THE RESIDUAL SPERMATOGONIA IN THE ADULT SALAMANDER, *HYNOBIUS LICHENATUS* BOUL. AND THEIR BEHAVIOR DURING THE SEASONAL CYCLE OF THE GERM CELLS[1]

BY

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(*6 Text-figures and 2 plates*)

Introduction

As to the investigation of the origin of the germ cells in an animal like the Amphibian, there are two different cases to be considered: one is concerned with the source of the primordial germ cells in ontogeny and the fate of these cells; the other deals with the origin of those germ cells in the adult which produce annually the crop of sexual cells in the seasonal cycle of the testes. It is the latter case that is to be dealt with in the present paper.

In adult Amphibians, the germ cells of one season are thoroughly transformed into spermatozoa which are discharged at the close of the breeding season. Thus every seminal tubule becomes entirely free from spermatozoa. This fact gives rise to a question: how is the new crop of germ cells produced for next breeding season?

With regard to this problem in the adult form, there are two opinions held among the earlier investigators. The first is that the new spermatogonia are produced by direct transformation from the somatic cells such as the epithelial cells lining the collecting duct of

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testis, while the second is that the new spermatogonia descend from the residual cells which had remained without further development during the preceding spermatogenetic cycle within the testicular tubules.

Unfortunately, the former investigations have not made clear many important points as to the problem of the origin of spermatogonia which play the principal rôle in the production of a new crop of germ cells after the close of the breeding season.

The present work is a part of the studies on Amphibian germ cells which have been continuously undertaken since the spring of 1929, under the direction of Prof. Dr. OGUMA. I wish to express my sincere gratitude to him for his constant encouragement and valuable criticism during the progress of the work.

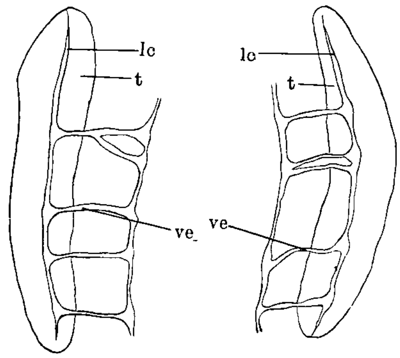
Material and Method

The material employed in this investigation consisted of the testes of adult salamanders, *Hynobius lichenatus* BOUL., which were collected in the vicinity of Sapporo during the years of 1929 and 1930.

The present species is the only Urodele Amphibian which inhabits Hokkaido and is terrestrial except during the breeding season. Early in April they gather at transitory pools of water formed by thawing snow, in the mountains and forests, and their mating as well as spawning occur simultaneously in these waters. At this period many specimens can be collected without difficulty. When the breeding season is over, they give up their aquatic life and return to terrestrial life again.

The animals were killed at various times from early April to November, including those prior to mating and those in hibernation. ZENKER's mixture and FLEMMING's solution of strong formula were chiefly employed for the fixatives. Paraffin imbedding, longitudinal and tangential sections of the testes were made. The latter case proved to be the most favourable for this study from the structure of the testis. Excellent results were obtained by staining in HEIDENHAIN's iron hematoxylin and counterstaining with light green.

Structure of the Testis



Text-fig. 1. Diagramatic drawings of right and left testes (t), showing the connection of longitudinal collecting duct (lc) and Vasa efferentia (ve).

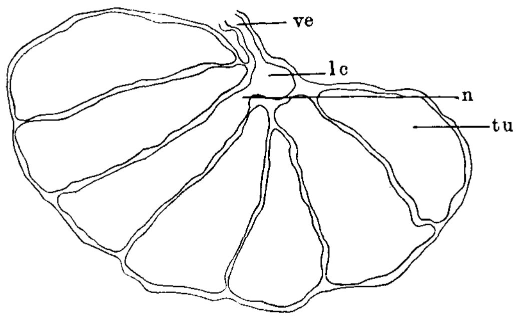
This organ is quite elongated and attached to the dorsal wall of the abdominal cavity by means of a mesorchium in which the vasa efferentia and blood vessels are imbedded (text-figs. 1, 2).

In the present species, the testis is not divided into several lobes as in *Salamandra*, *Desmognathus* and other genera of Urodelans, but consists of a simple club-shaped body, tapering towards the anterior end, as seen in text-figure 1.

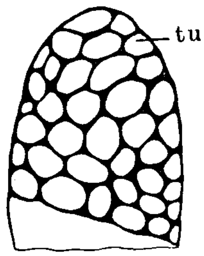
Text-fig. 2. Ventral view of the testes before breeding season. ca. ×2.5. t, Testis. m, Mesorchium. vd, Vas deferens.

The size and shape of the organ greatly varies according to season of the year, due to the seasonal changes of the germ cells contained. In general, it attains its maximum size at the season when the sexual products are ripe, being about 20 mm. in length and 5–3 mm. in diameter near the middle at this time. After the close of the breeding season, it gradually diminishes in diameter, owing to successive discharges of spermatozoa, until it attains one third its maximum size. The length, on the contrary, is not so conspicuously reduced as compared with the diameter; it becomes only 2 or 3 mm. shorter.

The Amphibian testis is composed of seminiferous tubules with different arrangement as compared with that of amniota. A great number of distinct tubules or ampullae are connected with a large collecting duct. As to the relations of the tubules to the main duct, the following three structural types were described by SPENGEL ('71); 1) the testis with a longitudinal collecting duct in the center, around which the tubules are arranged radially, 2) the testis with a superficial longitudinal collecting duct, whereby the arrangement of the tubules is fan-like, 3) the testis with tubules more spherical and terminating separately in the branches of a collecting duct.



Text-fig. 3. Diagramatic figure of a tangential section of an entire testis. lc, Longitudinal collecting duct. tu, Tubule. n, Short neck of tubule opening to collecting duct. ve, Vasa efferentia.



Text-fig. 4.

Ventral view of posterior end of testis. The peritoneum covering the surface is taken away. Diagramatic drawing. tu, Distal end of tubule.

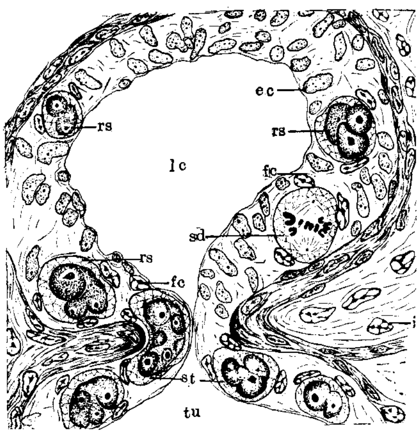
The testis of *Hynobius lichenatus* belongs to the second type, as the longitudinal collecting duct is quite superficial and the tubules arrange themselves in a fan-like fashion, as shown in text-figure 3. When we remove the peritoneum and observe the testis superficially, we find minute polygonal areas on the surface, spread over the entire organ. These denote the distal ends of the tubules (text-fig. 4). A tubule is a long cone in shape and contains a large number of cysts, each of which is formed from a single original spermatogonium by division. The tubules, separated from each other by connective tissue or interstitial tissue, are the structural units of the organ and each opens with a short neck into the longitudinal collecting duct (text-fig. 3). The contents of the tubules (spermatozoa) are gathered directly in the longitudinal collecting duct and then sent out by the vas deferens, which is connected to the former by means of 4 or 5 canals, vasa efferentia (text-figs. 1, 3).

Observation

The program followed was to examine critically how far the morphological changes took place in any part of the testis, in connection with the spermatogenetic cycle of a single season. The results of observations are summarized as follows.

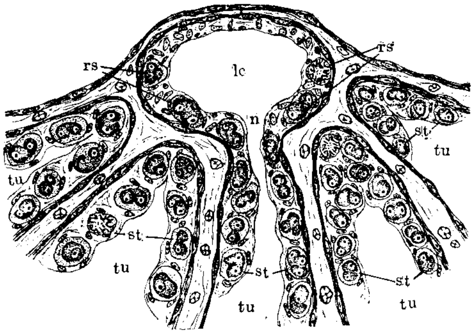
I. Just before and during hibernation

Figure 1 is a partial view of a tangential section of a testis, showing the collecting duct and proximal parts of the tubules. The testis was obtained from an animal killed just before hibernation at the end of October. The testis had attained its maximum size and was already filled up throughout with mature spermatozoa, provided for the next season.



Text-fig. 5. Schematic figure of the longitudinal collecting duct (lc) from a tangential section of the testis, showing the residual spermatogonia (rs) among the epithelial cells (ec) lining the duct. Taken at the end of October. Some of spermatogonia (st) are migrating towards the tubule. fc, Follicle cell. tu, Tubule. i, Interstitial cell. sd, Division of residual spermatogonium.

In the proximal portion or the neck of the tubules, where they are directly connected with the main collecting duct, the spermatogonia for use in the year after next can be seen lining the tubules to some extent (fig. 1 and text-fig. 6, *st*). These spermatogonia are sharply distinguished from follicle cells, interstitial cells and other tissue cells by having enormous size of cell body as well as their characteristic polymorphic nuclei (text-fig. 5). The collecting duct is empty, being destitute of spermatozoa, and the lining epithelial cells take rather irregular arrangement. Imbedded in these epithelial cells, a number of large isolated cells, surrounded by 3 or 4 follicle cells, are to be found (figs. 3, 4 and text-fig. 5, *rs*). They are separated by a delicate membrane from the epithelial cells, and possess a considerable amount of cytoplasm as compared with the latter kind of cells. Their nuclei always assume a bizarre or complicately lobulated form,[1] and contain irregular linin net-work, upon which faintly stained chromatin granules, can be observed, with numerous nucleoli. From a close comparison of these cells with the spermatogonia already observed in the proximal part or the neck of any tubule, it seems to be certain that they are cells of one and the same category with spermatogonia. Hence they are nothing other than cells playing no rôle through the present spermatogenetic cycle, and remain *in situ* as parent stock for future germ cells. These cells are usually called the residual spermatogonia.



Text-fig. 6. Schematic figure showing the longitudinal collecting duct (lc) and proximal parts of the tubules (tu), from a tangential section of the testis. rs, Residual spermatogonia. st, Migrated spermatogonia in the proximal parts of the tubules. n, Neck of the tubule opening to the duct.

The residual spermatogonia are mostly found among the epithelial cells of the collecting duct near the portion where the tubules are connected. They multiply by mitosis, since large mitotic figures are frequently seen among the epithelial cells (figs. 2, 3, 4 and text-fig. 5, *sd*). After this they migrate out of the collecting duct by amoeboid movement and arrange themselves as a lining of the tubule. In some preparations, sectioned through the transitional part of the tubules to the duct, we can distinctly observe how the large spermatogonia migrate out of the collecting duct (text-figs. 5, 6 and figs. 3, 4, *ms* and

1) A cytological study on the polymorphic nucleus of Amphibian spermatogonium will be reported under another title.

st). We conclude, therefore, without hesitation, that the spermatogonia which guard the proximal portions of the tubules are the descendants of these residual spermatogonia.

In the testes, taken on the 10th of November, I could seldom discover the residual spermatogonia in division and migration, but could find those in a state of rest with polymorphic nuclei among the epithelial cells. The animals, therefore, seem to spend the winter, in such a condition, without any advancement in the spermatogenetic course of the testis.

II. Duriug the breeding season

When examined during the breeding season of the following year, about the middle of April, quite similar conditions of the testis were to be observed microscopically. Namely, there were found a lot of spermatogonia, which had migrated from the collecting duct and aggregated at the area where every tubule opens into the duct, and the space left by the spermatogonia is filled by mature spermatozoa (fig. 5). Large residual spermatogonia, in resting stage, moreover, were observed among the epithelial cells of the duct (fig. 6, *rs*), as already observed before hibernation. The secondary sexual characters of the animal, as well as the absolute size of the testis show no difference between animals of both seasons. It is quite possible, therefore, to suggest that no marked changes occur in the testis during hibernation. The divisions of the residual spermatogonia among the epithelial cells of the collecting duct and their subsequent migration to the tubules may occur during this season to some extent, although not so frequently.

III. After the breeding season

When the breeding season is over, from the end of April to the beginning of May, the salamanders come back to terrestrial life after a temporary aquatic period for spawning. If the testis is examined at this time, the organ is found empty, owing to the discharge of the spermatozoa. On the other hand it is clearly observed, that the spermatogonia of a new seasonal cycle, which have been dormant at the mouths of tubules since the previous autumn, commence to multiply actively (fig. 7).

At the same time, the residual spermatogonia among the epithelial cells of the duct divide repeatedly by mitoses. When the collecting duct of testis is examined at this time, large mitotic figures of the residual spermatogonia can be seen scattered here and there among the epithelial cells (figs. 8, 10, 11, *sd*). In such case, the transformation of the epithelial cells of the duct into spermatogonia has been described by HARGITT ('24) in *Diemyctylus*, but careful examination disproves the occurrence of a similar phenomena in the epithelial cells, so far as the present material is concerned. As already stated, the residual spermatogonia can be strictly distinguished from the epithelial cells, in having their characteristic polymorphic nuclei, size, structure and a considerable amount of cytoplasm. On the contrary, the epithelial cells, the interstitial cells and follicle cells as well, are sharply characterized by their typical shape and structure. The distinction between these two kinds of cells is too obvious to be confused at any time. In the present species at least a transformation of differentiated epithelial cells into undifferentiated cells, i.e., spermatogonia, from a morphological and physiological point of view, does not take place whatsoever. At any rate, I could not find any configuration of cells which might suggest a transitional stage of the epithelial cells into spermatogonia.

As a result of repeated divisions of the residual spermatogonia, they increase in number prodigiously, so that a great number of spermatogonia become obvious among the epithelial cells, as shown in figures 8 and 9. At the same time, most of them migrate one after another towards the tubules through the neck of the latter (fig. 9, *ms*), while a few remain without migration, in their original positions, thus forming the residual spermatogonia (figs. 8, 9, *rs*). These phenomena are mostly observed in the testes of animals killed during May and June.

From the latter part of June to the beginning of July, a large proportion of the testicular space is filled up with the spermatogonia of a new sexual cycle. In all the tubules which constitute a testis, the germ cells develop synchronously and there is no cephalo-caudal direction of spermatogenesis—"a spermatogenetic wave"—as noted by numerous investigators in many other Urodelans, e.g., in *Salamandra* (MEVES, '96), *Amphiuma* (MCGREGOR, '99), *Desmognathus* (KINGSBURY, '02), *Proteus* (STIEVE, '20), *Necturus* and *Diemyctylus* (HUMPHREY, '21), *Diemyctylus* (HARGITT, '24).

While the tubules are being filled up with spermatogonia early in summer, further multiplication of spermatogonia seems to be closed, as any dividing and migrating figures of residual spermatogonia are not found. The residual spermatogonia, during summer time, which contain a considerable amount of cytoplasm and which represent the striking nuclear polymorphism, are found isolated from the epithelial cells (figs. 12, 13, *rs*). They remain in such a condition of rest, without becoming flattened as reported in *Desmognathus* (KINGSBURY, '02), until the time of hibernation.

The follicle cells (cyst cells), interstitial cells and peritoneal tissue cells outside the testis were also examined during these observations, but in the case of the present species, it has become obvious that they have no relation to the origin of spermatogonia.

Judging from the results obtained from the above observations which were carried out through the spermatogenetic cycle of the year, the following conclusion is drawn: a few spermatogonia, which always exist among the epithelial cells of the collecting duct, take no part in the annual spermatogenetic process, but persist as primordial germ cells or residual spermatogonia.

When the transformation of germ cells into spermatozoa is completed in autumn these residual spermatogonia recover their dividing activity first, and after the discharge of the latter at the breeding season in spring, begin to divide very actively among the epithelial cells of the duct. Most of their daughter cells migrate into the tubules through the neck of the latter, starting thereby the next sexual cycle. After the tubules are filled up by the germ cells of the new cycle thus produced, the residual spermatogonia cease their multiplication and remain unchanged with polymorphic nuclei among the epithelial cells of the duct in a state of rest until the next cycle commences.

General Consideration

As regards the origin of the germ cells of the adult Amphibians, in which the discharge of spermatozoa makes the organ empty at the breeding season, there are, as a whole, two opinions among earlier authors. One is that the spermatogonia are formed anew each season from the somatic cells such as the epithelial cells which line the collecting duct or the peritoneal cells. This was asserted by LA VALETTE ST. GEORGE ('76), MCGREGOR ('99), GATENBY ('16), OBRESHKOVE ('24), and HARGITT ('24). On the other hand, opinions of the residual spermatogonia have been maintained by HERMANN ('89), VOM RATH ('93), MEVES ('97), KINGSBURY ('02) NUSSBAUM ('06), STIEVE ('20), and HUMPHREY ('21, '22).

LA VALETTE ST. GEORGE ('76), in his work on Amphibian, concludes that the spermatogonia of the new cycle are derived from the 'Keimlager' which line the collecting duct. MCGREGOR ('99) says it is easy to demonstrate that the large spermatogonia of adult *Amphiuma* arise as proliferations from the epithelial lining of an irregular canal which runs along the centre of the testis. GATENBY ('16) is of opinion that in *Rana temporaria* the peritoneal cells are transformed into spermatogonia newly each season. More recently, HARGITT ('24), working on *Diemyctylus*, finds that the spermatogonia are formed anew each year from the epithelial cells which line the collecting duct or from the peritoneal epithelium which covers the testis. This opinion is supported by OBRESHKOVE ('24) from the evidences observed also in *Diemyctylus*. But the figures of HARGITT ('24) show no other cells than the typical germ cells which I believe to be the residual spermatogonia.

These figures do not explain how they have been transformed from the epithelial cells. Even in his highly diagramatic figures, the distinction between germ cells and epithelial cells is extremely clear. Indeed, he does not observe the actual transformation of the epithelial cells into spermatogonia. Probably, his conclusion may have been theoretically drawn on the presumption that, since the primordial germ cells in embryo are said to be derived from the body cells such as peritoneal epithelium, it is possible to say that the germ cells in the adult are formed from the epithelial cells of the duct and the peritoneal epithelium, i.e., the body cells. To support his view, he cites the germ-plasm theory of Weismann and the results of the studies on the embryological source of germ cells in Amphibians by many earlier investigators. A glance at the occurrences of large spermatogonia among the epithelial cells may recall to mind that the former is to be derived from the latter. But in the case of *Hynobius lichenatus*, so far as my own observations go, the spermatogonia which produce the cells of the new spermatogenetic cycle each season are not those transformed from the body cells, but are those which always remain among the epithelial cells lining the main collecting duct as residual spermatogonia during the year. HERMANN ('89), VOM RATH ('93) and MEVES ('97), who all worked on *Salamandra maculosa*, state that "die am oberen Pole des Hodens gelagerten indifferenten Keimzellen" furnish the cells for the next cycle of spermatogenesis. Both KINGSBURY ('02) and HUMPHREY ('21, '22), in *Desmognathus*, the former and the latter in *Necturus*, *Desmognathus*, *Cryptobranchus*, and *Diemyctylus*, all come to the same conclusion that a few primary spermatogonia in "the apex of the tubules" remain unchanged during spermatogenesis, and after expulsion of the spermatozoa these residual spermatogonia furnish the cells for the next season. They could never find, however, the residual spermatogonia which remain among the epithelial cell lining of the main collecting duct as they do in the present species.

It is quite obvious without the slightest doubt, that in *Hynobius lichenatus*, a few spermatogonia with polymorphic nuclei remain among the epithelial cells which line the collecting duct of the testis, and pass *in situ* up to time when annual spermatogenetic cycle commences. Then they multiply for the supply of new spermatogonia which undergo their maturation into spermatozoa. But still, some of them again stay in their original positions, without migrating into the seminal tubules, and thus remain as residual spermatogonia. The present species lends itself favourably to support this interpretation, due to the simple structure of the testes.

Summary and Conclusion

In the adult salamander, *Hynobius lichenatus,* there are a few residual spermatogonia in the main longitudinal collecting duct at any time of the spermatogenetic cycle. These spermatogonia are clearly distinguished from the epithelial cells near by, due to their size, structure, and characteristic nuclear polymorphism. They take no part in the annual spermatogenetic process, but persist always as primordial spermatogonia. In autumn, when the spermatozoa of next season have already been produced, these residual spermatogonia begin to increase in number by mitoses among the epithelial cells, and most of the daughter cells thus produced migrate into the proximal parts of the tubules. Some of them at the same time, do not migrate but remain in the duct. During hibernation no more division takes place, either in the tubules or in the duct. After the discharge of spermatozoa during the breeding season of the following spring, both the residual spermatogonia and their daughter cells divide actively and start the new spermatogenetic cycle.

December, 1930

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Plate II

Explanatory notes to Plate II

All figures of this plate are from microphotographs of sections of the testes. With the aid of Leitz, 'MAKAM,' tube length 170 mm.

Fig. 1. Partial view from a tangential section of the testis, fixed at the end of October. The longitudinal collecting duct and the proximal portions of tubules with spermatogonia of the new cycle filling with mature spermatozoa are shown. Leitz Obj. 3 × Oc. 8.

Fig. 2. Part of the longitudinal collecting duct from a tangential section of the testis, fixed at the end of October. The residual spermatogonia and their division are seen among the epithelial cells lining the duct. Leitz Obj. 7 × Oc. 5.

Fig. 3. Similar to figure 2, from the testis fixed at the beginning of November. Spermatogonia migrating from the duct to tubule are seen. Leitz Obj. 7 × Oc. 5.

Fig. 4. Similar to figure 3. A tubule with mouth open to the duct. Leitz Obj. 7 × Oc. 5.

Fig. 5. Partial view from a tangential section of the testis fixed in early April before mating. Compare with figure 1. Leitz Obj. 3 × Oc. 8.

Fig. 6. Enlarged view from figure 5, showing the longitudinal collecting duct and proximal portions of tubules. Leitz Obj. 7 × Oc. 5.

Abbreviations

- bl, Blood vessel.
- ec, Epithelial cell lining of collecting duct.
- fc, Follicle cell.
- lc, Longitudinal collecting duct.
- ms, Migrations of spermatogonia from duct to tubule.
- n, Neck of tubule opening to duct.
- rs, Residual spermatogonium.
- st, Spermatogonia migrated at the proximal part of tubule.
- sd, Division of residual spermatogonium.
- sz, Bundle of spermatozoa.
- tu, Tubule.

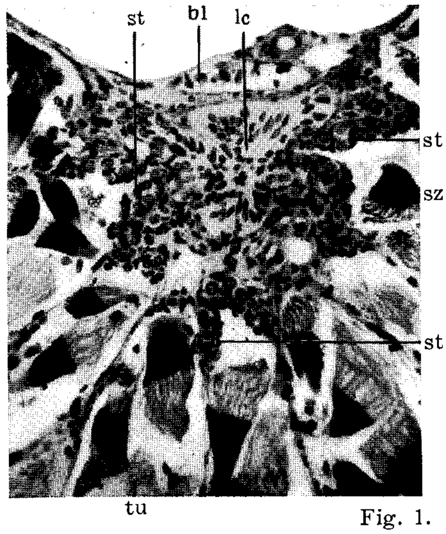

Fig. 1.

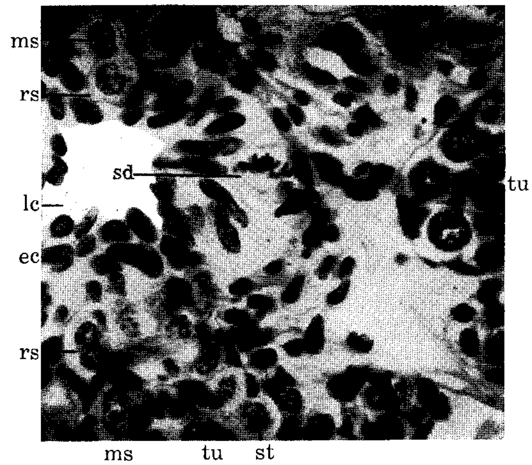

Fig 2.

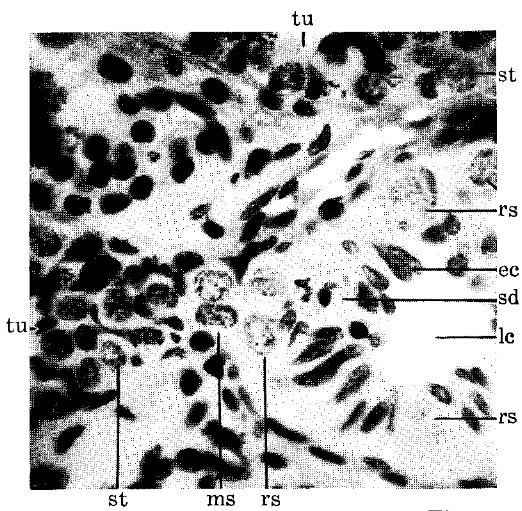

Fig. 3.

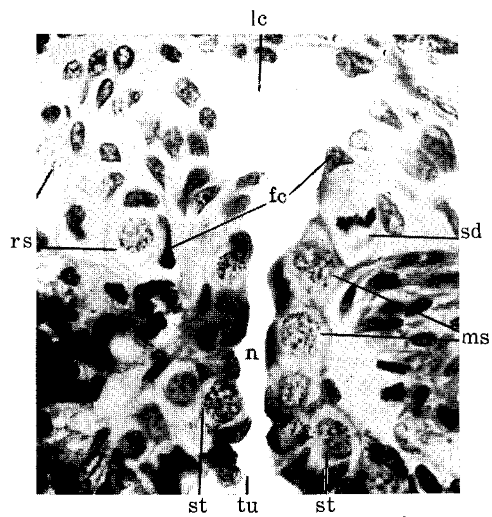

Fig. 4.

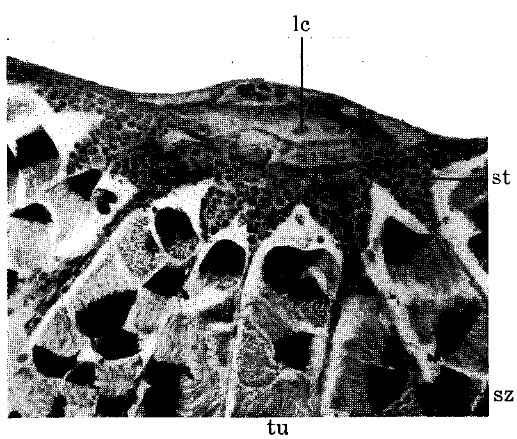

Fig. 5.

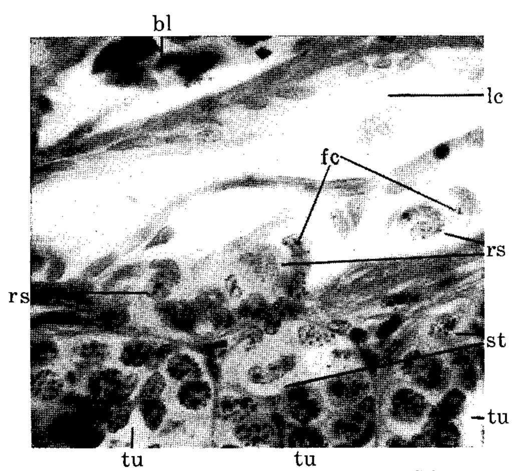

Fig. 6.

Photo. by S. Makino

S. Makino: The Residual Spermatogonia in the Adult Salamander, etc.

Plate III

Explanatory notes to Plate III

All figures of this plate are from microphotographs of sections of the testes. With the aid of Leitz, 'MAKAM,' tube length 170 mm.

Fig. 7. Enlarged view of the proximal portion of a tubule, showing the multiplication of spermatogonia migrated from the collecting duct. From a tangential section of the testis fixed at the end of April. Leitz Obj. 7 × Oc. 5.

Fig. 8. Part of the longitudinal collecting duct, showing the multiplication of the residual spermatogonia among the epithelial cells lining the duct. From a tangential section of the testis fixed at the beginning of May. Leitz Obj. 7 × Oc. 5.

Fig. 9. Part of the longitudinal collecting duct and proximal portions of tubules, from a longitudinal section of the testis fixed at the middle of May. A large number of the residual spermatogonia among the epithelial cells and their migrations to tubules can be seen. Leitz Obj. 6 × Oc. 5.

Fig. 10. Part of the longitudinal collecting duct, showing the division of the residual spermatogonium among the epithelial cells. From a tangential section of the testis, fixed at the beginning of May. Leitz Obj. 7 × Oc. 5.

Fig. 11. Similar to figure 10.

Fig. 12. Part of the longitudinal collecting duct, showing the residual spermatogonia remaining among the epithelial cells. From a tangential section of the testis, fixed at the beginning of July. Leitz Obj. 7 × Oc. 5.

Fig. 13. Similar to figure 12. Fixed at the end of August. Leitz Obj. 7 × Oc. 5.

All abbreviations of this plate are similar to Plate II.

Fig. 7.

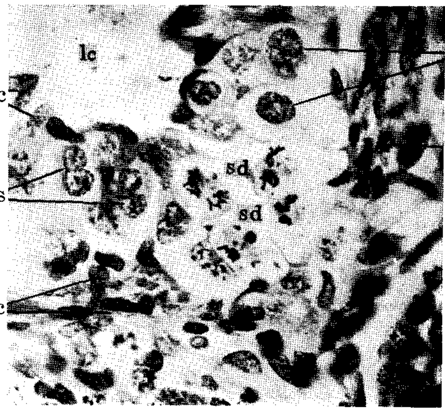

Fig. 8.

Fig. 9.

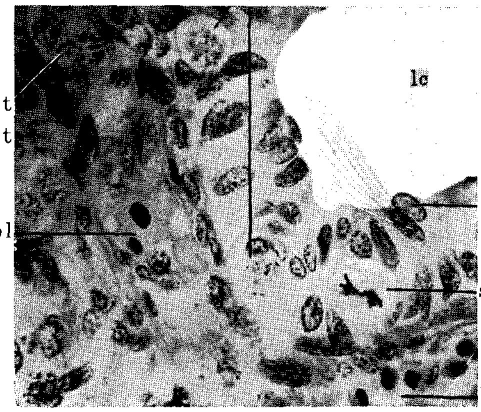

Fig. 10.

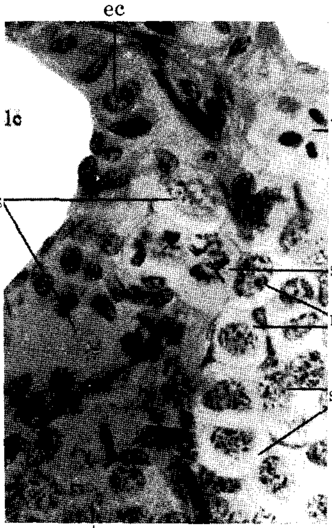

Fig. 11.

Fig. 12.

Fig. 13.

Photo. by S. Makino

S. Makino: The Residual Spermatogonia in the Adult Salamander, etc.