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MORPHOLOGICAL OBSERVATION ON REVERSAL PROCESSES OF SEX-DIFFERENTIATION IN THE GENETIC FEMALE GONAD OF THE MEDAKA, *ORYZIAS LATIPES*, BY ANDROGEN

H.Kobayashi and T.Hishida

Department of Biology, Asahi University School of Liberal Arts, Hozumi-cho, Motosu-gun, Gifu 501-02

ABSTRACT- Newly hatched fry of the genetic female (X^rX^r) medaka (*d-rR* strain) were daily given a methyltestosterone diet (50μg/g) until they grew more than 13mm in body length. Using both a light- and electron-microscope, morphological changes in the gonad undergoing sex-reversal were studied with special reference to initial transition steps in the reversal of germ cells from genetic female towards the phenotypic male direction. Special attention was also paid to the degenerating processes of oocytes differentiated from germ cells prior to and during sex-reversal. The first appearance of male gonial cells in the female gonad was in about the 9-10mm stage, when most germ cells have already transformed through oogonia into enlarged perinucleolar oocytes. It is most likely that male gonial cells have differentiated from some of the female germ cells, each of which is enclosed within a thin layer of somatic cells to form a distinct acinus of spermatogonia. As these acini increased in number and expanded in dimension by inner gonial proliferation, a typical testicular structure of tubular cysts was formed.

In inverse proportion to the progressive cystic (acinous) formation, oogenesis was greatly suppressed by exogeneous androgen. Consequently, oocytes previously differentiated were doomed to degeneration and finally transformed into complicated debris of large whorls or myelin-like structures displaying various abnormalities in form. These, together with spermatide debris degraded after spermiogenesis, were finally phagocytosed by thickened epithelial (Sertoli) cells lining the cysts, which were filled with many mature spermatozoa in 25-26mm body length stage when the gonad was established as a perfect testis. After oocyte degeneration, it was found that surrounding follicle (granulosa) cells gave evidence of much phagocytosis and suggested that they possibly play a principal role in the phagocytic exclusion of debris of degenerated oocytes.

INTORODUCTION

With the genetically analyzed *d-rR* strain of the medaka, *Oryzias latipes*, Yamamoto (1953, 1958) successfully induced functional sex-reversal of both sexes by oral administration of heterogeneous sex-steroids at juvenile stages. His work, which marked a major breakthrough, encouraged similar experimemts of other fish by other investigators (Dzwillo, 1962 ; Clemens *et al.*, 1966 ; Takahashi, 1975a,b ; Johnstone *et al.*, 1978 ; Nakamura, 1981), and also in other animals (Chang and Witschi, 1955 ; Kawamura and Yokota, 1959 ; Wolff and Haffen, 1962).

However, concerning the morphological changes during gonadal differentiation in the normal and sex-reversed medaka, work of Yamamoto and his collaborators has so far been carried out mainly with a light microscope. On the other hand, Satoh (1974) and Kanamori *et al.* (1985) reported the ultrastructural features of normal sex-differentiation processes in the gonad of the medaka, but there has been little research on the sex-reversal processes of the medaka gonad thus far.

Therefore, a preliminary attempt is made in this paper to elucidate detailed morphological processes in the transition stage of the genetic female gonad undergoing sex-reversal by administration of methyltestosterone, using both a light- and electron-microscope.

MATERIALS AND METHODS

Newly hatched fry, produced by crossing white (X^rX^r) females mated *en masse* with heterozygous orange-red (X^rY^R)males were used. They were the 16th and 17th generations of *d-rR* strain (Yamamoto, 1958). Daily hatchings (July 1-13) were divided into two groups. One was reared on a normal diet as a control group, while the other was fed the methyltestosterone diet as an experimental group, with the dosage level of a 50 μg/g diet, which proved to sufficiently induce a complete reversal in sex differentiation of the genetic female medaka (Yamamoto, 1958).

The two groups (experimental and control) of larvae were separately reared indoors in plastic aquaria (25×37×14cm) at room temperature (22-25℃) under natural light conditions until they grew to more than the 13mm stage (about 40 days), and then they were given the normal diet and reared outdoors in the same aquaria.

The normal diet used was No.2M, a commercial food for carp larvae (Nihon Haigo-Shiryo). The methyltestosterone diet, consisting of methyltestosterone crystals (Sigma, Co.) ground in a mortar was mixed with the normal diet. Special care was taken in order to obtain a homogeneous mixture of the hormone powder food.

Newly hatched fry reached 5.6-6.7mm in body length at the 7th day after hatching and 7.0-8.8 mm by the 14th day (Hisida, 1975). Thus, the fry do not grow at the same rate, so that gonads from the larvae of the same age greatly vary in degree of development. For this reason, it was deemed to be more appropriate for us to relate developmental changes to the body length of larvae rather than age after hatching. Therefore, gonadal development has constantly been evaluated in terms of the body length in our study.

When larvae reached the definite body lengths as follows, 9-10, 11-13, 15-18, 20-23, and 25-26mm, five groups of 10 larvae each were respectively selected, the trunk regions including the gonad, were excised under anesthesia in 0.015 % phenylurethane solution and pre-fixed in Karnovsky's fixative (1965) for 4-5 hrs at room temperature. After washing in 0.1 M cold cacodylate buffer (pH 7.3), they were post-fixed in 1 % cold OsO_4 buffered to pH 7. 3 with the same buffer for 4-5 hrs, and after thorough dehydration in an ethanol series, they were embedded in Spurr's resin (TAAB).

Thin sections were cut with a diamond knife on a Porter-Blum MT-2B ultratome, double-stained with uranyl acetate and lead nitrate, and examined by a JEM-100U electron microscope at 80 Kv. For light microscopic observation, thick sections were made at 0.5 μm in thickness with glass knives, stained with an equal mixture of 0.5% azur II and 0.5% methylene blue in 0.5% borax according to Hogan (1978).

OBSERVATIONS

On the way to sex-reversal in the direction of phenotypic male, androgen-adminstered genetic female gonads from the fish which had reached, respectively, 9-10mm, 11-13mm, 15-18mm, 20-23mm, and 25-26mm in body length were closely examined in each stage on at least five samples.

9-10mm stage

The male and the female gonads in normal and androgen-administered fish are illustrated in Fig.1. It was evident that under the deleterious effects of methyltestosterone, gonadogenesis in androgen-administered fish was remarkably suppressed in both sexes, as compared with those in normal fish (Fig. 1a, b) ; the suppressive degree was much greater in the female than in the male. Despite such effects of methyltestosterone, many germ cells in the female gonad had already entered the prosesses of oogenesis, part of them being further transformed into enlarged oocytes in the early perinucleolar stage (Fig. 1c). However, some of the remaining cells formed several clumps of distinct acini typical of early testicular structures, which were simultaneously observed also in the male gonad of both normal and administered fish (Fig. 1b, d). Thus, in the gonad of the administered genetic female fish, the first sign of sex-reversal was apparent at this stage. The electron microscope revealed that each formed acinus was usually composed of one large gonial cell and its tightly enclosing somatic cell

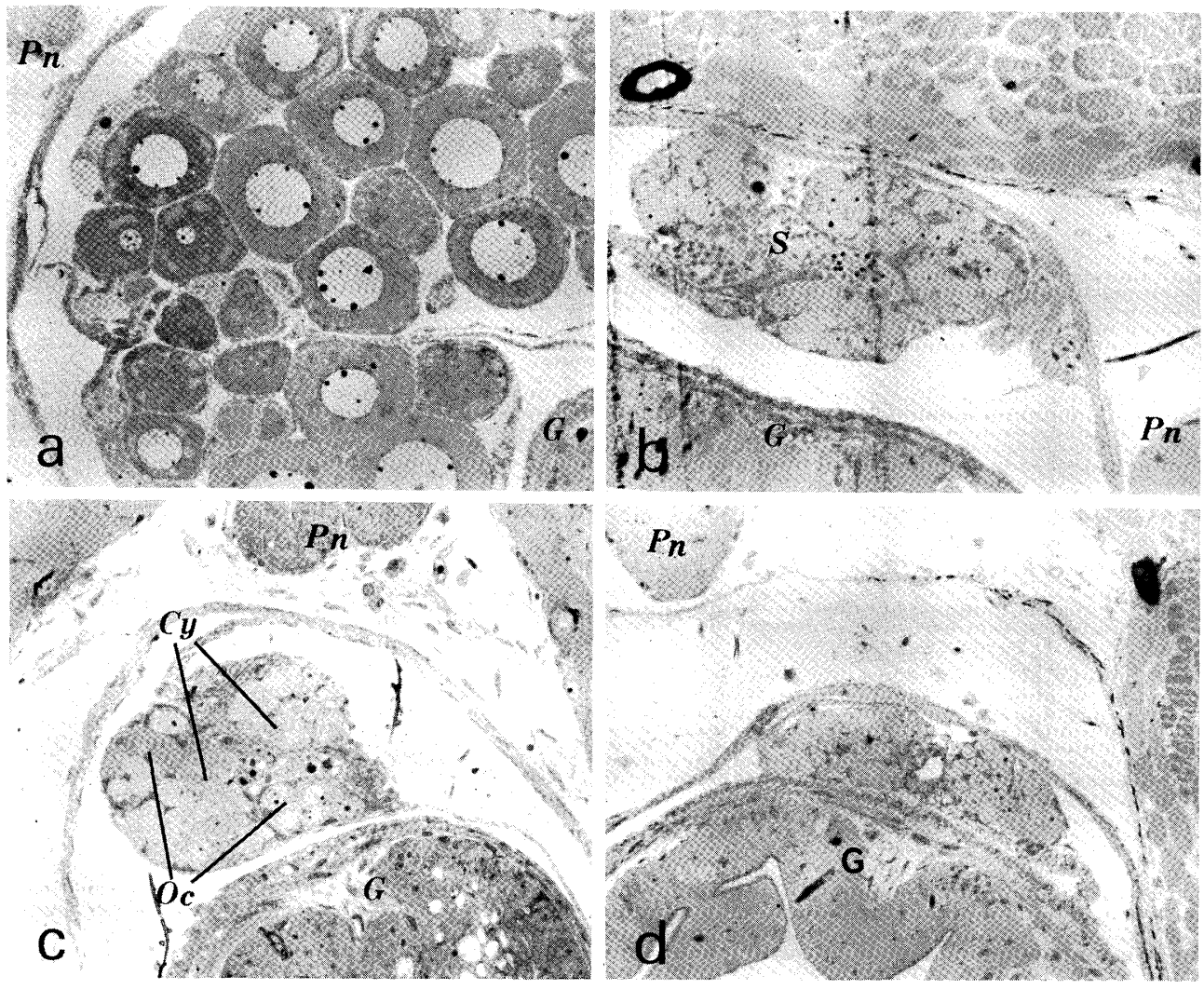

Fig.1 Cross section of the gonad of 9-10mm medaka. x 250. a. normal genetic female. b. normal genetic male. c. genetic female administered methyltestosterone (M.T.). d. genetic male administered M.T.. Cy,cysts of spermatogonia. Oc, oocytes. S, spermatides. Pn, pronephric duct. G, gut.

layer (Fig. 2a). The inner gonium had the same ultrastructures as those described by Satoh (1974) and Hogan (1978) for primordial germ cells (PGCs), such as a large cell size, a large nucleus with a prominent nucleolus, and specific cytoplasmic organella, i.e., annulate lamella, fenestrated sheets of endoplasmic reticulum, and germinal dense bodies sometimes associated with mitochondria. It was found that occasionaly some of these acinous gonia had mitotic figures (Fig. 2b), while others divided into smaller ones (Fig. 2c).

Therefore, it seems that through inner gonial proliferation, each acinus gradually expanded into the cysts of spermatogonia, where subsequent spermatogenesis and spermiogenesis proceeded. Judging from these observations, it has been proved that the transition stage from female germ cells to male spermatogonia corresponds to the time of acinus formation in the female gonad of the administered fish. Within the newly formed cyst, spermatogonia still preserved some of the specific organella described above in their cytoplasm and in addition, neighboring gonia intimately contacted with each other by intercellular bridges (Fig. 2d). Their nucleus, with a round contour, was reduced in size and their nucleoplasm became electron dense. But, only a part of them, slightly larger and less electron dense, were found to contain synaptonemal chromosomal complexes, indicating entrance into meiotic prophase.

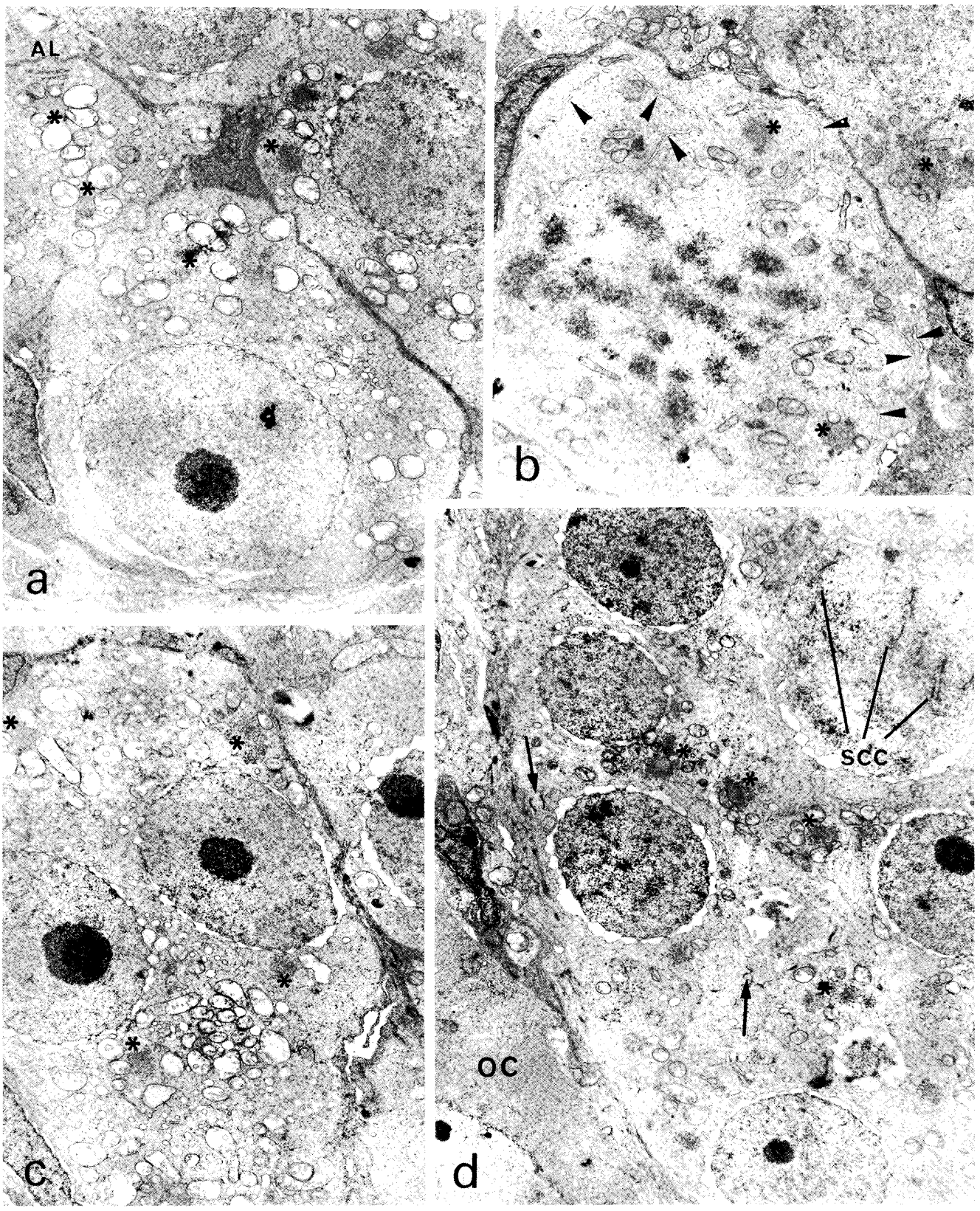

Fig.2 Electron micrographs of the early phases of cystic (acinous) formation in the gonad of 9-10mm genetic female medaka administered M.T. in Fig.1c. x 5300. a. A gonial cell encased by a thin layer of the somatic cells, forming a spherical cyst. b. A cyst encasing a gonial cell with mitotic figures. c. A cyst encasing two-divided gonial cells. d. A. portion of the newly formed typical cyst of spermatogonia. Al, annulate lamella. OC, oocyte. SCC, synaptonemal chromosomal complexes. Arrows, intercellular bridges. In the cytoplasm of gonial cells, characteristic organella such as fenestrated sheets of endoplasmic reticulum (arrowheads) and germinal dense bodies (asterisks) are frequently observed.

11-13mm stage

Although there was no appearance of new oocytes throughout the gonad in this stage, cystic (acinous) formation was still in progress, but at slow rate (Fig. 3). Several small cyts were observed sporadically in the interspaces among enlarged oocytes which had previously developed. Moreover, transformation of spermatogonia into spermatocytes was evident within these cysts, judging from the fact that each of them joined together by extending their intercellular cytoplasmic bridges (Fig. 3, 4).

On the other hand, it appeared that many of the enlarged oocytes had begun to degenerate, accompanied by various degrees of abnormalities, while some of them had already degenerated into large necrotic fragments. The most prominent feature in degenerating oocytes was the formation of round vesicles, sometimes of a large size, which were arrayed around the peripheral surface of their ooplasm. Neighboring vesicles fused together to make irregularly discontinuous spaces between the oocyte and the surrounding follicle layer. Another prominent feature was a marked distortion in the overall appearance of the oocytes. Rarely could unusual oocytes having two germinal vesicles be seen, as a result of the fusion of two degenerating oocytes. Ultrastructural observations also showed the occurrence of apparent necrotic changes at the subcellular level in the ooplasm of such oocytes and their degradates (Fig. 4). As the degeneration of oocytes advanced, it was found that the oocytes gradually shrank, detaching from the surrounding follicle cells which partially became thicker and more irregular in shape, being highly suggestive of phagocytic evidence.

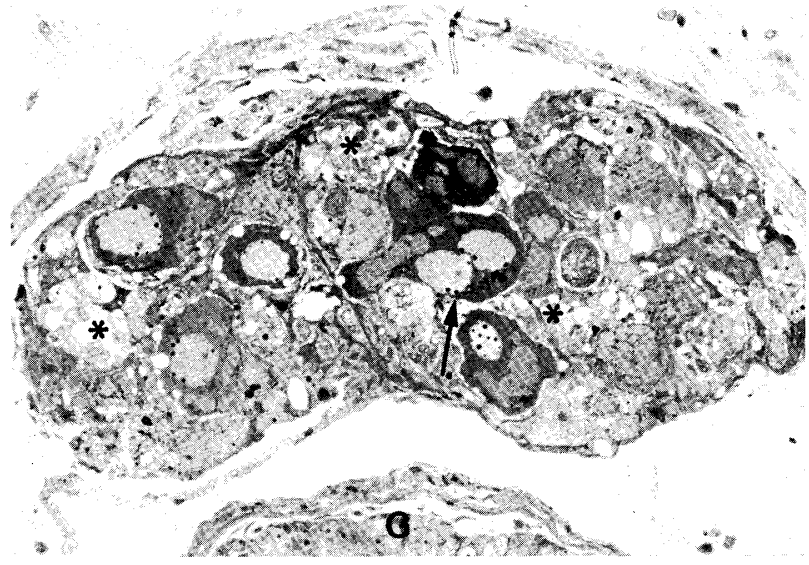

Fig.3 Cross section of the ovo-testis of 11-13mm genetic female medaka administered M.T.. x 250. Cystic formation of spermatogonia and spermatocytes advances, while oocytes begin to degenerate displaying various degrees of abnormalities. G, gut. An abnormal oocyte having two germinal vesicles is seen (arrow).

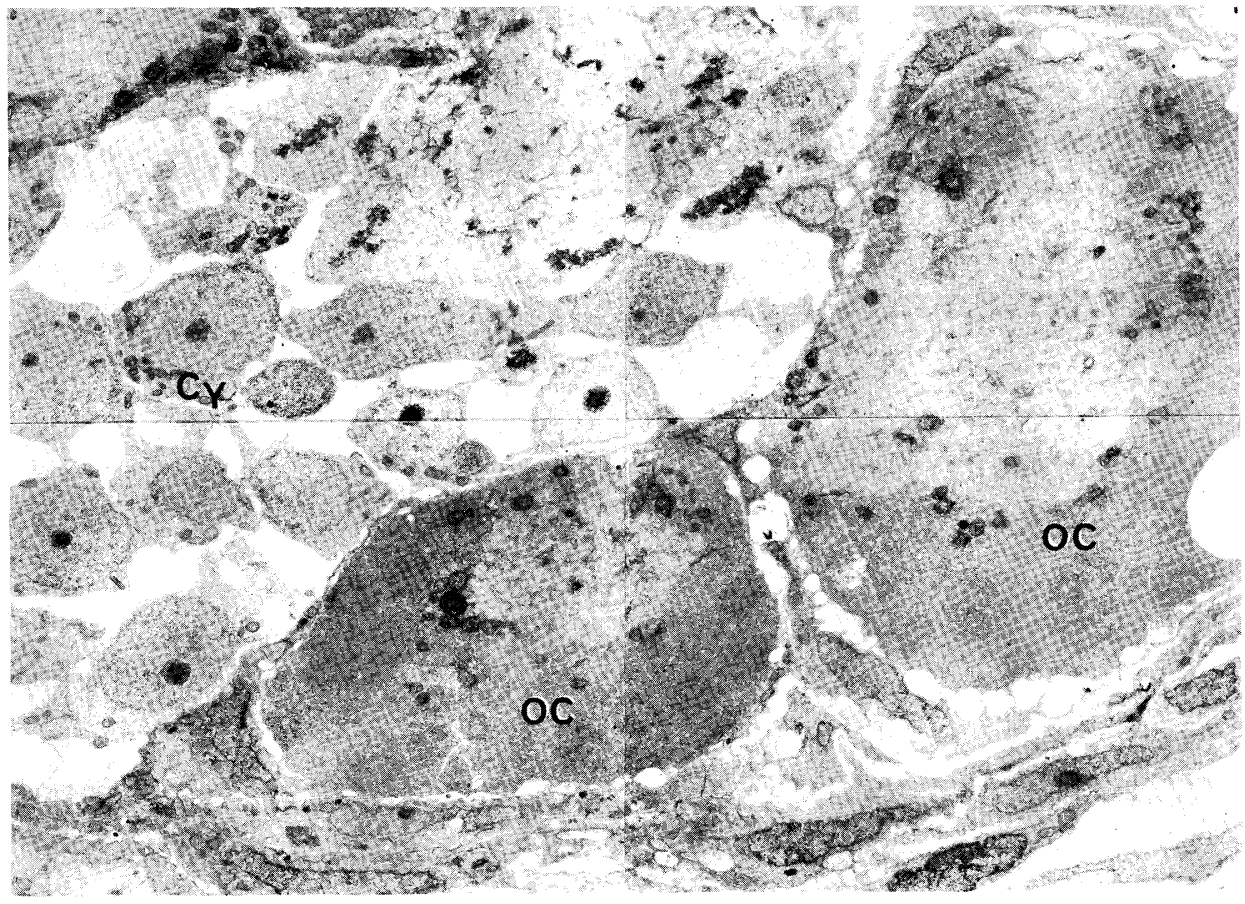

Fig.4 Electron micrograph of a portion of the spermatocytic cyst (Cy) and degenerating oocytes (OC) and their degradates in the gonad of 11-13mm genetic female medaka administered M.T.. x 3000.

15-18mm stage

Cystic structures of spermatogonia and spermatocytes became more obvious in the area among the oocytes than in the previous stage. Paralleling the subsequent spermatogenic processes, it was found that these cysts expanded remarkably in their dimension, and some of them joined together occupying a considerable part of the gonad. On the other hand, enlarged oocytes which seemingly had completely stopped their auxocytic growth and had been under degeneration, still remained in the gonad at this stage (Fig. 5a). Furthermore, possibly as a natural consequence after the termination of androgen administration in this stage, just nacent, a few small oocytes with synaptonemal complexes were often seen in the gonad (Fig. 5b).

20-23mm stage

Contrary to the disappearance of large degenerating oocytes, a great number of cysts containing various kinds of spermatogenic cells occupied almost the whole gonad becoming a form of adult testis with nearly typical structures. Within every cyst, development of spermatogenic cells was very synchronous and all in the same phase of spermatogenesis (Fig. 6). On the other hand, it is worthy of note here that a few of the tiny oocytes in the perinucleolar stage appeared again intercystically or intracystically. These oocytes were usually deeply stained with basic dyes, indicating degeneration. The electron microscope also proved apparent necrotic features in both the nucleoplasm and the ooplasm of such oocytes (Fig. 7). The surrounding follicle cells thickened significantly and were frequently seen to have captured various debris of sperm heads and remnant spermatide cytoplasm during or following spermiogenesis.

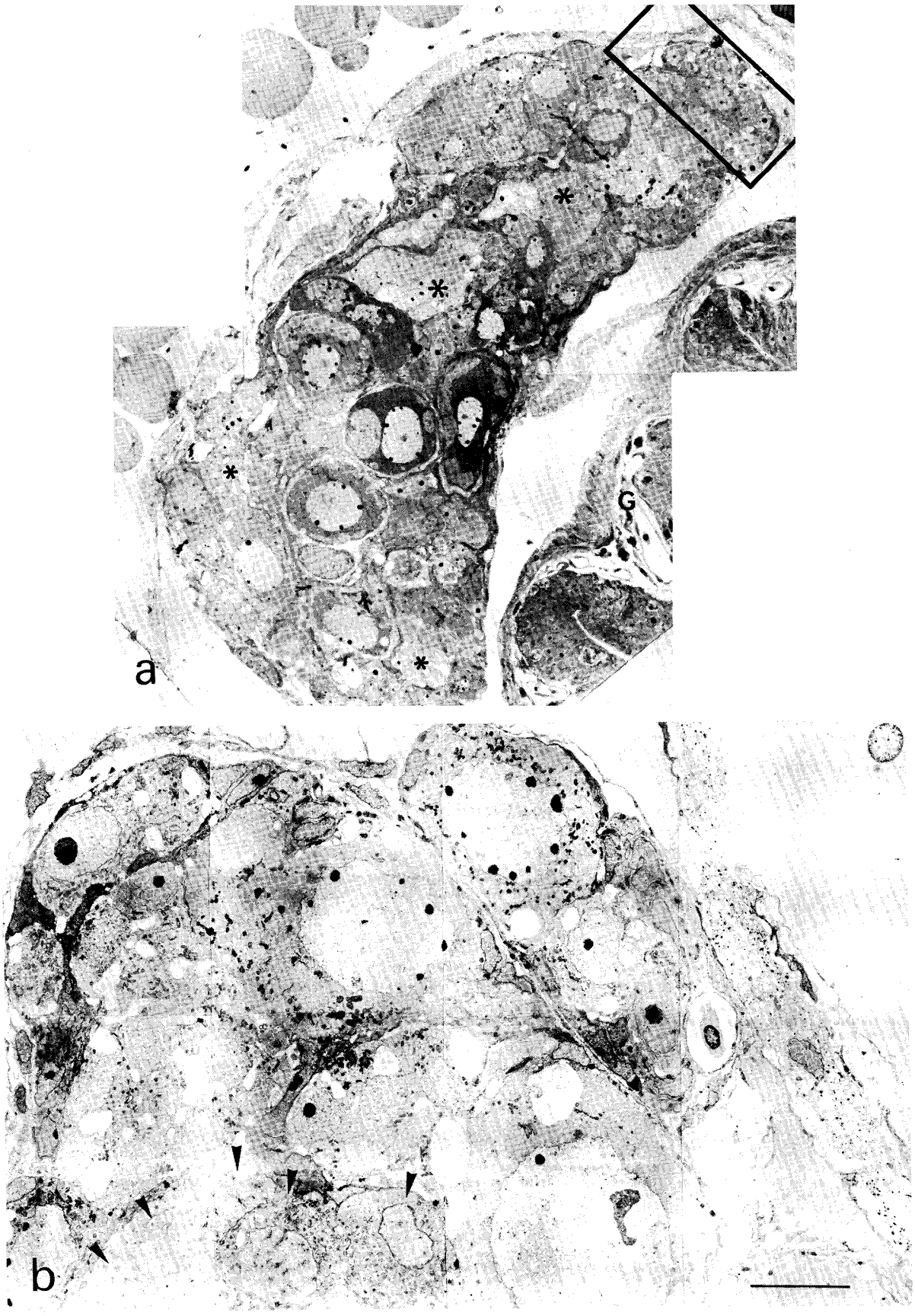

Fig.5a Cross section of the ovo-testis of 15-18mm genetic female medaka administered M.T. x250. Cystic structures (asterisks) of spermatogonia and spermatocytes become evident, though enlarged oocytes under degeneration are still remaining. G, gut.

Fig.5b Electron micrograph taken at x 3000 of the area within the rectangle shown in Fig.5a. Bar indicates 10 μm. An appearance of small premeiotic oocytes with synaptonemal complexes can be seen (arrowheads).

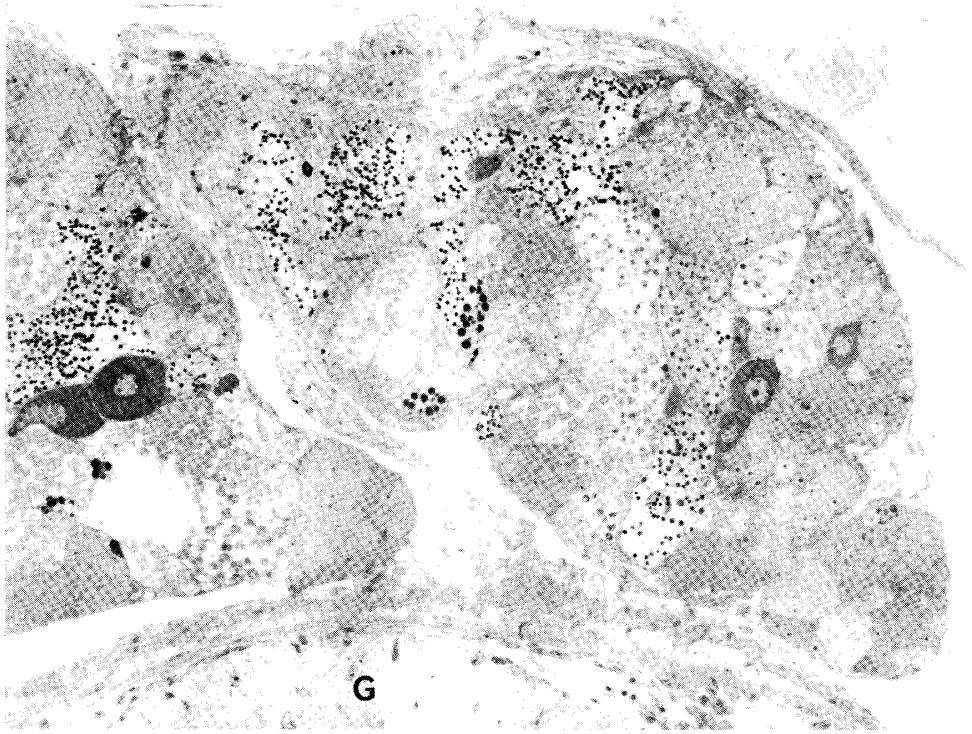

Fig.6 Cross section of the ovo-testis of 20-23mm genetic female medaka administered M.T.. x 250. G, gut. The gonad is almost completely occupied by a great number of cysts containing various kinds of spermatogenic cells. A few small perinucleolar oocytes, intercystically or intracystically, can be seen.

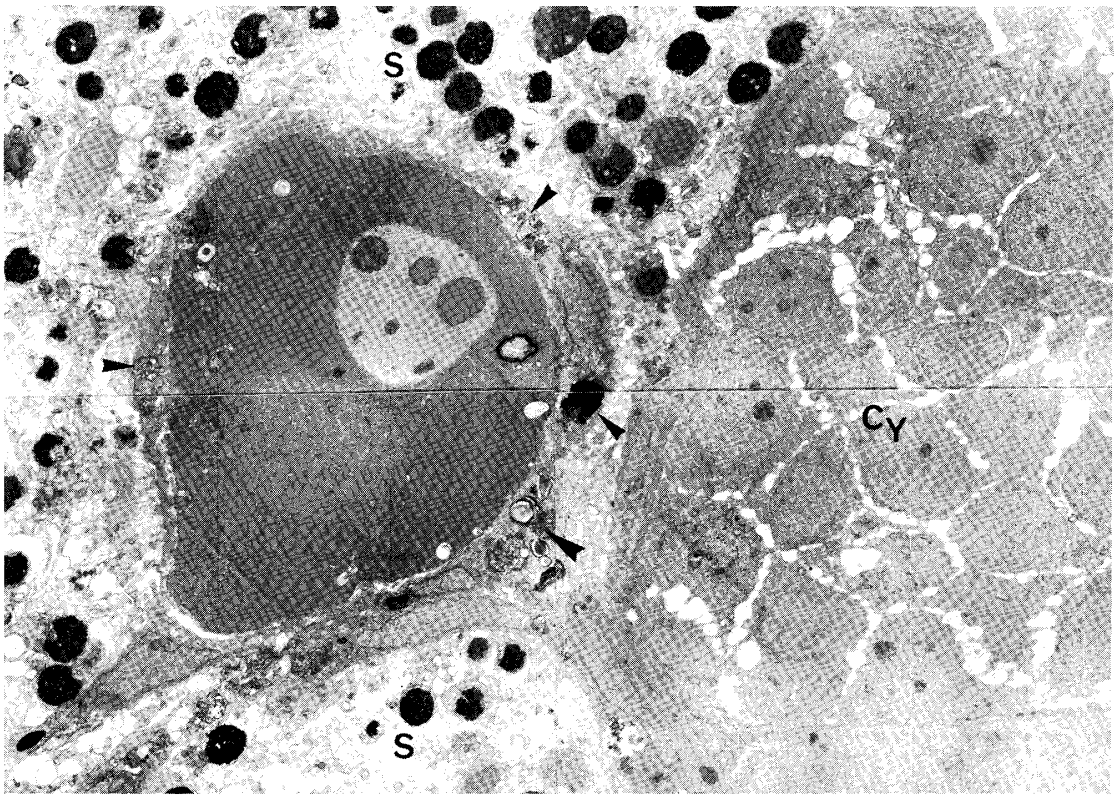

Fig.7 Electron micrograph of an atretic small oocyte under degeneration in the spermiogenic cyst in the genetic female gonad from 20-23mm medaka administered M.T.. x 3000. S, spermatides. Cy, spermatocytic cyst. Note that thickened follicle cells apparently phagocytose the debris of sperm head and residual spermatide cytoplasm (arrowheads).

25-26mm stage

All degenerating oocytes had completely disappeared by this stage, and a gonad free from oocytes was established as a perfect testis, marking the termination of sex-reversal (Fig. 8).

In the central region of the testis, a large lumen was formed by the rupture of expanded cysts of mature spermatozoa. However, there was an abundant amount, in various sizes and places, of necrotic debris and fragments, probably derived from degenerated oocytes during sex-reversal, and residual cytoplasm of spermatides after spermiogenesis. When examined with an electron microscope, these necrotic debris often exhibited a complicated structure with large whorls or myelin-like figures at this stage (Fig. 9). Some of them were phagocytosed by thickened cyst epithelial (Sertoli) cells to form various phagosomes in their cytoplasm. Moreover, on the apical cell surface of such cells, thin filopodia were frequently seen (Fig. 9a). Similar debris were also present in the cyst lumen and they were enclosed by several phogocytes derived from follicle cells (Fig. 9b).

A small cluster of cells with characteristic features of steroidogenic (Leydig) cells was observed around a blood vessel in the connective tissues lining the cyst epithelium (Fig. 9a). In their cytoplasm, there were intensive smooth-surfaced endoplasmic reticula and randomly arranged pleomorphic mitochondria with tubular cristae, as is usual for steroidogenic cells.

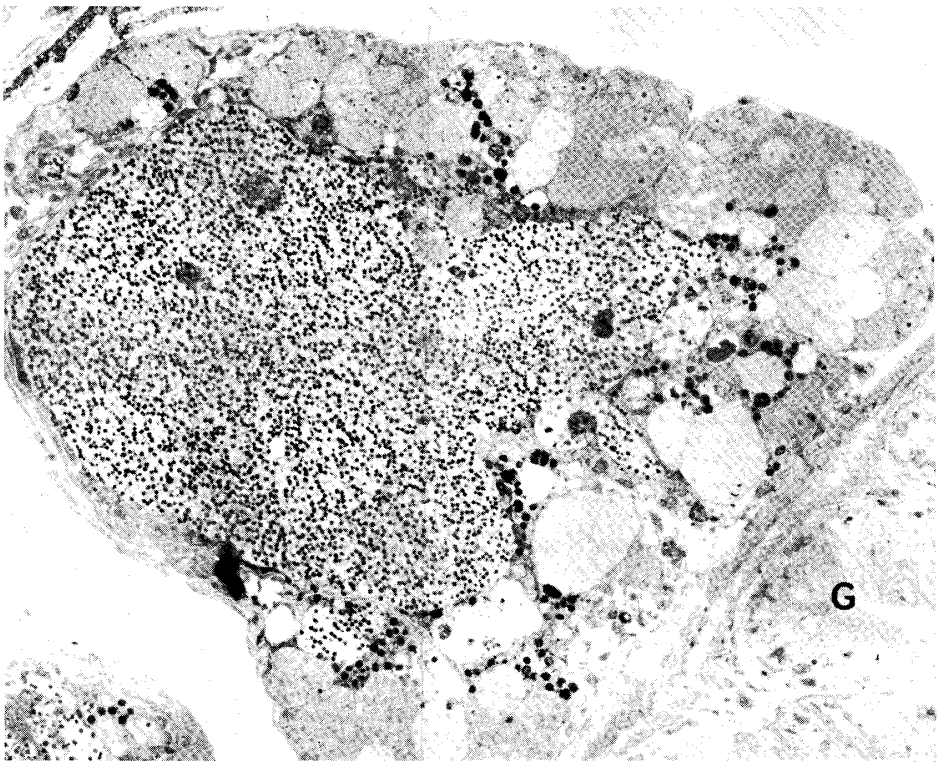

Fig.8 Cross section of the fully matured testis from 25-26mm genetic female medaka administered M.T.. x 250. G, gut. In the central region of the testis, a large lumen is formed by the rupture of expanded cysts containing mature spermatozoa. Various necrotic debris and fragments derived from degenerated oocytes are seen.

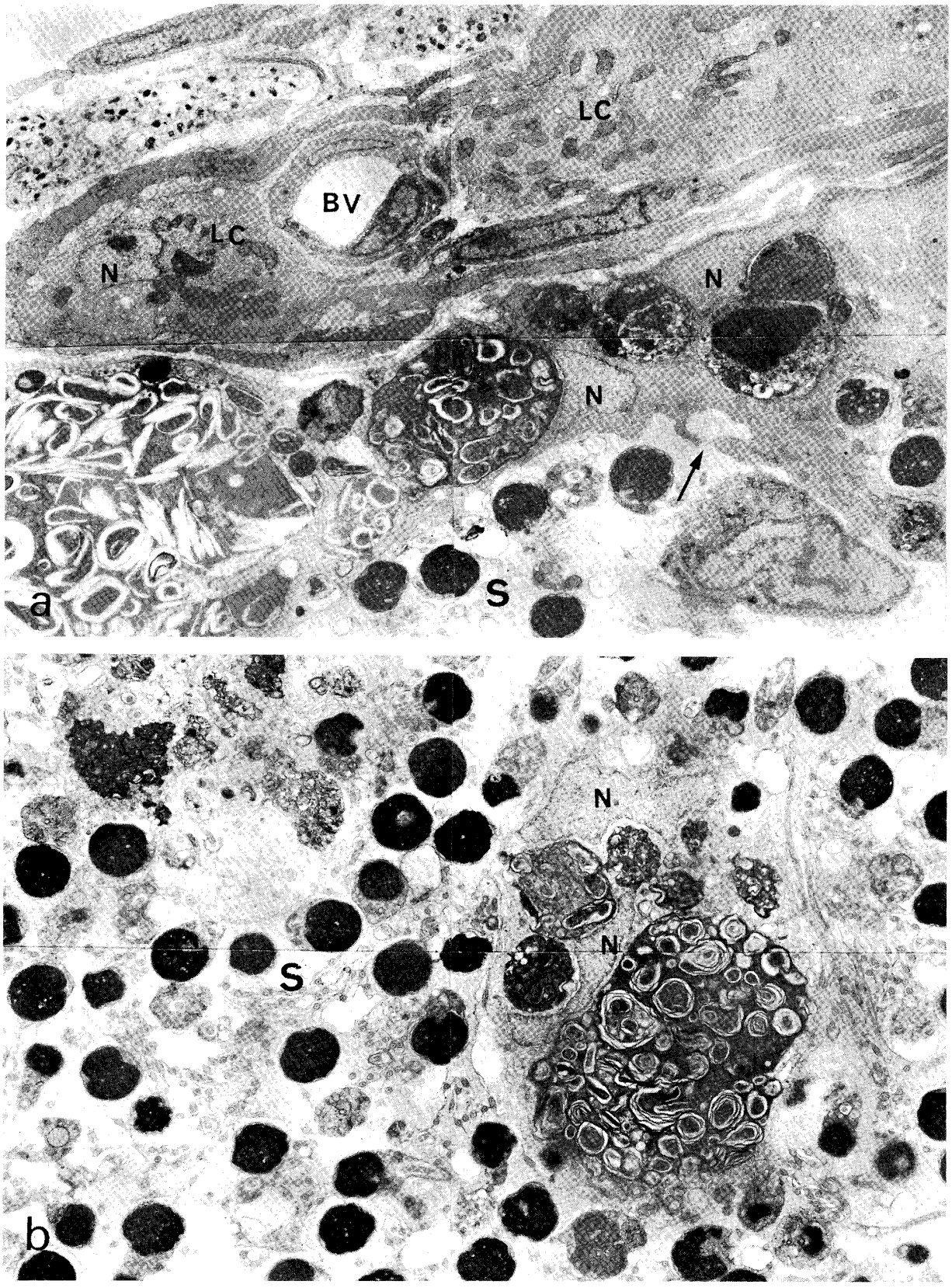

Fig.9 Electron micrograph of the testis from 25-26mm genetic female medaka administered M. T.. x 5000. a. A portion of the cyst epithelium. Large or small necrotic debris are frequently seen within the cytoplasm of epithelial (Sertoli) cells with filopodia (arrow) , indicating their active phagocytosis. In the connective tissue lining the cyst, a small cluster of Leydig cells (LC) are observed around a blood vessel (BL). N, nucleus. S, spermatozoa. b. A portion of the cyst lumen. There are also similar debris, some of which are sequestered by free phagocytic cells.

DISCUSSION

In the normal gonadal sex differentiation of the medaka, by aid of thorough studies of mitotic and meiotic activities of germ cells during early development, it has been revealed that the sex differentiation of germ cells occurs at the time of hatching (Tsuzuki *et al.*, 1966 ; Satoh and Egami, 1972 ; Hamaguchi, 1982). Although primordial germ cells proliferate in both male and female embryos during the few days before hatching, male germ cells cease to divide immediately after hatching, and further cell proliferation is delayed until about the 9-10 mm (in body length) larvae stage, whereas female germ cells continue to increase in number with some of them entering into meiotic prophase within a few days after hatching and continuing to grow thereafter (Satoh, 1974).

On the other hand, moderate (25 μg/g diet) and higher (50 μg/g diet) dosages of methyltestosterone, when administered from hatching time to the juvenile stage (about 12mm in body length) of the medaka, alter the direction of sex-defferentiation in the genetic female and also exert a definite suppressive effect on gonadogenesis in both sex genotypes, the degree of which varies with dosage levels (Yamamoto, 1958 ; Kawamoto, 1973 ; Hishida, 1975). The dosage of a 50 μg methyltestosterone/g diet used in the present experiments is the upper limit for functional reversal in sex differentiation of the medaka, beyond which the gonad becomes atypical or extremely rudimentary.

Therefore, in the androgen-adminstered fish of the present experiments, gonadal development was greatly retarded and gonadogenesis was remakably suppressed in both genetic sexes, as compared with those in normal fish. Furthermore, the suppressive degree was much greater in female than in male. Thus, in the 7-8mm stage, the genetic females had a much smaller gonad with far fewer oogonia and oocytes on the way to oogenesis than those in normal females. In the

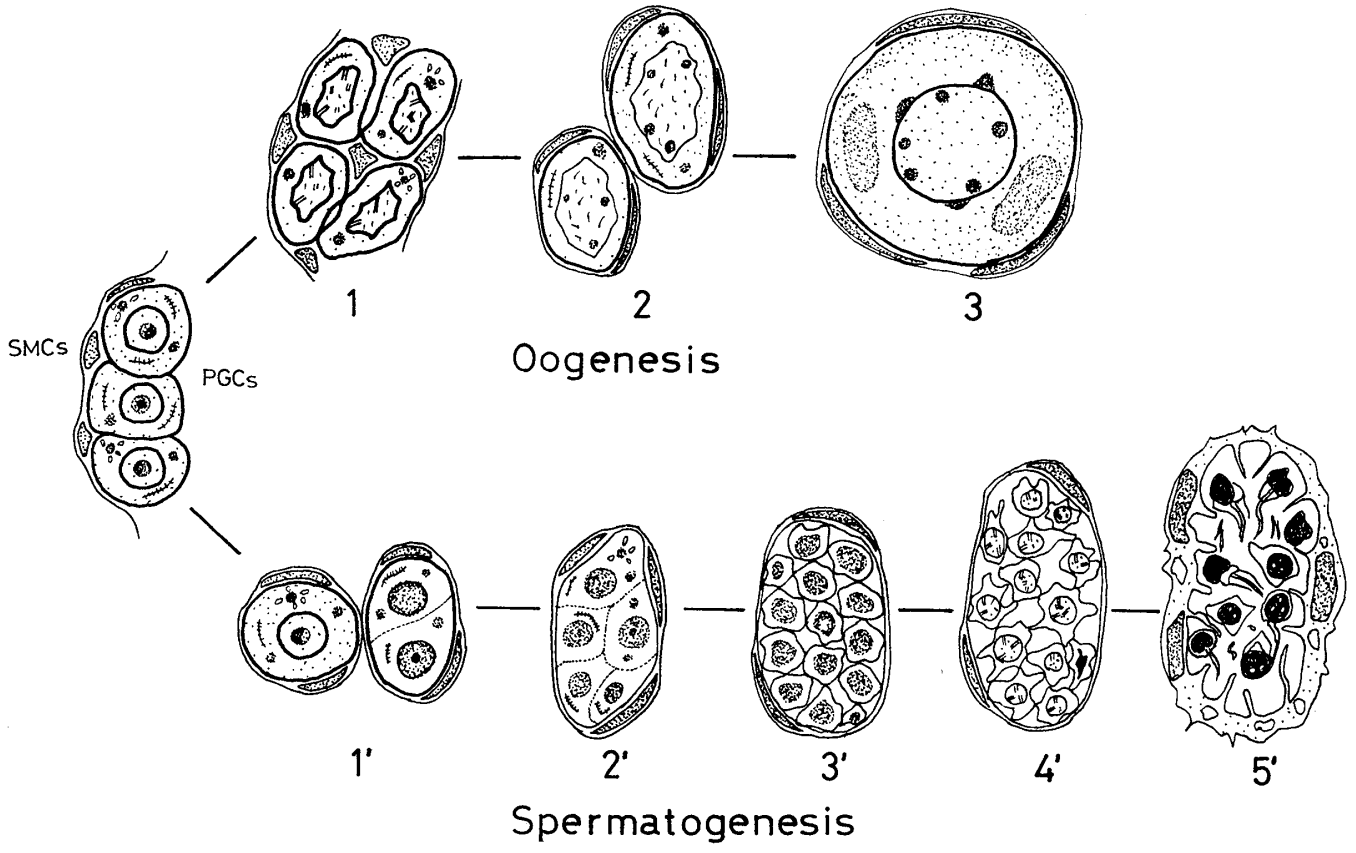

Fig.10 Schematic representation of both processes of oogenesis and spermatogenesis. 1. oogonia to premeiotic oocytes. 2. early perinucleolar oocytes. 3. late perinucleolar oocyte. 1', 2'. acinous spermatogonia. 3',4'. spermatocytes. 5'. spermatides to spermatozoa. PGCs, primordial germ cells. SMCs, somatic cells.

9-10mm stage, most of the germ cells in the androgenized female gonad had already entered into the process of oogenesis, part of them being further transformed into enlarged oocytes in the early perinucleolar stage (Fig. 1, 2), despite the deleterious effects of methyltestosterone. Some of the germ cells were found to form several clumps of distinct acini typical of early testicular structures, each of which was usually composed of one large gonial cell and its tightly enclosing somatic cell layer which, later on, formed the cysts of spermatogonia by gonial proliferation as schematically illustrated in Fig. 10. Afterwards, as gonial development advanced, enlarged late perinucleolar oocytes halted further growth before vitellogenesis, and were destined to follow the degeneration processes, displaying various degrees of abnormal structures.

Newly formed acinous cysts increased in number, expanded in dimension by inner gonial proliferation, and developed into acinous cysts of smaller gonia, and further into tubular cysts typical of adult testicular structures containing various kinds of germinal elements. Furthermore, at the time of hatching, when androgen administration was begun in the present experiments, germ cells in genetic females were assumed to have been in the transition stage to oogonia or in the oogonial stage.

Hence, in view of the above facts it justly follows that the germ cells in the genetic females, once determined to differentiate towards the direction of female sex appropriate to their genetic sex, develop through oogonia into oocytes and continue growing at the most to late perinucleolar oocytes, under suppressive action of the androgen. Thereafter, further growth is halted before vitellogenesis, and they are destined to degeneration by the deleterious action of this exogenous androgen. Thus, the inhibitory effect of the androgen on egg-formation is inferred to have probably been accomplished by two processes : first, by inhibition of conversion of germ cells into oogonia and the destruction of many oogonia, a small number of oogonia escape the action of the androgen and go on to become oocytes ; and, secondly, androgen inhibits vitellogenesis. Although oocytes rapidly grow and become late perinucleolar oocytes, they degenerate before the initiation of vitellogenesis.

After the androgen administration ended, premeiotic oocytes appeared in the gonad of 15-18mm larvae. Futhermore, a small number of tiny perinucleolar oocytes appeared, again intercystically or intracystically, in the gonad of 20-23mm young. These oocytes, however, were unable to grow further under overwhelming and unfavorable testicular circumstances, finally ending in degeneration. These facts suggest that the occurrence of many stem cells or germ cells remaining undifferentiated had been able to resist and patiently endure the inhibitory action of the androgen.

As for the reversal of sex differentiation, the first sign of the reversal seen thus far has only been in the appearance of several clumps of acini in the gonad of androgenized female larvae with a 9-10mm body length, which were usually composed of one large gonial cell and a tightly enclosing thin layer of the somatic cells. Thus, the transition stage from female stem cells or germ cells to male gonial cells is considered to correspond exactly to the time of acinous formation in the genetic female gonad undergoing sex-reversal. However, in what cellular level is the reversal from the female direction to the male one accomplished by male-inducing (androtermonic) action of the androgen, whether in the germ cell or in the gonial cell? This point remains to be resolved.

On the other hand, the critical period for the reversal of sex differentiation in the genetic females by this androgen, beyond which the reversal becomes impossible, is confirmed to be at about the 9mm stage (Hishida, 1975). This close coincidence, between the initial stage of acinous formation and the critical stage, strongly supports the assumption that androtermonic action of the androgen is effective only during a limited period of embryogenesis, in which the sex-determining genes are believed to be in an active state.

There has, thus far, been few descriptions concerning the degeneration processes of oocytes in the female gonad under sex-reversal

by androgen. It has been revealed from present observations that the degeneration begins with the occurrence of various necrotic changes in both the nucleoplasm and ooplasm of the oocytes which were doomed to degeneration by the deleterious action of the androgen, and terminates in the formation of the debris with complicated structures showing large whorls or myelin-like figures inside.

The degenetation processes of oocytes is conceived to submit solely to their autolytic degradation at first stage and, later on, to phagocytosis by cyst epithelial (Sertoli) cells. Follicule cells become greatly thickened at this stage, but have no participation in the phagocytosis of degenerating oocytes *per se*. However, they did have a role and were often observed to engulf the degraded debris and fragments of oocytes into their inner vacuoles.

With regard to the degeneration of male germinal elements, cyst epithelial (Sertoli) cells played a great part in phagocytosis of residual spermatide cytoplasm debris and dead cells during spermiogenesis, excluding them from the cell, as suggested by Gresik *et al.* (1973) in young of the medaka.

In the genetic female gonad under sex-reversal, follicle (granulosa) cells also took an active part in phagocytosis and the exclusion of sperm fragments and their debris.

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