A histochemical study of sex inversion produced by estradiol in chick embryos

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WITH ONE PLATE

INTRODUCTION

Gonadal inversion produced by sex hormones has been studied by many authors trying to understand sex differentiation and its hormonal control. The work of Dantchakoff (1935), Wolff & Ginglinger (1935) and Willier, Gallagher & Koch (1935), who studied sex inversion in genetically male chick gonads caused by the action of estrogens, as well as work done since then on birds and other vertebrates, dealt with the morphology of inverted gonads, while very few attempts have been made to analyse them functionally. Perhaps the most important work on this aspect has been that of Wolff & Haffen (1961), who demonstrated that inverted chick gonads are capable of producing feminizing substances.

It seems well established that hormonal production by normal chick embryonic ovaries is localized in medullary interstitial cells as judged from their high content in lipids and cholesterol (Scheib, 1959; Narbaitz & Sabatini, 1963) and Δ5-Δ3β-hydroxysteroid dehydrogenase (Narbaitz & Kolodny, 1964; Chieffi, Manelli, Botte & Mastrolia, 1964). It appeared to us of interest to know if cells with the same localization and histochemical characteristics are present in inverted gonads. Since at early ages these are undistinguishable from normal ovaries, their study was made possible through the help of simultaneous determination of genetic sex by study of the chromosomes.

MATERIAL AND METHODS

As shown in Table 1, a first series of experiments was made, in which forty-five White Leghorn chick embryos were injected at the 4th incubation day with 0.1 mg. of estradiol benzoate (Progynon, Schering) on the chorioallantoic membrane. Twenty-two of them were dissected on the 9th day, their left gonads

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being fixed in neutral formalin, sectioned and studied for cholesterol with digitonine, as described by Narbaitz & Sabatini (1963). The other twenty-three were dissected either on the 10th or on the 13th day, and the left gonads studied with a histochemical technique for \( \Delta 5-3\beta \)-hydroxysteroid dehydrogenase (Levi, Deane & Rubin, 1959). In both groups, a piece of liver was dissected from each embryo, cut into small pieces, treated for 10 min. in a sodium citrate hypotonic solution (3·7 mg./ml.) and fixed and stained with acetic-orcein for 10 min. Squashes were then made and studied with phase microscope (Narbaitz & Teitelman, 1963).

### Table 1

**Gonads from embryos injected on the 4th day and chromosomally sexed**

<table>
<thead>
<tr>
<th></th>
<th>Cholesterol (*)</th>
<th>( \beta )-Hydroxysteroid dehydrogenase (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injected males</td>
<td>+ (10)</td>
<td>+ (11)</td>
</tr>
<tr>
<td>Normal males</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Injected females</td>
<td>- (12)</td>
<td>+ (12)</td>
</tr>
<tr>
<td>Normal females</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Gonads dissected on the 9th day.
† Gonads dissected on the 10th or 13th day.
‡ Included in table for comparison: extracted from previous paper (Narbaitz & Sabatini, 1963).

Numbers between brackets indicate number of cases studied.

### Table 2

**Gonads from embryos injected on the 9th day**

<table>
<thead>
<tr>
<th>Material</th>
<th>Number</th>
<th>Digitonine</th>
<th>Schultze</th>
<th>Feigin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovaries dissected on the 12th day</td>
<td>38</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Ovaries dissected on the 15th day</td>
<td>20</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Adrenal glands of both previous groups</td>
<td>58</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Control ovaries (dissected on the 12th day)</td>
<td>20</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Testes and adrenals of injected males</td>
<td>15</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

In a second series of experiments shown in Table 2, 110 embryos were injected on the 9th day of incubation with the same dose of estradiol benzoate. Since differentiation has occurred by that time, no inversion could take place and a chromosome study was thus not necessary for sex determination. These embryos, together with thirty-five non-injected controls, were dissected on the 12th or 15th incubation day. All the ovaries and adrenals of those embryos which happened to be females, and a small part of the testis and adrenal glands of those embryos which were males, were fixed in formalin, sectioned and studied with the following techniques: (a) digitonine precipitation, (b) Schultze’s technique for cholesterol, and (c) Feigin’s technique for free cholesterol (Feigin, 1956).
PLATE

Fig. A. Gonad corresponding to a 13th-day male embryo. Technique for Δ5-3β-hydroxy-steroid dehydrogenase. Note the distribution of groups of cells with high enzymatic activity in the medulla. (x 160.)

Fig. B. Same gonad with higher magnification. A group of interstitial cells is shown. (x 600.)

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RESULTS

**Embryos injected on the 4th day**

Direct microscopic observation and the study of photomicrographs of chick embryo cells in mitosis permits the diagnosis of sex (Ohno, Kaplan & Kinosita, 1960). Male nuclei show two 'Z' chromosomes, while female nuclei only show one. These 'Z' chromosomes are easily identified as they are the fifth in size and the only metacentrics.

As shown in Table 1, ten of the twenty-two embryos used for cholesterol study were genetic males. The corresponding gonads showed birefringent precipitates, localized in their medullary region and with the same distribution and apparently with the same intensity shown by normal ovaries. Birefringence was not detected in the twelve gonads belonging to genetic females.

Eleven of the twenty-three gonads used for \(\Delta 5-3\beta\)-hydroxysteroid dehydrogenase belonged to genetic males. All gonads, both male and female, showed abundant groups of interstitial cells in their medulla with intense positive reaction for the enzyme and with the same distribution as that found in normal non-injected ovaries (see Plate, Figs. A & B).

**Embryos injected on the 9th day**

As shown in Table 2, thirty-eight embryos injected on the 9th day and dissected on the 12th day happened to be females and their ovaries did not show birefringence, although control ovaries of the same age were intensely positive. Ovaries dissected on day 15, i.e. 6 days after injection, were also positive.

Schultze's reaction was positive in all groups, including those in which birefringence was absent. Testes and adrenal glands of the injected embryos showed birefringence similar in localization and intensity to those of the controls.

Sections treated with alcohol-ether, after digitonine precipitation, gave negative results with Schultze's technique. This procedure, suggested by Feigin (1956), was only used in control ovaries.

DISCUSSION

Scheib (1959) established the presence of lipids and cholesterol esters in embryonic chick gonads. On the basis of her results with Feigin's technique she concluded that no cholesterol was present in the free form. The presence of cholesterol in chick embryonic gonads was also studied in our laboratory (Narbaitz & Sabatini, 1963) and we reached the conclusion that it appears in ovaries on the 8th, and in testis on the 10th, day. No effort was made at that time to establish if it was esterified or in free form. Our present results with Feigin's technique confirm those of Scheib and appear to indicate that all, or at least most, of it is in the ester form. If this were true, the use of digitonine technique would not be necessary because esters are spontaneously birefringent. Nevertheless, the other commonly used test, consisting in staining sections...
previously treated with digitonine with Sudan Black, appears to show that a part at least of the cholesterol is in free form. When digitonine technique is employed the precipitation of this fraction may increase the birefringence produced by esters.

In a previous work (Narbaitz & Sabatini, 1962) it was observed that the half of the 9-day gonads of embryos previously injected with estradiol gave negative reactions with the digitonine technique. On the basis of previous results with non-injected embryos, it was deduced that those gonads which gave negative results should belong to genetically male embryos. On the contrary, our present results demonstrate that gonads which show negative results after estradiol injection correspond to genetically female embryos. This conclusion is in agreement with Scheib's (1960) findings which show that lipogenesis in inverted male gonads follows the same pattern as in normal ovaries.

The disappearance of birefringence in female embryonic gonads injected with estradiol is an intriguing fact. Estrogens are known to diminish in mammals the amount of circulating cholesterol and Merola & Arnold (1964) believe that this is the result of an inhibition of cholesterol synthesis at the stage of mevalonate decarboxylation. Nevertheless, the fact that Schultze's reaction continues to be positive in the same gonads, appears to indicate that the lack of birefringence observed by us is not produced by a decrease in the amount of cholesterol. In addition, the effect is not generalized to cholesterol synthesizing tissues, being specific in respect of sex (females) and of tissue (ovaries and not adrenal glands). This is also a transitory effect as shown by the presence of birefringence in ovaries when dissected 5 days after injection. No final conclusion may be drawn until biochemical determinations are undertaken.

The most interesting result is undoubtedly the demonstration of interstitial cells with high amounts of cholesterol and \( \Delta 5-3\beta \)-hydroxysteroid dehydrogenase activity in genetically male embryos. This, together with the fact that, according to Wolff & Haffen (1961), inverted gonads produce feminizing substances, indicates with a high degree of probability that these cells are producing estrogens as interstitial cells of normal ovaries do. If so, we should face the intriguing fact of the differentiation of an estrogen-producing cell induced by the action of estrogens. We believe that the most logical explanation consists in admitting that the differentiation of these cells, which normally occurs in females, is in some way inhibited in genetically male embryos and that the action of injected estradiol may consist merely in counteracting this inhibition. Even if this interpretation were true it would remain as an open question whether the inhibition is conveyed by a substance acting as sex organizer (e.g., Witschi's medullarin) or whether it is an intracellular phenomenon (e.g., repressor gene).

**SUMMARY**

1. Forty-five White Leghorn chick embryos were injected on the 4th incubation day with 0·1 mg. estradiol benzoate. Some of them were dissected on the
9th day, their gonads being studied with the digitonine histochemical technique for cholesterol. The rest were dissected either on the 10th or on the 13th day and used for Δ5-3β-hydroxysteroid dehydrogenase histochemical determination. In both groups genetic sex was determined by a chromosome study made on liver cells.

2. The gonads belonging to male embryos contained interstitial cells with cholesterol and enzymatic activity comparable in amount and distribution to those found in normal ovaries.

3. The gonads of female embryos contained apparently normal enzymatic activity but gave negative results with the digitonine technique. Additional experiments were made in order to analyse this fact further.

4. The differentiation, in male embryos, of estrogen-producing cells through the action of estrogens is inferred from our results, and this is explained by supposing that in these cells differentiation is normally inhibited in males by some kind of genetically controlled factor. The action of estradiol would consist in counteracting this inhibition.

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