Growth and sexual differentiation in the gonads of chick and duck embryos

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SUMMARY

The sex-dependent morphological asymmetry between gonads of bird embryos is generally considered to represent the critical point at which the definitive pattern of sexual differentiation is established. A quantitative study of growth in gonads of chick embryos (6- to 11-day-old) and of duck embryos (7- to 11-day-old) has been carried out in order to clarify in what manner growth of gonads is involved in sexual differentiation. Growth has been estimated by the determination of total protein and DNA content, and of DNA synthesis ([3H]Tdr incorporation in vitro).

Increase of protein and DNA content follow different growth curves according to the sex and the laterality of gonads. As early as day 6 (chick) or day 7 (duck) significant differences of protein content exist between gonads: left > right in both sex embryos, and male > female on both sides. Differences in DNA content of gonads are large even at the earliest stages. In the 6-day-old chick embryo both testes contain less DNA than the left ovary and more DNA than the right ovary. In the 7-day-old duck embryo both testes contain the same amount of DNA as the left ovary and twice as much DNA as the right ovary. Consequently, the protein/DNA ratio has a different value according to the sex of the gonad.

In the 6-day-old chick embryo and in the 7-day-old duck embryo absolute values of DNA synthesis (cpm/gonad) are higher in left gonads than in right gonads, and higher in male than in female gonads. When calculated as a ratio to the protein content (cpm/protein) DNA synthesis is lower in the left ovary than in the three other types of gonads, both in chick and duck embryos. When calculated as a ratio to the DNA content of gonads (cpm/DNA), DNA synthesis is lower in the left ovary than in the three other gonads in the chick, and lower in the left ovary and in testes than in the right ovary in the duck.

The results show that growth of gonads is sex-dependent at a very early stage. The meaning of this sex character is discussed with special reference to the role of steroid hormone secretion.

INTRODUCTION

One of the earliest detectable differences between male and female bird embryos is the degree of morphological asymmetry between the gonads. In the chick, this sex-dependent asymmetry first becomes perceptible in 7-day-old embryos, when the difference in size between the ovaries in female embryos is greater than that between the testes in males. It is also at this stage that histological differences between the gonads of male and female chick embryos can first be clearly recognized (Venzke, 1954; Stahl & Carlon, 1973). This stage

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is therefore considered to represent the critical point at which the definitive pattern of sexual differentiation is established.

Growth rate differences between male and female gonads underlie their sex-dependent asymmetry. Anatomical and histological observations are not sufficient to compare precisely the differential development of male versus female, and of right versus left gonads. Only quantitative estimations are able to reveal the exact pattern of gonadal growth and to show in what manner this growth is involved in sexual differentiation. The purpose of this work was to describe gonadal growth quantitatively, in order to emphasize the role of growth as an early character in sexual differentiation.

Until recently gonadal growth has only been measured as increase in wet weight (Breneman, 1941; Venzke, 1943; Romanoff, 1967). These results are not reliable for younger embryos when the gonads are still very small. In a recent article Teng & Teng (1977) reported protein, RNA and DNA determinations in ovaries of the chick embryo. The lack of information about male gonads and the stages of development studied by these authors (days of incubation 8–18) preclude these results from shedding any light on the sexual differentiation of the gonads. However, measurements on gonadal volume by Mittwoch, Narayanan, Delhanty & Smith (1971) provide evidence in favour of the early sexual differentiation of gonadal growth.

The present investigation attempted to estimate the two main aspects of total gonadal growth, i.e. cellular proliferation and cellular differentiation, by examining DNA and protein. In rapidly growing organs, such as the embryonic gonads, cellular proliferation is high and DNA synthesis, which is a measure of this proliferation, is predominant. However, differentiation involves protein as well as DNA synthesis. Measurements of the total protein and DNA content, as well as the rate of DNA synthesis, have therefore been used in order to estimate gonadal growth during sexual differentiation, from day 6 to day 11 of incubation for the chick, and from day 7 to day 11 for the duck embryos. Before day 6 for the chick and day 7 for the duck it is not possible to separate intact gonads from the mesonephros on which they lie.

All determinations were performed on both chick and duck embryos to enhance the generality of our conclusion, as well as to point out some species differences.

MATERIALS AND METHODS

Animals. Eggs of both White Leghorn chick and Pekin duck (Anas platyrhyncos) were incubated at 38 °C in a humidified incubator. The sex of 6- and 7-day-old chick embryos and of 7- and 8-day-old duck embryos was determined by examination of the heterochromosomes in blood cells of the extra-embryonic circulation (Omura, 1970; Gasc, 1973). This technique does not disturb normal embryonic development. The sex of older embryos was identified by morphological examination of gonads.
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[3H]Thymidine incorporation. Gonads were separated from the mesonephros with a microscalpel and placed on a semi-solid culture medium. Contaminating pieces of mesonephric tubules were completely removed. Groups of gonads (groups of seven for 6-day-old chick and 7-day-old duck gonads and groups of five for older gonads) were transferred to a second culture medium (Wolff & Haffen, 1952) containing [3H]Tdr (10μCi/ml; 16 Ci/mM) and unlabelled thymidine (50×10^{-6} mM/ml). It has been previously demonstrated (Gasc & Lébart, 1972) that this medium is suitable for the incorporation of [3H]Tdr in gonads. When unlabelled thymidine is not added to the culture medium labelling is not homogeneously scattered in the whole organ, but an autoradiographic control showed that in our culture conditions [3H]Tdr incorporation is homogeneous even for older gonads (11-day-old). After a 4 h culture on this medium, the gonads were washed and homogenized in Tyrode (1 ml for each group, either 7 or 5). These homogenates were used for protein and DNA determinations and for radioactivity counting.

Protein determination. 0.1–0.3 ml of gonad homogenate was used for the protein determinations (Lowry, Rosebrough, Farr & Randall, 1951), with bovine serum albumin as a standard. Additional protein determinations were performed on other gonads, immediately after removal from the mesonephros. Despite the absence of the 4 h culture, protein contents were identical in both sets of measurements and mean values of protein were calculated from determinations of both sets.

DNA determination. 0.2–0.4 ml of gonad homogenate was stored at −18 °C. In order to reach the threshold of sensitivity for Burton's technique (Burton, 1956), aliquots of several experiments were pooled before being precipitated in cold PCA (final concentration 0.5 N). After centrifugation the supernatant was discarded and the sediment was twice hydrolyzed (15 min; 70 °C) in a small volume (0.2–0.5 ml) of 0.5 N-PCA. Both hydrolyzates were pooled and 0.3 ml reagent was added to a 0.150 ml measure of these. After an overnight incubation (30 °C) optical density (600 nm) was measured in 0.5 ml microcuvettes. Samples of 1–10 μg calf thymus were used as standards. Standard curves proved fully reliable between 2 and 50 μg, since the variation did not exceed 5 %. Additional DNA determinations were performed on gonads not cultivated prior to homogenization. Mean values of DNA content in gonads were calculated from these two sets of values.

Radioactivity counting. 0.5 ml aliquots of homogenate were precipitated with cold TCA (final concentration 5 %) and filtered on Whatman filter (GFB). Then filters were washed with 0.5 % TCA and dried. Radioactivity fixed on the filters (i.e. [3H]Tdr incorporated in DNA) was counted two or three times and mean values calculated. Values of [3H]Tdr incorporation are listed in tables and graphs in absolute terms (cpm/gonad), as well as relative to protein (cpm/μg of protein) and relative to DNA (cpm/μg of DNA). Each cpm/protein ratio was calculated from cpm and protein determinations performed on the same group.
of gonads. A standard deviation (S.D.) value could, therefore, be calculated for the mean of these ratios of $[^3H]$Tdr incorporation/protein (i.e. rate of DNA synthesis/protein). Since samples of several groups of gonads were pooled for each DNA determination, and additional DNA determinations without cpm measurement were carried out, cpm/DNA values could only be calculated as ratio of cpm and DNA mean values, without S.D. values.

Statistical analysis. Each protein, DNA, cpm and cpm/protein determination refers to one measurement performed with one homogenate of gonads. The mean value of determinations and the corresponding standard deviation were calculated (Lamotte, 1957). The statistical significance between mean values was evaluated by the Student's $t$ test. The correlation between right and left gonads was also calculated according to Lamotte (1957).

RESULTS

I. Protein and DNA content in chick gonads

The amount of protein and DNA in chick embryo gonads increases progressively between days 6 and 11 of incubation. Fig. 1 shows a sex difference, in that growth curves for protein and DNA are parallel for testes but not for ovaries. In both testes and in the right ovary, the amount of protein (Fig. 1a) increases regularly between days 6 and 11 (9–10 $\mu$g of protein per day), but in
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Fig. 2. Scatter diagram of the protein content in gonads of the 6-day-old chick embryo. Each point represents the mean amount of protein in the left gonad (abscissa) and in the right gonad (ordinate) of a sample of seven pooled gonads.

In 6-day-old chick embryo gonads, sex differences in DNA content are evident (Table 1). Both testes have almost the same DNA content \((P > 0.05)\), whereas the left ovary contains twice as much DNA as the right \((P < 0.01)\).
Table 1. Amount of protein (µg/gonad) and DNA (µg/gonad), and the ratio (protein/DNA) in gonads of the 6-day-old chick and 7-day-old duck embryos

Mean values ± S.D. (n), Number of determinations. Each determination of protein was performed with a sample of seven pooled gonads; each DNA determination with a minimum of 28 pooled gonads.

<table>
<thead>
<tr>
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<th>6-day-old chick embryo</th>
<th>7-day-old duck embryo</th>
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<tr>
<td></td>
<td>Left</td>
<td>Right</td>
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<tr>
<td><strong>♀ gonads</strong></td>
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<tr>
<td>Protein content</td>
<td>7.59 ± 1.59 (15)</td>
<td>5.07 ± 1.15 (15)</td>
</tr>
<tr>
<td>DNA content</td>
<td>0.192 ± 0.025 (3)</td>
<td>0.091 ± 0.010 (3)</td>
</tr>
<tr>
<td>Protein/DNA</td>
<td>39.5</td>
<td>56.0</td>
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</table>
Values of protein/DNA ratio bring further evidence of differences between male and female gonads (Table 1). The testes and the right ovary have the same value for this ratio (≈ 60) whereas the left ovary has a much lower value (39.5). Thus for the same amount of protein the left ovary has a higher DNA content than the other gonads.

II. Protein and DNA content in duck gonads

Growth curves (protein, Fig. 3a; DNA, Fig. 3b) for the right and left testis of the duck embryo are parallel, whereas growth curves of the right and left ovaries are quite distinct. Growth remains fairly steady until near the end of the period studied, when it slows down rapidly except in the left ovary. The amount of protein and DNA in the left ovary begins to exceed that in the testes between days 10 and 11 in the duck as compared to days 7 and 8 in the chick (Fig. 1). During the period of development that has been studied, growth of the testes appears similar to that of the left ovary in the duck and the right ovary in the chick.

Mean values of protein and DNA content in gonads of the 7-day-old duck embryo are reported in Table 1. The amount of protein is higher in the left gonad than in the right gonad, and higher in the male gonad than in the female
homologous gonad. The sex difference between left testis and left ovary, and between right testis and right ovary, is in both cases highly significant ($P < 0.001$).

The DNA content of gonads in the 7-day-old duck embryo also shows sex differences. Testes and left ovary contain at least twice as much DNA ($\approx 0.200 \mu g$) as the right ovary ($0.090 \mu g$). This similarity between testes and the left ovary has been noted previously (Fig. 3b). The difference between the right ovary and the right testis is significant ($P < 0.02$), as is the difference between the right ovary and the left ovary ($P < 0.01$).

A comparison between chick and duck embryos shows that in both species, the testes contain the same amount of DNA, but this amount is almost twice as high in the duck as in the chick. This difference between chick and duck gonads is also evident from the protein/DNA ratio (Table 1). This ratio is equal in the testes and in the left ovary of the duck embryo ($\approx 40$), whereas in the chick embryo the left ovary is the only gonad with a low ratio (39.5).

III. $[^{3}H]$Tdr incorporation in chick gonads

Between days 6 and 10 of incubation $[^{3}H]$Tdr incorporation (estimated in cpm/gonad, i.e. absolute value of incorporation) increases sevenfold in the left
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[³H]Tdr incorporation is higher in the left than in the right gonad of both male and female embryos, but this difference is always greater between ovaries than between testes (Fig. 4a). Incorporation is constant in both testes between 7 and 10, but increases in both ovaries.

In the 6-day-old embryo, absolute values of incorporation in the gonads depend on their sex and laterality (Table 2). The difference between the right ovary and the right testis is significant ($P < 0.01$), but that between the left ovary and the left testis is not.

When incorporation is calculated with respect to the protein content in gonads (i.e. rate of incorporation/protein), values are higher for right than for left gonads, in embryos of both sexes (Table 2). In the 6-day-old embryo the difference between right and left ovary is not significant ($P > 0.1$) whereas the difference between testes is significant ($P = 0.05$). The differences between ovaries and testes are not significant.

The lower part of Table 2 gives [³H]Tdr incorporation relative to the DNA content of the gonads. Values are almost equal in both testes and in the right ovary, but much lower for the left ovary. Thus the left ovary, which has (in the 6-day-old embryo) the higher DNA content (Table 1), has the lower rate of incorporation/DNA. Moreover, the three gonads (right ovary and both testes) which are relatively low in DNA content (see protein/DNA ratios in Table 1) have the highest [³H]Tdr incorporation rate/DNA. No s.D. value could be calculated for these ratios and therefore no statistical significance estimated (see Materials and Methods).

IV. [³H]Tdr incorporation in duck gonads.

[³H]Tdr incorporation in duck gonads increases between days 7 and 11 of incubation (Fig. 4b). In the testes the incorporation curves are parallel and achieve a level in the 9-day-old gonad intermediate between the right and left ovary curves. The incorporation level of the testes in the duck embryo plateaus later and at a higher level than in the chick embryo. The same species difference has already been observed for the protein and DNA content of gonads (Fig. 1 and 3).

Absolute values of [³H]Tdr incorporation (cpm/gonad) in gonads of the 7-day-old duck embryo show the same differences between sexes as in gonads of the 6-day-old chick embryo (Table 2). Incorporation is higher in testis than in ovary ($P < 0.01$). The rate of [³H]Tdr incorporation/protein in the left ovary of the 7-day-old duck embryo, as in 6-day-old chick gonads, is the lowest of the four gonads (Table 2).

Large differences of [³H]Tdr incorporation/DNA rates appear between the right ovary and the three other gonads in the 7-day-old duck embryo. Estimated in cpm/µg of DNA, this is twice as high in the right ovary as in the left ovary or the testes. The statistical significance of this difference could not be estimated since these rates are ratios of means (see Materials and Methods). Yet the
Table 2. $[^3H]Tdr$ incorporation (4 h culture) in gonads of 6-day-old chick and 7-day-old duck embryos

Incorporation is calculated per gonad (cpm/gonad), or as a ratio to the amount of protein (cpm/$\mu$g of protein) or DNA (cpm/$\mu$g of DNA). Mean values ± s.d. except for cpm/DNA. This latter rate is a ratio of mean values of cpm/gonad (1st line of the table) and of DNA/gonad (2nd line of table 1) without s.d. value. (n), number of determinations. Each determination was performed with a sample of seven pooled gonads.

<table>
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<tr>
<th></th>
<th>6-day-old chick embryo</th>
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<th>7-day-old duck embryo</th>
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<td>$\Phi$ gonads</td>
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<tr>
<td></td>
<td>Left</td>
<td>Right</td>
<td>Left</td>
<td>Right</td>
</tr>
<tr>
<td>Cpm/gonad</td>
<td>450 ± 121</td>
<td>363 ± 74</td>
<td>519 ± 71</td>
<td>482 ± 59</td>
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<td></td>
<td>(10)</td>
<td>(10)</td>
<td>(10)</td>
<td>(10)</td>
</tr>
<tr>
<td>Cpm/$\mu$g of protein</td>
<td>61·1 ± 8·9</td>
<td>68·7 ± 14·1</td>
<td>64·6 ± 10·3</td>
<td>77·7 ± 15·3</td>
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<tr>
<td></td>
<td>(10)</td>
<td>(10)</td>
<td>(10)</td>
<td>(10)</td>
</tr>
<tr>
<td>Cpm/$\mu$g of DNA</td>
<td>2345</td>
<td>3980</td>
<td>3820</td>
<td>3890</td>
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importance of the difference between the left and the right ovary, when compared to the equal values of this rate in both testes, suggests that this difference is actual.

The difference between chick and duck embryos for the rate of incorporation/DNA is conspicuous in Table 2: in the chick, the left ovary is the only gonad whose rate of incorporation/DNA is relatively low, and in the duck the right ovary is the only gonad whose rate is relatively high. This species difference supports our previous observation about the chick gonads: that is, gonads with the highest DNA content, which also have a low value for the protein/DNA ratio, have the lowest rate of [\(^3\)H]Tdr incorporation/DNA.

**DISCUSSION**

The data presented have implications concerning sexual differentiation in gonads of bird embryos. Before discussing these implications, three observations from the results should be pointed out.

(1) A difference has been shown between the linear growth pattern for protein in testes of the chick embryo, and the accelerated growth pattern of the left ovary (Fig. 1a). This difference brings a clear mathematical expression to the morphological observation of differences of growth between ovaries and testes.

(2) Figs. 1 and 2 show the continuous growth (protein and DNA) of the right ovary. Therefore the regression of this gonad has not started before day 11 of incubation, and growth is still significant at this stage.

(3) In the 6-day-old chick and the 7-day-old duck embryos, the left ovary has a lower rate of DNA synthesis than the other three gonads (Table 2). This seems paradoxical, since the left ovary will grow more than the other gonads during the following days. It is necessary, however, to consider the synthesis rates in relation to the amount of protein and DNA of the gonads; therefore, the absolute values of growth can be temporarily larger in the gonad with the smallest synthesis rates. Furthermore, rates in the following days strikingly decrease in all gonads except in the left ovary (unpublished results). Thus, the left ovary has low and slowly decreasing synthesis rates; the other gonads have higher synthesis rates, decreasing more rapidly.

Protein and DNA contents in gonads of the 6-day-old chick and of the 7-day-old duck embryo show sex differences (Table 1 and 2). Protein and DNA growth curves are also different in male and in female gonads (Figs. 1 and 3). Consequently the total growth of gonads depends on their sex as early as day 6 in the chick and day 7 in the duck embryos; this dependence was studied until day 11. The growth pattern differences show that, early in development, growth mechanisms are controlled by the genetic sex of the embryo.

Among these mechanisms one of the most important is DNA synthesis. Table 2 shows that DNA synthesis levels in gonads are dependent on the genetic sex. Sex differences of DNA synthesis levels between gonads are evident both
for absolute values and for rates. Gonads relatively rich in DNA (the left ovary of the chick, and the left ovary and both testes of the duck) have the lowest DNA synthesis rate. This observation suggests an effect of the genetic sex on mechanisms of gonadal growth more precocious than we report in this paper.

This assumption is supported by the work of Mittwoch et al. (1971) on the growth of chick embryo gonads. These authors showed that, as early as day 5 of incubation, the volume of the left gonad is greater than the volume of the right, in both sexes; while the testis of the 5-, 6- and 7-day-old embryo has a volume greater than the corresponding ovary in an embryo of the same age. This difference we observed in measuring protein content in the chick gonads from day 6 in the chick and day 7 in the duck.

According to Mittwoch (1971), growth is responsible for sexual differentiation in gonads: a gonad with high growth differentiates into an ovary and a gonad with low growth differentiates into a testis. Our results do not support this hypothesis. Indeed, although the left ovary of the 6-day-old chick contains more DNA than the testes, it contains less protein and its DNA synthesis rates are lower. Furthermore, in the duck embryo the testes have higher DNA synthesis and content values than the left ovary, and this persists until after day 9 (Fig. 4b) or 10 (Fig. 3a, b). At this time other sexual characters are well fixed. Contrary to the hypothesis of Mittwoch (1971), it does not seem possible to assert that growth has the predominant role in sexual differentiation of gonads. Rather we consider gonadal growth as one character among others.

Two of these other sexual characters have to be mentioned in this discussion. The first is the degree of asymmetry in gonocyte distribution between the two gonads of an embryo. According to the exhaustive study by van Limborgh (1968a, b; 1970), this asymmetry appears to be sex-dependent in gonads of young chick (4-day-old) and duck (5-day-old) embryos. The second is hormonal secretion. According to Scheib & Haffen (1974), steroid hormone secretion differs in male and female in 6- or 7-day-old embryo. Woods & Podczaski (1974) have reported a difference in androgen secretion between male and female gonads on day 5½ of incubation. These results do not come from accurate measurements but from estimations, and the differences between these values cannot be accepted without reservation until they become large enough to overcome the lack of precision, that is, in the 6- or 7-day-old embryo. In a more recent paper Woods, Simpson & Moore (1975) have reported a difference in plasma concentration of testosterone between male and female in the 6½-day-old embryo.

Therefore, steroid secretion exists in the early embryo but there is no clear evidence of a sex difference before day 6 of incubation (see reviews in Scheib & Haffen, 1974; and in Weniger, 1974). Thus, the sexual differentiation of hormonal secretion occurs no earlier than the differentiation of other sex-dependent characters, such as asymmetry of gonocyte distribution or growth. Hormone secretion may, therefore, not be the predominant and determinative factor of
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the early sexual differentiation of gonads. Further, the role of the steroid receptors must be considered in this new pattern of relationships, not merely the direct effect of hormones on tissues.

We are very grateful to Professor Et. Wolff who initiated this work on gonadal growth and provided us valuable advice and encouragement in the course of this study. The results were submitted on the 21st of January 1976 to the Faculty of Sciences of the Pierre and Marie Curie University (Paris, France) as a part of a doctoral thesis (C.N.R.S. A.O. 12042).

REFERENCES


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