 25—28. Polar views of the metaphase plate of the secondary spermatocyte; 11 diads, 6 large and 5 small, are seen in each plate. The black spots at the apices of V's are polar granules (Figs. 27 and 28).

Fig. 29. A side view of the secondary spermatocyte in the anaphase.

Figs. 30 and 31. A polar view of the daughter sets of chromosomes in the secondary spermatocyte anaphase; 11 monads are seen in each figure.



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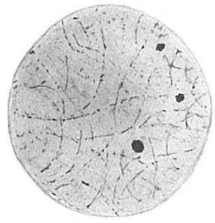

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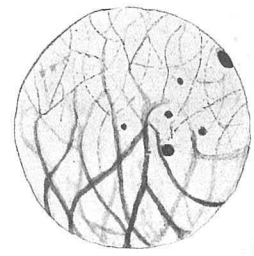

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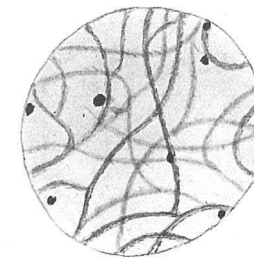

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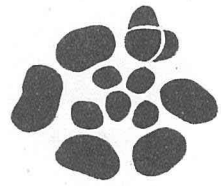

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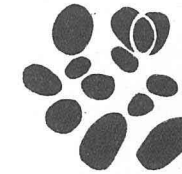

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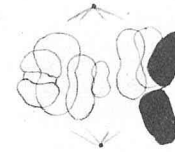

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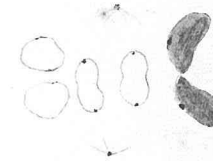

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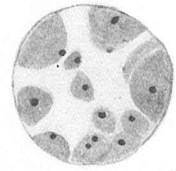

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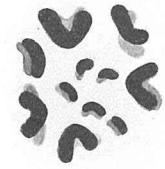

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