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# Studies on Amphibian Chromosomes

## I. On the Chromosomes of *Hyla arborea japonica* GUENTHER

By

SHIGEMORI IRIKI

Zoological Institute, College of Science, Kyoto Imp. Univ.

*With Plates I and II*

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## Contents

## INTRODUCTION

Since the early work of BORN (1881) and YUNG (1881), many observations have been made to elucidate the question of sex determination in amphibians. The most remarkable of these is probably the recent work by WITSCHI (1923). The results obtained from these studies seem to show that in amphibians sex is not definitely determined at the time of fertilization, but changeable during subsequent development.

A great deal of work has also been done on the cytology of this group. But neither the question of sex chromosomes nor the conditions underlying the problem of sex determination has been elucidated by these works. It is believed that sex chromosomes are not the sole sex determiners, although they are important agencies in sex determination. In any case, it is desirable to study the sex chromosomes of amphibans crucially and see whether or to what extent the findings are in harmony with the experimental results. In this series of reports I intend to describe the results of my study of the chromosomes of amphibians, especially those of anurans, found in Japan. I express my hearty thanks to Professor TAKU KOMAI to whom I am indebted for valuable criticism and for his great kindness in looking through the manuscript. To Mr. OSAMU MINOUCHI I am also under deep obligation for his kind assistance especially on the side of technique.

## MATERIAL AND METHODS

The frog which I used in the present study is *Hyla arborea japonica* GUENTHER, one of the commonest frogs in Japan. The material was gathered in June and July from the fields in the suburbs of Kyoto. They were killed by striking on the head. The testes were taken out from them as quickly as possible and cut into small pieces, not exceeding three millimeters in diameter, and dropped in the fixatives. For fixing the chromosomes, the following fixatives were used.

1) FLEMMING's strong solution without acetic acid.

2) The same fluid diluted to 1/2 or 2/3 strength.

3) CHAMPY's solution.

Of these, CHAMPY's solution proved to be the best. After 24 hours of fixation in this solution, the material was washed in running water for 24 hours. Then a series of alcohols (70, 90 and 100 percent) was used for dehydration. The absolute alcohol was replaced by creosote-toluol, this by toluol, this in turn by toluol-paraffin and then the material was imbedded in paraffin. The sections were cut into thicknesses of 8, 10 or 15 micra; and after bleaching in $H_2O_2$, they were put into the following mixture for 24 hours or more.

Glacial acetic acid.....50 parts.

Saturated picric acid.....50 parts.

This bath is a modified form of CHURA's bath, more strongly concentrated than in the original formula and employed by MINOUCHI (1927) in his study of mammalian chromosomes. The sections taken out from this bath were thoroughly washed in running water, and put in 10% solution of iron alum for 5–24 hours. After washing in running water, they were stained in HEIDENHAIN's iron-haematoxylin for 3–24 hours. For differentiation, 0.5%–1.0% solution of iron alum was used. After differentiation the sections were washed in running water for 2–12 hours and dehydrated with alcohol, cleared in creosote-xylol and mounted in canada-balsam.

By employing the above-mentioned method, the metaphasic chromosomes were well preserved and stained clearly. The only deficiency of this method lay in the difficulty of staining the chromosomes in all stages, especially those in the nucleus of the growth period. Therefore, FLEMMING's strong solution was substituted for CHAMPY's mixture for the observation of the chromosomes in the growth period, with good results.

## OBSERVATIONS

### 1. *Chromosomes of the Spermatogonium*

Figs. 1–5 are polar views of the equatorial plates of the spermatogonial divisions. The figures are arranged according to their sizes, Fig. 1 being the largest and Fig. 5 the smallest. As is clear from the figures, there is no morphological difference among them. I shall call the larger ones the earlier spermatogonia and the smaller the later spermatogonia. The earliest spermatogonia (=primary spermatogonia) are solitary and surrounded by a few follicle cells. They multiply by divisions of the ordinary equational type, until a spermatocyst containing a certain number of spermatogonial cells(=secondary spermatogonia) is produced. The cells become smaller with the division. Thus the equatorial plate of the earlier spermatogonia is invariably larger than that of the later, each chromosome of the earlier spermatogonial metaphase being longer than the corresponding chromosome of the later spermatogonial metaphase.

Fig. 1 shows the earliest (=primary) spermatogonium, which is solitary, largest in size, and surrounded by a few follicle cells, while Fig. 5 shows the smallest one I have observed, which is probably the ultimate spermatogonium.

As shown in those figures, the equatorial plates of the spermatogonia contain 24 chromosomes without exception. Many of these, especially the larger chromosomes, are atelomitic and V-shaped. In the equatorial plate the larger V-shaped chromosomes are arranged generally at the periphery, and the others are scattered about in the central space. Since the larger chromosomes lie with their points of flexure towards the spindle axis, a rosette is formed. Sometimes, however, the angle between the two arms of the V becomes very large so that the V is often converted into a long rod (Fig. 1).

Of all the chromosomes, the largest pair apparently represent the sex chromosomes. But it is often hard to sort out the largest pair, because they are situated frequently obliquely to the equatorial plate,

and the degree of inclination in relation to the plate is not always the same. All of the metaphase chromosomes are well separated and there is no danger of miscalculating the number.

## 2. *Chromosomes of the First Spermatocyte*

### *a. Growth Period*

In the resting stage the nuclear substance is resolved and the chromatin is in a diffuse condition. There are a few nucleoli which are stained deeply by haematoxylin (Fig. 6). In the next stage the chromatin appears in the form of a granular, much convoluted spireme (leptonema) and the nucleoli as deeply staining bodies. The threads are arranged in no definite way (Fig. 7). But sooner or later there occurs a slight convergence of the threads towards one pole of the nucleus (Fig. 8). This is the zygotene stage. The nucleoli can still be distinguished. As to the synizesis of the leptotene threads, I will not touch upon that subject for the present. Next comes a pachytene stage which can be distinguished clearly, as this stage is characterized by thicker threads (pachynema) and by a smaller number of the threads as compared with the preceding stages. The pachytene threads show no clear sign of duality and there is no more p oarization (Fig. 9). The nucleoli are hardly visible in this stage in material preserved in FLEMMING's strong solution (Fig. 9). The pachytene threads continue to increase in thickness (Fig. 10), and probably after the diplotene stage become the strepsitene threads, each of which is composed of a pair of threads spirally twisted about each other (Fig. 11). In the diakinesis the rings are clearly observed, which condense gradually, the threads increasing their thickness, until the characteristic feature of the tetrads becomes apparent. Besides the rings, there exists a V-shaped chromosome, which is the largest of all the chromosomes. Its later history shows that it is the sex chromosome without doubt. In the diakinesis all the chromosomes are scattered about in the peripheral parts of the nuclear space. I could make out

neither karyosome nor any phenomenon of heteropycnosis in the growth period. The nucleoli, which appear in the earlier stage of the growth period, disappear gradually in the later stage, and have no relation with the sex chromosomes. The detailed observations on synapsis and tetrad formation are left for a later study.

### *b. Metaphase and Anaphase*

In the equatorial plates of the first division there are always 12 tetrads (Figs. 14-16). The tetrads can not be sorted out into classes according to their sizes, because there is no sharp differentiation in size among them. Of the 12 tetrads eight or nine are arranged in a circle around a central space in which three or four tetrads are present. Generally, the larger tetrads lie at the periphery and the smaller ones in the central space; ocassionally, however, some larger tetrads are present in the central space and some smaller ones at the periphery. But the largest V-shaped tetrad, which represents probably the sex chromosomes, remains always at the periphery and stands vertical to the plate. This arrangement is, in principle, identical with that of the spermatogonial chromosomes, each tetrad representing a pair of homologous chromosomes.

In insects (Orthoptera, Hemiptera), annelids (*Tomopteris, Allolobophora*) and in lower vertebrates (Amphibia), the tetrads are vertical rings longitudinally cleft in the plane of the ring and showing cross-sutures at both ends. In the simpler types of the rings the longitudinal split may be obscure. According to WILSON (1925), the ring is composed of two synaptic mates widely separated in the middle, but united at both ends.

I did not observe the tetrad formation carefully, and I can not say whether this interpretation applies to the present study. It is very likely, however, that the tetrads in this frog are produced in the same way, but are condensed to such an extent that the ring shape is obscured or totally lost to view. In the side view the tetrads are

dumbbell-shaped and lie vertical to the equatorial plane with the spindle fibres attached to each half. The largest chromosome, however, stands on the plane with both arms oblique to the plane and directed outwards, so that it has the form of an open V in the side view (Figs. 17–18). Each of the arms looks apparently rod-shaped, but bends a little at the point where the spindle fibre attaches (Fig. 18). In the polar view this chromosome appears as a double structure, the arms partly overlying each other.

In the anaphase the tetrads divide each into two equal halves. The larger tetrads assume the form of a double V, but the precise form of the smaller ones can not be distinguished clearly. In this division the largest tetrad shows the characteristic behavior, namely, it leads the way in the march of the chromosomes to the poles. This behavior, known as "precession", is one of the special peculiarities of the sex chromosomes (Figs. 19–20). Fig. 21 shows the side view of the two daughter complexes in the anaphase of a spermatocyte, Fig. 22 *a* and *b* the polar views of the same, while Fig. 23 is one of the two daughter complexes in the polar view.

Anaphase features of all these tetrads show that the structure is identical, in principle, with the ring tetrad found in urodeles, in which the rings are divided into two half-rings, which in the anaphase assume the form of split V's.

### 3. *Chromosomes of the Second Spermatocyte*

In the anaphase of the second division, as noted already, most of the daughter chromosomes show duality (Figs. 21–23). With the interkinesis the chromosomes undergo further changes, during which the anaphasic duality is lost to view (Fig. 24). But the outline of each chromosome is obvious so that one can easily recognize 12 chromosomes.

A good many equatorial plates of the second division were examined, which all showed 12 chromosomes (Figs. 25–27). Of these, eight or nine chromosomes are in most cases arranged in a circle

around a central space in which three or four chromosomes are present. Generally the larger chromosomes lie at the periphery of the equatorial plate, and the smaller ones in the central space. This arrangement is not constant, but the largest chromosome, which is derived from the vertical V of the first spermatocyte, lies always at the periphery of the plate. This arrangement is, in principle, similar to that in the first spermatocyte and spermatogonium.

As noted already, the larger chromosomes of the spermatogonium are atelomitic and V-shaped; but it is not clear whether the smaller ones are telomitic or atelomitic. In the metaphase of the second division the larger chromosomes are double V's which are situated horizontally on the equatorial plane. It was impossible to decide whether the smaller ones are rod- or V-shaped, though there is no doubt that each consists of two superimposed monads. In every respect these metaphasic chromosomes are of the same shape as the anaphasic chromosomes of the first division. This fact makes it certain that the anaphasic duality of the first division foreshadows the duality which appears in the metaphase chromosomes of the second division. In the anaphase the dyads divide each into two monads (Fig. 28). Thus each spermatid contains the same set of 12 monads, some large and some small; the larger monads are V-shaped but the shape of the smaller ones can hardly be made out (Figs. 29 *a–b*).

## REMARKS

### 1. *Chromosomes of the Spermatogonium*

In the metaphase of the spermatogonium there are certain large V-shaped chromosomes besides smaller ones. The number of the chromosomes in anurans is not very large; but since many of them are large in size and naturally tend to overlie one another, it is often difficult to ascertain the diploid number with accuracy, especially when the chromosomes are not well preserved. This had led some previous authors to miscalculate the number of the spermatogonial chromosomes.

In the present material, however, the chromosomes on the equatorial plate are separated so sharply from one another, that there is no such danger, and in all the spermatogonial metaphase figures I could count 24 chromosomes clearly. There is no doubt then that the fundamental diploid number of chromosomes of this frog is 24.

### 2. *Chromosomes of the First Spermatocyte*

In the present study no special attention was paid to the behavior of the chromosomes during the growth period, and the problems of synapsis and tetrad formation are left for a later study. In the equatorial plate of the first spermatocyte there are always 12 tetrads. They are all well separated and not connected one with another by a strand-like material. In well-preserved material, the anaphasic chromosomes of the first division appear in identically the same shape as that of the metaphasic chromosomes of the second spermatocyte.

### 3. *Chromosomes of the Second Spermatocyte*

Chromosome counting in the second spermatocyte metaphase is not very difficult in anurans, as the number is always rather small. But no worker has succeeded in obtaining a beautiful metaphase figure of the second spermatocyte. This is evidently due to failure in fixation, because, though it is not very hard to fix the spermatogonial and the first spermatocyte chromosomes, the case is different with the second spermatocyte chromosomes. Most of the authors give the figures of the second spermatocyte chromosomes as dot-like or dumb-bell-shaped, and many of the spermatogonial chromosomes as atelomitic and V-shaped. In comparing these chromosomes with those of insects, one is ready to be sceptical about the validity of the observations of those authors. In the equatorial plate of the second spermatocyte of this frog there are 12 dyads, each composed of two monads. The larger dyads are V-shaped, corresponding with the long V-shaped spermatogonial chromosomes, though the former look firmer than the latter, but the exact shape of the smaller chromosomes could not be

determined. In any case it is certain that the division phases of the spermatogenesis of the frog are identical, in principle, with those in insects.

### *4. Sex Chromosomes*

The central interest of the present work lies in the question as to how the phenomenon of sex determination known in amphibians can be explained from the standpoint of chromosome theory. MINOUCHI (1927) has mentioned, in his study of mammalian chromosomes, that, in order to identify the sex chromosome, the following points must be made clear: (1) the behavior and structure of the chromosomes during the growth period, (2) the transformation of the chromosomes during the period from the diakinesis to the metaphase, (3) the structure, size and form of the chromosomes in the metaphase of the first spermatocytic division, (4) the behavior, size and form of the chromosomes during interkinesis, (5) the diploid number of chromosomes, (6) the haploid number of chromosomes in various stages during meiosis.

PAINTER (1921) has mentioned, in his study of the chromosomes of some lizards, the following five points as necessary to be determined for the sex chromosomes: (1) the number of the spermatogonial chromosomes for the male, (2) the number of tetrads in the first spermatocyte and their behavior, (3) the number of dyads in the second spermatocyte, (4) the number of chromosomes passing to the spermatid, (5) the diploid number of chromosomes for the female.

In addition to these five points, NAKAMURA (1928), in his study of the chromosomes of a snake, emphasizes a sixth point, namely, the behavior of the karyosome during the growth period.

Perhaps the most striking of all the characteristic behaviors of the sex chromosome is that which appears in the growth period of the spermatocyte. During this period, the sex chromosome usually becomes greatly condensed and stained intensely with basic dyes so as to appear as a karyosome or chromatin-nucleus, contrasting very sharply with the thread-like or difuse and lightly staining autosomes. This change

which is designated "heteropycnosis", is known not only in the above-mentioned vertebrates, but is widely spread in the animal-kingdom. In the early prophase of this frog no nucleolus which could be identified as a karyosome or chromatin-nucleolus was observed during the growth period, consequently no phenomenon of heteropycnosis came into view. It is true that there are observable a few nucleoli. However, these all disappear in the later stage, so that they are plasmosomes without doubt.

But, in the metaphase of the first division, there is a tetrad which is different in structure and behavior from the other tetrads in the features that (1) it remains always at the periphery of the equatorial plate, (2) it is of the shape of an open V lying on the equatorial plate with both arms pointing obliquely outwards, (3) in the anaphase it leads the way in the march of the chromosomes to the pole (precession) (Figs. 14–20), and (4) it is the largest of all the tetrads.

Minute study of the shape and size of this tetrad leaves no doubt that this has developed from the V-shaped chromosome in the diakinesis stage. In the metaphase of the first division, each arm of this tetrad looks apparently rod-shaped, but bends a little at the point where the spindle fibre attaches (Fig. 18). Apparently the arm represents a half-ring of a ring tetrad united at one end, but separated at the other.

All the features just mentioned apparently show that the tetrad in question represents the sex chromosome of this frog. There is no question that this corresponds with the sex chromosome found in the metaphase of the first spermatocyte reported by WITSCHI (1924) in *Rana temporaria* L. It lacks, however, the most striking characteristic of sex chromosomes, namely the heteropycnosis in the growth period. But this process is known to show a great deal of variation in respect to the period at which it occurs, and the extent to which it is carried out, and even a few cases are known where it does not occur at all. So that the absence of heteropycnosis in this case does not necessarily disprove the fact that these chromosomes are sex chromosomes.

In the anaphase stage the arms of this tetrad separate along the plane of the equatorial plate, and go to the opposite poles. Meanwhile each arm becomes a split V, and in the interkinesis it becomes diffuse like the autosomes and shows no special feature to distinguish it from the autosomes. In the equatorial plate of the second spermatocyte the chromosomes appear as V-shaped dyads consisting of two clearly superimposed monads lying horizontally and always at the periphery of the plate. Each of the these dyads divides in the anaphase of the second division into two V-shaped monads.

Since the spermatogonial chromosomes are exactly twice as many in number as the tetrads, each tetrad must be composed of two spermatogonial chromosomes. Further, it is clear that the pair of the largest chromosomes correspond with the largest tetrad.

The facts mentioned above make it evident that the sex chromosomes in the diploid complex are two, and after two maturation divisions, the resultant four elements are distributed among the spermatids. When two sex chromosomes exist in the diploid complex, the formula of the sex chromosome must be XY or XX. The Y chromosome, if present, is usually distinguished without difficulty from X by its smaller size, and often also by a difference in behavior during the growth period, such as, the rate and degree of condensation. The two sex chromosomes of this frog, however, are identical in size and shape, and there is no difference in behavior by which one can distinguish one from the other. Thus, so far as can be judged from the morphological evidence, the formula of sex chromosome in this frog is probably XX, and not XY. Consequently, the chromosome complex of this frog may be formulated as follows :—

Spermatogonium .....22+X X=24
First spermatocyte .....11+X =12
Second spermatocyte .....11+X =12
Spermatid ..... .....11+X =12

### 5. *Sex Chromosomes of other Amphibians*

KING (1912) has published apparently the only paper on the sex chromosomes of urodeles. She observed in the male of *Necturus maculosus* an X chromosome which is attached to one of the autosomes. She states that, since this X chromosome passes to one pole of the first maturation spindle, and slits in the second maturation mitosis, two classes of spermatozoa are produced. In the higher amphibians, according to her, the X chromosome has apparently formed a permanent union with one of the large chromosomes and can not be distinguished from the latter. PARMENTER (1919), in his study on *Ambystoma*, also mentions that there is a possibility that there exists a sex chromosome attached to a euchromosome.

In the anurans it has been a question for a long time whether the male or the female is heterozygous for the sex element. The fact that the frogs produced by artificial parthenogenesis can be of either sex gives support to the hypothesis that the female is heterozygous. On the other hand, WITSCHI's results (1923) obtained from his breeding experiments suggest that the male is heterozygous. Besides these, there are many evidences showing that the sex ratio in anurans is changeable according to the internal and external conditions, such as the age of the egg, food, temperature, etc., which all appear to show that the sex is determined by an agency other than the chromosome complex.

Much work has been done on the chromosomes of anurans, but few of the investigations have any direct bearing on the sex chromosomes.

LEVY (1915), in a study on *Rana esculenta*, observed an unpaired X chromosome which passes undivided to either pole in the first spermatocyte division. The spermatogonial chromosomes are approximately 25 in number, the chromosomes of the first spermatocyte are 13 and those of the second spermatocyte are 12 or 13.

SWINGLE (1917), while engaged in a study on the development of

*Rana pipiens,* found a frog possessing a marked hermaphroditic tendency. The gonads were true testes, though they contained many oöcytes, so that the animal was a modified male. Ten or twelve spermatogonial counts gave 25 chromosomes as the diploid number. In the first spesmatocyte division there appear 13 ring and dumbbell-shaped bodies of varying size. In the normal cases of the first division the dumbbell-shaped body migrates to one or the other pole of the cell, much in advance of the other chromosomes, and as a result an unequal distribution of chromatin to the secondary spermatocytes occurs, one cell receiving 12 chromosomes plus the dumbbell-shaped body, the other cell receiving 12 chromosomes only. At first he believed that this dumbbell-shaped chromosome is the sex chromosome; later he discarded this opinion (1921), and mentioned that the X of his earlier paper had been an ordinary chromosome.

WITSCHI (1924) worked on the sex chromosomes of *Rana temporaria.* According to him, the spermatogonial chromosomes in this species are 26 in number. In the metaphase of the first spermatocyte there are 13 tetrads, of which the largest one divides before the others do. Each tetrad divides into two equal parts and 13 chromosomes go to each pole. At this time the largest chromosome exhibits a compound constitution by the presence of a marked constriction in the middle which gives the chromosome a bilobed appearance. This chromosome is divided in the second division, the larger half going to one pole and the smaller to the other. Very often the chromosome shows a distinct tendency to lag on the spindle. WITSCHI concluded that we have here an unequal XY pair, of which the larger component is X and the smaller Y. Recently STOHLER (1926–1928) studied three species of *Bufo,* and reached the conclusion that there is no chromosome that can be designated the sex chromosome.

Thus there is much difference of opinion among the previous workers as to the conditions of the sex chromosomes in amphibians. And the result on *Hyla* described above supports none of the results of these workers.

## SUMMARY

1. In the equatorial plate of the spermatogonial division, there are always 24 chromosomes. Many of these, especially the larger chromosomes, are atelomitic and V-shaped, while it could not be determined whether the rest are rod- or V-shaped.

2. The number of tetrads present in the metaphase plate of the heterotypic division is 12. The tetrads are condensed to such an extent that the ring-shape, which is common in Urodela, is obscured or lost. But the largest tetrad is V-shaped and always lies at the periphery of the plate.

3. The tetrads divide each into two equal parts in the first division, so that each of the daughter complexes receives an identical set of chromosomes.

4. The number of dyads in the metaphase plate of the second division is 12. Each dyad consists of two superimposed monads. The larger dyads are V-shaped, but the shape of the smaller ones could not be made out.

5. The dyads divide each into two equal monads, so that each spermatid receives an identical set of 12 chromosomes.

6. In the metaphase plate of the first division there is a large V-shaped tetrad standing vertical to the plate. It divides in the first division into two equal parts (dyads) each of which divides again into two equal parts (monads) in the second division.

7. This pair of chromosomes are apparently the sex chromosomes. As there is no difference between the two members either in size or in behavior, the sex chromosomes in the male seem to be of the XX-type, and the male is homozygous in respect to sex.

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## EXPLANATION OF PLATES

### Plate I and II

All the Figs., ZEISS Obj. Apo. 1.5 mm.; Ok. K. 18; Tub. 160 mm.; ABBE's Zeichenapparat; paper 34 mm. lower than the stage.

Figs. 1–5. Polar views of the metaphase of spermatogonia. 24 chromosomes are seen in each plate.

Fig. 6. The first spematocyte nucleus in the resting stage.

Fig. 7. The same 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 later pachytene stage.

Fig. 11. The same in the strepsitene stage.

Figs. 12–13. The same in the diakinesis.

Figs. 13–16. Polar views of the first spermatocytes. 12 tetrads are seen in each plate.

Figs. 17–20. Side views of the first spermatocytes.

Figs. 21. Side view of the first spermatocyte in the anaphase.

Figs. 22 *a* and *b*. Polar views of the daughter complexes of the first spermatocyte in the anaphase. *a* represents lower and *b* represents upper sets, in one section.

Fig. 23. Polar view of one of the daughter complexes of the first spermatocyte in the anaphase. Better preserved than Fig. 22.

Fig. 24. The interkinesis.

Figs. 25–27. Polar views of the metaphase plate of the second spermatocytes. 12 dyads are seen in each plate.

Fig. 28. Side view of the second spermatocyte in the anaphase.

Figs. 29 *a* and *b*. Polar views of the daughter complexes of the second spermatocyte in the anaphase. 12 monads are seen in each figure.

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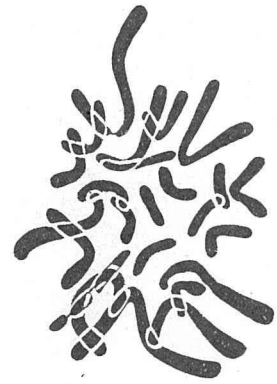

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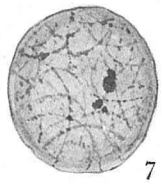

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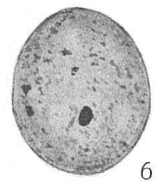

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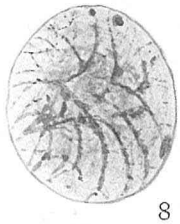

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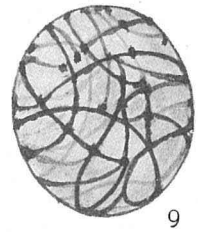

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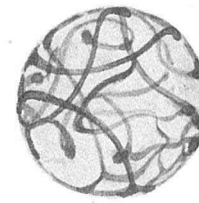

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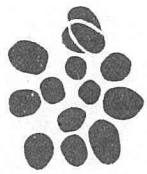

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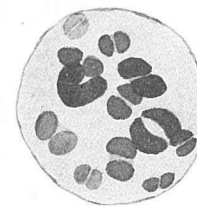

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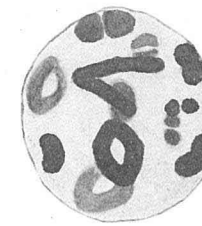

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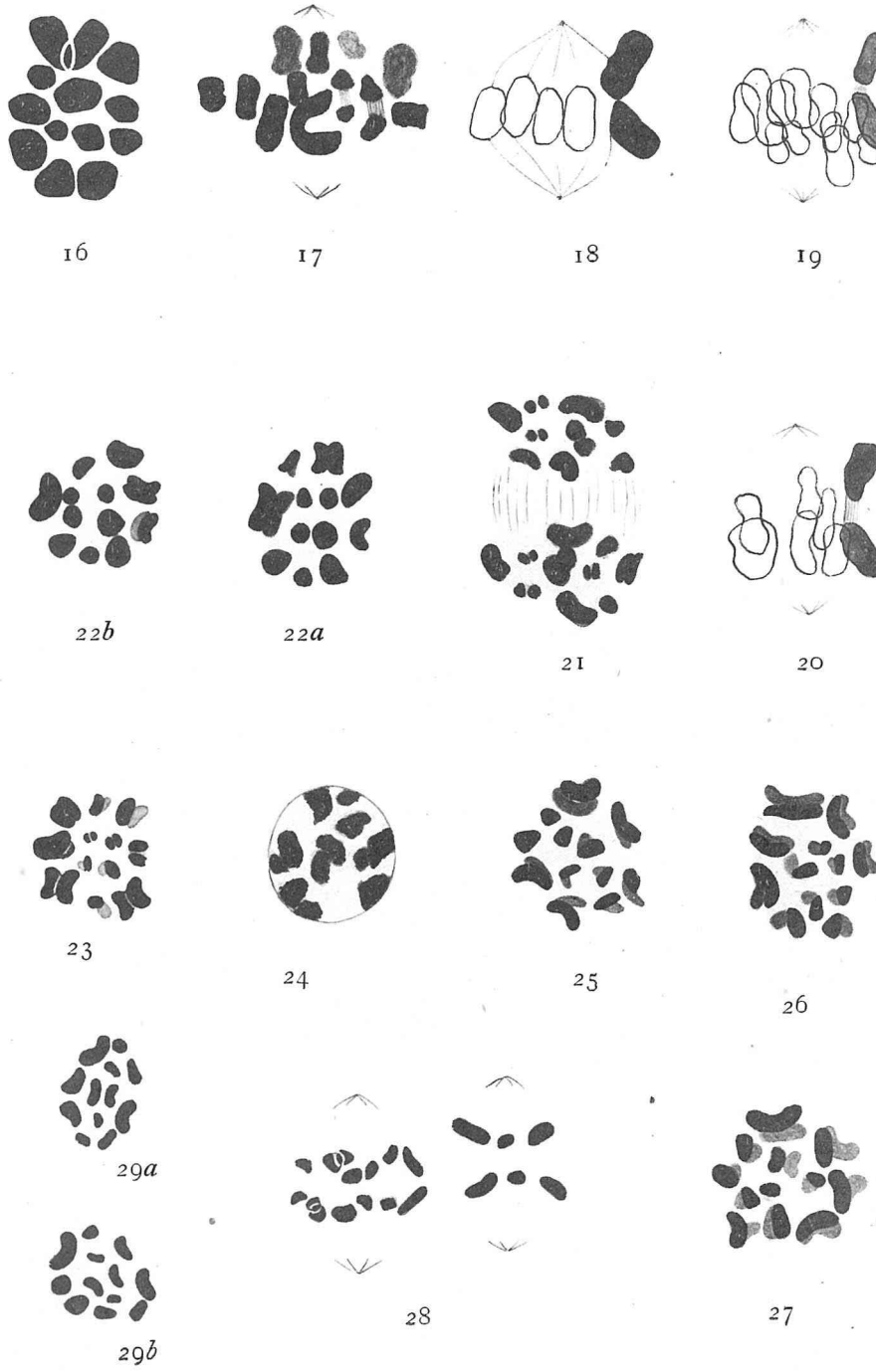


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