Effects of steroid sex hormones on chick embryo gonads in organ culture, with special reference to hormonal control of gonadal sex differentiation

J. JORDANOV, Pavlina ANGELOVA

Institute of Morphology, Bulgarian Academy of Sciences, Academician G. Bonchhev str., bl. 25, 1113 Sofia, Bulgaria.

Summary. At the initial stages of sex differentiation (7.5 and 8.5 days of incubation), chick embryo gonads were treated directly with testosterone or estradiol-17β in organ cultures. Chemically-defined media containing cholesterol as a steroid precursor were used. The differentiation of gonads in the 10 to 12-day controls, cultured in media containing no hormones, was close to that of gonads of equivalent age in ovo. Testosterone added to the medium exerted an inhibitory effect on the cortex of the female gonad and a masculinizing one on its medulla. The results of estradiol treatment confirmed the known feminizing effect of that hormone on the male gonad, the meiotic prophase in the genetically male germ cells being initiated in the induced cortex. These data may be interpreted in favour of a bihormonal theory of gonadal sex differentiation in birds, where the predominantly-synthesized male or female hormone in the gonad determines the male or female pattern of development of the corresponding gonad.

Introduction.

The significance of steroid sex hormones in the differentiation of secondary sex characteristics is relatively well elucidated in the higher vertebrates (Price et al., 1975; Jost, 1976). However, there is no unifying concept to explain the part played by these hormones in the differentiation of the gonad itself. Jost is of the opinion that in mammals « there is no definite evidence that the initial differentiation of the gonads is governed by hormones ». On the other hand, Wolff (1965) postulates that this process in the ovary of birds is regulated by estrogens which are produced early in the ovary. This assertion is based on numerous well known experiments by Wolff and collaborators who obtained sex reversal (feminization) of the embryonic male gonad under in vivo or in vitro estrogen action or under the action of ovaries secreting these hormones. Unsuccessful experiments to masculinize a female gonad using similar approaches with androgens led Wolff (1965) to conclude that the differentiation of the avian testis was androgen-independent.

Later, we established that embryonic female chick gonad could be masculinized when cultured in vitro in association with more adult testes of high
hormonal activity (Jordanov and Angelova, 1974, 1979). After that, Stoll et al. (1978, 1980) reported similar results obtained by early implantation of testes in female embryos. The reversal was ascribed by us to testicular androgens and by Stoll et al. to the Müllerian duct inhibitor of the same organ.

In the present work, we undertook experiments to establish whether the direct application of testosterone or estradiol to young chick gonads in culture would lead to their sex reversal.

In our opinion, such an approach under well-defined conditions, is a reliable and relatively physiological one for studying the hormonal control of gonadal sex differentiation, considering that: (1) a chick gonad differentiates in vitro according to its genetic sex, meaning that the factors of this process are within the gonad itself (Weniger, 1961; Grassi-Milano and Pitini, 1977; Carlon and Erickson, 1978; Jordanov and Angelova, 1979); and (2) its differentiation in ovo and in vitro is accompanied by steroid hormone synthesis with a prevalence of estradiol in the ovary and testosterone in the testis (Guichard et al., 1977; Angelova, Jordanov and Kanchev, 1980).

So far, only a few investigations have used a similar approach (Wolff and Haffen, 1952b; Stenger-Haffen, 1957; Weniger and Zeis, 1973; Carlon and Erickson, 1978), and those that did, contain certain methodological incongruities such as the use of high doses of hormone, short-term culture, inclusion into the medium of components (embryonic extract, serum) in which the presence or lack of hormones, or of their precursors, could lead to ambiguous or even controversial results.

We worked with both high and low (close to physiological) doses of hormones added to chemically-defined media. A method was applied allowing the culture of gonads up to 2 weeks.

Material and methods.

The left ovaries and testes of chick embryos (White Plymouth Rock female × White Cornish male hybrid) were explanted at 7.5 and 8.5 days of age. A slightly modified version of an already-described method (Jordanov, 1971) was used. Briefly, the organs were mounted on a highly permeable celloidin membrane having a plastic ring as a frame. Before they were mounted, a thin layer of warmed 1% agar solution was spread on the membrane; the organs (2 ovaries or 3 testes per membrane) were placed in this solution before it completely gellified. The membrane was introduced into a large excavated slide over about 0.5 ml of liquid medium. Every second day, the membranes with explants were transferred to fresh media, pH 7.5-7.6. The ovaries were cultured for 10-12 days and the testes for up to 13 to 15 days.

The following culture media were used:

Medium I: Eagle’s medium (modified MEM, Flow Lab.) supplemented with cholesterol 200 ng/ml, bovine serum albumin (BSA, Reanal) 3 mg/ml and penicillin and streptomycin at routine doses.
Medium I: TCM 199 (Difco) supplemented with BSA and antibiotics only; as known, TCM 199 contains 200 ng/ml cholesterol.

The two media (usually medium I) were used in control cultures and as basic media for the preparation of those containing the hormone. These media had been shown previously to ensure good growth and advanced differentiation of 7.5-day ovaries over a 10-day culture period, contrary to a medium without a steroid precursor (Eagle + BSA + antibiotics) in which growth and differentiation were poorly expressed (Jordanov and Angelova, 1982).

Medium II: Medium I or II with testosterone (Merck) added at three concentrations: 200 ng/ml, 2 000 pg/ml or 400 pg/ml.

Medium IV: Medium I or II with estradiol-17β (Merck) added at three concentrations: 200 ng/ml, 4 000 pg/ml or 2 000 pg/ml.

The lower doses of testosterone (T) and estradiol (E2) were determined according to data of Guichard et al. (1977) and Angelova, Jordanov and Kanchev (1980). They ensured a ratio of the two hormones for the female and male gonads in the in vitro system close to the ratio of hormones produced by the gonad of the opposite sex in ovo after day 8 of incubation.

Using the above method, the organs flattened (ovaries) or remained more turgid (testes) on the membrane. Supravital observations after a few hours determined whether the sex of the gonad had been correctly identified when taken from the embryo: the ovaries displayed a narrow bright peripheral band (the cortex) which embraced the broad medulla of unequal density having a mosaic aspect; the testes were transversally striated, having the appearance of a tiger hide. Changes in the gross morphology of the explants were also followed supravitally during culture (Pl. I, fig. 5; Pl. II, fig. 4).

For histological examination, the explants were fixed in Carnoy, embedded in paraffin, sectioned at 7 μm, and the sections were stained with Delafield’s hematoxylin. Sections of left ovaries and testes from 17.5-day old embryos were also examined for comparison.

Results.

A total of 160 gonads was cultured. Their distribution according to sex, age and type of culture medium is shown on table 1.

1. Gonads in control cultures (non-hormonal media I and II).

   a) Female gonads. During the first days after explantation, the 7.5-day old gonads manifested distinct growth, the cortex attaining a breadth of 1/3 the breadth of the organ towards the end of the culture period. At the same time, the mosaic appearance of the medulla became more pronounced. After 10-day culture, histological observation showed a well developed cortex (Pl. I, figs. 1, 3) covered by cubic epithelium and packed with a large number of germ cells, many of which were entering the meiotic prophase up to zygotene and pachytene (Pl. I, fig. 4). The medulla consisted of a more compact layer underneath the cortex, while in the remaining larger part, it presented a network of unorganized

Reproduction Nutrition Développement n° 3-84. — 2.
cords anastomosing with each other and separated individually by irregular lacunary cavities. The cords contained varying proportions of different cell types (Pl. I, figs. 2, 4): oogonia, interstitial (steroidogenic) cells having a typical clear cytoplasm and a small heterochromatic nucleus, and satellite and connective tissue cells. In general, the differentiation of the cortex of the ovaries resembled that of ovaries of equivalent age in ovo (17.5-day old embryos). However, their medulla, though having the same cellular composition, underwent no distinct regression with cord distension as in ovaries in ovo.

The same pattern of development was found in explanted 8.5-day old female gonads which showed more intense growth of both the gonad and its cortex.

PLATE I

FIG. 1. — 7.5-day-old ovary after 10-day culture in non-hormonal medium II (TCM 199). C : cortex, M : medulla. × 80.
FIG. 2. — Same as fig. 1 at higher magnification (× 320). A part of the medulla with unorganized cords.
FIG. 3. — 7.5-day-old ovary after 10-day culture in non-hormonal medium I (Eagle’s MEM). Well-developed cortex (C). × 100.
FIG. 4. — Same as fig. 3 at higher magnification (× 500). A border area between the cortex and the medulla. In the former, oocytes are at different stages of meiotic prophase; in the latter, nests of interstitial cells are visible (arrows). × 500.
FIG. 5. — 7.5-day-old ovary after 5-day culture in medium III (T : 2 000 pg/ml). The cortex (C) is poorly developed; the medulla has a testicular appearance. Supravital observation. × 80.
FIG. 6. — 7.5-day-old ovary after 10-day culture in medium III (T : 2 000 pg/ml). Tubular organization of the medulla. × 80.
FIG. 7. — Same as fig. 6 at higher magnification (× 500). A part of the medulla. Tubules lined with satellite cells; note the germ cells among them.
b) Male gonads. Up to the end of the 10-day culture period, male gonads manifested poor growth. Their transversal striation was slightly accentuated. Histological observations showed a cubic or columnar epithelium on the gonadal surface while, in the interior, densely packed tubular structures with a poorly developed stroma between them were seen. These structures surrounded by a basal membrane were lined with low-cylindrical (future Sertoli) cells with apical cytoplasmic portions forming the central axis of tubules in which the lumen was lacking. A considerably smaller number of spermatogonia was found among these cells. Usually, there was a trend towards differentiation such as that in a testis of equivalent age in ovo. However, the in ovo testis had the following differences: flat germinal epithelium; tubules possessing higher epithelial cells with an apical cytoplasm which was vacuolized and subject to disintegration in places with the formation of a discontinuous lumen; abundant stroma separating the tubules and making the in ovo organ much larger.

2. Gonads cultured in medium III containing testosterone.

a) Female gonads. In a great number of 7.5-day old gonads (some 100, 75 and 50 % of the ovaries cultured at doses at 200 ng, 2 000 pg and 400 pg/ml testosterone, respectively) the cortex enlarged insignificantly or slightly, contrary to the medulla which became massive and transversally striated, giving it a testicular aspect. These changes appeared as early as days 5-6 of culture (Pl. I, fig. 5). Some 80 and 60 % of the 8.5-day old explants developed in a similar manner when cultured at doses of 200 ng and 2 000 pg/ml testosterone, respectively. All these gonads were histologically examined at the end of the 10-day culture period. In most cases, the cortex observed was composed only of a 2 to 3-layered columnar epithelium with short cords and buds protruding from it towards the medulla. The cords and buds contained some germ cells at a gonial stage (Pl. II, fig. 1). In some cases, the cords fused into an almost continuous, but not broad, cortex containing a larger number of germ cells (Pl. I, fig. 6 ; Pl. II, fig. 2) which, in the case of 8.5-day old ovaries, sometimes entered meiosis up to leptotene. The well-developed medulla consisted of densely arranged anastomising tubular structures, oriented chiefly towards the hilum of the organ (Pl. I, fig. 6 ; Pl. II, figs. 1, 2). These structures were usually genuine tubules with a
narrow lumen, lined with a higher or lower cylindrical epithelium surrounded by a distinct basement membrane. Germ cells at a gonial stage were scattered among the epithelial cells (Pl. I, fig. 7). In other cases, no clear lumen was present in the tubules, their central axis being formed by confluent vacuolized apices of the epithelial (satellite) cells (Pl. II, fig. 2). The intertubular stroma was relatively less developed. Instead of epithelioid interstitial cells, typical of the ovary, the stroma contained fusiform or stellate cells adhering to the tubules and similar to the interstitial cells in the testis described by us (Jordanov et al., 1978).

The above description could be applied to ovaries cultured at a dose of 2,000 pg/ml testosterone. With a higher dose, the intertubular stroma was very sparse, it was difficult to distinguish the individual tubules, and there was usually a predominant number of either germ or satellite cells in the tubules. At the lowest dose, the cortex was relatively well developed, while the medulla contained fewer, but better-shaped, tubules having sufficient intertubular stroma among them.

b) Male gonads. As compared to the male gonads in the control cultures, those grown in a medium with 2,000 pg/ml testosterone showed features of further evolution close to that of male gonads of equivalent age in ovo (see 1b). The organs were visibly larger. Histological examination revealed tubules with sharper outlines due to a better-developed intertubular stroma; fusiform interstitial cells were more frequent in this stroma; the epithelial cells of the tubules were higher with vacuolized apical portions separated by a barely-formed lumen in certain sites. However, the number of spermatogonia in the tubules seemed to be the same as that in the control cultures and the germinal epithelium on the surface of the gonads was columnar (Pl. II, fig. 3).

3. Male gonads cultured in medium IV containing estradiol.

In a large percentage of the gonads (70% or more of 7.5 and 8.5-day old gonads at the three estradiol doses), a bright, homogeneous band appeared along the margin of the organ opposite to the hilum as early as about days 5-6 after explantation (Pl. II, fig. 4). This band grew larger in the following days, taking up 1/4 to 1/3 of the breadth of the organ. The rest of the gonad manifested a weaker or stronger effacement of its transversal striation.

Explants with the above pattern of change were histologically examined. Some of those cultured for 8 days consisted of lower or higher columnar superficial epithelium and large nests of germ cells protruding from it towards the interior of the organ, all of these cells at a gonial stage. The nests, separated by a small amount of stroma, almost formed a continuously broad cortex, the lower boundary of which was delineated by a basement membrane along its greater length. There followed a thin connective tissue layer underneath the cortex, while the rest of the organ contained chiefly unorganized cords which lent it a reticular appearance. These cords contained groups of clear epithelioid interstitial cells similar to those in an ovary. In certain places, the cords showed a more organized tubular structure. The histological aspect of explants undergoing more prolonged culture, up to a total in ovo and in vitro age of 22 to 24 days, resembled the one
described. Yet, many of the cortical germ cells were entering the meiotic prophase up to leptotene and even zygotene (Pl. II, fig. 5). The described transformation of male gonads under the action of estradiol was equally valid for all three doses of the hormone.

Discussion.

The present study shows that chick gonads of both sexes, taken at their initial stages of sex differentiation (7.5 and 8.5 days of incubation) and cultured for 10-12 days in a non-hormonal medium supplied with cholesterol as a steroid precursor, developed according to a pattern very close to that in gonads of equivalent \textit{in ovo} age. This was particularly true of the essential tissue and cell components of the organs, \textit{i.e.} ovary: formation of a thick cortex and germ cells entering into the early stages of meiotic prophase; testis: formation of organized structures of the semiferous cord-type and germ cells at the spermatogonial stage. The ovarian medulla developed in a tissue consisting of structurally unorganized cords and lacunae like those in the ovary \textit{in ovo} but without expressed regression. The interstitial (steroidogenic) cells in these cords were well represented by their epithelioid shape, typical of those in the ovary. The pattern presented was in contrast to that of ovaries cultured in the same media without a steroid precursor, where the development of the cortex was much reduced and cortical germ cells remained at a gonial stage (Jordanov and Angelova, 1982). These data appear to provide new indirect evidence for the claim that differentiation in chick ovary depends on the estrogens produced by it. They likewise explain the adequate self-differentiation of chick ovary \textit{in vitro} (see Introduction), where media containing serum and embryonic extract were used, both being good sources of steroid precursors.

Our experiments with cultures of 7.5 and 8.5-day old male gonads in a medium containing estradiol indicated that estradiol exerted a feminizing effect on a large percentage of them. This was shown by the formation of an almost continuous cortex and by a greater or lesser disorganization of the testicular cords in the organ. The results agree well with those of similar investigations by Wolff and Haffen (1952b) and Stenger-Haffen (1957). However, the data reported by these authors refer to duck testis and to the effect of unknown, probably high, hormone doses. We used low doses close to the physiological level, exerting the same effect on the male gonad. Moreover, when our organ cultures were prolonged to a total \textit{in ovo} and \textit{in vitro} age of 22-24 days, it was possible to establish the onset of the meiotic prophase in the genetically male germ cells of the induced cortex. A similar phenomenon has also been observed by us (Jordanov and Angelova, 1979) in testes feminized by their association \textit{in vitro} with older ovaries. This again supports the hypothesis we proposed at that time concerning the part played by estrogens in meiosis; we suggested that, after they induce a number of genetically-programmed divisions in germ cells, estrogens lead to the release of the meiotic prophase in these cells. When male gonads were cultured in a medium containing testosterone, organ differentiation
closely resembled that in ovo. However, in this case, the germinal epithelium was not completely flatted, but retained its columnar structure in certain sites; this phenomenon has also been described by Stenger-Haffen (1957). It is possible that a similar retention of the columnar epithelium could have been interpreted by Weniger and Zeis (1973) as « feminization » of the testis under the action of testosterone in their short-term (4 to 5-day) cultures and in a medium that was not very favourable at that.

We believe that our data on the culture of female gonads at their stages of initial differentiation in a medium containing testosterone are of fundamental interest. This hormone exerted a stronger or weaker inhibitory effect on the ovarian cortex in most of the gonads, but had a transforming effect on the medulla, leading to the formation of tubular structures similar to the testicular ones. It seems evident that, here, we are dealing with masculinization of the derivatives of the first proliferation of the ovarian germinal epithelium, which normally regresses in ovo. It should be noted that the interstitial cells in the reversed female gonad acquired a morphological aspect (fusiform) similar to that in a testis. All these facts support the claim that androgens secreted by the male gonad are responsible for the masculinization of chick female gonad, as observed in experiments where it was associated in vitro with older testes (Jordanov and Angelova, 1974, 1979) or where testes were implanted early in female embryos (Stoll et al., 1978, 1980).

Three questions arise in connection with the above data. First, did the morphological reversal of the ovary under the action of testosterone correspond to a functional reversal, as regards hormonal biosynthesis? Our preliminary radio-immunoassay studies (Angelova, Jordanov and Kanchev, 1983) demonstrated that chick gonads pretreated in culture with testosterone and having acquired signs of masculinization, enhanced their endogenous production of testosterone above that of estradiol, the T/E\textsubscript{2} ratio being raised above 1, as in a testis. A reverse phenomenon was reported by other researchers (Haffen and Cédard, 1968; Teng and Teng, 1977) in a testis feminized under the action of estrogens, where endogenous estradiol production increased above that of testosterone. Therefore, we can say that the morphological reversal of the ovary under the action of testosterone seemed to correspond to a functional reversal.

The second question is: how can one explain the discrepancy between our results and those of Wolff and Haffen (1952a) in relation to the evolution of the medulla of chick ovary cultured in non-hormonal medium? In their short-term (5 to 6-day) culture, these authors describe the « spontaneous » testicular transformation of medullary cords, resulting in the reversal of the ovary into an ovotestis. In our opinion, the discrepancy can be attributed to a difference between the capacities of the media used in our experiments and those of Wolff and Haffen. The two French researchers themselves, noting the insufficient development of the cortex in their explants, considered that the aberrant evolution of the ovary into an ovotestis might be due to the fact that their medium was not capable of maintaining a high production of female sex hormones in the gonad. Actually, the medium of Wolff and Haffen was a
strongly-diluted extract of 7-day old chick embryos, probably containing a minimal amount of cholesterol (see Needham, 1931). Later workers, who cultured chick ovaries in an extract of older, 8 to 11-day old embryos (Weniger, 1961; Grassi-Milano and Pitini, 1977) or in a medium containing calf serum (Carlon and Erickson, 1978; Jordanov and Angelova, 1979), reported and even emphasized that they had not observed a real reversal of the ovary into an ovotestis and described an evolution of the medulla close to that in the present studies. We would explain the differences between our results and those of Wolff and Haffen by the first of the three hypotheses proposed by them, i.e. that the aberrant evolution of the ovary into an ovotestis was due to the fact that the equilibrium between female and male hormones within the gonad was unbalanced in favour of the male hormones which exerted a masculinizing effect on the medulla.

The third question we wish to raise is: why were previous attempts to masculinize chick embryo gonads unsuccessful? As concerns negative results obtained by injections of androgens into the egg, we accept the suggestions of Scheib (1976) an Scheib and Haffen (1974) that the cause for unsuccessful masculinization could be the unstability of the androgens and the possibility of their metabolization into inactive products during their pathway to the ovary, or of their aromatization into estrogens in the ovary itself. Similar negative results, obtained in earlier experiments on the in vivo or in vitro association of a female with a male gonad, were explained by the fact that neither the ages of the associated gonads nor their relative hormonal activities were taken into account (Jordanov and Angelova, 1979). Lastly, the failure of experiments so far to reverse a female gonad in vitro with the aid of exogenous androgens could be due, in our opinion, to methodological incongruities (see Introduction).

In conclusion, our results support a bihormonal theory of avian gonadal sex differentiation, the causative factor of which would be the corresponding steroid sex hormone with its genetically determined prevalence in the gonad (estrogen in ovary, androgen in testis). In the process of the binding of the two hormones to their receptors — which are probably unequally distributed between the two main components of the gonad, the germinal epithelium and primary sex cords — and possibly also during their competition for these receptors, the quantitatively prevalent hormone imposes itself, determining a female or male pattern of development in the corresponding gonad.

An important role has been attributed to the H-Y antigen in the differentiation of the gonad of the heterogametic sex (the ovary in birds); it would be of interest to investigate whether in experiments such as the present ones the expression of this antigen would be suppressed in the female gonad during its masculinization by androgens, as reported by Zaborski et al. (1980) in the right ovary of chicken during its masculinization after left ovariectomy. In any case, our data support the view of Zaborski et al. that the expression of the H-Y antigen in birds is hormonally dependent and has an intermediary role in gonadal sex differentiation.

Acknowledgements. — We wish to thank Mrs. E. Donova for her careful technical assistance.


Des gonades embryonnaires de Poulet préllevées aux stades initiaux de leur différenciation sexuelle (7 j 1/2 et 8 j 1/2 de l'incubation) ont été soumises à l'action directe de la testostérone ou de l'oestradiol-17β en culture organotypique. On a utilisé des milieux nutritifs de composition chimique définie contenant du cholestérol comme précurseur des stéroïdes. Dans les cultures témoins de 10-12 jours (sans hormone dans le milieu nutritif) les gonades subissent une différenciation analogue à celle des gonades du même âge in ovo. La testostérone introduite dans le milieu de culture a un effet inhibiteur sur le cortex de la gonade femelle et en même temps un effet masculinisant sur la médullaire. Les résultats du traitement par l'oestradiol confirment l'action féminisante déjà connue de l'hormone sur la gonade mâle ; dans le cortex induit, on observe que les cellules germinales entrent en phase méiotique. Ces données sont en faveur d'une théorie bihormonale de la différenciation sexuelle des gonades chez le Poulet selon laquelle la prédominance quantitative de l'hormone femelle ou mâle synthétisée par la gonade détermine sa différenciation dans le sens correspondant.

References


Sex hormones and gonadal differentiation


