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Submicroscopical observations on the differentiation of chick gonads

By ROBERTO NARBAITZ & RUBEN ADLER[1]
From the Faculty of Medicine, Buenos Aires

The role of hormones in gonadal differentiation has not been fully elucidated. One of the main problems consists in determining the exact moment in which steroid synthesis begins. If, as has been claimed, sex hormones act as organizers and are responsible for the morphological changes which characterize gonadal differentiation, then they should appear before these changes take place.

Although the morphological differentiation of chick gonads is evident only after the eighth day of incubation small differences in epithelium height permit sex identification on the seventh day. Biological (Wolff, 1946), biochemical (Gallien & Le Foulgoc, 1961) and histochemical (Scheib, 1959; Narbaitz & Sabatini, 1963; Narbaitz & Kolodny, 1964; Chieffi, Manelli, Botte & Mastrolia, 1964) evidence suggests that estrogen synthesis takes place in embryonic ovaries after the eighth day. On the other hand, steroid production by embryonic testes has not been proven. Wolff (1950) showed that a testicular secretion is responsible for the atrophy of Müllerian ducts on the ninth day; however histochemical techniques have given contradictory results. Thus, lipids are present after the eighth day but cholesterol does not appear until the tenth day (Narbaitz & Sabatini, 1963); Chieffi *et al.* (1964) claim that 3β-hydroxysteroid dehydrogenase activity is present in 8-day testes, but Narbaitz & Kolodny (1964) obtained negative results with the same material. Regarding the undifferentiated gonads, the evidence for steroid synthesis is still scarce. Weniger (1961) claims that the feminizing action of embryonic ovaries described by Wolff (1946) is already present in 5-day gonads belonging to genetically female embryos, but no biochemical nor histochemical facts have yet supported this claim.

Since cells producing steroid hormones have special submicroscopic characteristics an electron microscopic study of differentiating chick gonads was undertaken in the hope of finding additional evidence that may throw light on these problems.

[1] *Authors' address:* Institutó de Anatomía General y Embriología, Facultad de Medicina, Paraguay 2155, Buenos Aires, Argentina.

MATERIALS AND METHODS

White Leghorn embryos were used in all cases. The left gonads from 6-, 7-, 8-, 9-, 11-, 13- and 16-day embryos were dissected. Macroscopic examination did not permit sex identification at 6 and 7 days; to ensure that gonads of both sexes were examined 25 embryos of these ages were used. At the other ages at least four gonads of each sex were studied.

Immediately after dissection the gonads were fixed in a 2 % osmium tetroxide solution in Periston (Bayer) (Polyvinylpyrrolidone, 6 g; NaCl, 0·55 g; KCl, 0·042 g; $CaCl_2$, 0·05 g; $MgCl_2$, 0·0005 g; dist. water, 100 ml) at pH 7·4 and 4 °C. After 2 h fixation they were transferred to 2 % uranyl acetate aqueous solution for 90 min, dehydrated by bringing through 50, 70, 80 and 96 % ethanol solutions for 15 min each, and allowed to remain in 100 % ethanol for 2 h with two changes. The gonads were then transferred to a solution consisting of equal volumes of propylene oxide and Epon 812 mixture, After 90 min the gonads were transferred to Epon 812 mixture. Embedding was performed in the usual way. Sections were made with a Porter Blum microtome and stained with lead citrate as described by Reynolds (1963). They were studied with a Siemens Elmiskop I.

RESULTS

Electron microscopic observation with low magnifications permitted the identification of the diverse histological structures described in classical studies on chick gonads differentiation (see Hamilton, 1952). Therefore, no special scanning procedure was needed in order to identify the different parts of the gonads.

Undifferentiated gonads. Plate 1, fig. A shows the aspect of a typical sex cord of a gonad of a 6-day embryo. The cells have an oval nucleus and their cytoplasm contains a large number of ribosomes and a fair number of mitochondria with well-developed cristae. In some of these cord cells characteristic vesicles are seen (*a*, Plate 1, fig. B). These are limited by agranular membranes and are irregularly distributed throughout the cytoplasm. Some of the vesicles contain a material of low electronic density (*b*, Plate 1, fig. B). The number of vesicles varies greatly from cell to cell. Some lipid droplets with wavy contour may be observed in some of the cells.

In the sex cords, gonocytes can be identified by their large, rounded nucleus with finely divided chromatin. The cytoplasm is similar to that of the other cells

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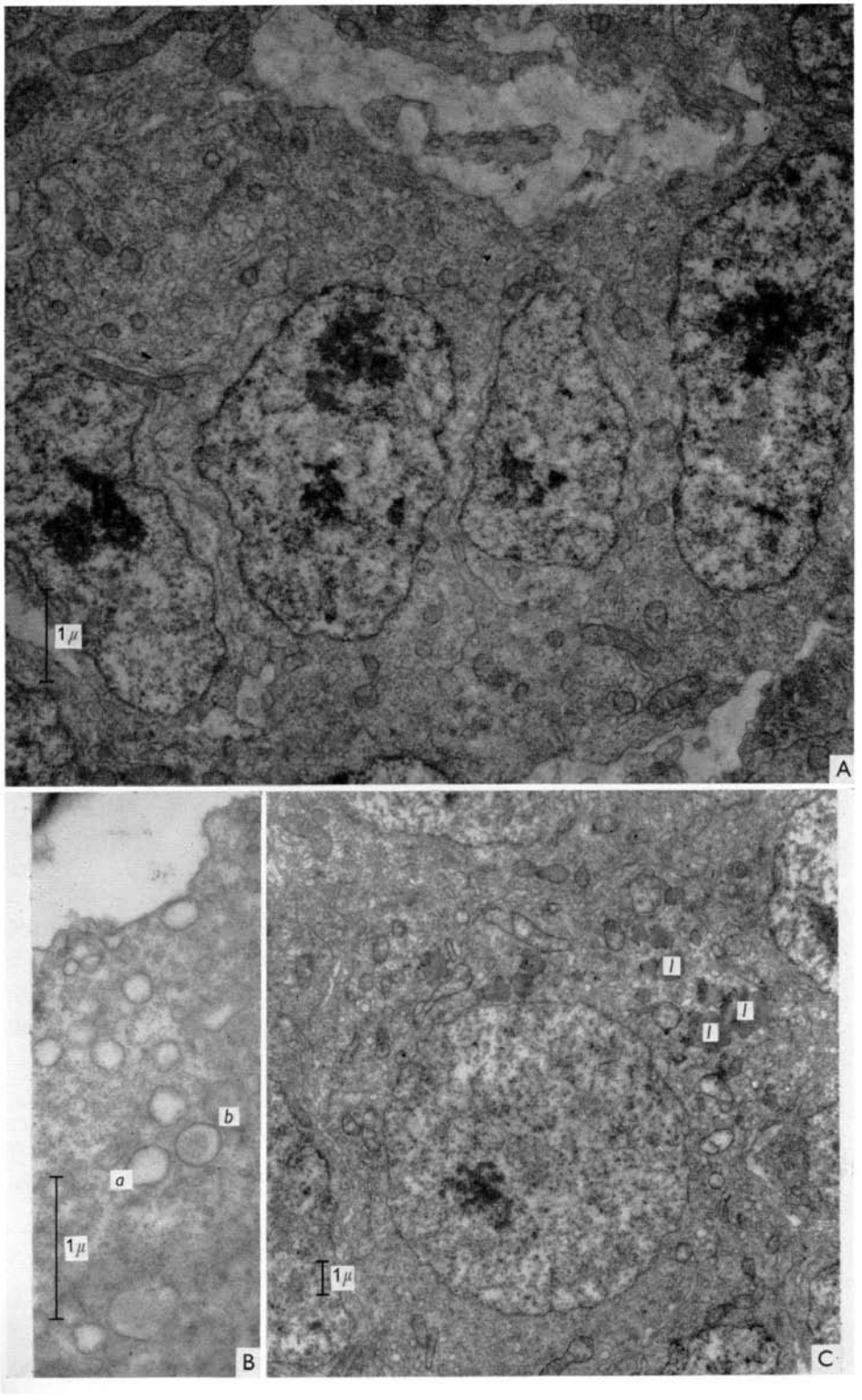
PLATE 1

Fig. A. Gonad of 6-day embryo: several cells in a sex cord are seen.

Fig. B. Same gonad at higher magnification. *a*, Vesicle surrounded by agranular membrane; *b*, vesicle with contents of low electron density.

Fig. C. Same gonad. Germ cell in a sex cord; several lipid droplets are seen (*l*).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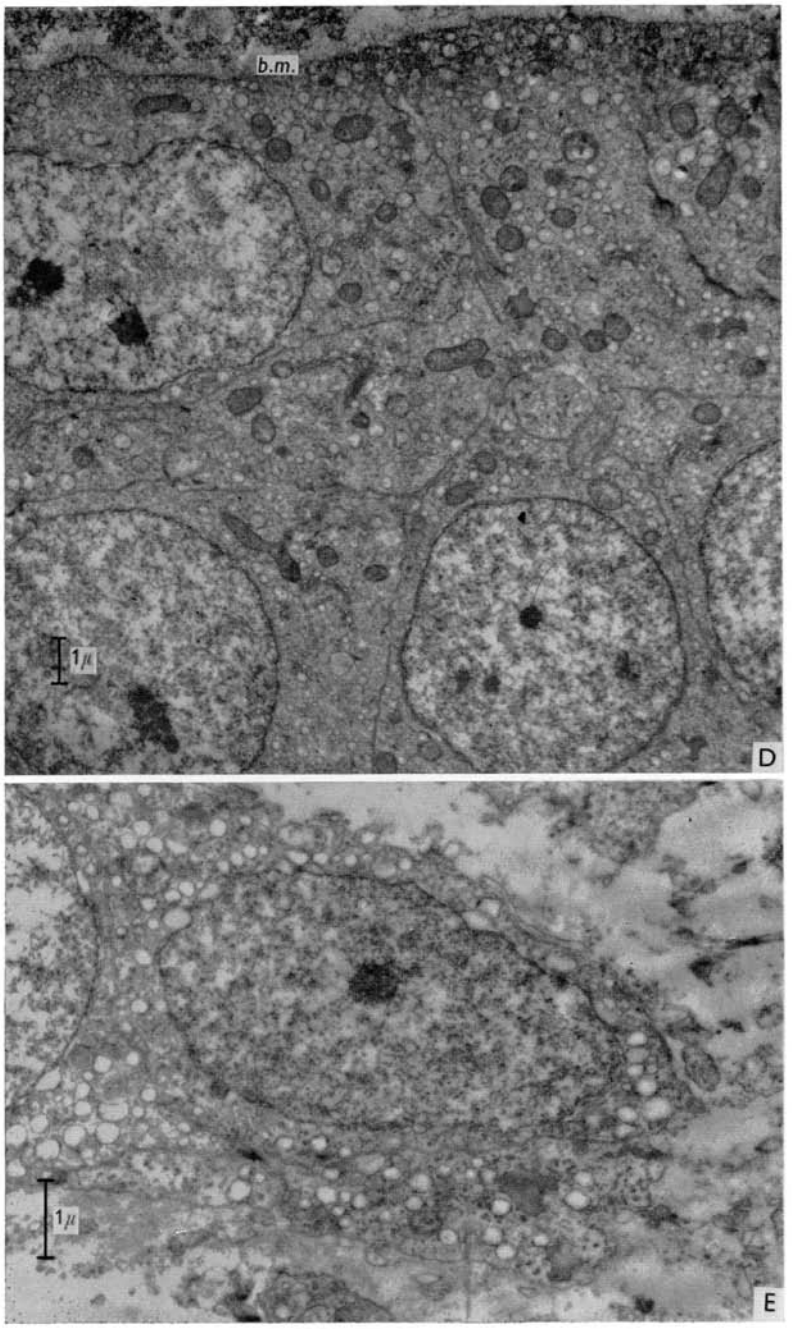

Fig. D. Nine-day testis. Testicular cord cells show many vesicles. Basement membrane is seen limiting the cord (*b.m.*).

Fig. E. Nine-day testis. Interstitial cell showing many vesicles.

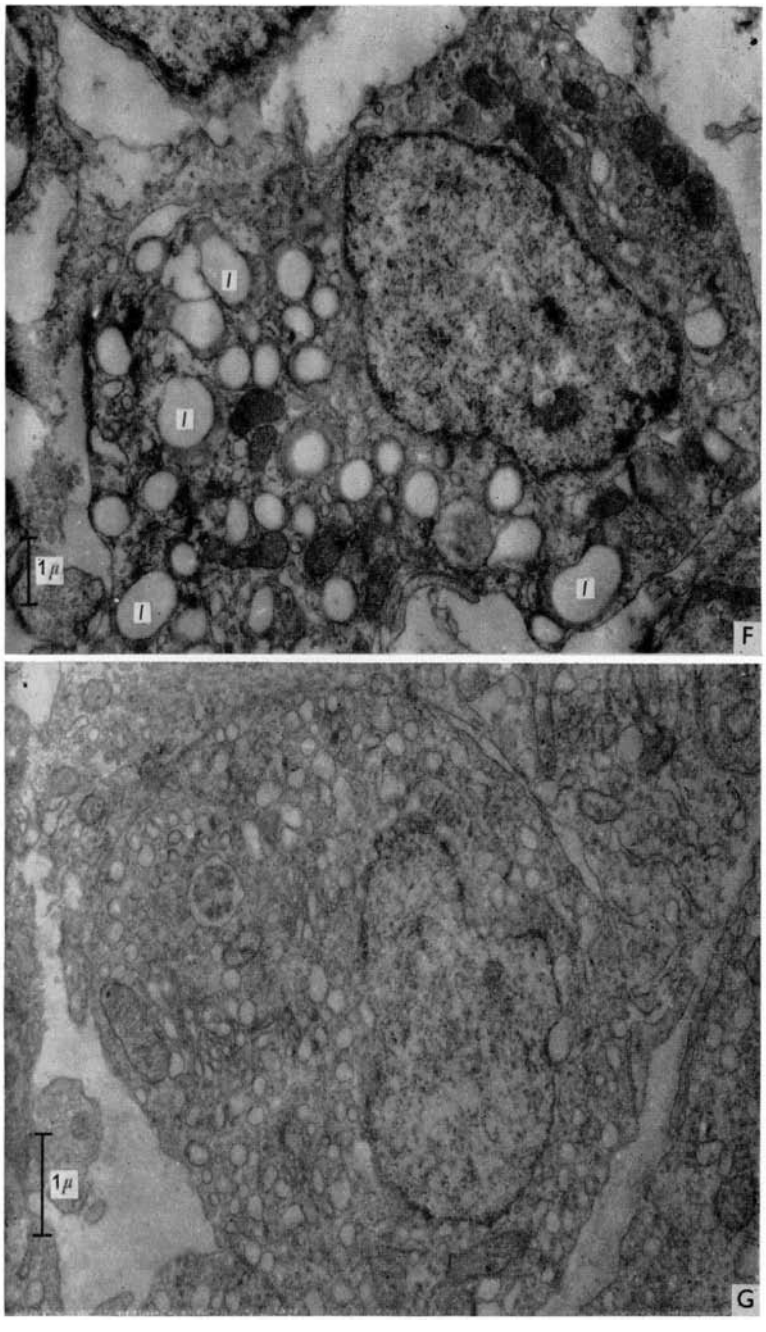

Fig. F. Sixteen-day testis. Interstitial cell showing vesicles and large lipid deposits with a central vacuole (*l*).

Fig. G. Nine-day ovary. Cord cell showing many large vesicles.

of the cord but with a larger content of lipid droplets (Plate 1, fig. C). Among the sex cords there are mesenchymal cells with an elongated nucleus and very sparse cytoplasm.

In the cortical zone of 6-day gonad two types of cells are recognized: gonocytes and epithelial cells. Their structure is similar to that described for gonocytes and sex cord cells in the medullary region. Mitoses are seen in all parts of the gonad.

Seven-day gonads are very similar to those of 6-day embryos, but in some of them some cord cells show an intense proliferation of vesicles.

Testes. Plate 2, fig. D shows the submicroscopical aspect of the cord in 9-day testis. There is a basement membrane separating the cord from the interstitium. A large number of vesicles are now seen in the cytoplasm of all the sex cords. In the 8-day testis vesiculization is found only in few cord cells but on the ninth day it is present in nearly all of them. After the ninth day many vesicles also appear in some cells of the interstitium (Plate 2, fig. E). Lipid droplets of wavy contour are seen in some of the cord and interstitial cells.

At the eleventh and thirteenth days the testes are very similar to those at the ninth day. The only difference observed is in the width of the basement membrane, which increases with age. In the 16-day testis some of the cord and interstitial cells appear filled with lipid deposits (Plate 3, fig. F). These deposits appear to have an empty vacuole in the center, the lipid being restricted to the peripheral zone.

Ovaries. In the 8-day ovary the medullary region shows a striking difference from that of the 7-day gonad. Sex cords have disappeared as such; parts of them have acquired a lumen and form lacunae, while other parts remain in closely packed cell groups. Mesenchymal cells appear intermingled between the lacunae and the groups of cord cells.

The cytoplasm of the cord cells undergoes striking changes. On the eighth day vesicles increase in size and number in some of them (Plate 3, fig. G) while in others large lipid deposits appear. On the ninth day the lipid deposits show a central vacuole (Plate 4, fig. H). Because of the increase in number and size of the vesicles and the growth of lipid deposits, the cytoplasm appears filled by them, and the cells appear highly vacuolated (Plate 4, fig. I). This process of differentiation is progressive; on the ninth day large groups of cells have been transformed and by the eleventh many of the cells which surround the lacunae have also undergone the same transformation.

The mesenchymal cells keep the same aspect described at previous ages (Plate 5, fig. J). After the eighth day a primitive albuginea of typical mesenchymal cells has formed between the cortex and the medulla.

Germ cells undergo an intense proliferation in the cortex while disappearing from the medulla after the eighth day. Their cytological aspect has not changed (Plate 5, fig. K). On the sixteenth day meiotic changes are evident in the nuclei and a crowding of mitochondria in a juxta-nuclear zone is observed. Epithelial

cells of the cortex surround germ cells and become follicular cells. Vesicles and lipid droplets have been observed in the cytoplasm of epithelial cells at all ages studied (Plate 5, fig. K).

DISCUSSION

In cells producing steroid hormones two submicroscopic structures appear to be constant: the agranular reticulum and the lipid deposits.

Agranular reticulum has been described in many types of cells. It is specially abundant in adrenal cells (Ross, Pappas, Lanman & Lind, 1958), lutein cells (Enders, 1962), Leydig cells (Christensen & Fawcett, 1961; Christensen, 1965) as well as in other tissues with high lipid metabolism. The membranes may appear in the form of tubules and vesicles. According to Fawcett (1964) this variation in the organization of membranes may depend on the procedure used for fixation and embedding, and the original organization is always tubular. In our material membranes appeared in the form of vesicles, although the other cytoplasmic constituents were adequately preserved.

Lipid deposits are specially abundant in adrenocortical cells (Lever, 1955; Sabatini & De Robertis, 1961), but are also present in lutein cells (Enders & Lyons, 1964) and Leydig cells (Christensen & Fawcett, 1961). Small deposits appear generally as droplets with a wavy contour while large ones are rounded and often show an empty vacuole in the central zone.

According to Cotte (1959) the lipids rendered insoluble by fixation are located in the peripheral portion, while those soluble are in the central portion and extracted during embedding. This suggestion seems to be confirmed by Idelman (1964), who found that when certain solvents are avoided during the processing, the lipid deposits show no vacuoles, but the central portion has a lower electron density than the peripheral zone.

Our observations show that agranular reticulum in the form of vesicles is present in chick gonads at all ages studied. Vesicles are seen in 6- and 7-day undifferentiated gonads, and increase in number and size in testes after that age. They are particularly abundant and large in some cells of ovaries after the eighth day and in testes after the sixteenth day. Lipid deposits are present in the form of small droplets in undifferentiated gonads and in testes up to the thirteenth

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PLATE 4

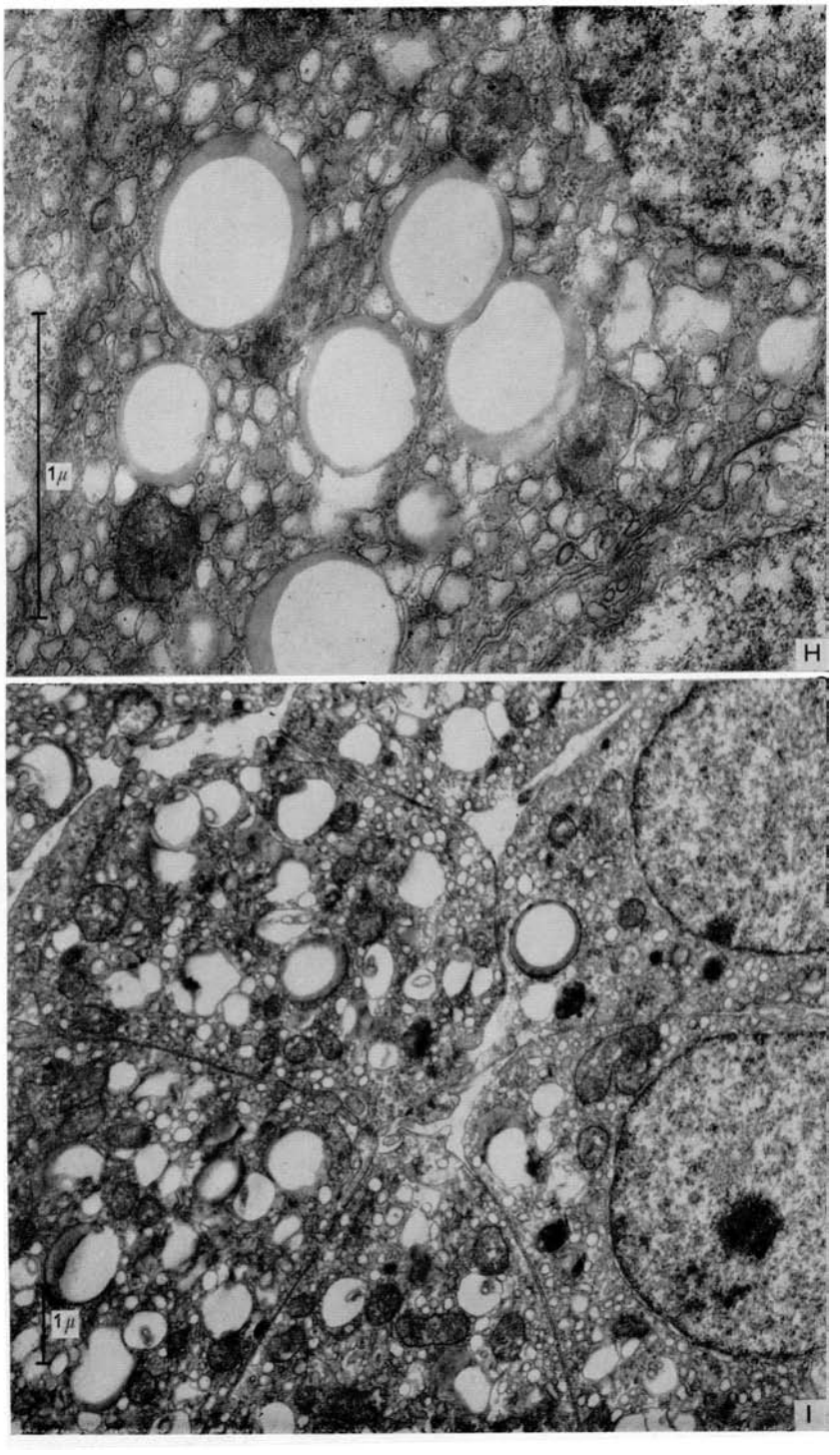
Fig. H. Nine-day ovary. Cytoplasm of a cord-derived cell showing large vesicles and lipid deposits with central vacuoles.

Fig. I. Nine-day ovary. Group of cord-derived cells fully differentiated.

PLATE 5

Fig. J. Nine-day ovary. Mesenchymal cells.

Fig. K. Twelve-day ovary. A germ cell with big rounded nucleus and a follicular cell with elongated nucleus are shown.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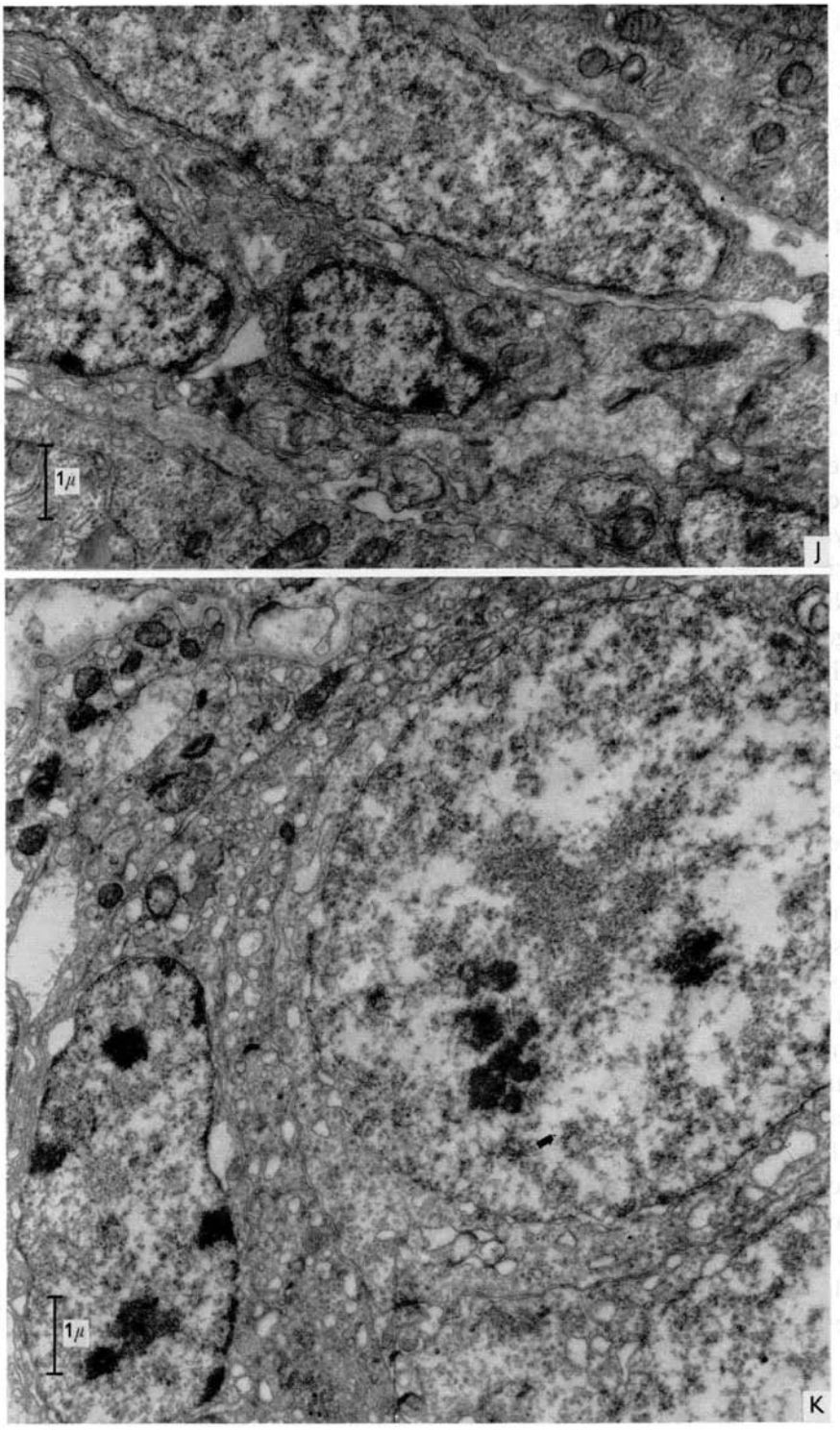
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day, while large deposits with central vacuoles are found in ovaries after the eighth day and in testes after the sixteenth day. The increase of agranular reticulum and lipid deposits with age appears to be related to the progressive differentiation of steroid-producing cells.

Estrogenic substances have been identified in chick embryonic ovaries by Gallien & Le Foulgoc (1961). The fact that cells with submicroscopical structure indicative of steroid production are present in the ovarian medulla after the eighth day, together with histochemical evidence showing the presence in these cells of lipids and cholesterol (Scheib, 1959; Narbaitz & Sabatini, 1963) and 3β-hydroxysteroid dehydrogenase (Narbaitz & Kolodny, 1964; Chieffi *et al.* 1964) indicates that these cells are the site of production of these steroids.

A fair number of agranular vesicles and some lipid droplets appear in testicular cord cells after the eighth day, although in smaller amount than in medullary cells of the ovaries of the same age. Scheib (1959) found that lipids and cholesterol accumulate in testicular cord cells, but Narbaitz & Sabatini (1963) detected cholesterol only after the tenth day. Also, the 3β-hydroxysteroid dehydrogenase histochemical technique, which gives clearly positive results in ovarian medullary cells, in testicular cord cells appears to be negative according to Narbaitz & Kolodny (1964) and positive for Chieffi *et al.* (1964). It could be suggested that testicular cord cells may be the site of steroid production; synthesis would occur at a low rate and this fact together with differences in the sensitivity of the techniques used would explain the contradictory results. This conclusion would support Wolff's (1950) claim in the sense that the testicular secretion responsible for the atrophy of Müllerian tubes is an androgenic steroid similar to adult male hormones. This conclusion should next be confirmed by biochemical determinations.

If androgens are produced by testicular cord cells, which originate from primitive sex cords, and the estrogen-producing cells in ovaries also derive from the same cells, then it can be concluded that the steroid-producing cells in both sexes derive from the same cell type in the undifferentiated gonad. The most important event in sex differentiation would consist in the differentiation of the primitive cord cells in one direction or the other. The presence of lipids and agranular reticulum in 6-day cord cells probably indicates that differentiation is already starting at this early stage.

After differentiation has taken place, cells with the same submicroscopical structure appear in testicular interstitium. Benoit (1950) has claimed that interstitial cells in the chick embryonic testis migrate from cords after the ninth day. Morphological details cannot indicate if they have migrated from the cords or if they form *in situ* from mesenchymal cells. Additional evidence is necessary in order to establish their origin and function.

SUMMARY

1. An electron microscope study of chick gonads of 6-, 7-, 8-, 9-, 11, 13- and 16-day embryos was made. Special attention was paid to cytoplasmic structures such as the agranular reticulum and lipid deposits which are considered to be regularly present in steroid-producing cells.

2. Six- and 7-day gonads have a constant number of agranular vesicles and few lipid droplets in their cord cells and gonocytes. Testes over that age showed in their testicular cord cells a large number of vesicles and few lipid droplets. Some interstitial cells had a similar appearance after the ninth day. Ovaries after the eighth day and testes after the sixteenth show cells with very large vesicles and a great accumulation of lipid deposits.

3. The results obtained, together with existing biochemical and histochemical evidence, show that estrogens are secreted by ovarian cells derived from primitive sex cords and that androgens are probably produced by testicular cord cells derived from the same source.

4. It is suggested that the most important step in gonadal differentiation is the differentiation of the primitive cord cell to produce either estrogen or androgen according to genetic instructions.

5. The possible origin of the interstitial testicular cell is discussed.

RÉSUMÉ

Observations inframicroscopiques sur la différenciation des gonades de poulet

1. On a étudié au microscope électronique les gonades d'embryons de poulet de 6, 7, 8, 9, 11, 13 et 16 jours. On s'est attaché particulièrement à l'examen des structures cytoplasmiques telles que le réticulum agranulaire et les dépôts de lipides qu'on considère comme régulièrement présents dans les cellules productrices de stéroïdes.

2. Les gonades de 6 et 7 jours ont un nombre constant de vésicules agranulaires et peu de gouttelettes lipidiques dans les cellules des cordons et les gonocytes. Les testicules, au-delà de cet âge, présentaient dans les cellules de leurs cordons un grand nombre de vésicules et peu de gouttelettes lipidiques. Quelques cellules interstitielles avaient un aspect semblable après le 9e jour. Les ovaires après le 8e jour et les testicules après le 16e jour montrent des cellules à très grandes vécicules et une grande accumulation de dépôts lipidiques.

3. Les résultats obtenus, conjointement aux données biochimiques et histochimiques existantes, montrent que les oestrogènes sont sécrétés par des cellules ovariennes dérivées des cordons sexuels primitifs et que les androgènes sont produits probablement par des cellules de cordons testicullaires de même origine.

4. On suggère que l'étape la plus importante dans le différenciation des gonades est la différenciation de la cellule du cordon primitif qui produit soit des oestrogènes soit des androgènes selon l'information génétique.

5. L'origine possible de la cellule interstitielle du testicule est discutée.

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