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Review

Embryonic sex hormones in birds

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Introduction

Even if they did not coin the term, the concept of embryonic sex hormone can be traced back to Bouin and Ancel (1903), who, after studying the histogenesis of the interstitial gland of the testis in the pig (Ancel and Bouin, 1903), came to the conclusion that «this gland conferred through internal secretion on the male organism during its intra-uterine life the essential characteristics of its sex». This concept was then applied by Lillie (1916) and Keller and Tandler (1916) to explain the freemartin phenomenon and finally verified experimentally by Burns (1925), who first performed parabiosis in *Amphibia*.

In birds, intersexuality was first obtained by sex hormone injections (Wolff and Ginglinger, 1935), before hormonal activity of the embryonic gonads was demonstrated by grafting experiments (Wolff, 1946-47). Studies on the chemical nature of the hormones followed (Weniger, 1969), relayed by research on the regulation of hormone secretion by gonadotrophins. This plan will be adopted in the present paper.

Grafting and coculture experiments

Wolff (1946-47) transplanted pieces of chick embryo gonads into the coelomic cavity of embryos at 50h of incubation (25 pairs of somites). In a female host, a testicular graft determined regression of the Müllerian ducts; in a male host, an ovarian graft determined feminization of the testes. These results clearly demonstrated hormonal activity of the chick embryo gonads. They have been confirmed many times, and not only in the chick (Malinowska and Weniger, 1965) but also in other avian species, e.g. the duck and the pigeon (Akram, 1969).

Similarly, in *in vitro* culture, hormonal activity of ovarian anlagen found expression in the feminization of cocultured testicular anlagen, while regression of cocultured Müllerian ducts proved the hormonal activity of testicular anlagen (Weniger, 1961). Intimate contact between inducer and receptor organs was not necessary; action still took place when there was a distance of several millimeters between the two organs on the gelified culture medium (Weniger, 1962). So, the hormones diffused into the culture medium, and one could try to isolate and identify them.

Isolation and identification of estrone and estradiol

Wolff and Ginglinger (1935) had formulated the hypothesis that embryonic sex hormones were not different from the sex hormones of adults. So, the search for androgen and estrogen was undertaken.

Culture media of chick embryo ovaries were subjected to ether extraction. The dried ether extract was dissolved in 50% aqueous glycerol, which was used in the Allen and Doisy test. The test was negative with control media, positive with media of ovaries. So, the ovaries had secreted an estrogenic substance into the culture media (Weniger, 1964, 1965b). When the crude ether extract was partitioned, the estrogenic activity was recovered in the fraction of the phenolic steroids (Weniger, 1965c). When this fraction was further divided into an «estrone-estradiol» fraction and an «estriol» fraction, the estrogenic activity was recovered in the former fraction (Weniger, 1966).

The next step was the identification of estrone and estradiol by radiochemical methods. Chick embryo ovaries were cultured in the presence of [1-^{14}C] sodium acetate, and radioactive estrone and estradiol formed were identified by radiochromatography and derivative formation (Weniger *et al.*, 1967). Definitive identification of both estrogens was based on recrystallization to constant specific activity (Weniger, 1969).

Is there a role for estrogen in ovarian sex differentiation?

The hypothesis of Wolff and Ginglinger (1935) not only stated that embryonic sex hormones were identical with the sex hormones of adults, but also that in normal development sex differentiation of the genetically female gonadal anlage into an ovary occurred under the influence of «folliculin», since «folliculin» — the name for estrone in those days — injected into the male embryo feminized the testes.

This would mean that estrogen was being secreted by undifferentiated genetically female gonadal anlagen. Estrogen synthesis from progesterone takes place in ovarian anlagen as early as 5 days old, when they are still undifferentiated (Weniger, 1968), and from sodium acetate in 5- to 6-day-old anlagen (Weniger and Zeis, 1969), but not in 4- and 5-day-old anlagen (Weniger and Zeis, 1971). On the other hand, testes feminized by estradiol secreted estrone and estradiol (Akram and Weniger, 1967, 1969), and the capacity to synthesize estrogen appeared very rapidly, within 24 h (Weniger and Zeis, 1975).

If estrogen is to be responsible for ovarian sex differentiation, one would expect that suppressing within the developing gonadal anlage the action of estradiol by tamoxifen — an estrogen antagonist — would prevent ovarian differentiation. Salzgeber *et al.* (1981) and Scheib and Baulieu (1981) have reported the formation of seminiferous cord-like structures in the ovarian medulla, after injecting tamoxifen into chick or quail embryos. However, these structures could not be observed by others (Weniger and Zeis, 1984; Koo *et al.*, 1985; Weniger and Samsel, 1985; Didier and Croisille, 1989). In addition, tamoxifen-treated ovaries secreted the same amounts of estrogens as control ovaries (Weniger *et al.*, 1982).

Similarly, suppressing estrogen synthesis by aminoglutethimide, an aromatase inhibitor, should impede ovarian differentiation. This was not the case, although estrogen secretion in the presence of

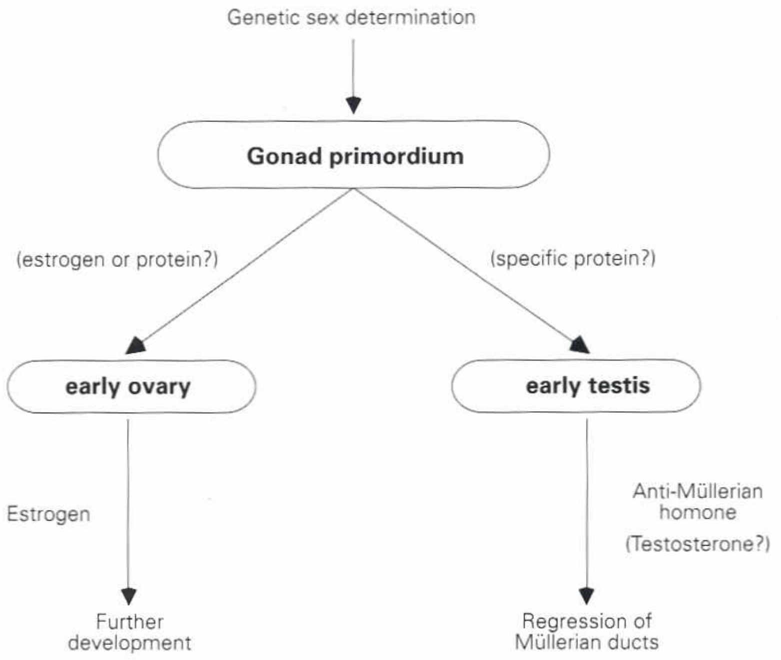

Fig. 1. Main steps in sex differentiation. *After sex has been genetically determined, an undifferentiated gonad primordium develops in both sexes. Under the influence of sex inducing substances (estrogen? protein?), it differentiates into an early ovary or testis. The early ovary secretes estrone and estradiol, the early testis, anti-Müllerian hormone and, according to some authors, testosterone. The significance of estrogen secretion in the female chick embryo is unknown. In the female duck embryo, estrogen is responsible for the differentiation of the syrinx and genital tubercle, two early somatic sex characters (Et. Wolff and Em. Wolff, 1951).*

the inhibitor was strongly reduced (Weniger *et al.*, 1985). So, all taken together, the evidence is not in favor of a role for estrogen in ovarian sex differentiation. Recall that Gasc (1978) had already stated that «hormone secretion might not be the predominant and determinative factor of early sexual differentiation of gonads». Recent work proceeds along a line which ascribes a role to sex-specific proteins in ovarian sex differentiation. Two protein spots peculiar to the ovary were detected in the cytosolic fraction after two-dimensional gel electrophoresis (Samsel *et al.*, 1986), and these same spots appeared on the male electrophoregram after estrogen treatment of the testis (Samsel *et al.*, 1988).

Search for testosterone

While the demonstration of estrone and estradiol secretion by the chick embryo ovary was easily achieved, demonstration of testosterone secretion by the testis encountered great difficulties. *In vitro* culture experiments revealed that the chick embryo testis did not have the same effects as the mouse embryo testis on target organs which responded to testosterone. For instance, growth and differentiation of the mouse embryo Wolffian duct were promoted by the mouse embryo testis, but not the chick embryo testis (Weniger, 1965a). Similarly, the rat embryo testis stimulated the chick embryo Wolffian duct, whereas the chick embryo testis did not (Chouraqui *et al.*, 1980; Weniger and Zeis, 1980). *In vivo*, contrary to the condition in the mammalian embryo, where testosterone secreted by the testes is responsible for the male sexual differentiation of the genital tract (Jost, 1946-47, 1950, 1953) and brain (MacLusky and Naftolin, 1981), these differentiation processes are not attributable to testosterone in the male avian embryo. Testosterone-induced epididymal differentiation does not occur before the third week after hatching (Maraud, 1963; Maraud *et al.*, 1975, 1980) and brain differentiation is not an active process in the male embryo but in the female, the active substance being estradiol secreted by the ovary (Adkins, 1978, 1979).

Interstitial Leydig cells, which supposedly secrete testosterone, were identified by ultrastructural criteria in the chick embryo testis by Scheib (1970). However, contrary to the ovarian interstitial cells, presumptive Leydig cells in the testis do not show the fine structure typical of active steroid-secreting cells (Carlon and Erickson, 1978). In the testis of the newly-hatched male quail, Leydig cells are poorly differentiated; they do not acquire the typical features of active steroidogenic cells before the third week (Scheib, 1973).

Contradictory results were obtained when radiochemical methods were used to identify testosterone produced by the bird embryo testis. While, according to Haffen and Cedard (1968), Galli and Wassermann (1972, 1973) and Guichard *et al.* (1973a), testosterone was formed from dehydroepiandrosterone, progesterone or pregnenolone by the chick embryo testis, as well as the quail embryo testis (Guichard *et al.*, 1973b; Scheib *et al.*, 1974), Weniger (1969, 1970), Weniger and Zeis (1973b, 1976, 1977) and Weniger *et al.* (1984) did not succeed in demonstrating testosterone production by the chick embryo testis in spite of numerous attempts. However, when attention was paid to the concentration of the radioactive precursors used, it was recognized that it was of paramount importance. When the substrate, androstenedione or dehydroepiandrosterone, was present in the culture medium at a concentration of 70nM, which is already a rather high concentration, the formation of testosterone could not be demonstrated. However, concentrations in the micromolar range yielded measurable quantities of testosterone, which increased to about 10% of the added substrate when the concentration was 70µM. In addition, at these very high concentrations, the capacity to form testosterone did not belong solely to the testis, but was shared by other organs such as the ovary or the mesonephros (Weniger *et al.*, 1985). It is concluded from these studies that, when exposed to high substrate concentrations, the chick embryo testis can form testosterone.

Determination of testicular and plasma testosterone concentration by radioimmunoassay gave incoherent results. For example, it is difficult to conceive that the adrenals contained more testosterone than the testes or the ovary (Tanabe *et al.*, 1986).

If one compares plasma testosterone levels found in the chick embryo, they are seen to vary widely from laboratory to laboratory and even in the same laboratory at different times (Woods *et al.*, 1975, 1983; Gasc and Thibier, 1979; Tanabe *et al.*, 1979, 1986). Again it seems illogical that, except at the stages of 12 and 16 days, the concentration should be higher in the female than in the male (Tanabe *et al.*, 1986). This was also the case in the duck embryo (Tanabe *et al.*, 1983). In the quail embryo, Ottinger and Bakst (1981) found values an order of magnitude higher than in the chick and duck with peak values over 1,5 ng/ml at 8 and 15 days.

As regards the amounts of testosterone released by chick embryo gonads into culture media, the values given by Guichard *et al.* (1977, 1979a, b) in three different papers differ markedly. According to unpublished, personal results, radioimmunoassayable testosterone was present in culture media in increasing amounts with advancing age of the cultured testes, and amounts augmented in the presence of LH. However, since the more specific radiochemical methods did not confirm the formation of testosterone when the radioactive substrates were used at physiological concentrations, one may wonder whether this radioimmunoassayable testosterone was true or spurious. As far as the present author is concerned, testosterone secretion by the chick embryo testis is either non-existent or insignificant.

Chemical nature of anti-Müllerian hormone

The hypothesis had long been defended that testosterone was the hormone responsible for the regression of the Müllerian ducts in the male chick embryo (Wolff *et al.*, 1952; Lutz-Ostertag, 1954, 1974, 1976a, b, 1977).

Without considering the fact that the chick embryo testis does not secrete testosterone, as we have seen, the following results contradict this opinion. Far from determining the regression of the cocultured chick embryo Müllerian duct, the mouse embryo testis, which secretes testosterone, stimulates its development (Weniger, 1963). Crystalline testosterone has the same effect (Weniger and Zeis, 1973a). When the Müllerian ducts from 8-day-old male embryos were cultured in the presence of testosterone solutions ranging from 0.1 mM-1nM, their regression, which had already begun at the time of explantation, did not continue (Weniger and Zeis, 1976). Testosterone added to the coculture of a chick embryo Müllerian duct with a chick embryo testis antagonizes the suppressive action of the anti-Müllerian hormone (Weniger and Zeis, 1982). Finally, using ultrafiltration membranes of different pore sizes, it was shown that the active substance has a molecular weight between 30,000 and 100,000 daltons (Weniger *et al.*, 1975; Weniger and Zeis, 1976). It is most likely a glycoprotein, as the mammalian anti-Müllerian hormone (Budzik *et al.*, 1983; Picard and Josso, 1984). It should be added that, if the chick embryo Müllerian duct does not regress when cocultured with a mouse embryo testis, it is most probably because the feminizing action of testosterone prevails over that of the anti-Müllerian hormone. Now in progress are experiments aimed at verifying whether, in the absence of testosterone, human recombinant anti-Müllerian hormone (Cate *et al.*, 1986) has an effect.

Regulation of estrogen secretion by the ovary

Twenty years ago, Woods and Weeks (1969) interpreted the 50% reduction in Δ_5-3ß-hydroxysteroid dehydrogenase activity in the ovaries of 13.5-to 19.5-day-old chick embryos as constituting evidence that the pituitary exerts an effect on this enzyme activity, and thus on steroid hormone synthesis, in the ovary of the chick embryo during the last half of the incubation period. However, this conclusion was perhaps overhastened, since subsequent work rather dismissed it. Ovaries from 17.5-day-old intact or hypophysectomized chick embryos synthesize the same amounts of estrone and estradiol from [^{14}C] sodium acetate (Akram *et al.*, 1973; Akram and Weniger, 1974). Ovaries of intact or hypophysectomized 16-day-old embryos did not release significantly different quantities of estradiol into culture media (Weniger and Zeis, 1987). Results were the same at 18 and 19 days of incubation (Weniger *et al.*, 1989, 1990a). When earlier stages were investigated, a rather unexpected result was obtained: at stages 10-13 days, the estradiol secretion rate was significantly lower in hypophysectomized than in control embryos (Weniger *et al.*, 1990b). Since LH is being secreted (Woods and Thommes, 1984; Tanabe *et al.*, 1986) and since the ovary responds to exogenous LH as early as 7 days of incubation (Teng and Teng, 1977; Guichard *et al.*, 1979a; González *et al.*, 1987; Weniger and Chouraqui, 1988), it seemed logical to attribute the diminished estradiol secretion rate in the hypophysectomized embryo to the lack of LH. So, pituitary control of estradiol secretion seems established at 10-13 days. It is most conspicuous at 10-12 days and ceases at 15-19 days. This does not mean that LH secretion ceases at these advanced stages. However, since sensitivity of the ovary to LH diminishes with age (Weniger and Chouraqui, 1988), stimulation of estrogen secretion is no longer apparent.

Conclusion

As a conclusion, some problems may be raised which deserve further attention. The most important one relates to the mechanism of gonadal sex differentiation (Fig. 1). If, at least in vertebrates, the sex of an organism is determined by the genetic constitution of the egg, what mechanism underlies the differentiation of the gonadal anlage into an ovary or testis? Steroids can disturb this mechanism, modifying gene expression. In this connection, the mechanism by which androgens feminize the chick embryo testicular anlage (Weniger and Zeis, 1973c and d, 1974; Weniger *et al.*, 1983) should be worth considering for itself. Are receptors still undifferentiated at this early stage, binding indifferently estrogens or androgens? The study of the antagonizing actions of anti-Müllerian hormone and androgens or estrogens at the cellular and molecular levels is another challenging problem.

So, studies on embryonic sex hormones in birds remain a fruitful field of research.

Summary

Hormone activity of embryonic gonads in birds was demonstrated by grafting and culture experiments. Anti-Müllerian hormone responsible for the regression of the Müllerian ducts in the male is most probably a glycoprotein. Whether the testis also secretes testosterone has long been disputed, but most arguments are against this possibility. From early stages of development, the ovary secretes estrome and estradiol. However, it could not be demonstrated unambiguously whether estrogen is identical with the sex inducing substance in the female. The hypophysis seems to control ovarian estrogen secretion at 10-13 days of incubation in the chick embryo.

KEY WORDS: *gonadal sex differentiation, androgen, estrogen, anti-Müllerian hormone, bird embryo*

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