# A CYTOLOGICAL STUDY ON THE MATURATION AND FERTILIZATION OF THE EGG OF *HYNOBIUS RETARDATUS* (AN URODELAN AMPHIBIAN)[1]

BY

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*With 29 Text-figures and 4 Plates (50 figures)*

## Contents



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## Introduction

A great many workers have published their observations on the phenomena of maturation and fertilization, since such studies were started on the eggs of sea-urchins and *Ascaris* more than half a century ago. Now-a-days our knowledge on this subject is not confined to such invertebrates, but has been far extended to many kinds of vertebrates. Even merely in Amphibia, one finds already several important memoirs as cited elsewhere in the present paper. Most of these studies, however, as compared with the knowledge regarding invertebrates, seem to be still incomplete, especially in respect to the manner of dissolution of the germinal vesicle and to the chromosome behavior after the meeting of the maternal and paternal pronuclei. To accumulate data on different species and to compare with the previous works, the present study has been undertaken using *Hynobius retardatus* as the material, which is found abundantly near Sapporo.

The morphological study on the chromosomes in the two maturation divisions of the egg will be published in another paper with the data obtained in the male side, involving some conceptions on the sex-chromosome.

The work has been carried on since 1929, under the direction of Professor Kan OGUMA, to whom the author wishes to record his sincere appreciation for many valuable suggestions and for a careful supervision and kindly criticism during the course of the work. Thanks are also due to all members of this institute for their kind aid in collecting material.

## Historical[1]

So far as the amphibian egg is concerned, O. SCHULTZE ('87) appears to be the first author to present an accurate account of the

1) The present general sketch makes no approach to completeness, dealing only with the main works concerning the maturation and fertilization phenomena of the amphibian egg.

ripening and fertilization of the egg, though the very phenomena had frequently been recorded prior to his study, by NEWPORT ('55), M. SCHULTZE ('63), Van BAMBECKE ('70), GÖTTE ('75), O. HERTWIG ('77) and others, both in Anurans and Urodelans.[1] SCHULTZE ('87) demonstrated the structure of the germinal vesicle and polar spindles in the egg of the urodele *Siredon*, while ROUX ('85, '87) made an accurate study for the first time on the routes of the pronuclei in the frog egg. BORN ('92, '94) studied the structure of the chromatin filaments in the germinal vesicle and the ripening phenomena in *Triton* eggs of various stages, and examined by experiments the susceptibility to fertilization in unlaid eggs. The elaborate and important memoir of FICK ('93) on the Axolotl contributed greatly to accurate knowledge concerning all phenomena of egg-maturation and fertilization. His work supplemented to a great extent SCHULTZE's observation ('87) and has at the same time furnished the foundation of our present knowledge on this subject in Amphibia at least. On the other hand JORDAN ('93) described some detailed observations on the structure and continuity of chromatin filaments in the germinal vesicle and on the formation of polar bodies in *Diemyctylus*, corroborating BORN's account, while MICHAELIS ('97) published a short paper on the fertilization of *Triton*. During the years 1899 and 1900, CARNOY and LEBRUN contributed a series of excellent and valuable memoirs dealing with the development of the germinal vesicle and the formation of the polar spindles in the eggs of various Batrachia, as *Triton*, *Alytes*, *Bufo* and *Rana*. Their view expressed as to these Amphibia, that the chromatin filaments in the egg arise from repeated resolutions of the nucleoli in the germinal vesicle, could not be endorsed by KING ('01, '05) who repeatedly studied on the formation of the polar bodies and on the phenomena of fertilization in the egg of *Bufo* in greater detail. JENKINSON's study ('05) on the maturation and fertilization of the egg of the

1) Originals of these classical works were unfortunately not accessible to the author. A complete bibliographic survey of classical works is given in the text-book by O. HERTWIG (1906).

Axolotl, obtained an excellent result on the structure of the sperm-aster and the formation of the fertilization spindle. In the embryological study of *Hynobius*, KUNITOMO ('10) recorded some account of the maturation and fertilization processes. SMITH ('12) recorded essential observations on the phenomena of the maturation and fertilization of *Cryptobranchus* and in a later paper ('19) he published an extensive investigation on the individuality of germ-nuclei in the cleavage eggs of the same species. Recently in a series of studies, FANKHAUSER ('25, '29, '31, '32) has undertaken experimental researches to analyse physiological polyspermy in the egg of *Triton*. More recently, NAKAMURA ('33) has brought forward new observations on the relation between monospermy and polyspermy in the artificially fertilized eggs of *Hynobius* and has stated his opinion that monospermy is normal in *Hynobius* egg supporting the view expressed by KUNITOMO in his above mentioned paper ('10). At the 9th Annual Meeting of the Zoological Society (1933), KUNITOMO communicated interesting information regarding fertilization phenomena in the Japanese giant salamander, *Megalobatrachus*. Further, KAKIYAMA ('31, '32, '34) made serious investigations concerning the maturation and fertilization in the eggs of some Japanese Amphibia (*Diemyctylus*, *Bufo*, *Hynobius*).

## Material and Methods

*Hynobius retardatus* DUNN which furnishes the material for the present study is the sole representative of urodelan Amphibian in Hokkaido. It was formerly considered and described as *H. lichenatus* BOULENGER by previous authors (HATTA, '10, '13; HASHIMOTO, '10; SASAKI, '24, etc) until the systematic investigations made by INUKAI ('32) and MAKINO ('32) from the anatomical and cytological stand points established the indisputable difference between them. In the vicinity of Sapporo, the breeding season of this animal begins after it awakes from its hibernation, generally, at the beginning of April.[1]

1) Concerning the breeding habit of *H. retardatus*, see SASAKI ('24) and MAKINO ('33).

Numerous adult specimens gather in transitory pools formed by melted snow. The pairing and spawning take place in the water at the same time. At this period the gravid females with eggs in various stages of maturation, and mature males are collected easily in the waters and under the snow.

Material for the study of the maturation process was secured in the following manner; females were captured as soon as possible after they come out of their hibernating places and were dissected immediately. The eggs found in the ovaries and taken from various parts of the oviducts were transferred into the fixing reagents at once, and thus a series of stages in maturation were prepared. For the study of fertilization, eggs fertilized under captivity were used. The gravid females were placed in large glass aquaria containing water with mature males under approximately the same condition as in the natural environment. The mating and spawning take place in the very early morning, and then by preserving a number of eggs thus deposited at required intervals during 16 hours after fertilization, a series of the fertilization processes was acquired. Artificial fertilization was easily performed in eggs obtained from the lower parts of the oviduct, but in the present study such material was not employed at all.

As generally known, the fixation of such large and heavily yolk-laden amphibian eggs presents considerable difficulty in obtaining unaltered figures of protoplasmic inclusions for cytological purposes and in securing unbroken serial sections. After extensive experimentation, the author arrived at the conclusion that a saturated aqueous solution of sublimate containing 1 per cent glacial acetic acid is very satisfactory for either preservation or sectioning.[1] Eggs left ten to fifteen minutes in this fixing solution were washed thoroughly in 70% alcohol adding sufficient iodine solution and then preserved in pure 70%

1) The mixtures containing chromic acid or potassium bichromate recommended in studying the egg of Urodelans by several investigators (SCHULTZE, '87; FICK, '93; BORN, '94; JENKINSON, '05; KUNITOMO, '10 and others), seem to be improper for the present material. Eggs preserved with such solutions always shrink and crumble in sectioning.

alcohol. It is necessary before fixation to take off the gelatinous envelopes, in which the eggs are enclosed, by means of scissors. This is easily done if the eggs are put in water for a few hours, since the gelatin becomes inflated and softened by taking up water. In eggs taken from the oviducts and in those taken immediately after laid, the outer gelatinous envelopes adhere so closely to the egg body that it is hardly possible to detach them from the egg-surface without any damage to the latter. So the eggs may be put into the fixatives without the removal of the envelopes, and thereafter immersed in 10% formalin for several hours, until the envelopes become brittle. Then they are quite easily taken away by using needles. The eggs thus treated were washed and preserved in alcohol as in the former case. Comparison of these two cases shows no essential difference in the results.

The difficulty which many investigators have encountered in sectioning the amphibian egg will warrant a somewhat detailed account of the method by which the author was enabled to obtain satisfactory results. The mode of procedure in sectioning is as follows: the eggs preserved in 70% alcohol are put into 90% alcohol for 2 hours; 95% alcohol for 1 hour; commercial absolute alcohol for $1^1/_2$ hours; and then immersed in cedar wood oil. The eggs are left over one night in the cedar oil, which is then replaced by a mixture of toluol and creosote. When the eggs become transparent, this mixture is changed to pure toluol (for about 30 minutes in each). Then they are put into a mixture of toluol and paraffin at 42°C for about 3 hours; soft paraffin (42°C) for 3–4 hours; hard paraffin (52°C) for 1 hour; and finally imbedded in hard paraffin.

For a study of the nuclei of the eggs in the maturation and fertilization processes, it is necessary that an adequate number of eggs should be cut in series in different thickness. To study the germinal vesicle and polar bodies, a thickness of 12 to 15 micra is generally satisfactory, but sometimes 40 micra is required, while to follow stages of the fertilization process thickness of 10 to 12 micra proved to be the best.

Most sections are stained with DELAFIELD's haematoxylin with counterstaining of eosin. This gives satisfactory results for general study. Sometimes material stained with HEIDENHAIN's or WEIGERT's iron-haematoxylin was prepared for the sake of comparison.

The text-figures, except numbers 1, 2, 18, 19 and 29, are drawn with the aid of a camera lucida on the desk level on which the microscope was set. In reproduction for printing they are reduced to the magnifications indicated in each.

## The Gametes

*The ovum.* The general aspects of the spawns of the present species were given in the papers of SASAKI ('24), INUKAI ('32) and MAKINO ('33).

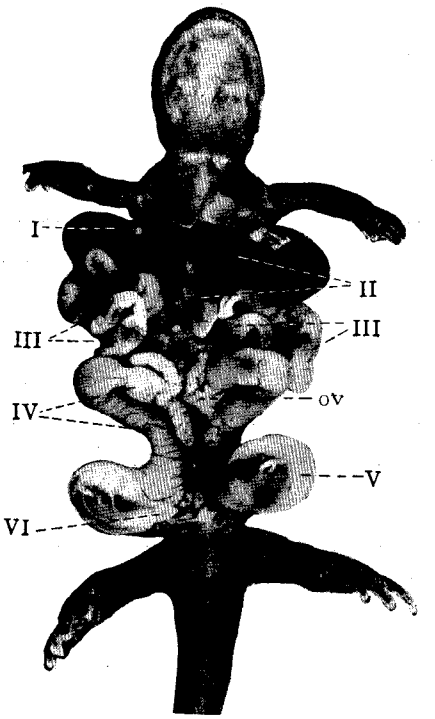
The egg body is perfectly spherical when fresh, measuring usually 2.5–3.0 mm in diameter including the chorion. It is chocolate brown in colour at the animal pole, fading to a gray or pale brown towards the vegetable pole. It is invested closely by the chorion, a very delicate, transparent and structureless membrane.

The yolk, nutritive elements of the egg, shows ovoidal granules microscopically. They take either basic or acidic stains. Their diameters vary greatly, ranging from 0.0025 mm to 0.019 mm in their long axis, the most usual ones being those ranging from 0.0075 mm to 0.0085 mm. In general, the granules are smaller near the animal pole, growing larger gradually towards the vegetable pole. The pigment granules, by which the egg is deeply coloured, are fine, short spine-like in shape and scattered about, aggregating into masses throughout the egg among the yolk granules.

The spawns are coated with jelly-like, gelatinous envelopes which are produced from the oviducts. These envelopes consist of the inner egg-capsules, directly investing the egg body, and the egg-sac, an elongated corrugated bag enclosing the eggs.

During their passage down the oviduct the eggs receive their gelatinous envelopes; a topographical sketch of their production in

the oviduct is as follows: at the uppermost part of the oviduct near the opening, the eggs collect in masses; a little further down they are arranged in solid rows (Text-fig. 1, I). In these positions, the oviduct is of very delicate transparent structure and eggs are free from covering. Following these parts, the oviduct walls become thickened step by step (Text-fig. 1, II); each egg therein acquires for the first time an elastic opaque capsule. In the middle parts of the oviduct, the eggs pass down one by one at some intervals, with the appearance of a string of beads (Text-fig. 1, III). While passing along these parts another layer of capsule, not so thick as the former, is produced in addition to the former one, to which it adheres intimately. Thus, in these portions the capsules evidently consist of two distinct layers. The part, posterior to the middle part above described, may be distinguished as the lower part in the entire length of the oviduct, because of the considerable increase of its diameter and of the remarkable thickening of the wall (Text-fig. 1, IV). Enclosed in this region of the oviduct the eggs are arranged into rows and a considerable amount of sticky gelatinous substance is secreted over the egg capsules in order to bind them to become at last a spindle-shaped egg mass. When the egg mass passes through the thickest and funnel-shaped portion of the oviduct (Text-

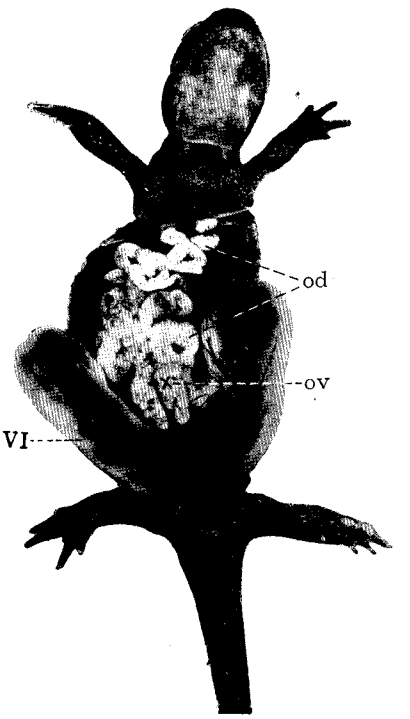



Text-fig. 1. Ventral view of the female reproductive system, showing the liberation of the eggs from the ovaries (ov) and their passage down the oviducts (I–VI). *ca.*×*1*.

fig. 1, V), it receives again a thin elastic membrane called the egg-sac, and thus the spawns are finally completed. Then the egg mass remains *in situ* for a considerable length of time until laying, at the lowest end of the oviduct which is of delicate membranous structure (Text-figs. 1–2, VI).

*The sperm.* The spermatozoon has been observed in smear preparations after staining with iron-haematoxylin or gentian violet.

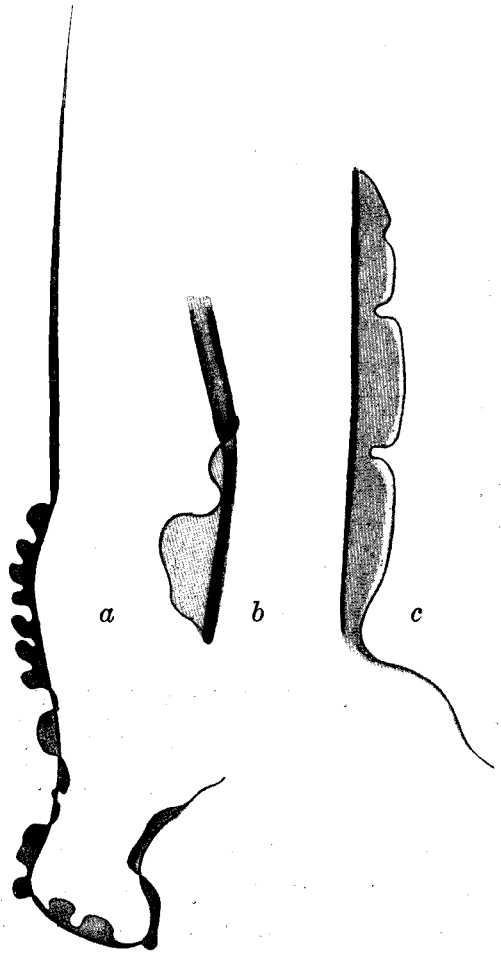
Generally the fresh spermatozoon measures about 0.185–0.190 mm in length. This dimension is, therefore, considerably reduced in the present species as compared with that of the Axolotl, which FICK ('93) described as 0.4-0.6 mm long, and rather allies to *Cryptobranchus*, which SMITH ('12) measured as about 0.225 mm long.




Text-fig. 2. Ventral view showing the egg masses just before laying, kept at the lowest parts of oviducts (VI). od : oviduct. ov : ovary. *ca.* ×1.

The head is typically lance-shaped, almost round in a cross section, as in all cases of salamanders, and 0.056–0.058 mm in length, the acrosome excluded (Text-fig. 3, *a*). The head stains intensely with either haematoxylin or gentian violet and appears to be perfectly homogeneous in structure with the entire body. The acrosome is 0.017–0.020 mm in length and assumes the shape of a sharply pointed spine, not provided with so prominent a barb as in, for example, *Molge*, *Salamandra*, *Pleurodeles*, and *Spelerpes* (see RETZIUS, '06). In staining it shows no affinity to either haematoxylin or gentian violet. In stained preparations as above mentioned, the entire head part

appears uniformly homogeneous, showing no otherwise stained part anywhere to be distinguished as the neck. The head, therefore, seems to be connected directly with the tail, at least in the stained preparations. In this respect the present species appears to differ from known Urodelans such as *Molge*, *Salamandra*, Axolotl, etc., in which RETZIUS ('06) and FICK ('93) demonstrated a distinct structure of the neck. However, a close examination of the fresh material with the dark-field microscope, reveals the actual occurrence of a 'neck' -like structure appended to the posterior region of the head (Text-fig. 3, *b*). It is a clear and strongly refractive part with a little smaller diameter in comparison with the head.




Text-fig. 3. *a*, general view of a spermatozoon. *ca.* × 800. *b*, neck-region after dark-field observation of fresh material. *Z. 2 mm : K 15×, ca.×2400. c*, terminal part of tail. *ca.* ×*2400.*

The tail is about 0.110–0.125 mm long, provided with a characteristic undulating membrane bordered by a contractile marginal filament (Text-fig. 3, *a*). The end of this filament always extends somewhat beyond the tip of the main piece constituting the apical piece of the tail (Text-fig. 3, *c*), as is generally found in other Urodelans (RETZIUS, '06; FICK, '93). The undulating membrane is colourless even in stained

preparations, while the main piece and marginal filaments stain lightly. After the dark-field observation of the fresh material one knows that the m rginal filament has its origin in a minute corpuscle lying between the head and the neck as illustrated in Text-fig. 3, *b*.

## Observations

### I. *Maturation*

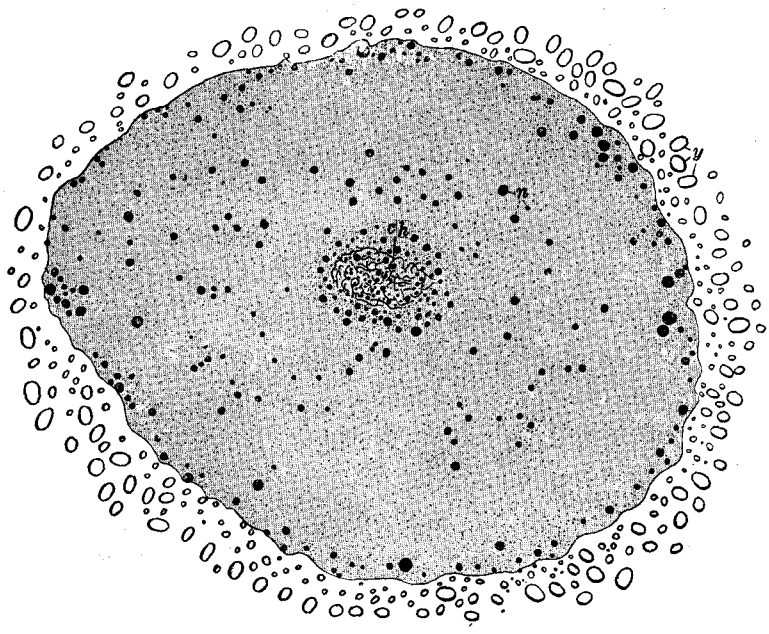
#### A. The germinal vesicle and its dissolution

1. *The germinal vesicle of the ovarian egg*

In the egg taken from the ovary of the animal during the breeding season, the germinal vesicle does not occupy the central part of the egg but a position about midway between the centre and the periphery at the animal pole (Fig. 1). The germinal vesicles of this period are almost oval in outline. They measure 0.38–0.48 mm by 0.47–0.65 mm in the meridional diameter. In *Bufo*, KING ('01) measured 0.24 mm by 0.34 mm and in Axolotl FICK ('93) gave 0.72–0.80 mm in the long axis and 0.30–0.32 mm in the short axis. The ground-substance appears homogeneous under a low power of microscope, but exhibits, in a high magnification, a fine but dense granular structure; that is, minute microsomes, stained very faintly with haematoxylin, are scatteringly imbedded in the homogeneous hyaloplasm. Enclosing the germinal vesicle there is recognized a membrane of delicate structure, not perfectly smooth but wavy in contour (see Figs. 4 and 6).

An enormous number of nucleoli, counted by hundreds, are distributed throughout the germinal vesicle. Particularly, they are found in greater number both in the peripheral region, adhering very closely to the membrane and in the central part of the vesicle (Text-fig. 4). In the latter position, they are generally found surrounding chromatin threads, which are crowded intertwining with one another, or in the interspaces between them (Text-fig. 4 and Fig. 5). The nucleoli are almost spherical in shape and take the

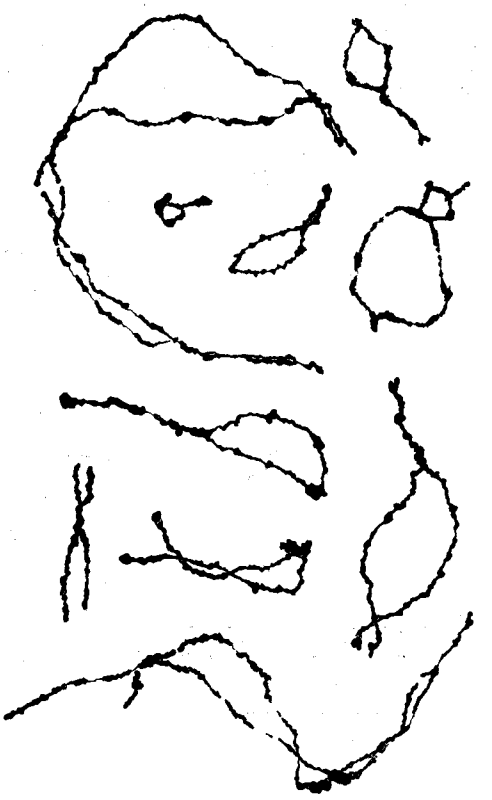
same stain as the chromatin, showing homogeneous structure. They vary in a great degree in their size fluctuating between 0.008 mm and 0.001 mm in diameter. In general, the smaller ones, 0.001–0.003 mm in diameter, are distributed among the chromatin threads, and stain very faintly, while the larger ones, 0.005–0.007 mm in diameter, surround the chromatin threads and stain deeply. It is the latter kind of nucleoli that are scattered through the entire space of the germinal vesicle towards the periphery.




Text-fig. 4. General view of the meridional section of the germinal vesicle from an ovarian egg, showing the distribution of nucleoli and the chromatin threads crowded at the central region. *n*, nucleolus. *ch*, aggregated chromatin threads. *y*, yolk granules. *ca.*×*110*.

The chromatin threads which aggregate in the central region of the germinal vesicle are very long and intertwined with one another as already mentioned (Text-fig. 4 and Fig. 5). It is impossible to count their number accurately owing to their intertwining on account of which they break into fragments upon sectioning. As shown in Text-fig. 5, the chromatin threads are stained intensely with haemat-

oxylin, varying in shape and length, the longest one measuring almost 0.065 mm and the shortest about 0.0075 mm. An individual chromatin thread is in reality composed of two finer ones which are twisted with each other to some extent. Taking this structure into consideration it seems to be highly probable that the chromatin threads in this kind of nucleus have already assumed a bivalent nature.



Text-fig. 5. Various forms of individual chromatin threads, from the germinal vesicle of the ovarian egg.
*Z. 2mm : K15×, ca.×1200.*

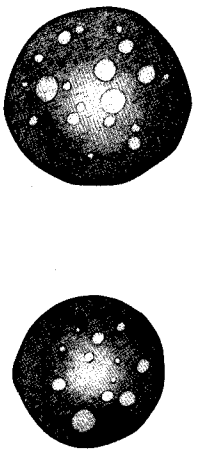
### 2. *The dissolution of the germinal vesicle*

At the time when the egg is about to fall into the body cavity from the ovary, the germinal vesicle has migrated further up to a position close to the periphery of the egg (Fig. 2). During the migration, however, the germinal vesicle seems to show little change in structure except that the mass of chromatin threads which also alters its position to a more peripheral one as compared with the preceding stage, as seen in Fig. 4.

When the germinal vesicle has reached a position near the periphery of the egg as stated above, the first step of its disintegration processes is begun: the border of the germinal vesicle nearest the periphery of the egg is first broken down showing irregular indentation (Fig. 7), and thereby the nuclear substance is allowed to flow out as shown in Fig. 2. In the meantime the

process of dissolution of the border extends to all sides of the germinal vesicle, firstly from the upper to the lateral parts and at last to the lower portions. Thus the demarkation between the germinal vesicle and the surrounding cytoplasm is completely lost and the vesicle keeps its original form no longer (Fig. 3).

While the vesicular wall is fading in the way just described, the nucleoli seem to be disintegrated. At first they all migrate gradually towards the peripheral regions of the vesicle and then vacuolation takes place in every one which assumes a compact appearance (Text-fig. 6). The vacuoles are small and less in number at the commencement of this process but rapidly increase in number, until the nucleoli become completely filled up with them. They no longer show any affinity for stains when they take the appearance just described and enter the final processes of disintegration.



Text-fig. 6. Nucleoli in the stage of disintegration, showing the numerous vacuoles within them. *Z. 2mm: K15×, ca.×1900.*

The second step in the dissolution processes of the germinal vesicle is indicated by the occurrence of a change in its ground substance. It is no longer in a state of homogeneous and granular texture as it was previously, but presents the appearance of a reticular meshwork (compare the texture shown in Fig. 5 with that in Fig. 8). The meshwork is not uniformly constructed. The part surrounding the central region where the chromatin threads are aggregated, shows a fine, dense fibrous structure, or intermediate form between granular and reticular, but in parts distant from the centre the structure becomes coarse and rough. The fibres of the mesh, moreover, are arranged radially as readily evinced in Fig. 8. By this time, the nucleoli have all completely disappeared.

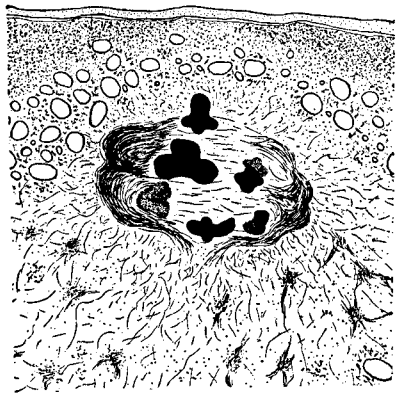
In parallel to the dissolution of the germinal vesicle and the disappearance of the nucleoli, there happens another important change in the chromatin threads. Up to this time the chromatin threads always remain in filamentous form as shown in Fig. 5. But when the ground substance of the dissolved vesicle takes the reticular appearance described above, the threads are found without exception completely transformed into chromosomes of bivalent structure, instead of fine threads. Probably this change takes place with considerable rapidity, since in none of the sections did the author succeed in discovering any stages intermediate between threads and condensed chromosomes. A similar condition was also noted by JORDAN ('93) in *Diemyctylus* and SMITH ('12) in *Cryptobranchus*.

The chromosomes of this period lie aggregated at the central part of the ruptured vesicle at the animal pole, surrounded by protoplasmic meshwork (Fig. 8). They are no doubt the tetrad, characteristic to the first maturation division, as the crosses or the rings are clearly observable. Unfortunately the author failed in an attempt to count the actual number of them, because some longer ones are very often cut into several fragments. Still the number of chromosomes shows approximately the same with the reduced or the haploid number[1]. From these evidences it may be concluded that this stage corresponds to the 'diakinesis' in the spermatogenesis, the stage just prior to the metaphase.

In the egg of the next advanced stage which is taken out of a part near the opening of the oviduct, further changes are to be noticed in the débris of the germinal vesicle. The structure of the meshwork becomes more coarse in appearance throughout its entire region and no longer shows the radial arrangement of the composing fibres. This fibrous structure gradually disappears too in the course of time and the débris of the vesicle is replaced by yolk granules which invade from the surroundings. At last the débris area is remarkably reduced (Fig. 9).

1) Concerning the chromosome number of *Hynobius retardatus*, see the previous papers published by the author (MAKINO, '32, '33).

During these changes, there commences, on the other hand, a gradual movement of chromosomes towards the upper part of the egg, accompanied by the formation of the spindle, the axis of which lies at right angles to the direction of the movement (Text-fig. 7). Once arrived at the extreme peripheral part of the egg (Fig. 9), the chromosomes with spindle cease further movement and seem to rest in preparation for the coming division. This stage is usually encountered in the sections of the egg taken from the uppermost parts of the oviduct, about the portion marked 'I' in Text-fig. 1.



Text-fig. 7. Section through the animal pole, just before the formation of the first polar spindle, showing the condition of chromosomes.
*Z. 4mm : K×15, ca. ×800.*

All these changes of the disintegration of the germinal vesicle, as seen in the foregoing descriptions, take place successively during a quite short time when the egg has left the ovary and just entered the oviduct. Since the similar phenomenon is noted in other Amphibia, *e.g.*, *Bufo*, *Triton*, *Diemyctylus*, Axolotl, *Cryptobranchus*, etc., by earlier authors, it seems to be a characteristic common to all amphibian eggs.

## B. The first polar spindle

Sections through the egg taken from the upper parts of the oviduct, at about the portion marked 'II' in Text-fig. 1, invariably show the metaphase stage of the first polar division. The first polar spindle lies close to the egg periphery, with its axis coinciding with that of the polarity of the egg, though sometimes deviating to a greater or less degree from the latter. Eventually it becomes oblique (Figs. 10–12). Therefore, there should occur a quick turning of the spindle axis, since in the previous stage it was approximately parallel

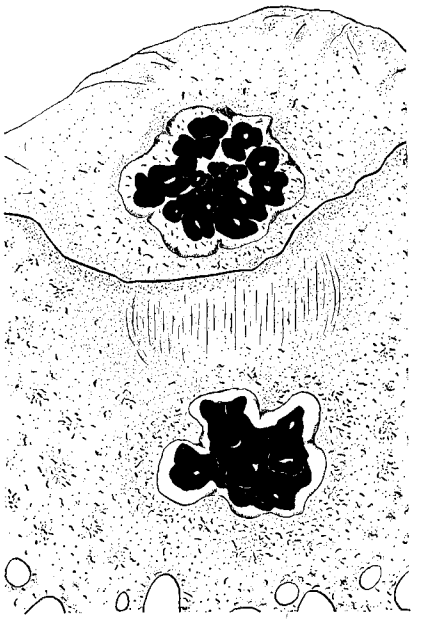
to the surface of the egg (Text-fig. 7 and Fig. 9). The polar spindle shows distinct spindle fibres and is surrounded by a small amount of cytoplasm with a deep deposition of pigment, which is contiguous to a dense accumulation of heavy yolk granules. Under low power of magnification, the polar spindle appears merely like a minute light spot, imbedded in opaque yolk granules at the periphery of the animal pole. In his study on Axolotl, JENKINSON ('05) states that, in some cases, at the inner pole of the first polar spindle, the spindle fibres undoubtedly converge into two separate points. However, in the present study it was impossible to find cases that prove the actual occurrence of bipolar structure of the spindle; on the contrary it was unexceptionally unipolar. There is no trace of centrosome or of any other like structure in the centre of convergence of the fibres at either spindle pole.

In every equatorial plate of this division, there are found, without exception, twenty tetrads.[1] The mode of arrangement of the tetrads on the equatorial plate is quite the usual one; the larger tetrads occupy the peripheral position surrounding the smaller ones at the central region.

In the anaphase, each tetrad separates into two equal dyads which go to opposite poles (Figs. 13–14). Thus, in the telophase, twenty chromosomes are again counted at either pole.

After the separation of the chromosomes, the outer pole of the polar spindle is raised up from the egg surface exhibiting a disc as shown in Fig. 15. Later, this disc is constricted off from the egg surface crossing the middle part of the spindle to form the first polocyte which sinks down, then, in a shallow depression produced at the place from where it was previously constricted (Fig. 16). Meanwhile, the spindle fibres become gradually indistinct and appear as a granular structure. After the polocyte is completely extruded, either in the polocyte or within the egg, the chromosomes are soon fused together into condensed and irregular masses (Figs. 16–18 and Text-

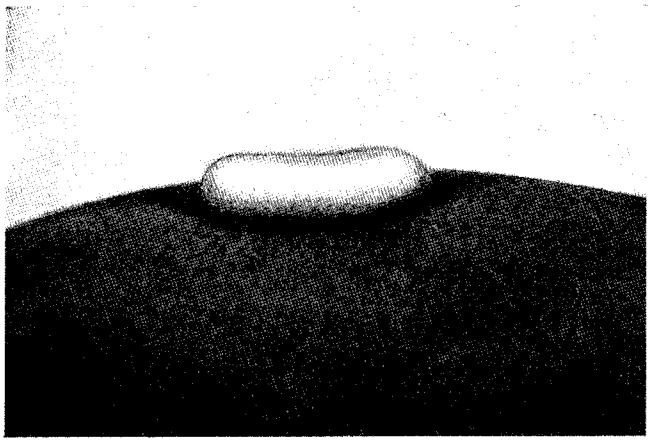
1) A preliminary account on the oocyte chromosomes has already been published (MAKINO '33).



Text-fig. 8. The first polar division telophase, somewhat oblique view. At either pole, the chromosomes begin to fuse together and become invested with a thin membrane.
*Z. 2mm : K15, ca. ×1500*

fig. 8). When the sister chromosomes lose their individuality by fusion a delicate membrane comes into view enclosing them (Text-fig. 8 and Fig. 17). The first polar division is thus accomplished.

The first polocyte is disc-shaped with an oval or sometimes round outline and lies in a shallow depression in a clear area devoid of yolk granules on the egg surface, as already stated. In fresh and living condition the first polocyte looks like a white pad placed upon the surface of the egg at its animal pole (Text-fig. 9). Such examples are easily found in eggs taken from the upper part of the



Text-fig. 9. External view of the first polocyte, lateral aspect. From fresh material. *W.I. 40 : K10×, ca.×600.*

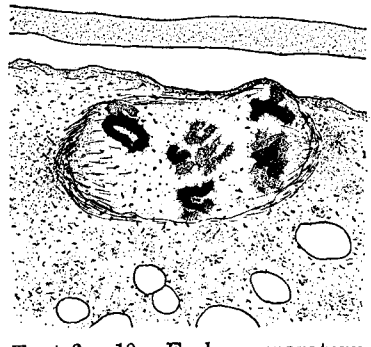
oviduct. The polocyte measures 0.042–0.050 mm in long diameter when fresh.

Generally speaking, the first polar division commences when the eggs are at the upper parts of the oviduct (about the portion marked 'II" in Text-fig. 1) and advancing in its course during their passage through the middle parts of the oviduct (the portions between, marked 'III' and 'IV' in Text-fig. 1) it is accomplished about the time when the eggs reach the lower parts of the oviduct (about the portion marked 'IV' in Text-fig. 1). There is found no case in which a later division actually occurs in the first polocyte so far as the present material is concerned, though FICK ('93) and JENKINSON ('05) described its occasional occurrence in Axolotl.

In the first polar division of *Cryptobranchus*, SMITH ('12) observed a 'contact disc', a disc-shaped body with an irregular cross-striated structure, which overlies the polar spindle close to the cell wall. In the present observation, all methods of fixation and staining disprove the presence of such a peculiar structure as the 'contact disc'.

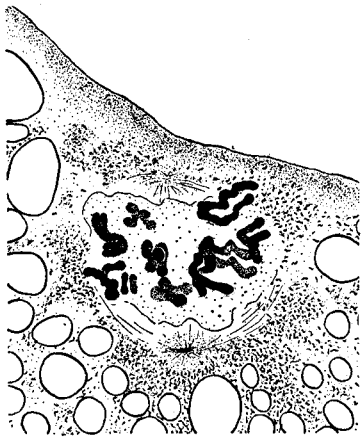
## C. The second polar spindle

After the first polocyte has been extruded, as previously described, the sister group of chromosomes left in the egg crowds together into an irregular condensed mass and becomes inclosed by a membrane (Fig. 18). In the nucleus thus vacuolized, the chromosomes quickly recover their individuality. The chromosomes with indistinct outline again become separated from one another and are widely distributed inside the nuclear membrane (Text-fig. 10). Pigment granules are heavily accumulated in the perinuclear part of protoplasm. The nucleus usually lies with its long axis parallel to the



Text-fig. 10. Early preparatory stage to the second polar division (interkinesis). Notice the formation of vesicular nucleus, lying just beneath the egg surface, with the long axis parallel to the latter.
*Z. 2mm : K15×, ca.×1500.*

egg surface and adheres very closely thereto. In the meantime, definite spindle fibres appear at either pole and soon the nuclear membrane is broken down.[1] The chromosomes gradually assume definite forms showing strong affinity for stain and the distinct dual structure of dyads. At the same time there occurs a quick turning of the axis of spindle in like manner to that observed in the preceding division. Or, in other words, by turning the spindle axis now comes at right angles to the egg surface (see Text-fig. 11). Then the chromosomes begin their equatorial arrangement.



Text-fig. 11. Following stage to Text-fig. 10, just before the formation of the second polar spindle. The dyads assume definite forms, distributed through the nucleus.
*Z. 2 mm: K15×, ca.×1500.*

Changes as just described seem to occur within a quite short time when the eggs arrive at the lower parts of the oviduct (about the portion marked 'IV' in Text-fig. 1). The eggs then pass down, without any advanced changes, through that part of the oviduct until they find themselves in the lowest end part of the oviduct, where they acquire an elastic egg-sac. They stay there for a rather long time before spawning takes place. Therefore, eggs taken from the parts of the oviduct, lower than the portion marked 'IV' in Text-fig, 1, invariably are in metaphase of the second polar

1) With regard to the formation of the vesicular nucleus between the first and second polar divisions as described here, none of earlier authors has noted in either Urodelans or Anurans, so far as the author is aware. Since it has been considered to be common that in the animal egg there is generally formed no vesicular nucleus between two maturation divisions in the egg ripening, the present result offers an extreme exception, probably a stage characteristic to this material. This stage of nuclear reconstruction seems to be the one corresponding to 'interkinesis', the resting stage between the first and second spermatocyte divisions in the spermatogenesis.

division[1] (Fig. 19). So the second polar division is arrested in metaphase for a considerable length of time until the spermatozoon penetrates into the egg after spawning.

Usually the liberation of the eggs from the ovary and their passage down the oviduct are completed during the day immediately before spawning occurs, and in the majority of cases, the eggs have arrived at the extreme lowest end of the oviduct (Text-fig. 2, VI) in the evening of the day provided for spawning. At this time these eggs are invariably in metaphase of the second polar division. In view of the fact that the metaphase of the second polar division proceeds to the anaphase only by insemination of spermatozoon, there arises an important question whether the metaphase actually continues *in situ* for such a long time as from evening to the next morning when spawning and fertilization take place. To ascertain this point the author undertook the following examination: three pregnant females, in which the eggs have arrived at the lowest end of the oviduct ready to spawn in the following morning, were dissected in the living condition, and a few eggs were taken out from the bodies of different individuals at intervals (of about every two hours) and preserved[2]. Results of microscopical examination in sections are tabulated as follows:

Table 1.

| Date | Time of fixation | Number of eggs examined | Results of observation |
|---|---|---|---|
| | | Individual A | |
| 1932, March 29 | 5:15 P.M. | 5 | IInd polar division metaphase |
| " | 7:00 P.M. | 3 | " |
| " | 10:00 P.M. | 4 | " |
| " | 11:50 P.M. | 5 | " |
| " March 30 | 2:10 A.M. | 5 | " |
| " | 4:00 A.M. | 7 | " |
| | | Individual B | |
| 1932, March 29 | 8:20 P.M. | 3 | IInd polar division metaphase |
| " | 11:55 P.M. | 5 | " |
| " March 30 | 2:15 A.M. | 4 | " |
| " | 4:00 A.M. | 5 | " |
| " | 10:00 A.M. | 5 | " |
| | | Individual C | |
| 1932, March 31 | 5:30 P.M. | 5 | IInd polar division metaphase |
| " April 1 | 9:30 A.M. | 7 | " |

1) The eggs under this condition are capable of fertilization and if inseminated artificially in the usual way, they develop normally.

2) The process of polar division in the eggs is synchronous in both sides of the oviducts.

As is obvious from the table, the examined cases without exception show the metaphase of the second polar division. From this evidence, therefore, one can hardly be in doubt that the second polar division is arrested in the state of metaphase over the night until spawning and fertilization take place in the following morning[1].

The chromosomes in the metaphase of this division exhibit a distinct dual nature taking a radial arrangement (Figs. 19–20). In every metaphase plate, twenty dyads of various shapes are easily counted. In all other general features the second polar spindle seems to show no marked difference from the first polar spindle, except that the former is generally somewhat more slender in appearance than the latter. At the poles of the spindle the centrosomes are not to be seen just as in the previous case.

## II. *Fertilization*

### A. Formation of the pronuclei

#### 1. *Further changes in the second polar spindle*

During the first about 20 minutes after spawning and insemination have occurred, the second polar spindle persists in the stage of metaphase (Fig. 21), and then it gradually advances in further course of division. The process of division is very slow. About 1 to $1^1/_2$ hours after insemination, the second division is completed, and the second polocyte is found fully formed about $1^1/_2$ to 2 hours after insemination. The course of divisions is shown serially in Figs. 22 to 24. The manner by which the second polocyte is formed seems to be similar to that described in the case of the first polar division.

The second polocyte is of flat disc shape with round or slightly oval outline, somewhat smaller than the first and surrounded by a

1) After the experiment was finished, the remaining eggs left in the bodies of each, experimental animal were artificially inseminated and all the eggs thus treated developed normally. This fact is sufficient to indicate that all the eggs employed in the experiment have shown no regressive changes during the course of the experiment, but are in the normal condition.

thin delicate membrane (Figs. 23–24, lateral views and Fig. 25, dorsal view). It contains a considerable amount of cytoplasm with agglomerated pigment granules and occasionally some yolk granules. The chromosomes are seen in a mass of irregular shape at the central region as in the first polocyte. The position where the second polocyte is situated varies in different eggs: sometimes it is found just beneath the first polocyte (Fig. 22), and in another case it lies some 0.07 mm distant from the first (Fig. 21).

From facts hitherto stated, it is evident that the later stages of the second polar division occur only when the egg is inseminated. Therefore, the late stages of maturation are overlapped by the early stage of fertilization. In this respect, all the Amphibia so far described in the literature[1], appear to be in harmony.

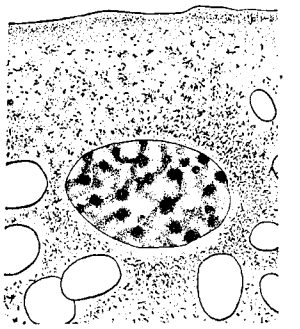
2. *Formation of the female pronucleus*

After the extrusion of the second polocyte is completed, the sister chromosomes left in the egg converge at their apices and tend gradually to fuse together into a compact mass of irregular outline, apparently losing their individuality (Text-fig. 12). This mass lies in a small accumulation of granular substance surrounded densely by pigment granules and locates very closely to the egg surface as shown in Fig. 26. Then a nuclear membrane appears enclosing the chromosome mass. In the next step the chromosome mass becomes disintegrated into irregular bodies, vague in contour, scattered throughout the nucleus which is not yet sharply outlined (Text-fig. 13).

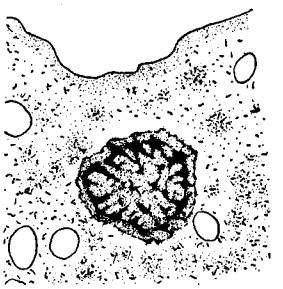
Text-fig. 12. The sister chromosome group left in the egg after the second polar division is completed, oblique view. *Z. 2 mm : K15×, ca.×1500.*

1) In Anurans, KING '01 (*Bufo*); HERLANT '11 (*Rana fusca*); PARMENTER '33 (*Rana pipiens* and *R. palustris*) etc., and in Urodelans, BORN '92, MICHAELIS '97, FANKHAUSER '32 (*Triton*); FICK '93, JENKINSON '05, (Axolotl); JORDAN '93, LEBRUN '02 (*Diemyctylus*); SMITH '12 (*Cryptobranchus*); KUNITOMO '34 (*Megalobatrachus*), etc.

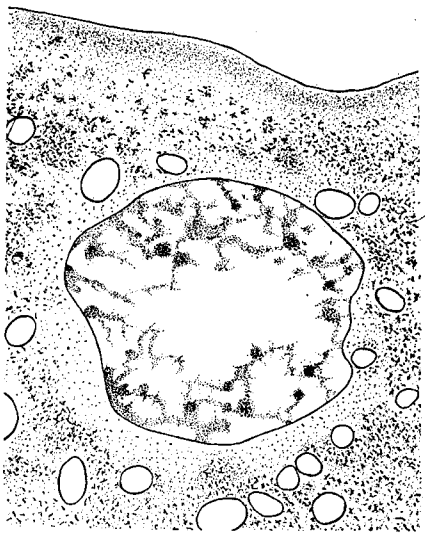
Meanwhile, the chromatic bodies lose much of their capacity to take stain: accordingly the interior of the nucleus becomes clearer in appearance. In parallel to the transformation of the chromatic bodies now mentioned the nucleus grows probably by accumulation of nuclear sap, and expands greatly to attain a smooth circumference (Text-fig. 14). This is a female pronucleus of complete form, and it takes, at this period, an ellipsoid shape with approximate diameters 0.01 mm × 0.013 mm. The whole process is completed usually in about $2^1/_2$ to 3 hours after spawning and pairing[1].



Text-fig. 14. The female pronucleus just metamorphosed, lying close to the egg surface, about 0.01 mm by 0.013 mm in diameter. About 3 hours after insemination. *Z. 2mm : K 15×, ca. ×1500.*



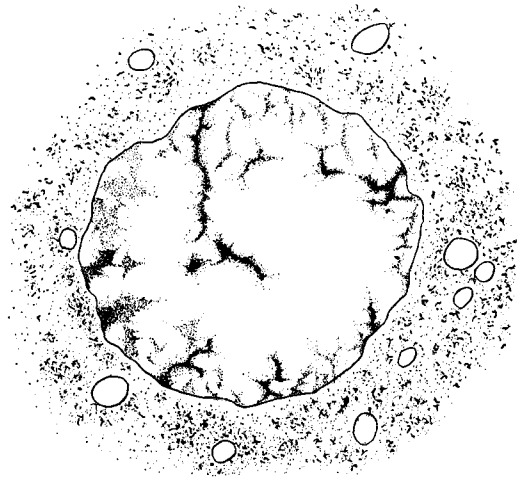
Text-fig. 13. The early stage in the formation of the female pronucleus. The disintegration of chromosomes begins in the nucleus. About $2^1/_2$ hours after insemination. *Z. 2mm : K 15×, ca. ×1500.*



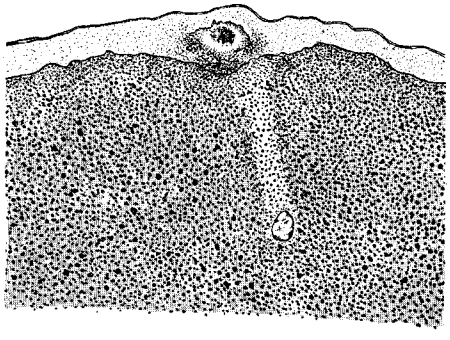
Text-fig. 15. The female pronucleus at the time of commencement of migration from the surface, about $3^3/_4$ hours after insemination. 0.021 mm in long diameter. *Z. 2 mm : K 15×, ca. ×1500.*

1) The process of formation of the pronucleus and its later migration vary in time in different individuals, owing probably to environmental influences.

The female pronucleus thus produced begins to move towards the deeper part of the egg. At the same time it grows considerably, and its form becomes almost spherical (Text-fig. 15 and Figs. 27–28). There are no longer found any chromatin threads in such a swollen pronucleus, except minute irregular pieces of chromatin bound together by delicate linin fibres, adhering closely to the nuclear membrane as shown in Text-fig. 16 and Figs. 27 to 30. Strictly speaking, the size of the female pronucleus shows individual variations by this time, but on the whole, it usually measures from 0.021 mm to 0.028 mm in diameter. The pronucleus lies generally in naked state among the yolk granules, densely surrounded by pigment granules. It is accompanied by neither the idiosome nor any other like structure which may direct its migration.

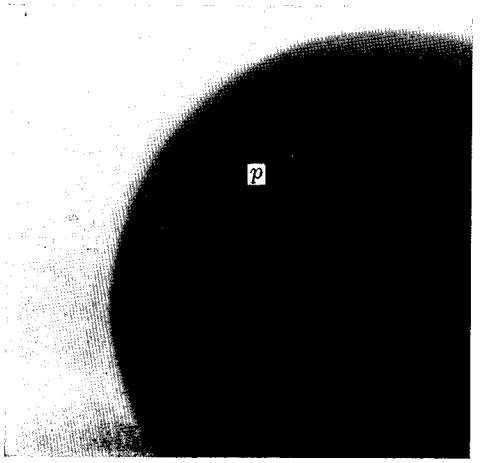


Text-fig. 16. The female pronucleus, located about one-third of the distance from the surface to the egg centre. About $4^1/_2$ hours after insemination. 0.028 mm in diameter. *Z. 2mm : K 15×, ca.×1500.*



Text-fig. 17. Section of an egg about 4 hours after insemination, showing the second polocyte, the movement of the female pronucleus and its route. *ca.×200.*

As a rule, at about $3^1/_2$ to 4 hours after insemination, the female pronucleus is found located about one-third of the distance from the centre to the surface of the egg where the second polocyte has been




Text-fig. 18. Surface view of the animal hemisphere of an egg, about $1^1/_2$ hours after insemination, showing the entrance pit ($p$) of a spermatozoon. The dark spot at the right hand shows a slight depression at the animal pole. From the living egg. *ca.*×*30*.

extruded (Text-fig. 17). The route of migration is indicated by a yolk-thin region marked by a trail of pigment granules, extending from the female pronucleus for a short distance towards the surface.

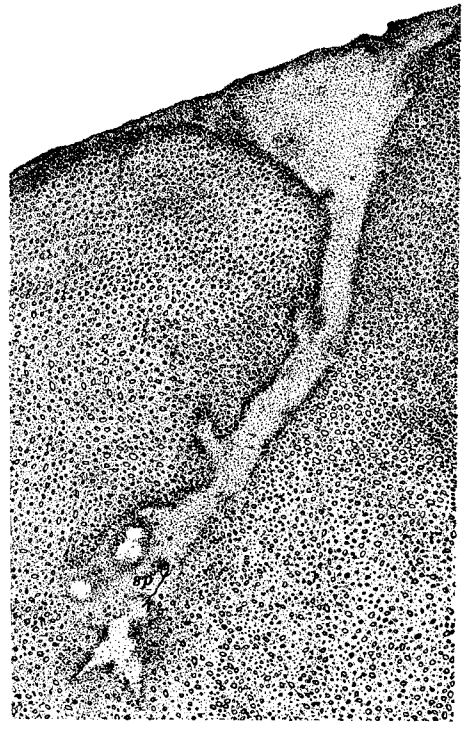
3. *The entry of the spermatozoon and the formation of the male pronucleus*

In the fresh egg, about 40 minutes after insemination, a minute pit can generally be found on the surface of the egg. This is a scar made by the entrance of a spermatozoon (Text-fig. 18, $p$) and one is able to discover it by close observation at low magnification. During 1 to $1^1/_2$ hours after insemination, this pit is visible most sharply but thereafter it gradually fades away. It is, therefore, only possible during these hours to determine from the superficial observation the locality where the spermatozoon enters the egg. Actual examination of 60 eggs naturally fertilized, shows that, in more than 50% of examined cases, the entrance of the spermatozoon is found in the equatorial zone between the animal and vegetable hemispheres of the egg. It seems probable, therefore, that the

Text-fig. 19. Section of an egg about 15 minutes after insemination, showing that a spermatozoon has penetrated through the chorion into the egg. *Photomicrograph*, ×*550*.

equatorial area of the egg may have a condition favourable for penetration of the spermatozoon.

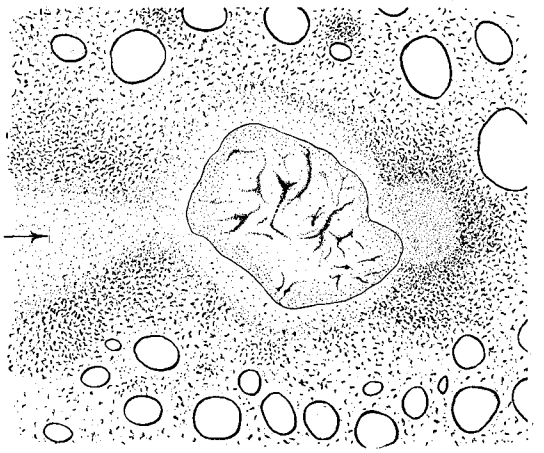
From the study of sections, it appears that the spermatozoon penetrates into the egg as early as 15 minutes after insemination, since in the eggs fixed 15 to 20 minutes thereafter, one can find the spermatozoon which has already gotten into the egg under the chorion as shown in Text-fig. 19. It is remarkable that the spermatozoon can pierce through the tough gelatinous capsule in so short a time. In the section of the egg, about 30 minutes after insemination, there is found a clear zone of cytoplasm in the midst of the yolk, extending inwardly from the surface of the egg at the animal pole. It indicates the route of a spermatozoon which is finding its way towards the centre of the egg (Text-fig. 20). The pigment accumulated underneath the egg surface flows into the deeper part surrounding this route and thereby the latter becomes quite conspicuous in appearance. Occasionally the tail of the spermatozoon is recognizable near the inner extremity of this clear zone imbedded in the cytoplasm (Text-fig. 20, *sp*). At the outer




Text-fig. 20. The yolk-free, clear area on the surface of the egg, caused by entry of a spermatozoon and its route of penetration is demonstrated by a track of pigment flow. The tail of the spermatozoon is seen at the bottom (*sp*). About 30 minutes after insemination. *ca.*×*200*.

extremity this zone widens remarkably so as to assume a funnel shape more or less depressed. It is this part that appears as a minute pit when observed in surface view of the fresh egg. There is no formation of an entrance cone or the like at all in the present material, though SMITH ('12), in the egg of *Cryptobranchus*, found a micropyle-like structure and called it a "pseudo-micropyle".

The spermatozoon enters the egg with its entire body, without breaking off[1] the tail. This is a wide-spread occurrence among the Amphibia so far studied (FICK '93 and JENKINSON '05 in Axolotl, KING '01 in *Bufo*, SMITH '12 in *Cryptobranchus*, KUNITOMO '34 in *Megalobatrachus*). It is highly probable that the tail acts as an efficient organ for propulsion by movement of the undulating membrane so far propelling it through the deutoplasm. Even when the head has metamorphosed into the male pronucleus the tail still remains unaltered in the egg. So, one can frequently clearly discover the sperm-tail which lies very closely to the conjugation-nuclei (Figs. 38–39 and Text-fig. 24, *sp*) or to the spindle of the first cleavage division (Fig. 44 and Text-fig. 28, *sp*)[2]. In this respect the present species evidently differs from



Text-fig. 21. The male pronucleus at beginning of migration, 0.017 mm by 0.012 mm in diameter. The idiosome is seen situated ahead of the nucleus, surrounded thickly by pigment granules. About 1 hour after insemination. The direction of movement is indicated by an arrow.
*Z. 2mm : K15×, ca.×1500.*

1) Recently KAKIYAMA ('34), working on *Hynobius nebulosus*, stated that the sperm-tail may be cast off outside the egg at the entrance. There seems, however, to be no positive observation upon which to base this statement.

2) In the bat, Van der STRICHT ('09) noted a similar example, in which the sperm-tail may clearly be seen in one of the first two blastomeres.

Axolotl (FICK '93), *Bufo* (KING '01) and *Cryptobranchus* (SMITH '12), in which the tail of the spermatozoon remains only for a short time after entry and then is absorbed.

As early as 1 hour after insemination, the spermatozoon is converted into the male pronucleus at the inner extremity of the route of penetration (Text-fig. 21). It is usually more or less oblong in outline with a smooth membrane measuring 0.017 mm by 0.012 mm in its diameters, and filled with clear, colourless nuclear substance. There are found no stained chromatin threads in the nucleus except fine and faintly stained linin fibres, showing no reticular structure. Then the male pronucleus commences to approach to the female pronucleus. At the same time it increases markedly in volume attaining nearly spherical form (Figs. 31–32). At this period, the male pronucleus ordinarily fluctuates between 0.021 mm to 0.026 mm in diameter. The male pronucleus, therefore, now becomes utterly indistinguishable from the female pronucleus so far as its structure and size are concerned. There is present, however, a remarkable character peculiar to the male pronucleus, which enables one readily to distinguish them. That is the new appearance of the idiosome which takes its position immediately ahead of the male pronucleus and consists of finely granulated protoplasm with a spherical outline, free from yolk granules, containing a few vacuoles and faintly taking acidic dyes (Text-fig. 21). Surrounding the idiosome pigment granules are accumulated in a thick layer.

Preceded by the idiosome, the male pronucleus progresses further until it finds the female pronucleus with which to conjugate. The route of the male pronucleus up to conjugation is also marked by a well-defined trail of pigment granules, as is the case in all other amphibian eggs in general.

4. *Polyspermy*

It has been long known that polyspermy is of normal occurrence in the egg of urodelan Amphibia. As a matter of fact, the literature describes the occurrence of polyspermy in various forms of Urodelans,

*e.g.*, Axolotl (FICK '93; JENKINSON '05), *Diemyctylus* (JORDAN '93: KAKIYAMA '31), *Triton* (MICHAELIS '97: BATAILLON '29, '30: FANKHAUSER '25, '32) *Amblystoma* (SMITH '11) and *Cryptobranchus* (SMITH '12). Contrary to this view, KING ('01) in *Bufo* and KUNITOMO ('10) and NAKAMURA ('32) in *Hynobius*, asserted that in these forms of Amphibia, monospermy is natural and polyspermy is to be considered pathological. In the present study, a few cases showed that two or sometimes several spermatozoa are found metamorphosed within an egg. As an extreme case, two male pronuclei conjugated with the female pronucleus to form a triple conjugation (Fig. 49) as described in detail later. As to whether polyspermy is physiological or pathological in the present material, however, it can not yet be decided, since several important problems associated with this subject still remain unsolved.

B. Movements and routes of the pronuclei

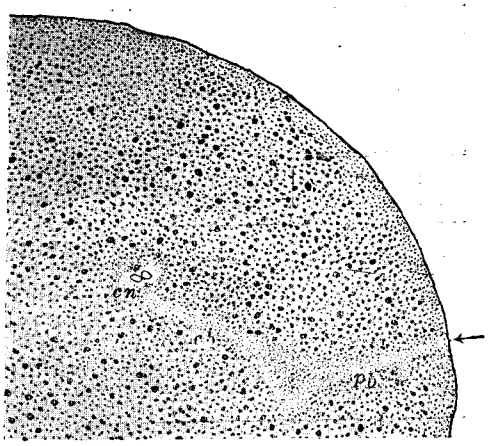
After metamorphosis, the male and female pronuclei begin their migration through the yolk granules towards the interior part of the egg up to the place where they meet.

The route of the spermatozoon is indicated distinctly from its beginning by a trail of pigment granules carried from the peripheral part of egg. It prolongs into the egg centripetally at first, marking the penetration route (Penetrationsbahn), as described by ROUX ('87) in the frog's egg, which is nearly vertical to the surface (Text-figs. 20 and 22, *pb*), and the spermatozoon metamorphoses at its terminal point. Then it curves abruptly towards the female pronucleus, thus trailing the copulation route (Copulationsbahn) of ROUX (Text-fig. 22, *cb*). These two routes often form a considerably wide angle as seen in Text-fig. 22.

The penetration route shows, of course, no constant relation to the position of the female pronucleus and is approximately, though never exactly, radial toward an imaginary point near the centre of the egg. On the contrary, however, the copulation route should be directly affected by the position of the female pronucleus. So it

becomes clear that the copulation route may be modified by the point from which the spermatozoon enters. Moreover, it is not exactly straight in most cases, but more or less curved or undulated, due probably to the movement of the female pronucleus.

The course of movement of the female pronucleus is also marked by a pigment track, though not so remarkable a one as that of the male. All of ten cases so far examined in this study, show the fact that the female pronucleus descends vertically from its original position at the animal pole towards the centre of the egg, approximately along a radius connecting the second polocyte with a point near the centre (Text-fig. 17). The present author still hesitates, however, to decide this point and will return to it again later. At any rate, the course of the female pronucleus does not show a great deviation from the radius and appears to be less extensive than that of the male.




Text-fig. 22. Half-diagramatic figure, showing the route of the male pronucleus, reconstructed from 3 adjacent sections. About 6 hours after insemination. Arrow indicates the point at which the spermatozoon enters. *pb*; the penetration route. *cb*; the copulation route. *cn*; the conjugation-nuelei. *ca.*×35.

In his study of Axolotl, FICK ('93) considered that the two pronuclei approach each other by amoeboid movements, stating, "Die Ortsbewegung geschieht offenbar durch eigene amöboid Bewegungen, denn man sieht häufig am Kern pseudopodienartige Fortsätze". But in the present species its actual occurrence is disproved as KING ('01) stated in *Bufo*. In this respect, WILSON ('25) says, "The causes that determine the movements of the pronuclei during fertilization of the egg are unknown. It was assumed by some of the earlier observers that

approach and union of the nuclei were determined by some kind of attraction between them; but this assumption is very insecurely based and the same may be said of the assumption that they are passively drawn together by the rays of the sperm-aster or by protoplasmic currents in the oöplasm."

## C. Conjugation of the pronuclei

Generally speaking, about 5 to 6 hours after the egg is inseminated, the male and female pronuclei have already come in contact (Figs. 34–42). They lie side by side with the nuclear membrane in intimate contact. The time of meeting of the pronuclei seems to be variable, owing probably to some environmental influences. The individual results examined in thirteen groups of different egg-sacs, under the condition of captivity in the laboratory[1], are summarized in Table 2.

Table 2.

| Time after insemination | Occurrence of conjugation |
|---|---|
| 4½–5 hours | 2 cases |
| 5–6 hours | 7 cases |
| 6–7 hours | 3 cases |
| 7–8 hours | 1 case |

As seen in the table, it appears to be most common that under captivity the meeting of the pronuclei first occurs within 5 to 6 hours after insemination has taken place.

The position at which the two pronuclei meet is never at the geometric centre of the egg, but is eccentric towards the animal pole (Fig. 33, *c* and Text-fig. 22, *cn*). Though not definitive, the following conclusion may be induced from the results obtained by examination of a number of different cases: the two pronuclei usually meet at a distance of about one-quarter to one-third of the egg diameter from the animal pole, and further this meeting-point lies approximately but never exactly along a radius joining the second polocyte and a point near the centre of the egg. The fact that the position

1) The water temperature of the aquaria was kept under about 8°C–9°C, during at least the first 5 hours, while in nature it ranged 6°C–7°C as the most usual environment.

of the conjugated nuclei is inclined to show a somewhat constant relation with regard to the egg-axis, can naturally be suggested, on the one hand, from the evidence that the female pronucleus descends only vertically from its original position at the animal pole towards the centre of the egg. The centrospheres appear on opposite sides in the plane of contact of the nuclei.

The pronuclei thus conjugated are quite alike apparently in their structure, size and staining capacity at the time of conjugation and there are found no characteristic indications by which they may be distinguished from each other (Figs. 34–36). It is hardly possible to tell with certainty, therefore, which is the female pronucleus and which is the male. When favourably preserved, both pronuclei are approximately round in outline with smooth membranes. In some cases, the nuclear surface is irregularly wrinkled and indented (Fig. 36). This irregularity of outline, however, is no doubt due to the mechanical effect of fixing.

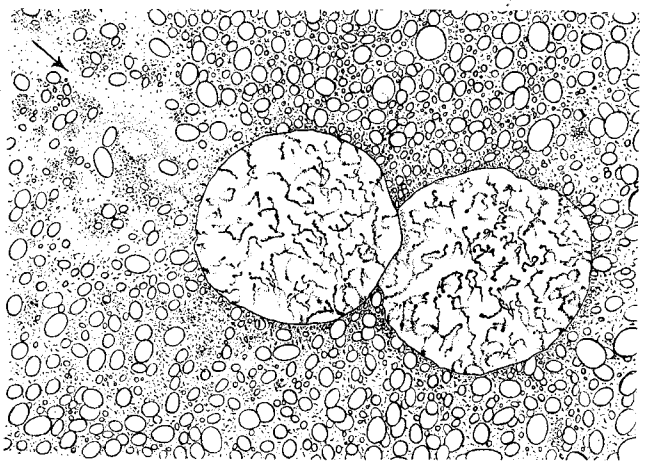
At the period of meeting, both of the pronuclei are seen much larger than in course of migration, measuring from 0.038 mm to 0.042 mm in diameter as the usual case. As an extreme case there are found pronuclei of as huge size as twice the diameter of the ordinary case, as seen in Figs. 37 and 40. Such exceptional occurrences in the size of the pronuclei, as naturally supposed, seem to be due only to individual variation.

### D. History of the chromosomes after karyogamy and formation of the first cleavage spindle

After the two pronuclei, the paternal and the maternal, come in contact, no actual fusion of them occurs at all, but they keep themselves in close contact distinctly separated by nuclear membranes, though the contacting surface attains a large dimension. Still more this condition remains unaltered throughout the whole period in preparation for the first cleavage.

As previously noted, at the time of meeting the male and female pronuclei are so alike in appearance that it is usually impossible to

distinguish them. In the early stage of conjugation the two pronuclei are stained rather faintly and the astral system is not yet developed (Text-fig. 23 and Fig. 34). They always lie in a plane parallel to the equator. The nucleus is filled with clear nuclear substance in which the chromatin threads, coiled to a more or less degree, are irregularly scattered along the nuclear membranes, as illustrated in Text-fig. 23. Surrounding the conjugation-nuclei[1], the yolk granules come directly in contact, with a thick deposition of pigment granules

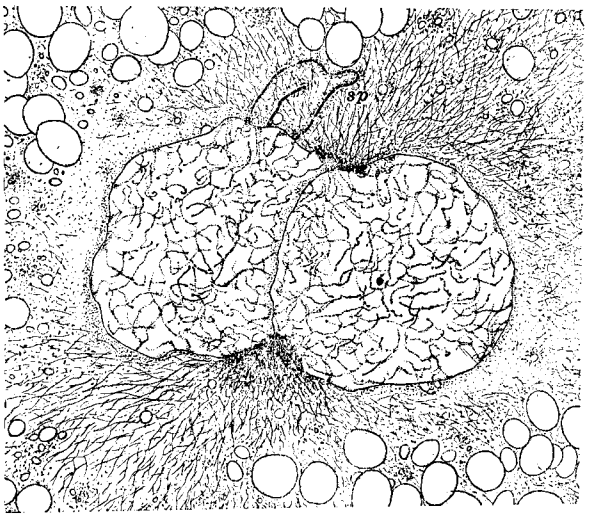


Text-fig. 23. The conjugation-nuclei at the earliest stage; the astral system not yet developed. Arrow denotes the route of the male pronucleus. About 6 hours after insemination. The same as Fig. 34 (Pl. X).
*Z. 3mm: K10×, ca.×700.*

on their surface. Sooner or later the astral system develops out of the two centrospheres, which possibly descended from the idiosome appended with the male pronucleus, and take positions on opposite sides of the conjugating plane of the nuclei (Text-fig. 24). The astral rays develop out radially in every direction from the centrospheres,

1) The term 'conjugation-nuclei' is employed in the present paper to denote the nuclei which never fuse into a single nucleus after karyogamy, in contrast to the 'fusion-nucleus' (WILSON '25, p. 396) in which a complete fusion takes place.

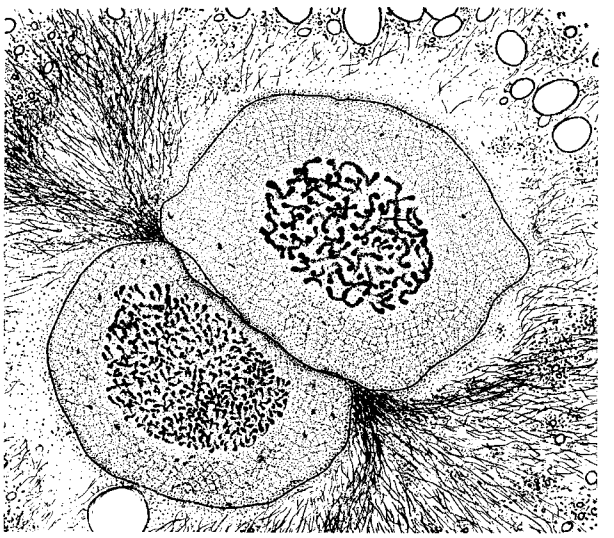
as if they would push away the yolk and pigment granules (Figs. 35–37.) By this time the chromatin threads in each nucleus become more distinct and sharply defined by taking stains. In the succeeding stage, a remarkable and characteristic change takes place in the conjugation-nuclei; the chromatin threads thicken a little and become aggregated into a confined central area of each nucleus (Text-fig. 25 and Fig. 40). The threads seem to be extremely long, and convoluted




Text-fig. 24. The conjugation-nuclei at a little later stage; the astral system has developed from two centrospheres which take positions on opposite sides of the conjugation-nuclei, close to the region of contact. The sperm-tail (*sp*) is visible at this meeting place. About 6 hours after insemination. *Z. 3mm : K 10×, ca.×700.*

with one another. By no means is one capable of counting the actual number. In this condition, the threads undergo gradual shortening and thickening in correspondence with the increase in staining capacity (Fig. 41). The process of transformation of spiremes mentioned above does not take place simultaneously in the two pronuclei, of which one is usually somewhat in advance of the other (see Text-fig. 25 and Fig. 40). When the condensation process advances further

in the spiremes there appear narrow chromosomes of uniform thickness (Text-fig. 26 and Fig. 42). The chromosomes are varied in length to a considerable degree, and still present convolutions and twisting as in the preceding stage. The number of chromosomes can possibly be counted at this stage, showing approximately the haploid number in each pronucleus respectively. Then the chromosomes begin to lie on the equatorial plane embraced by the two centrospheres (Text-fig. 27, see the nucleus to the right). Shortly

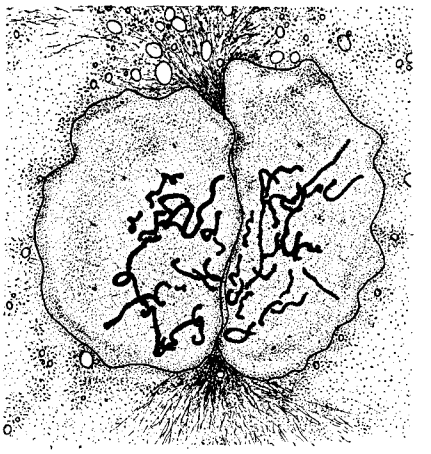


Text-fig. 25. The conjugation-nuclei in preparation for the first cleavage mitosis, horizontal section. The right hand nucleus is in a more advanced stage than the other. About $6^1/_2$ hours after insemination. The same as Fig. 40 (Pl. XI). *Z. 3mm : K10×, ca.×700.*

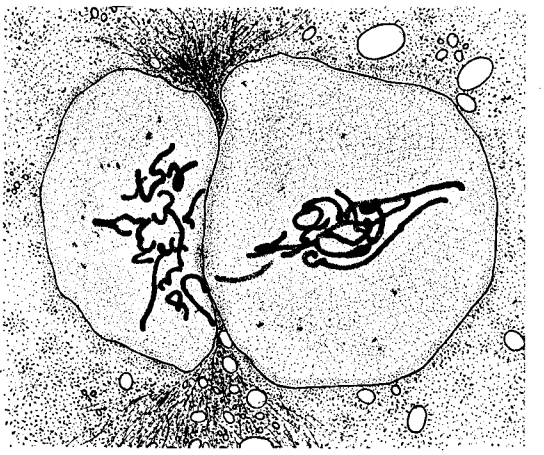
later, the nuclear membrane disappears, and the formation of the spindle is completed as the metaphase of the first cleavage division sets in (Fig. 43). The chromosomes are formed quite independently in each pronucleus, paternal and maternal, but they are arranged at last upon one and the same equatorial plane in connection with the common centrospheres. This has been determined beyond question

in a number of cases and there are not involved any conflicting evidences.

In the fully formed metaphase figure two different groups of chromosomes, paternal and maternal in their origin, still remain distinctly separated as is most clearly recognizable in lateral aspect (Text-fig. 28 and Fig. 44). In the anaphase, each group of chromosomes has divided into equal halves respectively, through the longitudinal splitting of individual chromosomes. In the telophase the individual chromosomes become enclosed in the vesicles, thus forming the karyomeres, and by the fusion of the latter the telophase nucleus is reconstructed (Fig. 45). Each daughter nucleus thus produced, again reveals double nature to some extent because of its distinct bilobed structure which suggests the paternal and maternal groups of chromosomes respectively (see Figs. 46 to 48).

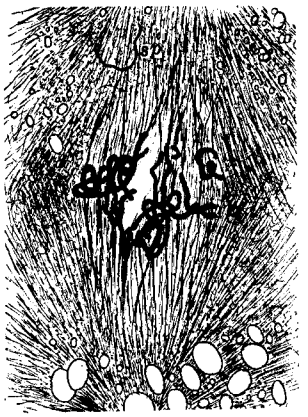


Text-fig. 26. The conjugation-nuclei in preparation for the first cleavage mitosis in a more advanced stage than the former figure, horizontal section. About $6^1/_2$ hours after insemination. The same as Fig. 42 (Pl. XI). *Z. 3mm : K10×, ca.×700.*



Text-fig. 27. The conjugation-nuclei just before the formation of the first cleavage spindle, horizontal section. In the nucleus at the right hand, the chromosomes begin to take the equatorial arrangement. About 7 hours after insemination. *Z. 3mm : K10×, ca.×700.*

Usually the first cleavage spindle has been found in the process of division in an egg fixed about 7 hours or more after insemination of the spermatozoon. About 8 to 9 hours after insemination the first cleavage furrow has ordinarily appeared. The furrow begins first as a pit at the animal pole, then it gradually grows towards the vegetable pole of the egg (Fig. 50).



Text-fig. 28. The first cleavage spindle, meta-anaphase, side view. The chromosomes are separated into two distinct groups. A piece of the sperm-tail (*sp*) is observed near the spindle. About $7^1/_2$ hours after insemination. The same as Fig. 44 (Pl. XI). *Z. 3 mm*; *K10×*, *ca.×700.*

### III. *Anomalies*

During the course of investigation, the author has encountered two cytological anomalies; one is the triple conjugation of pronuclei and the other is the multipolar spindles in the cleavage mitoses.

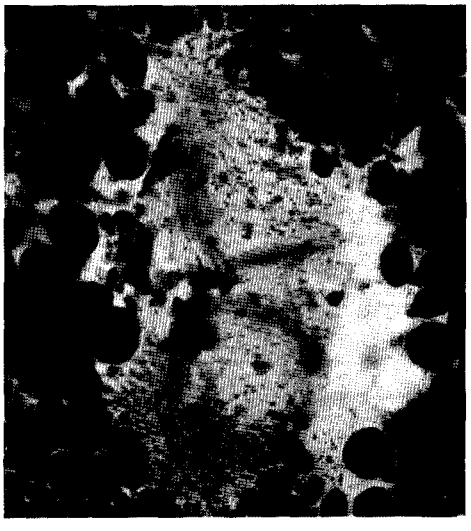
1). The case of the triple conjugation was found in an egg about 7 hours after insemination; three pronuclei come in contact simultaneously (Fig. 49). As in the normal case, the chromosomes develop independently in each pronucleus. The position occupied within the egg, does not differ from that of the normal case. As generally supposed, one of the three pronuclei is likely to be of female origin and the remaining two to be male. It is not inappropriate to consider that this triple conjugation may be caused by polyspermic fertilization. In fact, as previously noted, the supernumerary spermatozoa are occasionally found metamorphosed within an egg, and it may be a possible occurrence that two of these male pronuclei should come in contact with the female pronucleus.

If the egg under this condition should be capable of further development, it is still a matter of controversy whether the triple conjugation-nuclei would explain the origin of the triploid animal,

since it is exemplifi d by the sea-urchin egg[1] that the triploid or polyploid fusion-nucleus caused by pathological polyspermy, gives rise to the tripolar or quadripolar mitotic figures, resulting in the irregular distribution of chromosomes.

So far as the author is aware, such a case as the present triple conjugation, two male pronuclei and one female pronucleus, has been described by no authors in Amphibia, although the physiological polyspermy has usually been known in the urodelan egg. In the latter case, the supernumerary male pronuclei undergo degeneration without further development, as proved in *Triton* by FANKHAUSER ('32). On the other hand, pathological polyspermy was demonstrated in the frog's egg by HERLANT ('11) who concludes that the male pronuclei conjugate with one another resulting in harmful influence upon the development of the egg.

2). The two examples of quadripolar mitosis were found in the blastomeres of a single egg in the 8-cell stage which is naturally fertilized. In both cases, the four poles always lie nearly in the same plane (Text-fig. 29).



Text-fig. 29. Quadripolar mitosis, from the egg of the 8-cell stage. *Photomicrograph. ca.*×*550.*

The possible causes that give rise to the multipolar mitosis are utterly unknown in the present case. In the remaining blastomeres except the abnormal two, the division is normal. Recently, SMITH ('29) reported a case of quadripolar spindle in the segmenting egg of *Cryptobranchus*, describing it as caused probably by some injury to the egg.

1) For details, see WILSON ('25), p. 419.

## Discussion

### I. *The dissolution of the germinal vesicle and the formation of the first polar spindle*

In one of the series of memoirs dealing with the maturation phenomena of the amphibian egg, CARNOY and LEBRUN ('99) followed carefully, in the egg of *Triton*, the history of the chromosome formation of the first polar spindle through the disintegration stages of the germinal vesicle. According to their view, when the membrane of the germinal vesicle disappears, the chromatin threads, which have already appeared in the germinal vesicle, break into small chromatin granules. Then they are carried to the "*plage fusoriale*", a special portion of the germinal vesicle, which first lies at the lower pole of the germinal vesicle, and later produces radial rays. Next, these chromatin granules fuse into a large mass of irregular shape lying in the "*plage fusoriale*". A little later the large chromatin mass gives rise to filamentous chromosomes of a definite number, which are very irregular in shape at first, but gradually convert into bivalent chromosomes of regular shape, and scatter along the spindle fibre for provision of the first polar division. So far as the egg of *Hynobius retardatus* is concerned, the chromatin threads do not show, during corresponding stages, such complicated changes, as described by CARNOY and LEBRUN, but they persist without breaking up into granules. There is not found, in any stages so far examined, any slightest evidence of clumping of the chromatin elements into an irregular mass as figured by CARNOY and LEBRUN ('99). The chromatin threads in the present material seem to convert directly by shortening and thickening into tetrad forms before dissolution of the geminal vesicle, in quite a similar way to what is generally observed in the maturation process of the male germ cells.

KING ('01) stated, in the study concerning the maturation of the toad's egg, that before the disintegration of the germinal vesicle begins, a portion of the egg cytoplasm near the lower pole of the nucleus forms the "line of radiation", a thick and fibrous band.

She traced the structural changes occurring in the "line of radiation" and its rôle during the dissolution stages of the germinal vesicle. In the present investigation, however, no such particular structure is found in any of the stages of dissolution in spite of careful observation. From all evidences, the changes observed during the dissolution stages of the germinal vesicle in the egg of *Hynobius retardatus*, rather resemble those recorded by FICK ('93) in the egg of Axolotl.

## II. *On the karyogamy in the Amphibia*

WILSON ('25) classified the mode of fertilization into two main extreme types with respect to the behavior of the male and female pronuclei after conjugation in the egg; the one is the sea-urchin type represented by the sea-urchin egg, in which the pronuclei conjugate immediately after entrance of the sperm and apparently fuse completely to form a fusion-nucleus. The other is *Ascaris* type illustrated by *Ascaris megalocephala* (Van BENEDEN, '83–'84, see WILSON '25), in which no direct mingling of structural contents occurs between two pronuclei in spite of intimate contact, but they remain in distinct groups as paternal and maternal elements enclosed separately by nuclear membranes throughout the whole fertilization process.

Looking over the literature one finds that two different modes of fertilization are described as occurring in the amphibian egg by previous observers. MICHAELIS ('97) seems to be the first author who notes the complete fusion of two pronuclei in the egg of *Triton*. In the toad's egg, KING ('01) described how, soon after meeting, two pronuclei fuse completely. Similar statement is given in *Hynobius* egg by KUNITOMO ('10), stating as follows: "Bei dem noch weiter entwickelten Eie erscheinen die Kernmembranen an der einander berührenden Partie in schwindendem Zustand und am übrigen Teil faltig. Nach kurzer Zeit erfolgt dann die Vereinigung der beiden und weiter entsteht der neue Furchungskern".[1] On the contrary, FICK ('93)

1) A similar description has been given by KAKIYAMA ('31, '32), in the study on *Diemyctylus* and *Bufo*. But these two authors (KUNITOMO and KAKIYAMA), in spite of their description, seem not to have observed directly the actual figure of the fusion-nucleus in their material.

and JENKINSON ('05) in Axolotl, and SMITH ('12, '19) in *Cryptobranchus*, maintained that the male and female pronuclei do not actually fuse after coming in contact, but remain side by side with nuclear membranes intact. Still further, SMITH ('19) traced the individuality of the paternal and maternal nuclei throughout the cleavage as late as the gastrula, in the egg of *Cryptobranchus*. In brief, *Triton* (MICHAELIS '97), *Bufo* (KING '01) and *Hynobius* (KUNITOMO '10) belong to the sea-urchin type, while Axolotl (FICK '93; JENKINSON '05), *Cryptobranchus* (SMITH '12, '19) and the present *Hynobius* to the *Ascaris* type.

WILSON ('25) says "the difference between the two foregoing types (the sea-urchin type and the *Ascaris* type of fertilization) is determined mainly by the time element, *i. e.*, the length of the interval between entrance of the sperm and conjugation of the pronuclei". In the sea-urchin type the sperm enters the egg after the completion of the maturation processes; consequently the interval between entrance of the sperm and nuclear union is extremely shortened. In the *Ascaris* type, on the contrary, this interval is greatly prolonged owing to the precocious penetrance of the sperm within the egg, before the extrusion of polocytes. In such a case, the later stages of maturation should naturally be overlapped by the earlier stages of fertilization. So WILSON's conception may be paraphrased as "the difference between the sea-urchin type and the *Ascaris* type is correlated to the time of polocyte formation and entrance of the sperm".

Next, the relations between maturation and fertilization must be considered in the amphibian egg. In all cases observed, either in Anurans or in Urodelans, entry of the sperm takes place in the metaphase stage of the second polar spindle after the first polocyte has been extruded from the egg[1]. The metaphase stage of the second polar spindle can proceed in its further course only after the

1) This condition seems to be of rather widespread occurrence among the vertebrates, *e.g.*, *Amphioxus* (SOBOTTA '97), *Petromyzon* (BÖHM '88), the trout (BEHRENS '98), the mouse (SOBATTA '95), the rabbit (YAMANE '30) and the bat (Van der STRICHT '02).

sperm enters the egg. That is, the formation of the second polocyte absolutely depends upon the entrance of the sperm. Therefore, the maturation and the fertilization eventually overlap with each other in the amphibian egg.

Now, the problem will be renewed here whether both different types of fertilization exist in reality in Amphibia as the literature shows. Considered from the fact, on the one hand, that the difference between these two types of fertilization is due to the relations between the time of polocyte formation and entry of the sperm, and from the evidences, on the other hand, obtained from various forms of vertebrates as well as invertebrates,[1] the present author inclines to adopt the opinion that the amphibian egg belongs in general to the *Ascaris* type in mode of karyogamy. In fact, in most cases hitherto observed in Amphibia under favourable condition the processes of maturation are known to be overlapped by fertilization as in the case of the present study. It will follow then that the mode of karyogamy may consequently be the same as the present case, in which the *Ascaris* type was established.

So far as *Hynobius retardatus* is concerned, the present observations establish beyond a question the complete separation of paternal and maternal conjugation-nuclei, throughout the preparing stages for the first cleavage mitosis. To prove the double nature of the conjugation-nuclei the horizontal sections of eggs should be used, since one likely overlooks its occurrence, so long as only vertical sections were employed. In this respect, the results recorded by

1) Examples of the *Ascaris* type, and the relation between polocyte formation and sperm entrance in them are as follows: The sperm enters the egg, in *Ascaris* (BOVERI '87), *Cyclops* (RÜCKERT '95), *Polystomum* (GOLDSCHMIDT '02), *Nereis* (LILLIE '12), before either polar division has been accomplished; in *Crepidula* (CONKLIN '01), *Sagitta* (BOVERI '90), *Physa* (Von KOSTANECKI '96), *Chaetopterus* (LILLIE '06, MEAD '98), *Oploryotrocha* (KORCHELT '95), after the first polar spindle is fully formed and advances as far as the metaphase or sometimes the anaphase and then there is a pause until the sperm enters the egg; in *Thalassema* (WILSON '25, p. 402), *Amphioxus* (SOBOTTA '97), Axolotl (FICK '93), *Diemyctylus* (JORDAN '93), *Mus* (SOBOTTA '95) rabbit (YAMANE '30) bat (Van der STRICHT '02), when the first polocyte has been extruded from the egg and the second polar spindle formed. Details and review of the literature, see KORSCHELT und HEIDER '03 and WILSON '25.

MICHAELIS ('97), KING ('01) and KUNITOMO ('10) seem not to be satisfactory.

### III. *On the double nature of the cleavage nucleus*

There have been found in animals some evidences in the earlier stages of development in which the double structure of the nuclei is observable, each of the components being considered to consist of paternal and maternal elements respectively. The most classical and well-known example is that discovered in certain copepods of the genus *Cyclops*. HÄCKER ('95) and RÜCKERT ('95) found that in the species of *Cyclops* the two groups of paternal and maternal nuclei remain separated not only during the first cleavage, but also in later stages. In the early development of the gasteropod *Crepidula*, CONKLIN ('01) proved conditions quite identical with those observed in the copepods stating, "I have observed the double character of the nucleus in the telophase of every cleavage up to the 29-cell stage, and in several of the later cleavages up to the 60-cell stage, though it becomes more difficult to see as the nuclei grow smaller." BEARD ('02) likewise found in an Elasmobranch, *Raja batis*, a double structure of the resting stages of the nuclei in the embryonal cells later than the gastrula stage. Recently SMITH ('19) has ascertained, without the slightest doubt, in an urodelan Amphibian, *Cryptobranchus*, the complete separation of maternal and paternal germ-nuclei up to a late blastula stage.

In *Hynobius retardatus* the paternal and maternal nuclear elements not only remain separate groups enclosed by different nuclear membranes, to which they adhere very closely, during the stages of the first cleavage mitosis, even in the equatorial plate, but also give rise to a bilobed daughter-nucleus, each of which lobes indicates the original two kinds of the nuclear components. These bilobed nuclei of the 2-cell stage again divide and thereafter the bilobed nuclei are produced again in the 4-cell stage and occasionally even in the 8-cellstage. Though impossible to follow in absolute clearness in the

stages later than the 8-cell, this bilobed structure of the nuclei seems to become gradually inconspicuous after repeated segmentation. Whatever the case may be, the duality of the conjugated nuclei appearing in the earlier stages of development, may be no more than the exhibition of "a tendency on the part of the chromosomes to remain in separate maternal and paternal groups during a part of the earlier development" as WILSON ('25) considers.

## Summary

I. *Maturation*:

1. In the egg taken from the ovary at the breeding season of the animal, the germinal vesicle has generally occupied a position about midway on a radius through the animal pole. It is almost oval in outline and finely but densely granular in appearance. The slender chromatin threads, intertwined with one another, are found aggregated near the central part of the vesicle. The individual thread is made up of two finer ones which twist about each other at one or more points, showing the bivalent nature. A large number of nucleoli of various sizes are distributed almost throughout the vesicle.

2. At the time when the egg is about to leave the ovary, the germinal vesicle has located close to the periphery of the egg. The disintegration of the germinal vesicle is initiated by breaking down of the border at a certain part nearest the periphery of the egg, and this rupture causes the outflow of the vesicular contents. During these changes the nucleoli likewise undergo disintegration and fade away.

3. The débris of the germinal vesicle changes into a reticular structure.

4. In parallel to dissolution of the germinal vesicle, the chromatin threads begin to convert into bivalent chromosomes of definite shape. The chromosomes are confined to a certain flattened area

and in this condition they move nearer to the egg periphery in preparation for the first polar division.

5. The first polar spindle is formed when the eggs are at the upper part of the oviduct (Text-fig. 1, II) and advancing in course of division during their passage down the middle parts of the oviduct (Text-fig. 1, III-IV) the division is completed about the time when the eggs reach the lower parts of the oviduct (Text-fig. 1, IV). The spindle lies close to the egg periphery, with its axis perpendicular, or somewhat oblique, to the latter.

6. The first polocyte is found in a shallow depression on the egg surface and is strongly flattened with round or oval outline, having a diameter of 0.042–0.050 mm when fresh.

7. There is a resting stage of short duration between the first and second polar divisions; at this time the vesicular nucleus is formed.

8. The second polar spindle appears when the egg reaches the lower part of the oviduct (Text-fig. 1, IV) and advances as far as the metaphase. In this condition the eggs pass down the oviduct to the lowest part where they persist until spawning takes place (Text-fig. 2, VI).

9. The second polar division is arrested in metaphase for a considerable length of time, usually extending from the evening to the following morning, until spawning and entry of the spermatozoon occur.

10. The haploid number of chromosomes is twenty either in the first or in the second polar divisions.

11. In both maturation divisions, there is found no trace of centrosome at the centre of convergence of the spindle fibres.

II. *Fertilization*:

1. About 1 to $1^1/_2$ hours after insemination of the spermatozoon the second division is completed and half an hour later the second

polocyte is fully formed. The second polocyte is apparently similar in shape and feature to the first polocyte but somewhat smaller.

2. About $2^1/_2$ to 3 hours after entry of a spermatozoon, the female pronucleus is found metamorphosed at the periphery of the egg. It is of oval shape, with clear contents enclosed by a smooth nuclear membrane.

3. As the later processes of maturation advance only after penetrance of the spermatozoon, they are naturally overlapped by the earlier stages of fertilization.

4. After metamorphosis, the female pronucleus begins to move back towards the centre of the egg. During this migration it gradually undergoes a considerable increase of size.

5. The spermatozoon seems to enter the egg as early as 15 minutes after insemination. There is no evidence of the formation of an entrance cone.

6. The spermatozoon enters the egg with its entire body, the tail being taken into the egg too. The sperm-tail is frequently found near the meeting place of two pronuclei or the first cleavage spindle.

7. The male pronucleus is found already metamorphosed within 1 hour after penetrance. The migrating male pronucleus is entirely like in its structure and appearance to the female pronucleus, but is able to be distinguished from the latter by the presence of the idiosome. During migration it grows to a considerable degree.

8. The penetration route of the spermatozoon and the copulation route of the male pronucleus are marked by a trail of pigment granules among the yolk granules; the former is nearly perpendicular to the egg surface and forms a wide angle to the latter.

9. The conjugation of the pronuclei generally occurs during a period of 5 to 6 hours after insemination. The position in which the two pronuclei meet, is eccentric towards the animal pole; they meet at the distance of about one-quarter to one-third of the egg diameter

from the animal pole, along approximately the egg-axis through the second polocyte and the centre of the egg.

10. At the time of conjugation the pronuclei are quite similar in their structure, size and staining capacity; they are nearly round with smooth nuclear membranes. They vary from 0.038 mm to 0.042 mm in diameter.

11. After the two pronuclei come in contact, they do not actually fuse, but lie side by side in close contact separated by the nuclear membranes. The paternal and maternal nuclear elements remain in distinct groups during the preparatory stages for the first cleavage division and the chromosomes are formed independently in the respective nuclear vesicles.

12. In the egg about 7 hours after insemination, the first cleavage spindle is usually found in the process of division. One or two hours later the first cleavage furrow ordinarily appears.

13. In a few cases supernumerary spermatozoa are found metamorphosed in the egg.

14. Two cytological anomalies are encountered; the one is the triple conjugation of pronuclei and the other is the multipolar spindles in the cleavage blastomeres.

*May, 1934*

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# Plate VIII

## Explanation of Plates VIII–XI

All figures are photomicrographs of sections of the eggs:

### Plate VIII

Fig. 1. Meridional section through the animal pole of an ovarian egg, showing the position of the germinal vesicle. ca. ×50.

Fig. 2. Meridional section through the animal pole of an egg from the body cavity, showing the position of the germinal vesicle and the first step of its disintegration. ca. ×60.

Fig. 3. Meridional section through the animal pole of an egg, showing the germinal vesicle just ruptured. ca. ×60.

Fig. 4. Magnified view of the germinal vesicle located near the egg periphery, showing the position of the chromatin threads and the nucleoli. ca. ×100.

Fig. 5. Chromatin threads aggregating at the central position of the germinal vesicle of the ovarian egg at the stage of Fig. 1 and numerous nucleoli scattered among threads. ca. ×400.

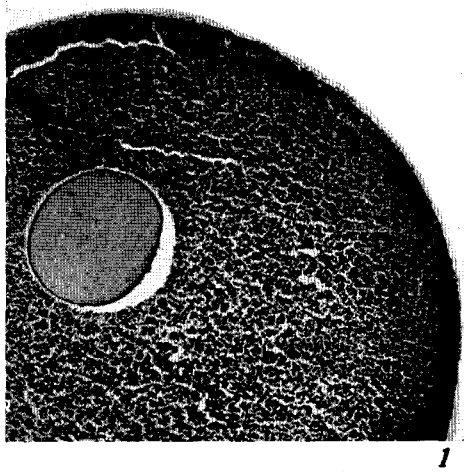
Fig. 6. Magnified view of a portion of the germinal vesicle of an ovarian egg, showing the border of the vesicle before its disintegration. ca. ×430.

Fig. 7. Magnified view of a portion of the germinal vesicle, showing disintegration occurring in the border. ca. ×400.

Fig. 8. Enlarged view of the upper portion of an egg just after the rupture of the germinal vesicle, showing the distribution and nature of the nuclear substance, and the condition of the chromatin elements. The same stage as Fig. 3. ca. ×400.

Fig. 9. Succeeding stage to Fig. 8, showing the further disintegration of the débris of nuclear substance and the condition of the chromosomes just before the formation of the first polar spindle, lying close to the periphery. From an egg taken from the uppermost part of oviduct. ca. ×400.

Fig. 10. Section of an egg taken from the upper part of oviduct, showing the first polar division metaphase, oblique view. Successive stage to Fig. 9. ca. ×400.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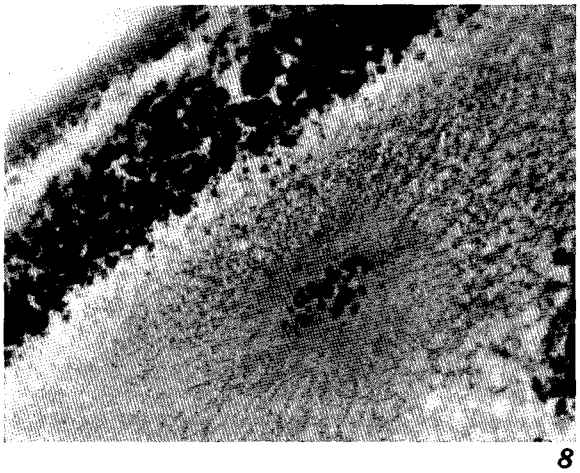
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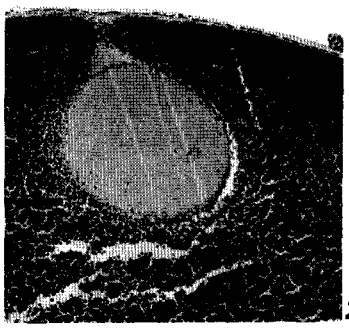
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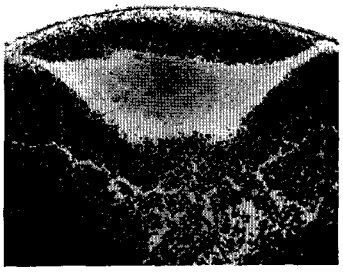
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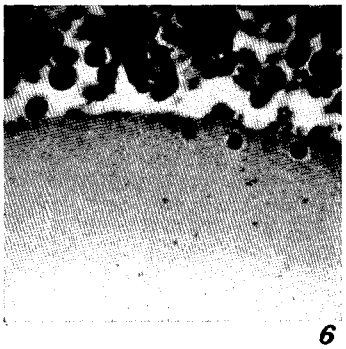
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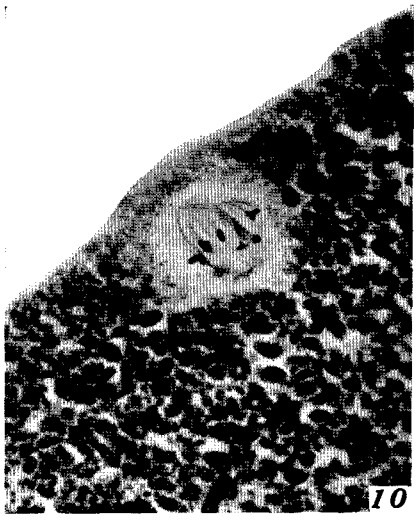
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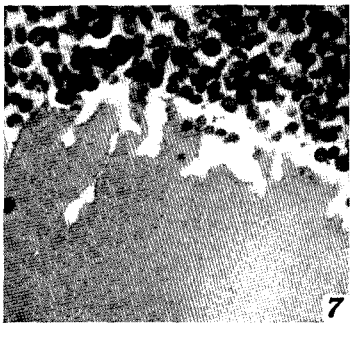
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*S. Makino photo.*

# Plate IX

## Plate IX

Figs. 11–12. Sections of eggs taken from the upper parts of oviduct, showing the first polar division metaphases, oblique views. Successive stage to Fig. 9. ca. ×400.

Figs. 13–15. Sections of eggs taken from the lower parts of oviduct, showing the first polar division telophases. Fig. 15 shows the polocyte about to constrict off, in which the clumping of the chromosomes has taken place; the mid-bodies are seen in the middle part of the spindle. The pigment granules are migrating to the polocyte. ca. ×400.

Fig. 16. The successive stage to Fig. 15. The first polocyte is completely separated. The chromosomes fuse together into a condensed mass. ca. ×400.

Fig. 17. Somewhat oblique view of the first polocyte just extruded. The chromosomes are about to fuse. The membrane surrrounding the polocyte is distinctly visible. ca. ×400.

Fig. 18. A condensed mass of sister chromosomes (telophase nucleus) in the egg after the first polocyte is extruded, polar view. ca. ×400.

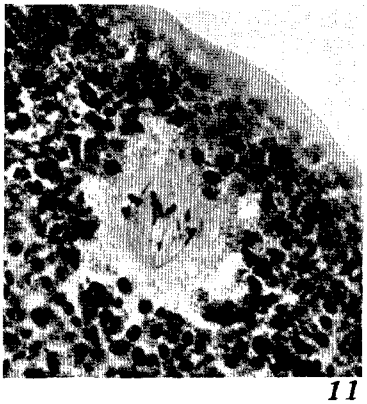
Fig. 19. Section of an egg taken from the lowest end part of oviduct, showing the second polar division metaphase, side view. ca. ×400.

Fig. 20. The same, showing the polar view of the second polar division metaphase. The chromosomes are arranged radially in the equatorial plate. ca. ×400.

Fig. 21. Section of an egg about 20 minutes after insemination, showing the metaphase stage of the second polar division. The first polocyte (I) is seen in course of disintegration, lying in the slight depression of the egg surface, apart about 0.07 mm distant from the second polar spindle. ca. ×400.

Figs. 22–24. Telophase stages of the second polar division, lateral views. In Fig. 22, a scrap of the first polocyte (I) is seen overlying the second polar spindle. In Figs. 23-24, pinching off of the second polocyte is demonstrated. From eggs about 1½ hours after insemination. ca. ×400.

Fig. 25. Dorsal view of the second polocyte, 0.038 mm in long axis. The chromosomes are seen fused at the central region. Pigment granules and yolk granules are contained. From an egg about 1½ hours after insemination.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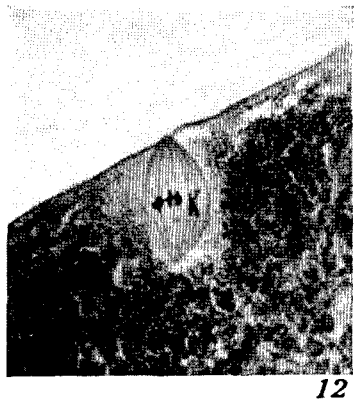
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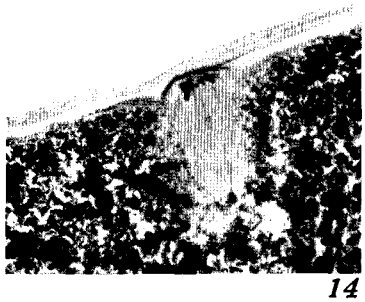
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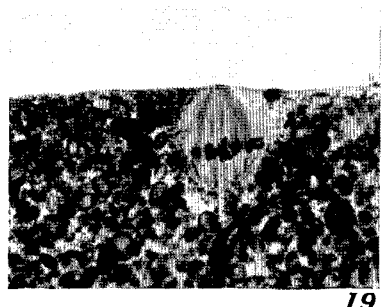
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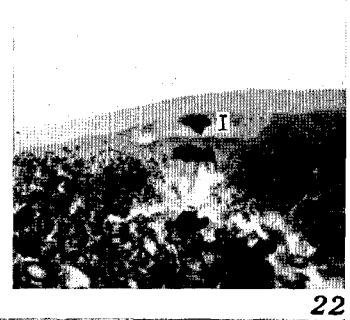

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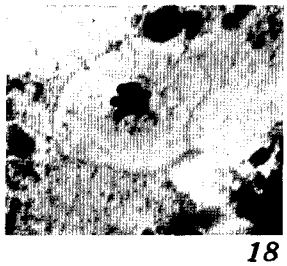
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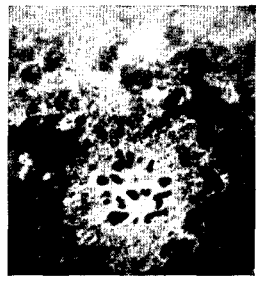
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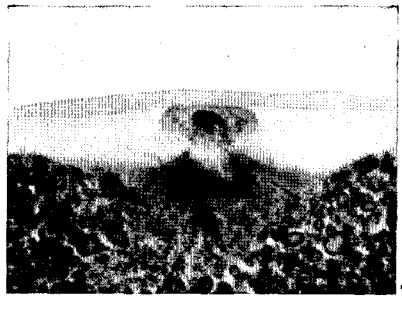
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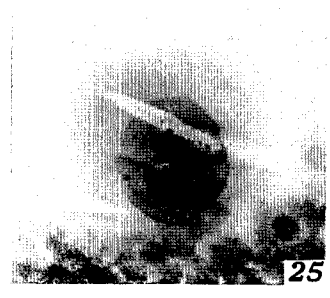
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S. Makino photo.

# Plate X

## Plate X

Fig. 26. Section of an egg about $2\frac{1}{2}$ hours after insemination, showing the sister chromosome group left in the egg, after the second polar division is completed. The chromosomes are found fused together into a mass lying close to the egg surface. The stage previous to the formation of female pronucleus. ca. ×630.

Figs. 27–28. The female pronuclei which have migrated a little inward from the surface. From sections of eggs about $3\frac{1}{2}$ hours after insemination. The black spot denoted by × in Fig. 28 is caused by a piece of dust in the preparation. ca. ×430.

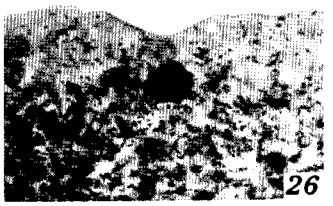
Figs. 29–30. The female pronuclei at the migration stage. From sections of eggs about $3\frac{3}{4}$ hours after insemination. ca. ×430.

Figs. 31–32. The male pronuclei at the migration stage. From sections of eggs about 4 hours after insemination. ca. ×430.

Fig. 33. Meridional section through the animal pole of an egg, showing the meeting of the pronuclei (*c*) which lie eccentrically towards the animal pole. About 6 hours after insemination. ca. ×60.

Figs. 34–36. The male and female pronuclei in close contact (conjugation-nuclei). Fig. 34 shows the earliest stage of conjugation; arrow indicates the route of the male pronucleus. Figs. 35 and 36 show a little later stage; the astral system has developed. From eggs about 6 hours after insemination. ca. ×430.

Figs. 37–38. Two successive sections, showing the conjugation-nuclei. Fig. 37 shows an exceptional case of nuclear size, nearly twice the usual one in diameter; one of the conjugation-nuclei is partly cut off. Fig. 38 shows the sperm-tail (*sp*), lying in the immediate proximity of the meeting place. From an egg about 6 hours after insemination. ca. ×400.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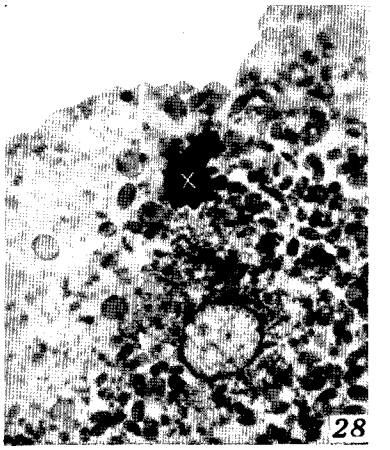
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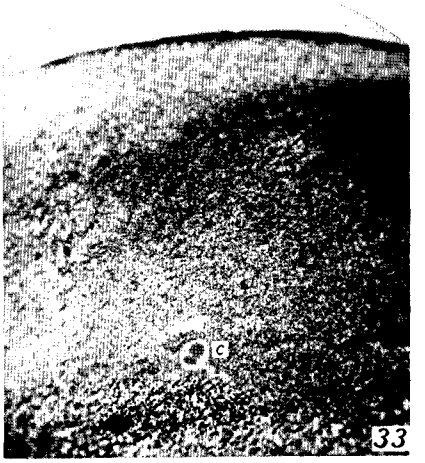
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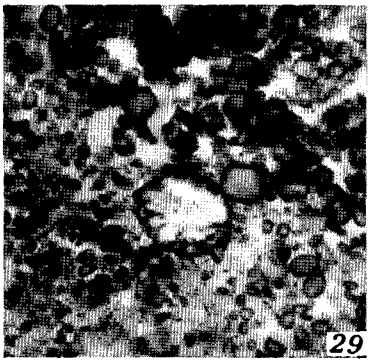
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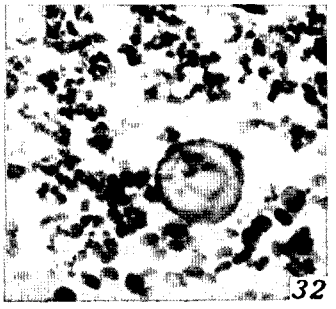
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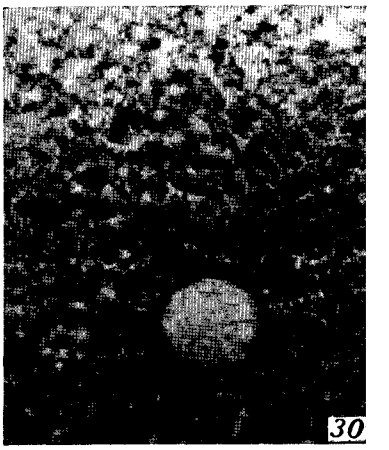
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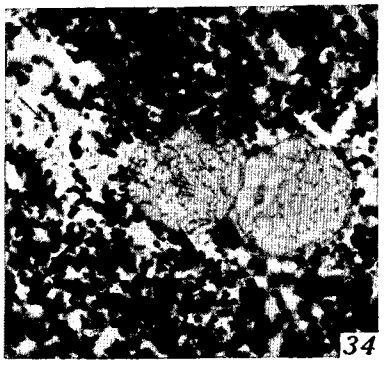
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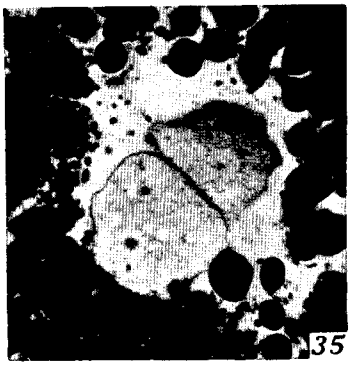
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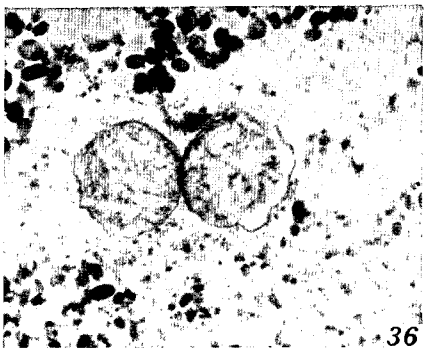
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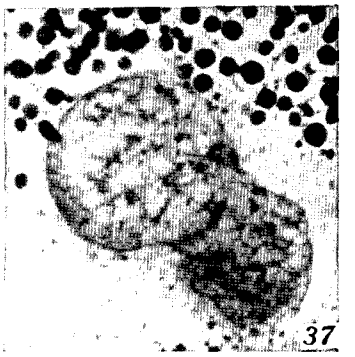
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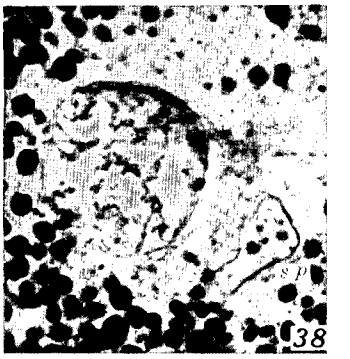
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*S. Makino photo.*

*S. Makino: A cytological study on the maturation and fertilization, etc.*

# Plate XI

## Plate XI

Fig. 39. The conjugation-nuclei. At the left side is seen a piece of the sperm-tail (*sp*). From an egg about 6½ hours after insemination. ca. ×400.

Fig. 40. The conjugation-nuclei, preparing for the first cleavage mitosis; horizontal section. The stage successive to Figs. 35-36. The nucleus shown at the right is in a more advanced phase than the other. From an egg about 6½ hours after insemination. ca. ×400.

Fig. 41. The conjugation-nuclei, preparing for the first cleavage mitosis, at a more advanced stage than Fig. 40; horizontal section. From an egg about 6½ hours after insemination. ca. ×400.

Fig. 42. The conjugation-nuclei, preparing for the first cleavage mitosis; horizontal section. The stage successive to Fig. 41. From an egg about 6½ hours after insemination. ca. ×400.

Fig. 43. The first cleavage spindle metaphase, lateral view. From an egg about 7 hours after insemination. ca. ×400.

Fig. 44. The first cleavage spindle meta-anaphase, side view. The chromosomes are seen segregated into two distinct groups, probably of paternal and maternal origin respectively. A piece of the sperm-tail (*sp*) is visible in the vicinity of the spindle. From an egg about 7½ hours after insemination. ca. ×630.

Fig. 45. The nuclear reconstruction in the telophase of the first cleavage mitosis. From an egg about 8 hours after insemination. ca. ×430.

Fig. 46. The daughter nucleus after the first cleavage division, the resting stage. The bilobed nature of nucleus is visible. From an egg about 8 hours after insemination. ca. ×430.

Figs. 47-48. The two daughter nuclei preparing for the second cleavage mitosis. The bilobed nature is distinctly visible in each. From an egg about 9 hours after insemination. ca. ×430.

Fig. 49. Abnormal case; three pronuclei come in contact, lying in the same horizontal plane. From an egg about 7 hours after insemination. ca. ×430.

Fig. 50. Meridional section through the animal pole of an egg, showing the appearance of the first cleavage furrow. About 9 hours after insemination. *n*, daughter nucleus. ca. ×50.

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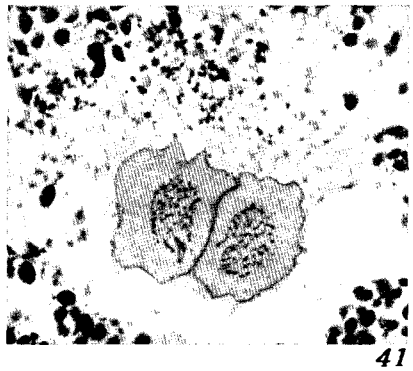
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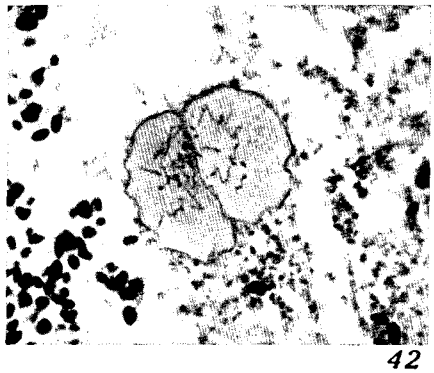
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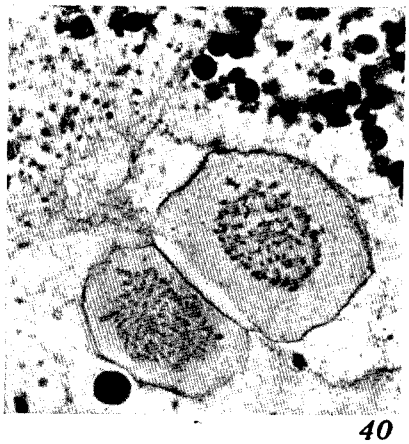
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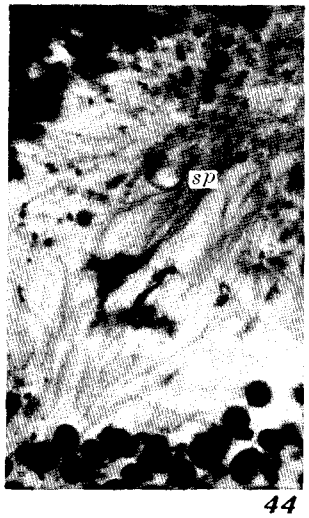

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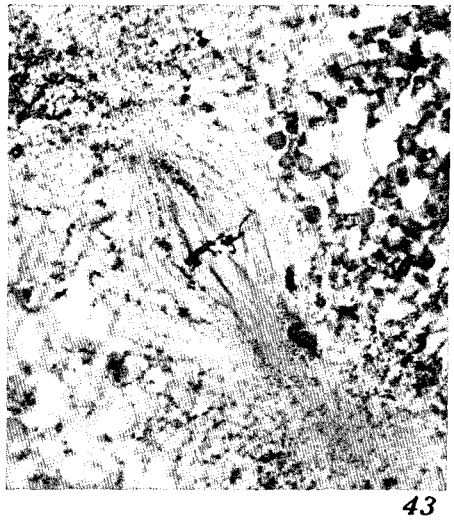
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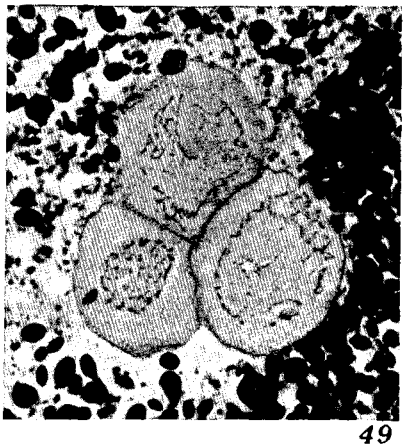
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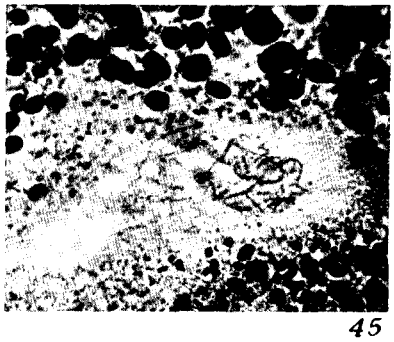
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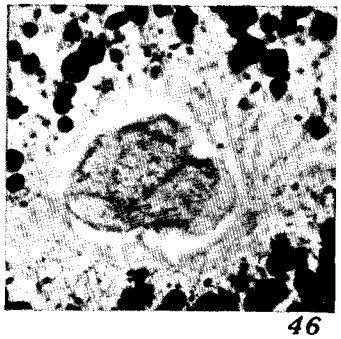
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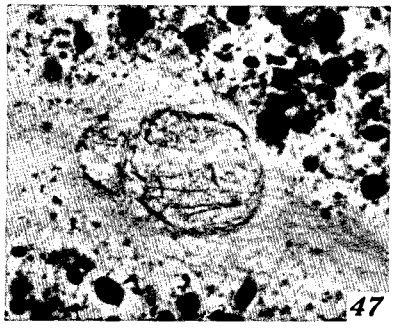
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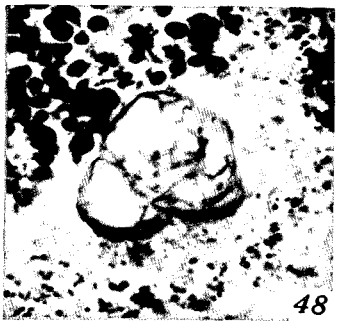
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*S. Makino photo.*

*S. Makino: A cytological study on the maturation and fertilization, etc.*