A Study of Chromosomes in the Two Sexes of *Hynobius retardatus* (an Urodelan), with a Consideration on the Chromosomes and Sex[1]

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(With two plates and one textfigures)

During these years the sexual differences of chromosomes in higher vertebrates have progressively been established on a good basis by a number of recent investigations. In mammals it is beyond any discussion that the male is heterogametic. Thanks to the extensive researches of Oguma ('34, '37, '38), Sokolow, Tiniakow & Trofimow ('36), and Yamashina ('41, '43, '44, '46), it became conclusive that in birds and reptiles, heterogamety occurs in the female, contrary to mammals, since a sex-determining chromosome was discovered in the female cell. Though a large amount of work has been cytologically done in amphibian, however, the question of sex determination and also of the sex chromosomes has not yet been decidedly cleared up in any species so far, the available data being very meager to make any conclusion. An item of importance to this subject is to research the chromosomes of both male and female sexes by way of comparison. The present paper which contains the results of a comparative study of chromosomes in the two sexes of *Hynobius retardatus* (an urodelan), with special regard to the question as to whether the sexual difference of chromosomes morphologically occurs or not, will contribute something to the field of the research concerning sex problems.

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Material and Methods

For the male chromosomes: The testes of different seasons were used for the material with favourable results. The observations of the spermatogonial chromosomes were based on the material obtained in April, just after breeding season, while the meiotic divisions were capable of observation in the testes of the animals secured from late Summer to early Autumn. For fixation of the testes, Flemming's strong solution without glacial acetic acid were exclusively employed. The technique in detail may be referable to the author's previous paper (Makino '32 c).

For female chromosomes: It is known that in higher vertebrates the multiplication of oogonial cells occurs actively in the gonads of embryos or of the young just after birth. According to Oguma ('34, '37), in the lizard and tortoise the oogonia are found actively divided in the ovaries of the young at birth or of some days before birth. In amphibians, at least in the case of the present species, however, the circumstance appears to be a little different. It was found by Hanaoka ('34) and Uchida ('35) that *Hynobius retardatus* belongs to the semidifferentiated race in regard to its gonadic development; the gonads maintain an undifferentiated condition during the larval stages before metamorphosis, when the most active multiplication of germ cells occurs. By this fact it is impossible, therefore, to determine which are the oogonia or the spermatogonia in the germ cells from such material. For this reason, the chromosomes of the oogonial cell were missed to observe in this study, and the present observations were exclusively concerned with the chromosomes of the first and second polar spindles in the maturating ova. For the technical procedure to prepare the maturating ova the reader can be referable to the author's previous paper (Makino '34 b).

Observations

I. *The chromosomes of the male*

The spermatogonial division: Concerning the chromosomes of the spermatogonial division a detailed account has been given in the previous paper (Makino '32 c). The definitive number of the chromosomes is determined to be forty as the male diploid complex. The chromosome complement, as shown in Fig. 1, is composed of twenty-two V-shaped chromosomes with median or submedian attachment, arranging at a peripheral position of the equatorial plate, and eighteen rod-shaped ones with varying lengths which are found occupying the central space of the equatorial plate. When these chromosomes are aligned in pairs according to the approximate order of their size and shape (refer to Makino '32 c, Pl. V. I), they can be arranged into twenty pairs made up of homologous mates, without there being either a solitary unpaired element or a pair of unequal size. In view of this finding it can be said that there is no indication for the existence of any particular element in the male diploid complex.

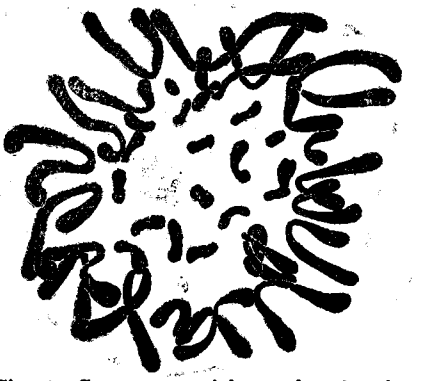

Fig. 1. Spermatogonial complex showing 40 chromosomes. ×3000.

The meiotic divisions: Through the growing stages the occurrence of the particular chromosome, different in nature from others, could not be proved in the meiotic nuclei as shown in Figs. 2 and 3.

As expected from the diploid complement, there were found twenty bivalents in the metaphase plate of the primary spermatocyte (Figs. 4–7). The larger bivalents were found occupying the outer circle of the plate surrounding those of smaller size in the central area. The larger ones being eleven in number, are of an atelomitic nature with a compound-ring structure, consisting of two or more chiasmata. In the later stage of metaphase, however, the structure of bivalents becomes obscure by and by, staining into massive bodies, owing to thickening of the composing chromatids, as seen in Fig. 7.

In the ensuing division every one of the bivalents separates

into two identical halves. The process of separation proceeds in a similar way as that described in the case of *Cryptobranchus allegheniensis* (Makino '35 b). At the end of separation there emerge two identical double V-shaped dyads corresponding with each other in the case of the atelomitic bivalent. And in the case of the telomitic bivalent, each daughter halves after segregation appear as double rod-shaped ones. In this way there are produced two corresponding sets of chromosomes, with an identical complex at anaphase of this division. Fig. 8 indicates the lateral view of the separated groups of chromosomes in eayly anaphase. Fig. 9 a and b are drawings of the sister phromosome complexes at late anaphase, observed from a pole by adjusting the focus in a single section. It is evident from these figures that every chromosome set resulted by division contains twenty elements including eleven atelomitic and nine telomitic ones, all of them corresponding exactly to each other pairwise. Thus is proved the non-existence of the heterochromosomes like the X or XY chromosomes.

The metaphase plate of the secondary spermatocyte assumes a quite similar configuration of chromosomes with that met with at anaphase of the first division. There are counted twenty chromosomes of dyad nature, eleven being atelomitic and nine telomitic (Figs. 10–11). In division every dyad separates into identical daughter halves (Fig. 12). Fig. 13 a and b show the anaphase complex of the second division, either of which contains twenty chromosomes, composed of eleven V-shaped and nine rod-shaped ones, corresponding pairwise.

II. The chromosomes of the female

The following description was based on observations upon the oocyte chromosomes in the maturation divisions of ova.

Sections of the ovum obtained from the upper portion of the oviduct in the female of the breeding season invariably show metaphase of the primary oocyte, the stage just before the formation of the first polar body (refer to Makino '34 b). The equatorial plate shows without exception twenty bivalents, as is the case with that of the primary spermatocyte division. Since the dimensions of the first polar spindle are much larger than those of the primary spermatocyte division, the individual chromosomes are seemingly

voluminous as compared with those of the male germ-cell. But the general feature of the chromosomes is not different by the two sexes. Eleven larger and nine smaller bivalents are distinguishable (fig. 14). An unfavourable swelled appearance of chromosomes, due probably to the effect of the fixing reagent adopted,[1] makes the structure of chromosomes obscure and does not permit a precise study of the morphology of bivalents as done in the spermatocytes. Even under these circumstances, however, the following observations are possible that the chromosomes of larger size are atelomitic V-shaped and the smaller ones are of telomitic nature. This evidence can be ascertained further by examining the anaphase configuration of the primary oocyte as given in Figs. 15–16, showing that there are discernible eleven large V-shaped elements and nine small rod-shaped ones. The presence of the chromosomes of a heteromorphic nature could not be proved here at all.

At anaphase of the first division, all of the bivalents separate into equal halves, and there is no element showing asymmetrical separation or extraordinary behaviour. As the result, there are produced two identical sets of chromosomes in both sides of the egg proper and the first polocyte. This evidence may clearly be referable to Fig. 16, in which the lateral view of the chromosomes in the first oocyte division is given, indicating that each sister halves of separated chromosomes correspond exactly with each other. In both of the sister complexes of chromosomes there are found twenty elements consisting of eleven V-shaped and nine rod-shaped ones of dyad nature. In the light of this finding it can be stated that all of the twenty chromosomes of the primary oocyte are to be regarded as ordinary bivalents, and no particular elements showing size difference between the component halves exist in so far as shown by the first division. The conclusion may be made here that it is difficult to detect among the chromosomes any special element, unusual in structure or in behaviour.

The eggs taken from the end part of the oviduct invariably

1) To obtain a satisfactory result in a microtome-sectioning, the ova were fixed with sublimate-acetic solution (see Makino '34 b), but this method of fixing dees not give on the one hand any fovourable result for preservation of the chromosomes. The solution containing osmic acid, adequately employed for fixation of chromosomes as usual, if applied for such yolk-laden eggs, makes the eggs brittle and they are of no use for further treatment.

show the metaphase stage of the second polar division. The metaphase plate of the secondary oocyte shows twenty dyads with a dual nature (Figs. 17–18); there are again discernible eleven V's and nine rods. In division all of the dyads split into two identical monads, as seen in Fig. 19 a and b in which the sister sets of chromosomes after separation were indicated. The distribution of chromosomes was thus quite identical in both of the egg proper and the second polar body, so that each of them contains eleven V-shaped and nine rod-shaped elements, repectively, which are comparable to a complete half constitution of the oogonial chromosomes. The evidence is suggestive of that, so far as the chromosomes are concerned, only one kind of the ovum should be produced.

III. Some remarks on sex determination and on sex chromosomes in amphibians

Because of the large size of the germ cells and of the ease in securing material in amphibians, a great amount of work has long been done in the fields of cytology as well as of experimental zoology, but the questions of various complicated phenomena regarding sex have not yet been completely cleared up in any amphibians so far.

The mechanism of sex determination in amphibians has been inconclusively shown until at present. In anurans there has been raised the question as to whether the male or the female is heterogametic. Studies in the genetics on sex determination in *Rana temporaria* involving breeding experiments with hermaphroditic frogs, carried out by Crew ('21) and by Witschi ('23, '29), appear to demonstrate that it is the male that is heterogametic. On the basis of these experiments parthenogenetically developed frogs would be expected to be females. Recent experiments by Kawamura ('39) with *Rana nigromaculata* and *R. japonica* seem to suggest this to be the case. Parmenter ('25), on the basis of cytological observations upon Loeb's parthenogenetic frog material (*Rana pipiens*), inclined to believe that the male is heterogametic. In toads, however, the results of breeding experiments with sex-reversed males are not consistent. Harms ('26) obtained results which suggest the occurrence of male heterogamety in *Bufo vulgaris*, while Ponse ('31) with similar experiments showed a fact that indicates a homogametic condition of the male in the same species of *Bufo*.

In connection with these evidences in anurans, it would be interesting to see the effects of polyploidy on sex observed in urodelans. In studies on the gonads of triploid newts (*Triturus viridescens*) Fankhauser ('38, '40) indicated that the male possesses typical testes while the female has very rudimentary ovaries. In the group of 15 triploid newts (*Triturus viridescens*) Griffiths ('41) found that all 15 specimens were females carrying rudimentary ovaries. On the basis of these results these two authors conclude that in newts triploidy has a pronounced effect on the female sex only, probably comparable to that observed in triploid moths. In animals with a definite sex chromosome mechanism, triploidy shows no effect on the homogametic sex, while the heterogametic sex is changed to an intersexual condition (the genic balance theory of Bridges '21). Concerning triploid newts as above noted, it is the female sex that is affected. Fankhauser and Griffiths are in view that this seems to mean, on the basis of the genic balance theory, that newts have the same type of sex determination as moths, suggesting the female sex to be heterogametic. If this be true, the condition is in a striking contrast to anurans where male heterogamety is very probable.

Under these circumstances it would be interesting to review the chromosome mechanism in relation to sex determination in amphibians so far reported. We have at present no conclusive cytological demonstration concerned with sex determination in this group of animals, although there are some informations that are relative to the sex chromosomes.

In anurans, there are indications that the male sex is cytologically heterogametic, though no conclusive evidences have been presented so far. Levy ('15) imformed that the male of *Rana esculenta* has an odd X which goes undivided to one pole in the first division. A similar conclusion was reached by Swingle ('17) in the study of *Rana pipiens*, but he has since been in doubt in regard to that odd element, reporting that the body in question is a precociously dividing ordinary chromosome, one-half of which sometimes migrates toward the pole more quickly than does the other half of the opposite pole. As mentioned by Swingle, it is clear that the questionable body described by Levy as the odd X is in all probability no other than a preciously dividing chromosome or an abnormally

extruded one out of the spindle, caused by inadequate method of preservation. Later the chromosomes of *R. esculenta* and those of *R. pipiens* were carefully reinvestigated by Galgano ('21, '33 a, b) and Parmenter ('25, '33), and the even number of 26 was ascertained to be contained in diploid without finding any odd element. Indeed it is now a highly established fact as the results of recent investigations that the male frogs of the Ranidae uniformly possess an even number of chromosomes, being 26 in diploid for any species (Witschi '24, Iriki '32 a, Makino '32 a, b, Sato '33, '34 b, Galgano '33 a, b, Parmenter '25, '33). Thus the situation strongly emphasizes that a single X-0 condition of sex chromosome cannot actually be present.

The breeding experiments of Witschi ('23, '29) with hermaphroditic frogs (*Rana temporaria*) indicate that the male sex is heterogametic. The cytological demonstration was shown by him to be consistent with this conclusion, and the XY pair was demonstrated in the male frog which exhibits some particular traits during the spermatocyte divisions (Witschi '24). He found a large chromosome with a bilobed appearance at the first anaphase, and interpreted it as of a compound constitution. This element is separated in the second division, the larger lobe going to one pole and the smaller to the other. Witschi ('24) presumed the larger component to be the X and the smaller the Y. In the study of 1933 concerning *Bufo*, he mentioned the similar condition of the sex chromosomes to occur in five American species of *Bufo*. In every case of *Rana* and *Bufo*, however, there is presented no positive proof for the important point that the bilobed element in question is of a compound structure in the second spermatocyte. The cytological phenomena given by Witschi ('24) are much confused and there is found no sufficient reason to assume that the bilobed chromosome be a sex chromosome of the XY type. His interpretation for this chromosome is, as it seems, rather artificially distorted. Later the chromosomes of *R. temporaria* were reexamined by Makino ('32 b) and Galgano ('33 b) with special regards toward the presence or the absence of this particular chromosome. The conclusions reached by these authors are as follows: all the chromosomes are divided equationally in meiotic divisions without such peculiar manner as precession or succession of some chromosomes as Witschi observed, and further there is no trace of existence of an unsual bilobed element lagging

on the spindle of the second division as given in Witschi's paper. Being considered all cases, it seems fairly certain that the sex chromosomes of the XY type described by Witschi ('24) are doubtful.

Working on *Bufo*, *Hyla* and *Rana*, Minouchi & Iriki ('31) and Iriki ('30, '32 a, b) described a large bivalent of a remarkable V-shape in the primary spermatocyte which is distinctive from others by its shape and behaviour. This chromosome was presumed by them to be the XX pair of sex chromosomes which designates homogamety of the male, on account of the fact that the component elements are seemingly identical in size and shape. But, without considering the genetic condition in these forms, it may be premature to interpret this bivalent as an XX pair, even though its components are morphologically identical. In this connection, we know remarkable examples—for instances, *Drosophila virilis* and some other *Drosophila* species—in which the XY mechanism of sex determination was indisputably established from the basis of genetic experiments, whereas the sex chromosomes show no morphological size-difference between the X and Y. However we cannot overlook a striking fact that a bivalent having a distinguishable shape and behaviour, as first noticed by Minouchi & Iriki ('31) and Iriki ('30, 32), is found widely distributed among anurans, namely *Rana*, *Polypedates*, *Rhacophorus*, *Cacopoides*, *Hyla* and *Bufo* (Minouchi & Iriki '31, Iriki '30, '32 a, b, Makino '32 a, b, Galgano '31, a, b, '33 a, b, Sato '33, '34 b, '36 b). Though the evidence is not sufficient to make any decisive statement at present, however, the wide distribution of this chromosome among anurans seems to suggest a possibility for its interpretation as a sex chromosome.

As is the case with anurans, the problem of the sex chromosomes of urodelans is also a very confused one. King ('12) observed in the male of *Necturus maculosus* that an X chromosome is attached to one of the autosomes. According to her descriptions, this X passes undivided to one pole in the first division and segregates equationally in the second division. She further suggested the presence of the Y element also attached to an autosome. Since then Parmenter ('19) suggested similar conditions to occur in *Amblystoma tigrinum*. Recently Carrick ('34) inclined to a similar conclusion claiming a possibility of an X chromosome associated with an

autosome in the same species. The results of these observations are not entirely clear-cut and involve no conclusive data which are available for the final solution of the problem. The question of the attached X chromosome of *Amblystoma* remarked by Parmenter and Carrick, was settled to a successful solution by a thorough investigation of Galgano ('33 b, '38). Galgano found the chromosome number to be 28 in diploid and 14 in haploid, and concluded that there was no indication for the presence of the attached X and also of the X and Y elements with unequal size. It should be mentioned here that the recent investigations contributed to the chromosomes of various forms of forms of urodelans, such as *Megalobatrachus japonicus*, *Cryptobranchus allegheniensis*, *Triturus pyrrhogaster*, *T. ensicauda*, *Pleurodeles waltlii*, 11 species of *Hynobius*, *Pachypalaminus boulengeri*, *Salamandrella keyserlingii* (Iriki '32 c, d, Makino '32 c, '33, '34 a. b, '35 a, b, '39, Galgano '33 b, '38, Sato '32, '34 a, '36 a), reached to the consistent conclusion that the number of chromosomes was always even in the males of any species, and further that there was observable no particular chromosome which from its shape or behaviour could be characterized as the heterochromosome, throughout the course of spermatogenesis. And further, the results of the present study involving the chromosome analysis between two sexes of *Hynobius retardatus* point to the fact that the chromosomes do not show morphologically any sexual difference, since there are found either in form or in behaviour no particular chromosomes by which the sexes are cytologically discernible. There appears to be no heteropycnosis, no lagging or precession, or any other feature which might suggest the presence of heterochromosomes of any sort. At least in *H. retardatus*, therefore, it is essentially difficult to decide which sex is cytologically heterogametic.

Looking over the facts remarked in the foregoing descriptions, the present status of study appears to indicate that the problem of sex determination has not yet been conclusively cleared up in any forms of amphibians. But we have a remarkable cytological phenomenon in amphibians that can not be overlooked; it is the fact that in urodelans there occurs no special chromosome distinguishable from others in both of the male and female chromosome groups, while anurans possess in the male group a pair of particular chromosomes characterized by its remarkable shape and behaviour. The

wide distribution of this special element among anurans gives support in its interpretation as the sex chromosome, even though it is not certain whether it is the XX pair or XY pair in its essential (genetic) constitution. In the light of this fact the following statement might be allowed to be emphasized in regard to the sex chromosomes of amphibians: the sex chromosomes of urodelans are in a very lower state of differentiation being entirely indistinguishable from the other autosomes, so that it is difficult to identify the sex chromosomes among the autosomes; while in anurans the differentiation of the sex chromosomes advance a step forwards, so that they exhibit some differential characters distinguishable in shape and behaviour from the autosomes. To the author's view, the sex chromosomes of urodelan amphibians are very close in nature to those of fishes. As emphasized by the author in the study of the sticklebacks (Makino '34 c), the sex chromosomes of urodelans as well as of fishes may be in the state of the lowest differentiation among vertebrates in the evolution of the sex chromosome. In this connection, it is interesting to know the evidence that, in these lower vertebrates, genetic sex determination is relatively labile and may be influenced by various secondary factors.

Summary

The chromosomes of an urodelan, *Hynobius retardatus*, were studied comparatively in the two sexes with special regards to the question as to whether the sexual difference of chromosomes is morphologically apparent or not. The number of chromosomes in the male was confirmed to be 40 in diploid and 20 in haploid by observations of spermatogenesis. In the female the haploid number of 20 was ascertained in both of the primary and secondary oocyte divisions. The chromosomes thus show no numerical difference in the two sexes. Morphologically speaking, the constitution of the chromosomes is also identical between the two sexes and there is no indication for the existence of any particular chromosome distinguishable in form and behaviour from the other elements. Throughout the courses of both spermatogenesis and oogenesis there occurs no heteropycnosis, no lagging or precession, or any other feature of chromosomes which might suggest the occurrence of

particular chromosomes of any kind. The conclusion reached is that the chromosomes exhibit morphologically no difference by sexes, so far as the scope of the present study is concerned.

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Explanation of Plate XXXII

All are camera-lucida drawings of the chromosomes of spermatogenesis. 2–3, ×2100. 4–13, ×3000.

Figs. 2–3. Nuclei at early and late diakinesis. No heteropycnotic element is observable.

Figs. 4–7. Metaphase groups of primary spermatocytes, showing 20 bivalents in each.

Fig. 8. Lateral view of the anaphasic spindle of the first spermatocyte division showing the separation of the chromosomes.

Fig. 9 a–b. Sister complexes of chromosomes at anaphase of the first spermatocyte division.

Figs. 10–11. Metaphase groups of secondary spermatocytes, showing 20 dyads in each.

Fig. 12 Lateral view of the anaphasic spindle of the second spermatocyte division.

Fig. 13 a–b. Sister chromosome groups at anaphase of the second spermatocyte division.

Explanation of Plate XXXIII

All are camera-lucida drawings of the chromosomes of oogenesis. ×2100.

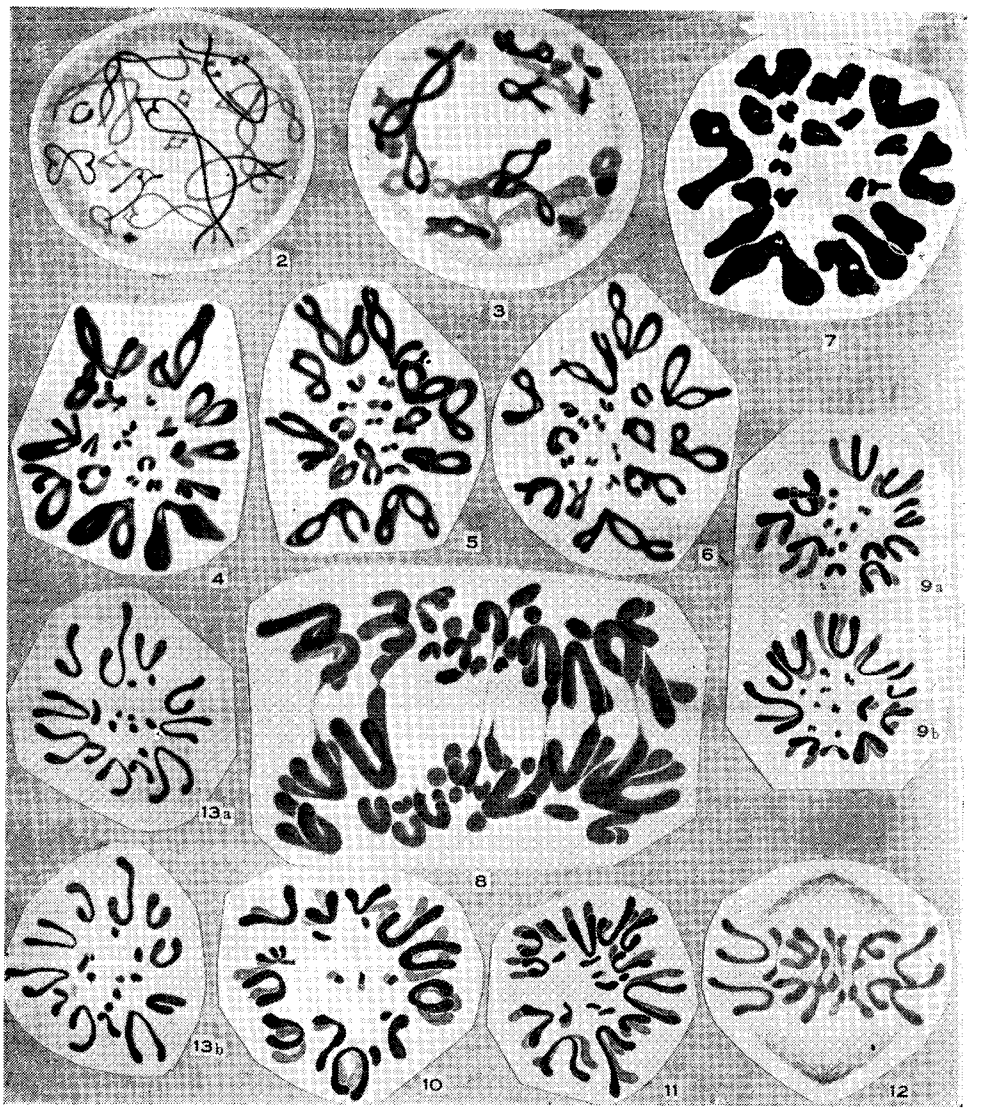
Fig. 14. Metaphase group of primary oocyte, 20 bivalents being shown.

Fig. 15 Lateral view of the anaphasic chromosomes in the first oocyte division. 20 elements are observable.

Fig. 16. Lateral view of the anaphasic spindle of the first oocyte division. Sister complexes contain in each 20 chromosomes (dyads) which correspond with each other in shape and size.

Figs. 17–18 Metaphase groups of the second oocyte division, 20 dyads being in each.

Fig. 19 a–b. Sister complexes of chromosomes at anaphase of the second oocyte division.



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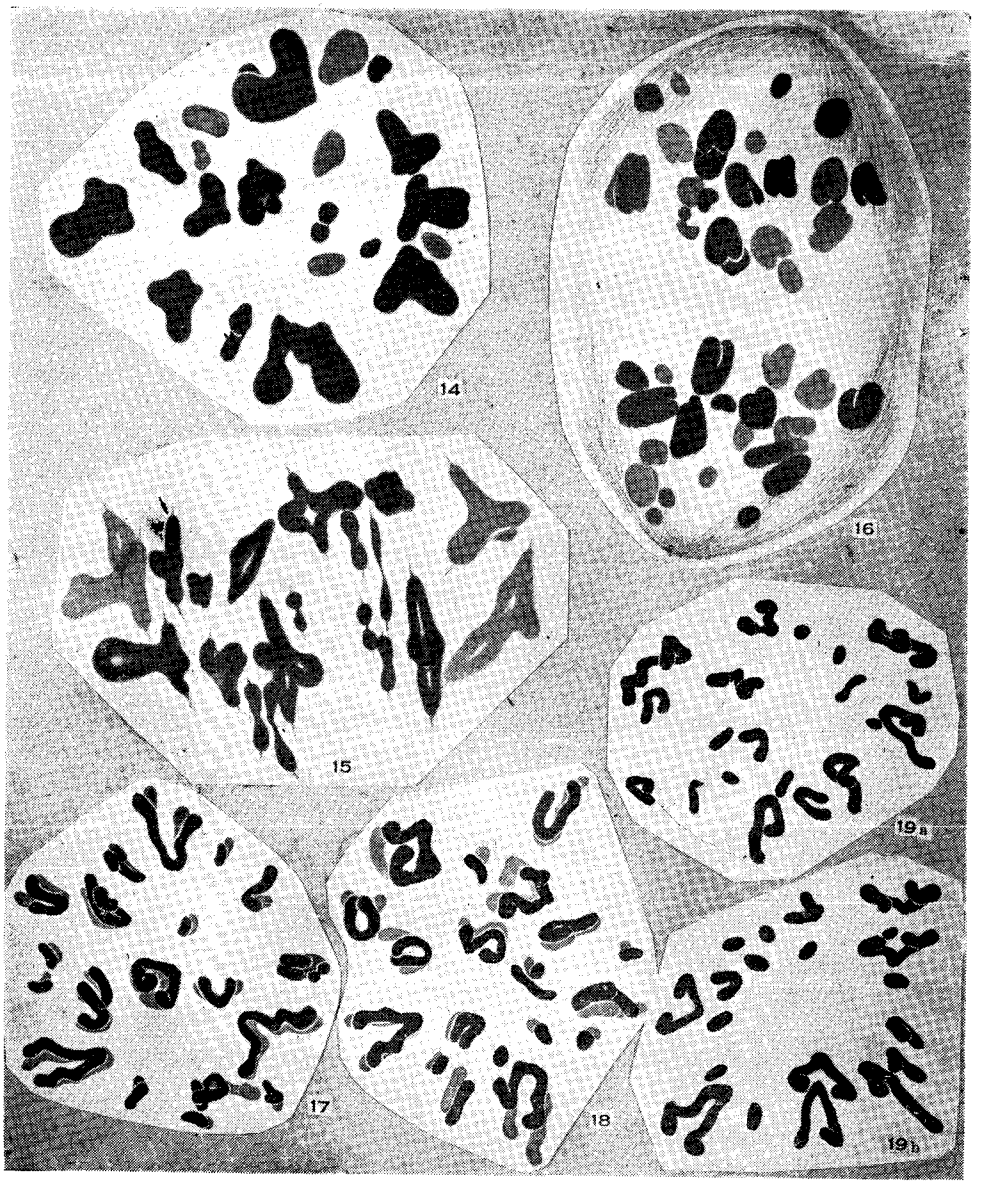

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