

Steroid-Producing Cells in Chick Intersexual Gonads¹

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Eggs of a sex-linked cross were injected on the fourth day of incubation with 100 μ g of estradiol benzoate. Both injected and control chicks were sacrificed at varying intervals after hatching and a histochemical study of their gonads was conducted. Medullary cells, rich in lipids and with a high 3β -hydroxysteroid activity, were present in newly hatched inverted embryos with a localization similar to that found in normal female gonads. These characteristics persisted in the inverted gonad throughout the whole period of sex reversion. As these cells are supposed to secrete estrogens, it is suggested that the regression of cortex in inverted gonads is not due to lack of estrogen stimulation. Rapid proliferation and differentiation of testicular tissue in the medullary region takes place after the cortex has started its involution and appears to be a consequence of this process, rather than a cause.

Three groups of workers (Wolff and Ginglinger, 1935; Willier, Gallagher, and Koch, 1935; Dantchakoff, 1935) reported simultaneously that female hormones produce sex inversion when injected into genetically male chick embryos. According to the dose used, left gonads may become partially or completely transformed, giving rise to ovotestes or apparently normal ovaries. These results have been confirmed repeatedly, using different estrogenic hormones and various techniques of injection (Gaarenstroom, 1939; Snedecor, 1949; Pincus and Erickson, 1962; Narbaitz and Sabatini, 1962).

Wolff and Haffen (1961) demonstrated that the inversion obtained consists not only in morphological but also in functional changes, by showing that the inverted left gonads are capable of feminizing other embryos if grafted into their coelom. Using a histochemical technique

for the demonstration of the $\Delta 5-3\beta$ -hydroxysteroid dehydrogenase activity, Narbaitz and Teitelman (1965) detected in the medullary region of inverted gonads the presence of steroid-producing cells very similar to those found in the same location in normal ovaries. In addition, Akram and Weniger (1967) proved that feminized gonads are capable of producing estrone and estradiol.

However, even though complete both from the morphological and functional standpoints, the feminization produced by the injection of estrogens is not definitive, and if embryos are allowed to hatch, the gonad reverts to its original sex: the ovarian cortex undergoes atrophy and a testis develops in the medullary region (Wolff, 1936; Gaarenstroom, 1939).

The mechanisms responsible for this regression of the inverted gonad to its original sex are not clearly understood. The present study seeks to contribute to the understanding of this process by following the evolution of steroid hormone-producing cells in inverted gonads during the post-hatching period.

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MATERIALS AND METHODS

A total of five hundred eggs of a sex-linked cross (Rhode Island male X Barred Plymouth Rock female; purchased from Nelson Hardy and Son, Essex, Massachusetts) were used in our experiments. In this particular cross, males are recognizable at the time of hatching by a small white spot on their heads. Inversion was produced by the injection of 0.1 mg of estradiol benzoate in oil solution (Progynon, Schering) into the air chamber of each egg during the fourth day of incubation. Over 300 eggs were injected and 90% of them hatched and survived until sacrifice. Chicks were killed at Days 1, 5, 7, 15, 20, 30, 40, 60, and 90 days after hatching. At least 15 injected animals and 10 uninjected controls of each age and sex were sacrificed simultaneously. Although measurements were not made, the growth of combs in both experimental and control groups was carefully observed.

Gonads were dissected and studied according to one of the three following procedures: (1) fixation in Bouin's fluid, embedment in paraffin, sectioning and staining with hematoxylin and eosin; (2) fixation in a 10% solution of formalin containing 1% calcium chloride, embedment in gelatin, sectioning with the freezing microtome, and staining with Sudan black B (for the identification of lipids) or according to Schultz' test (for the recognition of cholesterol); (3) quick-freezing, sectioning at 15 μ with a cryostat, and incubation of sections in a medium for the demonstration of Δ^5 -3 β -hydroxysteroid dehydrogenase activity (medium of Levy, Deane, and Rubin, 1959, but substituting propyleneglycol with *NN'*-dimethylformamide).

RESULTS

A noticeable growth of combs was observed in all males after Day 15. The amount of growth observed in males injected with estradiol during the embryonic period appeared to be intermediate between the one showed by male uninjected controls and the one of females.

Cystic oviducts were found both in male and female injected chicks. In some cases the entire oviduct was transformed into a large cyst containing a clear fluid; in other cases two or three cysts were formed from one oviduct. These occurred either on the left or right side, or most frequently, on both sides simultaneously. The size of cysts increased with age. A histological study of the wall of the cysts as well as a

biochemical study of the fluid contained in them is underway and will be published.

The macroscopic aspect of gonads from inverted males was clearly different from that of normal females: the right gonad had the aspect of a typical testis, while the left one was similar to an ovary but clearly smaller. These differences were evident at all ages examined.

In accord with this, the histological and histochemical study of the gonads treated with estradiol showed that at the time of hatching all right gonads were typical testes and left ones typical ovaries. Thus, seminiferous cords with a normal population of germ cells were present on the right side, with histochemical tests showing steroid-producing activity both in the cords and in the interstitial tissue, as it occurs in normal testes. By the same token, a thick cortex with numerous germ cells, all in meiotic prophase, and a bulky medulla with many lacunae and groups of medullary interstitial cells were characteristics of the left gonads. In 4 of 18 newly hatched injected males, a small number of seminiferous cords were present in the hilar region of the left gonad; the rest were undistinguishable from normal ovaries.

This similarity continued up to Day 5. Figure 1A shows the presence of lipids, and C and D show the steroid dehydrogenase activity found in the medullary interstitial cells in left, inverted gonads at the age of 5 days. The distribution of these cells and the intensity of the reactions observed were similar to those observed in ovaries of injected and control females of the same age.

After Day 5, inverted gonads followed a very different evolution from the one observed in normal ovaries. In the normals, germ cells became surrounded by cortical somatic cells forming follicles; in the inverted gonads, they gathered in compact groups surrounded by fibroblast-type cells. In subsequent days these germ cells started a very quick process of degeneration. Very few follicles, if any, appeared in inverted gonads.

After Day 10, large groups of seminiferous cords were found in the hilar region

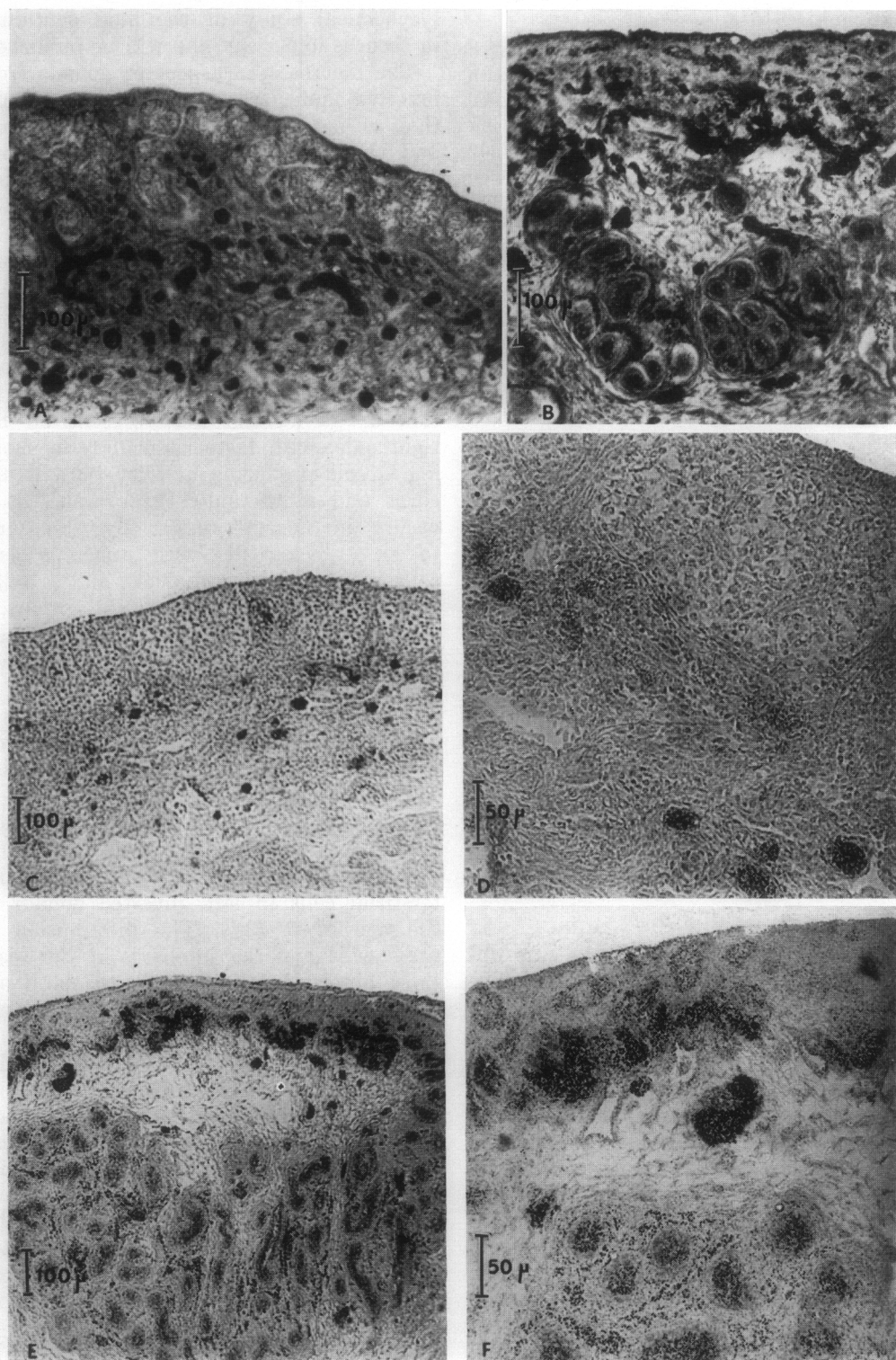


FIG. 1. A: Left gonad of 5-day-old male chick, injected *in ovo* with estradiol. Sudan black B. Medullary

of all left inverted gonads. Three clearly delimited zones were thus distinguished: an outer layer constituted by the degenerating cortex, an inner core formed by the seminiferous cords and an intermediate layer formed by the primitive ovarian medulla with typical interstitial cells. While in normal ovaries medullary interstitial cells become incorporated to the cortex forming the steroid-producing cells of the theca (Narbaitz and De Robertis, Jr., 1968), in the inverted gonads this evolution is not possible because follicles do not form and the cortex degenerates. As shown in Fig. B, E, and F these medullary interstitial cells remain in the outer part of the gonad. However, their content of lipids, cholesterol, and Δ^5 - 3β -hydroxysteroid dehydrogenase activity continues to be comparable to those observed in normal ovaries of similar ages.

With the development of seminiferous cords in the hilar region of the gonad, new types of steroid hormone-producing cells have now appeared. As shown by Fig. B, E, and F, both seminiferous cords and the groups of testicular interstitial cells located among them are loaded with lipids and demonstrate steroid dehydrogenase activity. The localization of these reactions in the testicular portion of the inverted gonad is similar to that observed in normal testes.

After Day 20, most of the volume of the inverted gonad consists of seminiferous tubules and interstitial tissue. However, a narrow cortex, with a few isolated follicles, and an intermediate layer, with medullary interstitial cells, persist at the periphery, separated from the seminiferous tubules by a layer of connective tissue. These peripherally located medullary cells continue to show the same histochemical reactions described previously. Even in gonads of 90-day-old chicks, in which tubules have started active spermatogenesis and in some

cases reached the stage of containing mature spermatozoa, they continue showing signs of active steroid secretion.

DISCUSSION

The proportion of total inversions observed in newly hatched embryos is clearly higher than those observed by previous workers (Wolff, 1936; Gaarenstroom, 1939) and is probably due to the fact that we have used higher doses of estrogens.

Secretion of estrogens in the chick ovary starts during the embryonic period (Gallien and Le Foulgoc, 1961; Cedard and Haffen, 1966; Weniger *et al.*, 1967). The synthesis of these substances appears to take place in the groups of medullary cells with histochemical and electronmicroscopic characteristics proper of steroid-producing cells (Scheib, 1959; Narbaitz and Sabatini, 1963; Narbaitz and Kolodny, 1964; Chieffi *et al.*, 1964; Narbaitz and Adler, 1966).

Male left gonads inverted by estrogen injection are also capable of producing estrogens in the embryonic period (Akran and Weniger, 1967; Haffen and Cedard, 1967) and medullary cells, with distribution and characteristics identical to those of the female gonad, are responsible for their synthesis (Narbaitz and Teitelman, 1964).

In normal ovaries these so-called medullary interstitial cells persist after hatching and most of them become incorporated into the theca folliculi (Narbaitz and De Robertis, Jr., 1968). Our present results show that, in the inverted gonads medullary cells persist after hatching, although they cannot become incorporated into follicles since very few of these are formed. Between Days 5 and 10 posthatching, during which time the cortex is showing the first signs of regression, the medullary cells in inverted gonads are comparable both in their number and in the intensity of their histochemical reactions to those in

cells are loaded with lipids. B: Left gonad of a 20-day-old male chick, injected *in ovo* with estradiol. Sudan black B. Medullary cells loaded with lipids are now located in the periphery beneath the degenerating cortex. Seminiferous tubules and testicular interstitial cells are also rich in lipids. C: Left gonad of a 5-day-old male chick, injected *in ovo* with estradiol. Steroid dehydrogenase activity. Medullary cells with strong enzymatic activity D: Same section as Fig. 1 C but with higher magnification. E: Left gonad of a 15-day-old male chick, injected *in ovo* with estradiol. Steroid dehydrogenase activity. Activity is present in medullary cells, in seminiferous tubules and in interstitial testicular cells. F: Same section as in Fig. 1E but with higher magnification.

normal ovaries. Should these cells continue to secrete estrogens, and their high enzymatic activity shows that they are capable of at least certain steps in steroid synthesis, then it would appear that the involution of the cortex is not the result of a lack of estrogenic stimulation. This idea agrees with the fact that repeated estrogen injections cannot prevent the regression of the cortex (Wolff, 1936).

Gaarenstroom (1939) had indicated that degeneration of the cortex could be due to the action of androgens secreted by testicular tissue developing in the medullary region. Our results indicate, however, that significant development of testicular tissue occurs only after Day 10 in most of the inverted gonads. In addition, we have found that the injection of high doses of androgens (20 mg of testosterone enanthate) into newly hatched females does not interfere with cortical development (unpublished observations).

According to the previous arguments, regression of the cortex of inverted left gonads could be due to an intrinsic deficiency and not to alterations in hormonal secretion. This conclusion fully agrees with those of Wolff and Haffen (1961) and Haffen.

After Day 10, testicular tissue develops very quickly in the central part of the gonad, and in a very short time it occupies most of the gonadal volume. Our results show that both testicular cords and testicular interstitial cells present the histochemical characteristics of steroid hormone-producing tissues as seen in normal testes. With few exceptions, this rapid growth and differentiation of testicular tissue started at a later time than degeneration of the cortex. This fact suggests that the growth of testicular tissue could be the result of the release of the medullary zone from cortical inhibition. If this is the case, the mechanism would be comparable to the one producing testicular transformation of right ovaries after sinistral ovariectomy (Domm, 1927; Benoit, 1950). Gardner, Wood, and Taber (1964) have indicated that a non-estrogenic gonadal inhibitor is secreted by the ovary. The degenerating cortex of in-

verted gonads could possibly be deficient in the production of this inhibitor, and this, in turn, would release medullary tissue and permit its growth.

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