Int. J. Dev. Biol. 33: 467-475 (1989)

Original Article

Detection of sex-specific proteins in chick embryo gonads and mesonephros: effects of estradiol benzoate or tamoxifen on their expression

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ABSTRACT Gonadal and mesonephric protein patterns from 19 day old normal chick embryos were investigated by two-dimensional polyacrylamide gel electrophoresis. Under these conditions, several sex-specific polypeptides were detected. As concerns gonadal extracts, four sex-specific polypeptides, all restricted to the cytosol, were present in the testis, whereas three sex-specific polypeptides, two localized in the cytosol, the other being membrane-bound, were identified in the ovary. Among the ovary-specific polypeptides two proved to be estrogen-dependent. They appeared in the left testis of embryos after early estradiol benzoate treatment and their expression was reduced in the ovary after early exposure to the antiestrogen, tamoxifen. Mesonephros extracts of both sexes also differed in their protein composition since three additional polypeptides (one in both the cytosolic and membrane fractions, the others in the cytosol) not found in females were found to be present in males. None appeared to be affected after either estradiol or tamoxifen treatment.

KEY WORDS: *2-D electrophoresis, gonads, mesonephros, sex-specific proteins, hormones*

Introduction

The morphological, cytological, cytochemical, histochemical and enzymological changes accompanying gonadal differentiation have been well documented, as have the onset, nature and rate of hormonal secretions during development (Gasc, 1974; Scheib and Haffen, 1974; Wolff, 1978; Scheib, 1983; Wartenberg, 1983; Scheib *et al.*, 1985; Weniger, 1987). However, little is known about the exact mechanisms and factors initiating the differentiation of the embryonic gonad into a testis or an ovary.

Surprisingly, despite the general agreement that the production of organ-specific proteins is of primary importance during organogenesis, there is apparently very little information concerned with the synthesis of sex-specific proteins during gonadal development. Regarding the latter aspect of gonadal differentiation, Muller *et al.* (1984) reported the presence of cytosolic and membrane-bound sex-specific proteins in the gonads of the rat foetus. More recently, similar results have been obtained by Samsel *et al.* (1986) in the chick embryo. However, data concerning the existence of sex-specific membrane proteins in the chick gonads are still lacking. The present paper is therefore concerned with a comparative analysis of the protein patterns observed after two-dimensional electrophoresis of the cytosolic and membrane fractions of gonadal extracts from 19 day old chick embryos.

Moreover, since various experimental studies (Wolff, 1950, 1978; Scheib, 1983) indicated that estrogen hormones appear to play an important role in avian ovarian differentiation, the protein patterns of gonadal extracts from estradiol benzoate or tamoxifen-treated embryos have also been analyzed. Tamoxifen, a nonsteroidal antiestrogenic drug, is known to compete with estrogens at the receptor site level (Sutherland *et al.*, 1977, 1980).

Furthermore, since after hatching certain mesonephric tubules escaping degeneration undergo very different sex-dependent changes (Maraud, 1963; Volle and Beaumont, 1964; Budras, 1972; Croisille *et al.*, 1978), the question also arises as to whether sex-specific proteins are elaborated in

Abbreviations used in this paper: MW, molecular weight; IEP, isoelectric point; kDa, kilo Daltons; NEPHGE, non-equilibrium pH gradient electrophoresis; NP 40, nonidet P-40; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate.

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0214-6282/89

TABLE 1

APPARENT MOLECULAR WEIGHTS AND ISOELECTRIC POINTS OF THE SEX-SPECIFIC PROTEIN SUBUNITS IN GONADS AND MESONEPHROS FROM 19 DAY OLD NORMAL CHICK EMBRYOS

Protein subunit number	Molecular weight (x10-3) MW (kDa)	Isoelectric point IEP
01	57	7.4
02	28	4.7
03	52	5.9
T1	104	7.2
T2	55	7.0
T3	41	8.6
T4	16	5.7
M1	104	7.0
M2	104	7.2
M3	38	7.9

that organ at the end of embryonic development in the chick.

Results

Protein patterns of gonadal and mesonephric membranes and cytosol in control embryos

After silver staining, the cytosolic and membrane preparations were found to have about 600 and 450 polypeptide spots respectively. Their isoelectric points (IEP) ranged from 4.2 to 9 and their apparent molecular weights (MW) from 10 to 150 KDa.

A thorough comparative analysis of the protein subunit patterns revealed the presence of sex-specific polypeptides in male and female gonads as well as in the mesonephros. The apparent molecular weights and the isoelectric points of the sex-specific subunits are shown in Table I. As regards the gonadal protein patterns, four polypeptides (T1 to T4) appeared to be testis specific and three (O1 to O3) ovary specific (Figs. 1, 3). Male specific proteins were detected only in the cytosol (Fig. 1a), whereas in female gonads two polypeptides (O1 and 02) were found in the cytosol (Fig. 1b) and another (O3) was restricted to the membrane fraction (Fig. 1d). In the mesonephros, three additional polypeptides were discovered in male samples in comparison to female samples (Figs. 2, 3). Two of these were observed only in the cytosolic fraction (M1 and M3) (Fig. 2a), whereas the third (M2) was present in both the cytosol and membrane fractions (Figs. 2a, 2c).

A comparison of the gonadal protein patterns with those of the mesonephros revealed that a polypeptide having the same molecular characteristics (MW and IEP) (28 kDa / 4.7) as the ovary protein subunit O2 was present in the cytosolic fraction of male and female mesonephros (Figs. 2a, 2b). On the other hand, a sex-specific polypeptide with identical properties (104 kDa / 7.2) was found both in the testis (T1) and male mesonephros (M2) (Figs. 1a, 2a). The other qualitative differences appeared to be organ and sex specific.

Effects of estradiol benzoate on the polypeptide patterns of the left gonad and mesonephros from male embryos.

After estradiol benzoate treatment, the expression of sex-specific polypeptides was modified in the left gonad of 19 day old male embryos. Among the four polypeptides (T1 to T4) that are present in the testis of control embryos, only one (T1) was found to occur in these gonads (Fig. 4a). Moreover, polypeptides O2 and O3, normally absent in the testis but present in the ovary, appeared in the left gonad of males after exposure to estradiol benzoate (Figs. 4a, 4c). However, the intensity of protein subunit O2 was less pronounced than in control ovaries.

No qualitative differences were found between mesonephroï from treated and control males.

Effects of tamoxifen on the polypeptide patterns of the ovary and mesonephros from female embryos

The ovaries of tamoxifen-treated chick embryos did not show any qualitative changes as compared to controls. The three sex-specific polypeptides (O1 to O3) were found to be present (Figs. 4b, 4d). However, the intensity of polypeptide spots O2 and O3 (more particularly that of O2) was reduced. Furthermore, none of the four testis polypeptides (T1 to T4) was detected in these gonads.

The polypeptide patterns of the mesonephroï from treated and control females did not show any significant differences.

Histological examinations

The early application of estradiol benzoate resulted in the feminization of all the males. The left testis became transformed into an ovotestis which was covered by a large cortex containing germ cells, and in the medulla testicular structures with spermatogonia could be observed (Fig. 5b).

In tamoxifen-treated female embryos, the ovary was reduced in size. Upon histological examination, the cortex was generally less developed and frequently missing in some parts of the gonad (Fig. 5d). As to the medulla, no significant differences could be observed in comparison to control female gonads (Fig. 5c).

Discussion

Comparison of the gonadal polypeptide patterns revealed qualitative differences between male and female preparations from control chick embryos. These observations agree with those reported by Samsel *et al.* (1986) showing that sex-specific proteins were present in the gonadal cytosol fraction at various stages of development. It should be noted, however, that owing to the use of different separation and detection procedures, only spots O2 and T4 can be compared to two out of the seven (five in the male, two in the female) sex-specific components described by Samsel *et al.* (1986) at a comparable developmental stage. By extending the pH gradient in the present research, several not yet described sex-specific protein subunits (O1, T1, T2, T3) were identified in the cytosolic

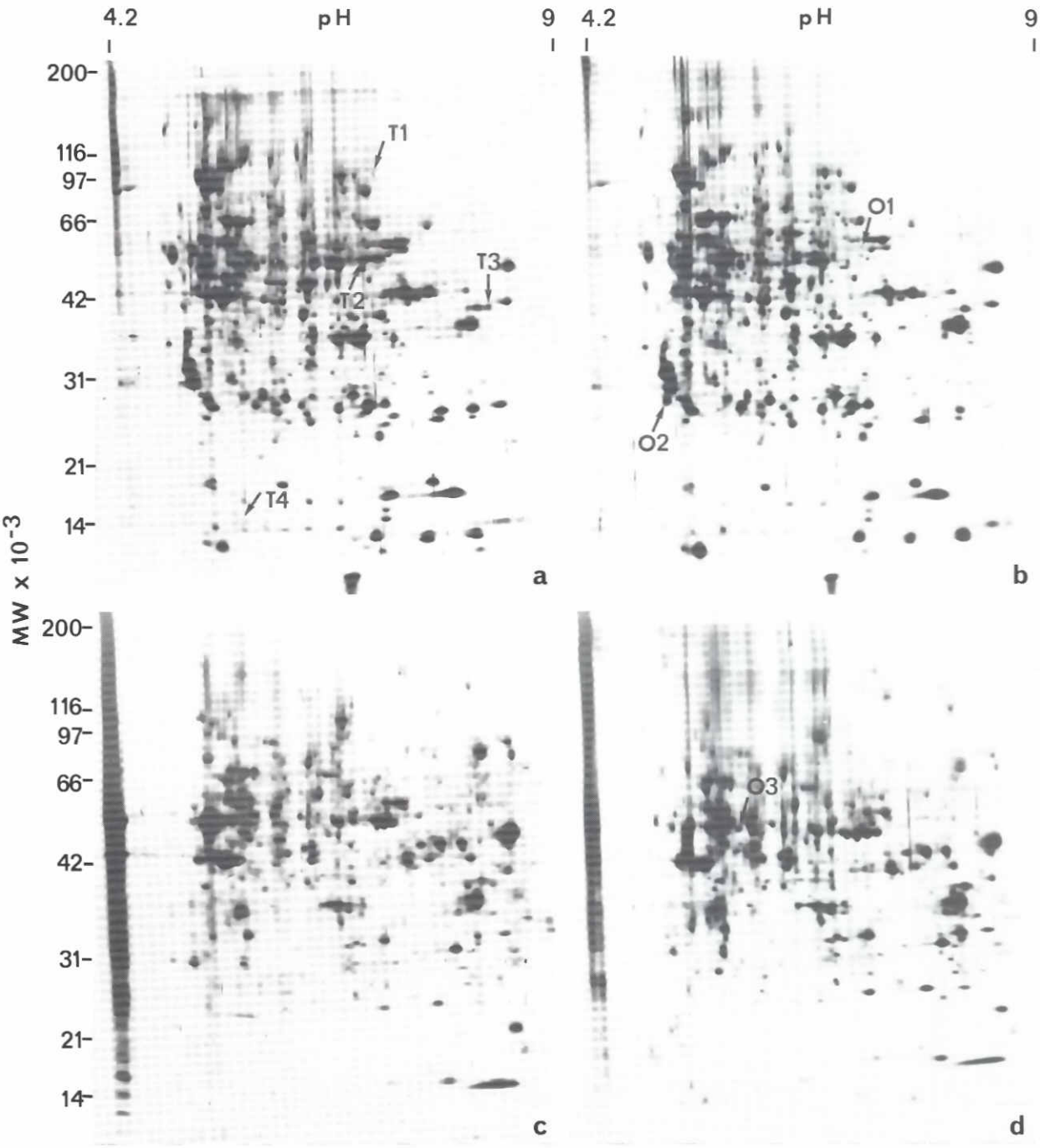

Fig. 1. Gonadal protein patterns in normal chick embryos. *Two-dimensional gel electrophoresis of cytosol* **(a, b)** *and membrane* **(c, d)** *fractions of the gonads from male* **(a, c)** *and female* **(b, d)** *19 day old normal chick embryos. Arrows indicate spots that are present in one sex only.*

fraction. Furthermore, the analysis of the membrane-bound proteins allowed the detection of an additional sex-specific polypeptide (O3).

By comparing gonadal and mesonephric profiles it appeared that a component featuring the same MW and IEP as O2 is also present in the mesonephros of both males and females. On the basis of similarities in MW, IEP and organ distribution, the latter component may be compared to one of the protein subunits (protein 47) described by Samsel *et al.* (1986). In addition, a component having the same characteristics as T1 in the testis shows up in the mesonephros of males (M2). Since the gonads had been carefully cleaned free of adjacent tissues and since O2 is not common to the gonads of both sexes, a possible contamination of the gonadal samples with mesonephric remnants can be excluded. Whether the protein subunits featuring

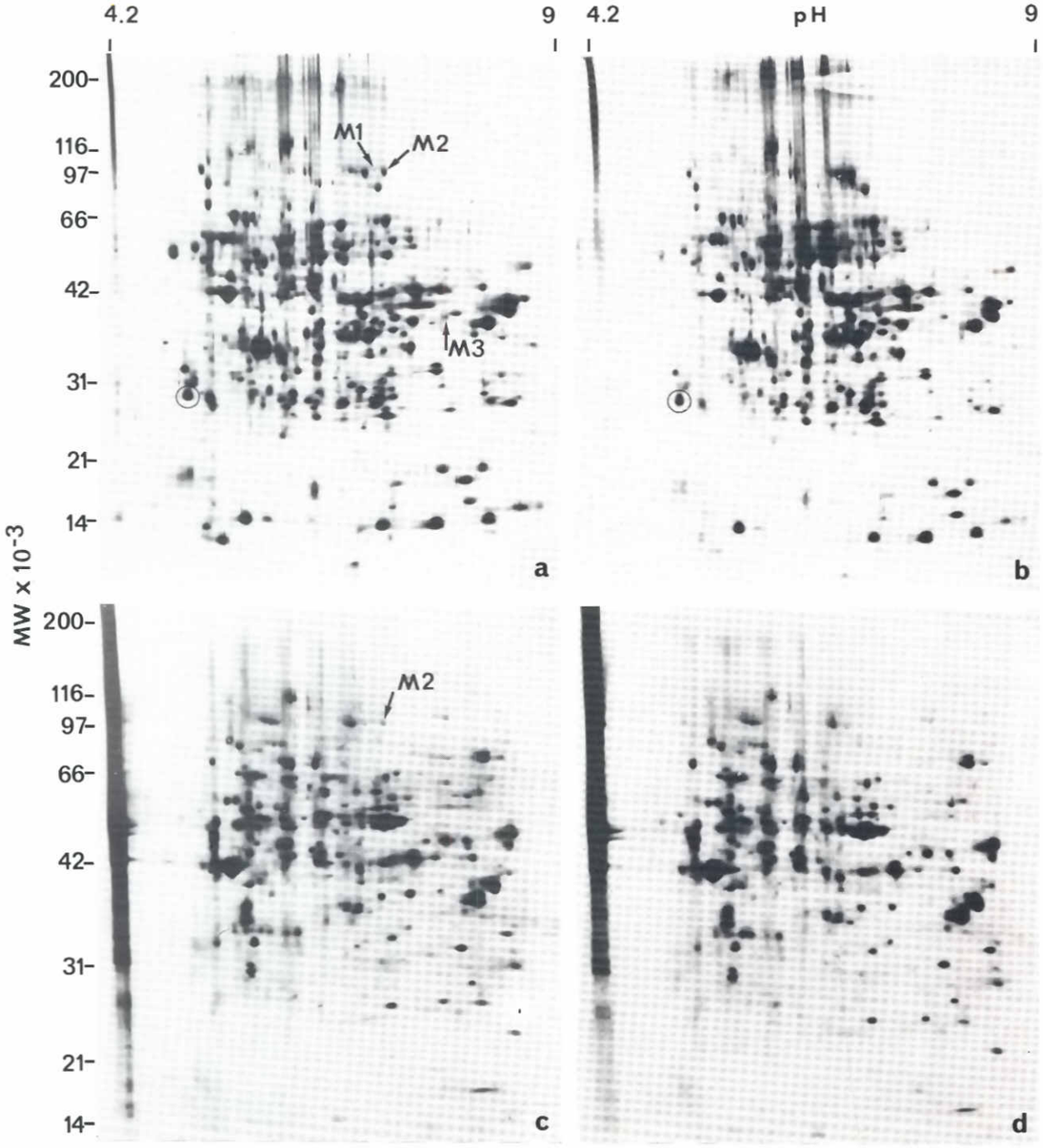

Fig. 2. Mesonephric protein patterns in normal chick embryos. *Two-dimensional gel electrophoresis of cytosol* **(a, b)** *and membrane* **(c, d)** *fractions of the mesonephros from male* **(a, c)** *and female* **(b, d)** *19 day old normal chick embryos. Arrows indicate spots that are present in one sex only. Circles indicate the spot corresponding to the component which shows the same characteristics as ovary protein 02.*

the same MW and IEP found to be present in both the gonads and the mesonephros are identical cannot yet be decided. Further comparative studies on the immunochemical properties should help in settling this question.

The histological observations made on the left gonad of estrogen-treated males agree with previous findings (Haffen, 1969; Haffen and Wolff, 1977; Scheib and Reyss-Brion, 1979; Scheib, 1983) and show that early treatment of chick embryos with estradiol benzoate promotes the feminization of this gonad in genetic males. The presence of target cells for estrogen hormones has been demonstrated in the left germinal epithelium of both male and female embryos

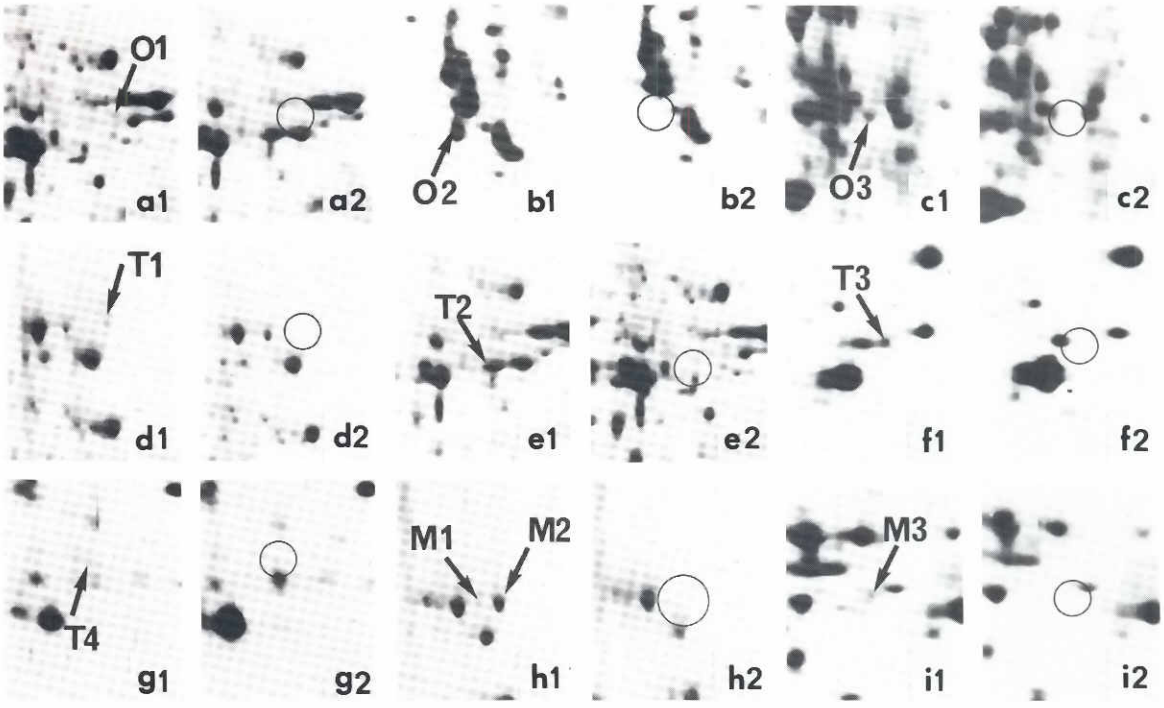

Fig. 3. Cuts of gels from 19 day old normal embryonic female and male chick gonads and mesonephros. *a1, b1, c1 depict spots 01, 02, 03 (see Fig. 1) that were found in the female gonad only (arrows). The corresponding areas in male gonads are marked by circles (a2, b2, c2). d1, e1, f1, g1 show polypeptides T1, T2, T3, T4 (see Fig. 1) that were present in the male (arrows), but absent in the female gonad (circles, d2, e2, f2, g2). h1, i1 indicate spots M1, M2, M3 (see Fig. 2) that were present in male mesonephros only (arrows). Circles mark the corresponding areas in female mesonephros (h2, i2).*

(Gasc, 1980) and the response of the left testis to hormonal treatment is in keeping with these data.

The electrophoretic studies of the polypeptide patterns complete the available information concerned with the feminization of the left testis in bird embryos. The ovotestis that differentiates in response to estradiol treatment expresses some of the ovary-specific polypeptides (O2 and O3). These observations suggest that their appearance depends, either directly or indirectly, upon estradiol action. Furthermore, since in the left gonad of estradiol-treated males these two protein subunits appear along with the development of a typical ovarian type cortex, it seems likely that they are normally associated with that structure. It must also be noted that, although numerous testicular cords were present in the medulla, only one out of the four polypeptides usually found in control testes was present in the gonads of estrogen-treated males.

Regarding the effects of tamoxifen on ovarian differentiation, divergent observations have been reported. According to Salzgeber *et al.* (1981), Scheib and Baulieu (1981) tamoxifen modified the development of the female gonads, with the ovary showing a dense medulla with numerous cords and a reduced cortex. According to Weniger and Zeis (1984), Weniger and Samsel (1985), Koo *et al.* (1985), however, tamoxifen had no histologically detectable effect on the differentiation of the left ovary. Injecting comparable doses of tamoxifen, we observed that in the ovary the cortex was less developed than in control females. However, no testicular cords were found to occur in the medulla. Furthermore, electrophoretic studies did not reveal any qualitative differences between the ovarian polypeptide patterns of control and tamoxifen-treated embryos. In the latter, none of the testis specific polypeptides was found. As to the ovary specific components, the expression of polypeptide O1 remained apparently unchanged whereas the intensity of the spots corresponding to polypeptides O2 and O3 was lower, thus further indicating that the synthesis of both these polypeptides is under estrogen control. Moreover, the parallelism between the decrease in intensity of spots O2 and O3 on the one hand, and the reduction of cortical development on the other hand, further supports the idea of the cortical localization of both polypeptides.

The analysis of the mesonephric protein patterns also showed sex-related differences since, in comparison to females, three additional polyeptides were found to be present in males. These observations may be related to the further evolution of the mesonephros in males and females. It is well established, indeed, that in birds as in other amniote vertebrates the mesonephros regresses with the exception of a number of tubules whose fate is sex-dependent. In the female, the latter remain as a small organ, the epoophoron (Budras, 1972). In the male, however, they undergo profound transformations and participate in the

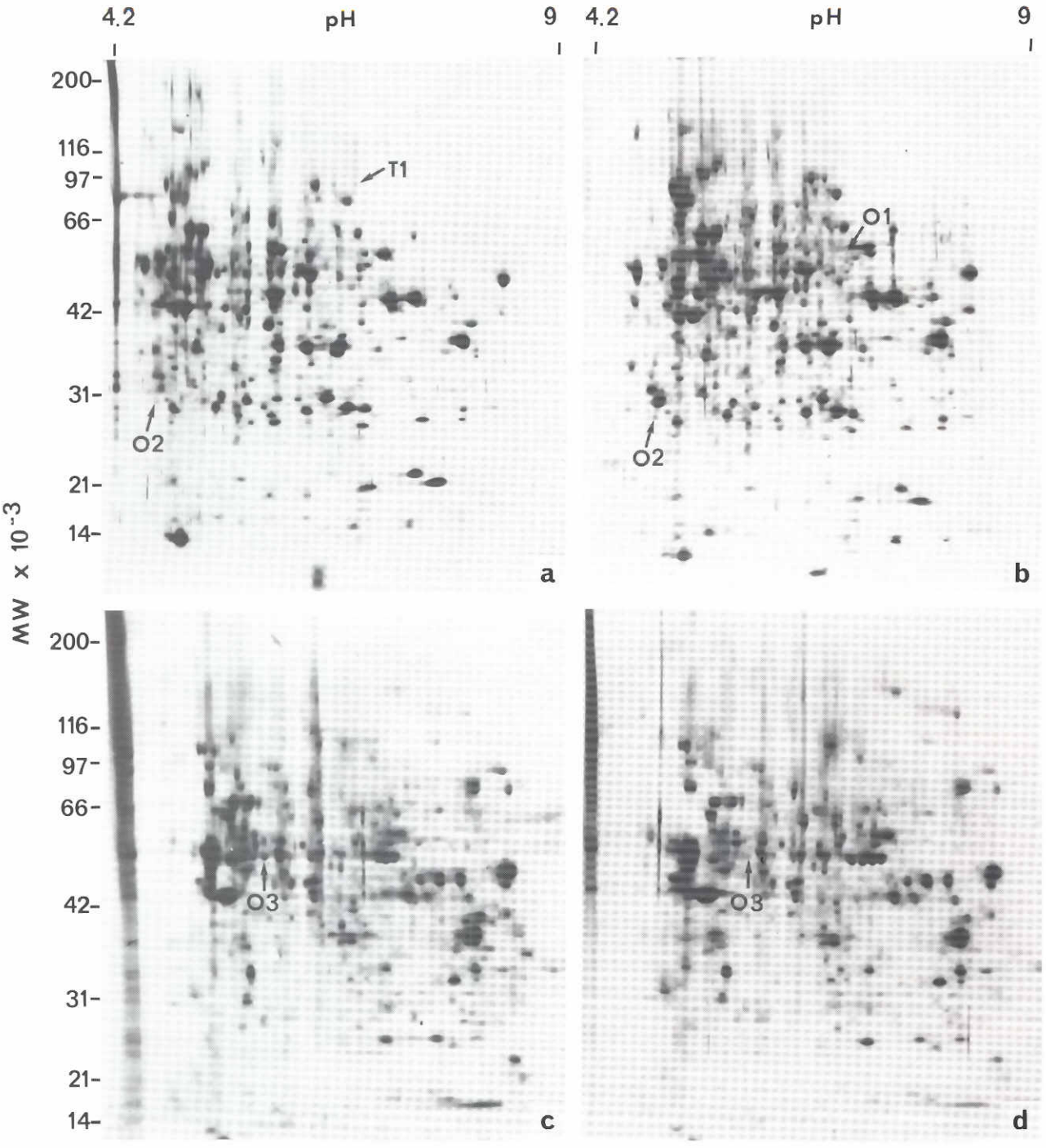

Fig. 4. Gonadal protein patterns in estradiol benzoate and tamoxifen-treated chick embryos. *Two-dimensional gel electrophoresis of cytosol* **(a)** *and membrane* **(c)** *fractions of the testis from estradiol benzoate-treated embryos, and of cytosol* **(b)** *and membrane* **(d)** *fractions of the ovary from tamoxifen-treated embryos. Sex-specific polypeptides are indicated by arrows.*

formation of the epididymis (Croisille, 1981; Maraud, 1963). Therefore the presence of sex-specific proteins in the male mesonephros can perhaps be regarded as an early indicator of such very different developmental fates. Differences in the protein patterns have recently been reported to exist not only between gonads but also between mesonephroï of male and female neonates of the European pond turtle (Dorizzi *et al.*, 1986).

The treatment by either estradiol or tamoxifen did not alter the polypeptide patterns of the mesonephros. Interestingly the intensity of spot O2 was not affected, an observation which, unless separate control mechanisms are

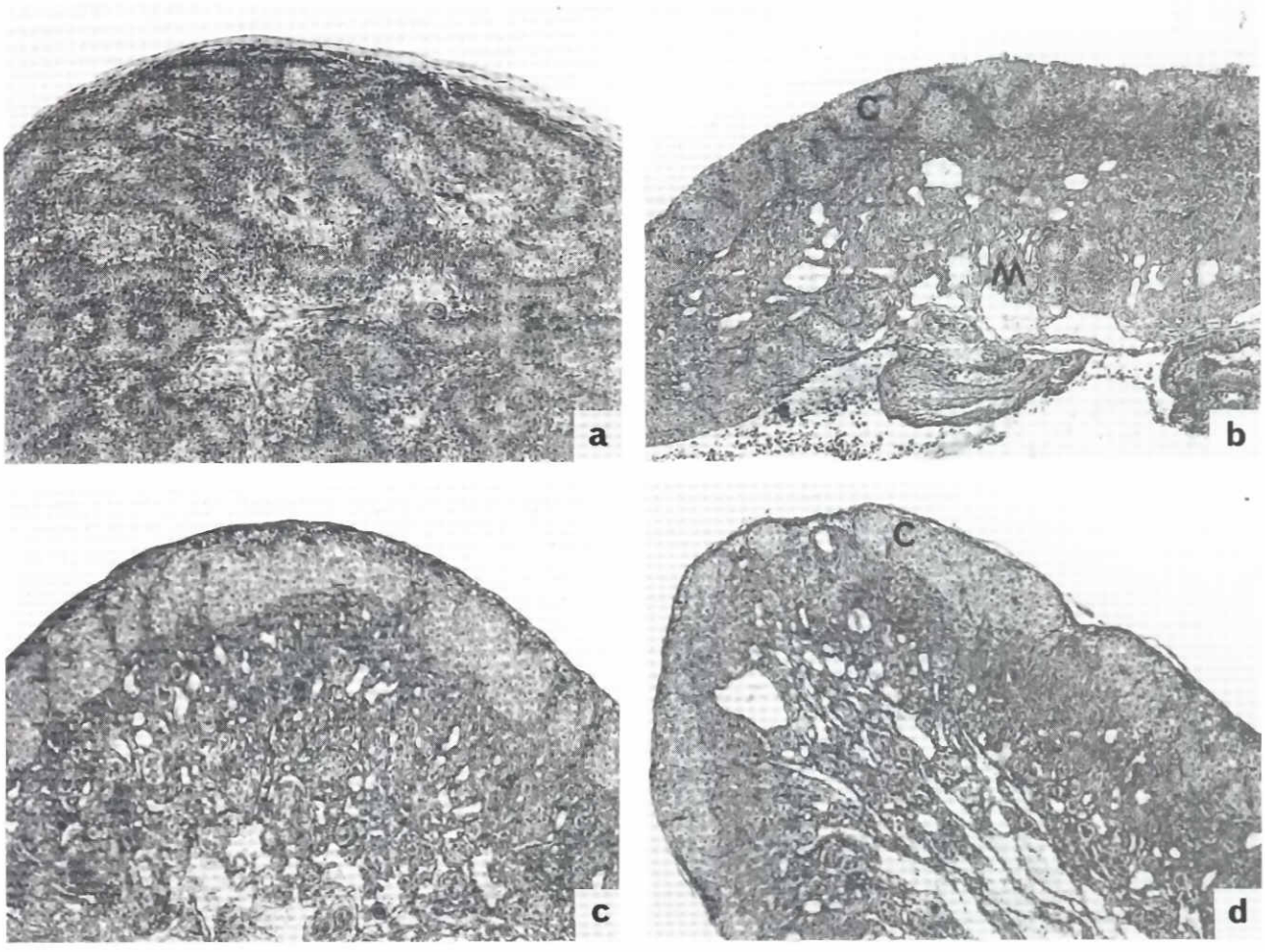

Fig. 5. Histological survey of left testis and ovary from a 19 day old chick embryo. (a) *control testis;* **(b)** *testis from estradiol benzoate-treated embryo: note the presence of a superficial cortex (C) and an underlying medulla (M) with testicular cords;* **(c)** *control ovary;* **(d)** *ovary from a tamoxifen- treated embryo: the ovary is reduced in size and features a thin cortex (C). x96.*

involved, argues in favor of the idea that, although they share the same apparent molecular weights and isoelectric points, components O2 detected in the ovary and the mesonephros are in fact different.

In summary, sex-specific proteins can be detected in the gonads as well as in the mesonephros of 19 day old chick embryos. However, further studies are needed to clarify the developmental significance of these findings.

Materials and Methods

Animals and treatment of embryos

Fertilized eggs of domestic fowl (*Gallus gallus domesticus*, Malvoisine strain) were purchased from a local supplier and incubated at 38°C in a humidified air chamber.

Estradiol benzoate (Benzogynestryl, Roussel) and tamoxifen (Sigma) were injected into the yolk sac of 3.5 day old embryos in 50 µl sterilized olive oil. The doses, chosen according to those used by previous investigators (Haffen, 1969; Haffen and Wolff, 1977; Salzgeber *et al.*, 1981; Weniger and Zeis,1984; Weniger and Samsel, 1985), were 200 µg per egg for estradiol and 1.5 mg per egg for tamoxifen. Controls were treated with 50 µl olive oil only.

After 19 days of incubation, the embryos were sacrificed by decapitation and dissected in Tyrode's solution. Testes, the left ovary and mesonephroï from males and females were separately collected in Tyrode's solution chilled on ice, cleaned free of adjacent tissues and weighed. After injection of estradiol benzoate, only the left gonad was removed from the male embryos.

Preparation of cytosol and membrane fractions

Tissue samples were homogenized using a glass-teflon homogenizer in cold 10 mM Tris-HCl buffer pH 7.6 (3 ml/g of tissue) supplemented with a cocktail of protease inhibitors (phenyl methyl sulfonyl fluoride: 1 mM; leupeptin: 10 µg/ml; aprotinin: 10 µg/ml) and sonicated at low energy four times for 10s with a MSE 60-W sonicator. The sonicated preparations were then centrifuged at 140,000 x g for 90 min at 4°C in a Beckman ultracentrifuge. The supernatant, constituting the cytosol fraction, was carefully collected and stored in aliquots at -70°C. The pellet was resuspended in Tris-HCl buffer and centrifuged once again at 140,000 x g for 90 min. The supernatant was discarded and the pellet stored at -70°C until used.

Preparation of samples for electrophoresis

Cytosol samples, 0.1ml, were prepared in tubes containing 144 mg of urea, 13 µl of 2-mercaptoethanol, 13 µl of pH 3.5-10 ampholine (LKB, Bromma, Sweden) and 26 µl of 20% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and layered on the top of the first dimension gel.

Pellet samples were resuspended at the rate of 10 mg wet weight in 150 µl of a solution containing 9.5 M urea and 2% CHAPS. The suspension was then sonicated on ice (2 x 15 s), centrifuged at 140,000 x g for 90 min at 18°C and the supernatant collected. After addition of 2-mercaptoethanol (final concentration 2%) and ampholine pH range 3.5-10 (final concentration 2%), the samples were subjected directly to the first dimensional analysis.

Protein determination

Protein concentration of the samples was determined by the method of Bradford (1976) using Bio-Rad Laboratories (Richmond, USA) reagents and bovine serum albumin as the standard.

Two-dimensional gel electrophoresis

In order to examine basic as well as acidic proteins, the non equilibrium pH gradient electrophoresis (NEPHGE) system of O'Farrell *et al.* (1977) was used for the separation in the first dimension with minor modifications. NP 40 was replaced by CHAPS, a zwitterionic derivative of cholic acid that improves resolution by inhibiting the formation of charge aggregates (Perdew *et al.*, 1983). Only pH 3.5-10 ampholytes were used. A protein sample of 70 µg was applied to the top of the gel (12 cm x 0.25 cm i.d.) and focusing was performed at 400V for 12 h. The gels were extruded and stored frozen at -70°C. A gel designed for measurement of the pH gradient was sliced into 1 cm pieces, each of which was placed in a test tube containing 2 ml of degazed distilled water. The tubes were stoppered, left for 2 h with occasional shaking, and the pH determined using an Orion Research pH-meter.

Electrophoresis in the second dimension was performed on slab gels (8-15% gradient acrylamide, 16 cm wide, 14 cm long, 0.15 cm thick) using the discontinuous system of Laemmli (1970). Frozen tube gels were equilibrated in O'Farrell's equilibration buffer (O'Farrell, 1975) for 30 min and carefully placed on the top of the stacking gel. Gels were run at 20 mA/gel until the front marker reached the surface of the separating gel and then at 30 mA/gel. They were calibrated with a set of "low" and "high molecular weight" marker proteins (Bio-Rad Laboratories, Richmond, USA) ranging from 14,400 to 200,000 Daltons.

Polypeptide patterns were evaluated directly on the gels and on photographs. For each sample at least seven gels were examined. A polypeptide spot was considered to be sex-specific if it appeared on most gels of a given sample from one sex but was absent on all gels of the corresponding sample from the opposite sex.

Staining

Staining was carried out with the silver nitrate method as described by Gorg *et al.* (1985).

Histology

Gonads were fixed in Bouin's solution and paraffin embedded sections (7µm) were dewaxed in toluene, cleared through decreasing concentrations of ethanol and stained with hematoxylin and eosin.

Acknowledgments

This work was supported by the Centre National de la Recherche Scientifique and the Ministère de l'Education Nationale.

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Accepted for publication: November 1989