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THE PRESENCE OF A COMMON EMBRYONIC BLASTEMA FOR OVARIAN AND TESTICULAR PARENCHYMAL (FOLLICULAR, INTERSTITIAL AND TUBULAR) CELLS IN CATTLE, *BOS TAURUS**

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Summary. The early embryonic gonadal development in the cattle is characterized by the appearance of an alkaline phosphatase positive blastema. Its derivatives in gonads of both sexes, follicular cells in the female and interstitial cells in the male, also show positive alkaline phosphatase reaction. Primordial germ cells are equally alkaline phosphatase positive, but loose this activity when they later transform to oögonia and oöcytes, or to spermatogonia respectively. Using the enzyme activity as label to trace these constituents in the developmental steps of the bovine gonads, the following results were obtained.

Differentiation processes leading to the appearance of the sex cords take place *in situ* within the gonadal blastema which occupies the main central part of the gonadal fold. It is essentially a segregation process of the follicular cell cords or of the interstitial cells and the tubular primordia from the undifferentiated common anlage.

The so-called "germinal epithelium" is not involved in the differentiation of sex cords. Its participation — if any — in the gonadal development is restricted to a very short and rather early period. Secondary sex cords (Pflügers cords) do not occur. In the cattle there is no reason to assume a cortico-medullary antagonism in the sex determined gonadal development.

It can be assumed that the follicular cells in the ovary and the interstitial cells in the testis are homologous. This applies possibly also to the tubular cells of the testis. Homology should be admitted also for the rete structures, which remain small and undeveloped in the ovary while in the male they show considerable development.

In the ovary the follicular cell cords differentiating within the central blastema match in a junctional zone with the peripheral layer of oögonia. These are taken up by the most peripheral branches of the follicular cell cords, thus transforming to ovigerous cords. During the downward movement within these cords the germ cells transform to oöcytes which for their part proceed through first meiotic prophase and reach the dictyotene stage. The maturation of the germ cells seems to be controlled by the follicular cells and may even temporarily get out of control until an adequate number of follicular cells is found in vicinity of individual oöcytes to form primordial follicles.

The alkaline phosphatase reaction reveals the presence of numerous persisting remnants of follicular cell cords in the developing and even adult ovary.

It is suggested that the findings in the cattle gonads can be applied also to other mammals, mainly to those with longer gestation periods like man.

In mammals and other vertebrates, differentiation of the gonadal ridges begins soon after the migration of primordial germ cells is completed. The sex chromosome constitution of primordial germ cells, however, does not appear to be a

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deciding factor in directing the development of indifferent gonads either into a testis or into an ovary. Surgical removal of the functional left ovary of the female domestic fowl *(Gallus gallus domesticus)* invariably induces compensatory development of the residual right gonad into a testis (BENOIT, 1923). The germ cells of the right gonad are transformed into functional spermatogonia while retaining the heterozygotic sex chromosome constitution characteristic of the avian female (MILLER, 1938).

Faced with this apparent neutrality of primordial germ cells with regard to gonadal development, WITSCHI (1951, 1962) introduced the concept of cortico-medullary antagonism in sex differentiation. From the phylogenetic as well as the ontogenic viewpoint, gonads are essentially hermaphroditic. Experimental data on sex reversal indicate that vertebrate gonads display marked bipotentiality early in development (WOLFF, 1962). WITSCHI's hypothesis claims that indifferent gonads of vertebrate embryos contain both cortical and medullar primordia. The differentiation of the ovary is supposed to be characterized by the prevalence of the cortex over the medulla, while in the male the medulla predominates, and a testis results. When this view is used at the cellular level, the cortex brings forth the follicular cells with their endocrine function of producing estrogenic steroid hormones, while the medulla furnishes the interstitial cells which play the major role in the androgenic endocrine system. This concept implies that follicular cells and interstitial cells are derived from different progenitors which are antagonistic to each other. Specifically, follicular cells were thought to be derived from the proliferating surface epithelium of the gonad, the so-called "germinal epithelium". Indeed, the cortical origin of ovarian follicular cells appears to have been the general consensus for many years (FELIX, 1911; GILLMAN, 1948; WATZKA, 1961; FRANCHI et al., 1962).

However, a recent investigation of fetal cattle gonads combining cytological observations with histology (OHNO and SMITH, 1964) revealed that initially deeper parts of the female gonad were endowed with more follicular cells than the superficial layer, and that an area directly beneath the "germinal epithelium" was always deficient in follicular cells. This observation cast some doubt on the cortical origin of follicular cells and tended to support the view originally expressed by FISCHEL (1930) that follicular cells also are derived from a blastema situated deep in the central part of an undifferentiated gonad.

Without an appropriate marker, any attempt to trace the ontogenic ancestry of a particular cell type is an hazardous venture. The histochemical reaction for alkaline phosphatase helped to clarify the original site and migration route of primordial germ cells in various mammalian species, including man (MCKAY et al., 1953; CHIQUOINE, 1954; MINTZ, 1957). During these studies on fetal cattle gonads of various stages, intense alkaline phosphatase activity was noted not only in the primordial germ cells of early embryos, but in fetal follicular cells and fetal interstitial cells as well. This enabled us to trace the ancestry of both cell types to a common blastema situated within the morphologically indifferent gonad.

Material and Methods

Fresh cattle embryos and fetuses of various stages (Holstein-Friesian breed) were collected from local slaughterhouses, brought to the laboratory in refrigerated containers within three

Table

Coll. No.	Group	Gest. age*	Size	Sex	Remarks**
56	I	Approx. 30 to 50 days after breeding	15 mm	♂	Liver cult.: XY
43			20 mm	♀	Liver cult.: XX
54			20 mm	♀	Liver cult.: XX
39			27 mm	♂	Liver cult.: XY
27			30 mm	♂	Liver cult.: XY
41			30 mm	♂	Liver cult.: XY
18			35 mm	♂	—
40			35 mm	♀	Liver cult.: XX
50	II	Approx. 50 to 60 days after breeding	43 mm	♂	Liver cult.: XY
16			45 mm		Liver cult.: XY
26			45 mm		Liver cult.: XY
33			45 mm		—
35			50 mm		—
52			52 mm		Liver cult.: XY
36			40 mm	♀	Liver cult.: XX
51			47 mm		Liver cult.: XX
31			50 mm		Liver cult.: XX
37			50 mm		Liver cult.: XX
30			55 mm		Liver cult.: XX
29	III	Approx. 65 to 75 days after breeding	70 mm	♀	—
38			70 mm		—
25			90 mm		—
22			95 mm		—
32	IV	Approx. 80 to 85 days after breeding	110 mm	♂	—
42			110 mm	♀	—
14			115 mm		—
23			115 mm		—
15a, b			130 mm		Dizyg. ♀-twins
34			130 mm		—
13a, b	V	Approx. 100 to 150 days after breeding	170—180 mm	♂	♂-twins
3			180 mm		—
11			200 mm		—
20a			235 mm		♂-twin of pair of unlike sex
21a			240 mm		♂-twin of pair of unlike sex
53a			250 mm		♂-twin of pair of unlike sex
62a			270 mm		♂-twin of pair of unlike sex
9a			270 mm		—
6			300 mm		—
24a			300 mm		♂-twin of pair of unlike sex
19a			305 mm		♂-twin of pair of unlike sex
5			370 mm		—
17			190 mm	♀	—
10			220 mm		—
8			260 mm		—
12			270 mm		—
2			300 mm		—
7			370 mm		—
28a	VI	Approx. 225 days after breeding	710 mm	♂	♂-twin of pair of unlike sex
64			710 mm		—
4a		Approx. 205 to 260 days after breeding	620 mm	♀	normal ♀-twin of pair of unlike sex (no vascular anastomosis)
65			880 mm		—

* After H. H. Dukes, The physiology of domestic animals, 5th ed., p. 646. Ithaca (N. J.): Comstock Publ. Co. 1943 [see also C. W. Nichols, Amer. J. vet. Res. *5*, 135 (1944)].

** In the small embryos, sex was determined by chromosomal analysis of cells in liver cultures (s. beneath). The results are recorded in this table simply as XY and XX according to the presence of XY- or XX-chromosomes in these cells.

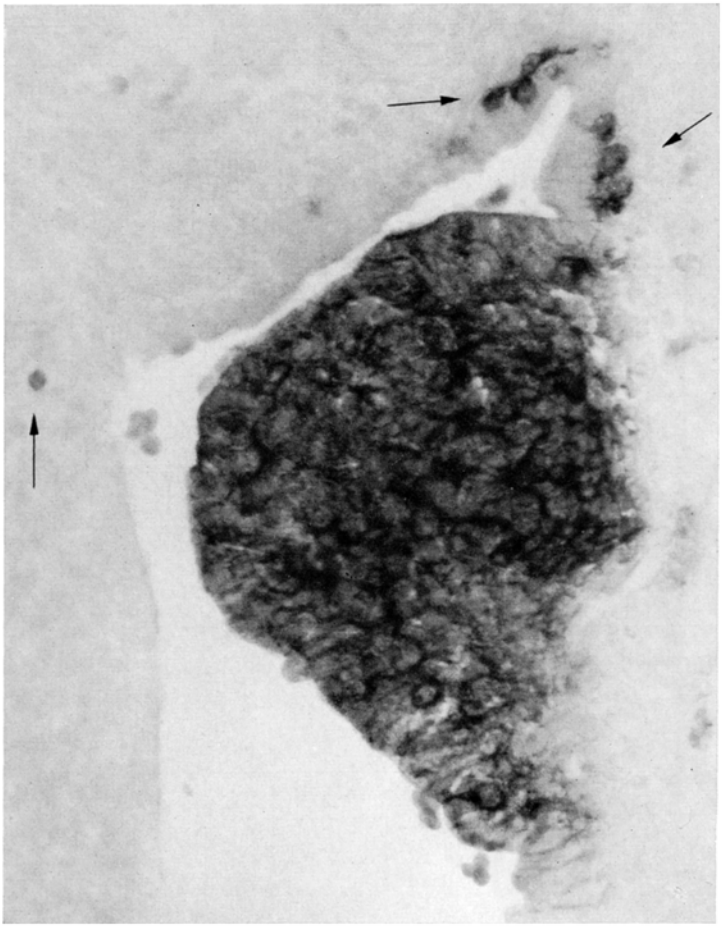

Fig. 1a and b. Gonadal fold of embryo No 56, ♂, 15 mm. Alkaline-phosphatase reaction (a) and Hematoxylin-Eosin (b). Gonadal blastema and surface lining strongly positive. Migrating primordial germ cells (arrow) also alkaline-phosphatase-positive. Magnification 220 ×

hours, and processed immediately. A total of 53 embryos or fetuses ranging in crown-rump length from 15 to 880 mm (approximately 30 days after breeding to nearly full-term) were used for the present study. They are recorded in Table according to size, and arranged in groups in relation to gross periods of gonadal development.

The smaller embryos of Group I were fixed *in toto* in acetone. From all other embryos one gonad was fixed in acetone, the other in Carnoy's fluid. In order to preserve the genital ducts most gonads of Group I and II were left together with the mesonephroi. Tissue fixed in Carnoy's fluid was used for hematoxylin-eosin, and also PAS-stained sections, while material fixed in acetone was used for the histochemical demonstration of alkaline phosphatase.

Alkaline Phosphatase Reaction. Whole embryos or embryonic organs were fixed overnight in ice-cold acetone, but no longer than 18 hours. They were processed through six succeeding changes of fresh acetone, and two changes of benzene, 30 min each. Embedding was made after two hours in paraffin with one change (melting point, 56° C). The blocks were stored in a deep freezer until cut, usually the next day. Deparaffinized sections were processed from acetone to water, followed by an incubation of one and/or two hours in freshly filtered incubation mixture of α-naphthylacid phosphate (Sodium salt, Sigma) and Fast Blue RR (Dajac Lab.) in 0.1 mol Tris-buffer, pH 9.2, temperature 20° C. For counterstaining, safranine was used.

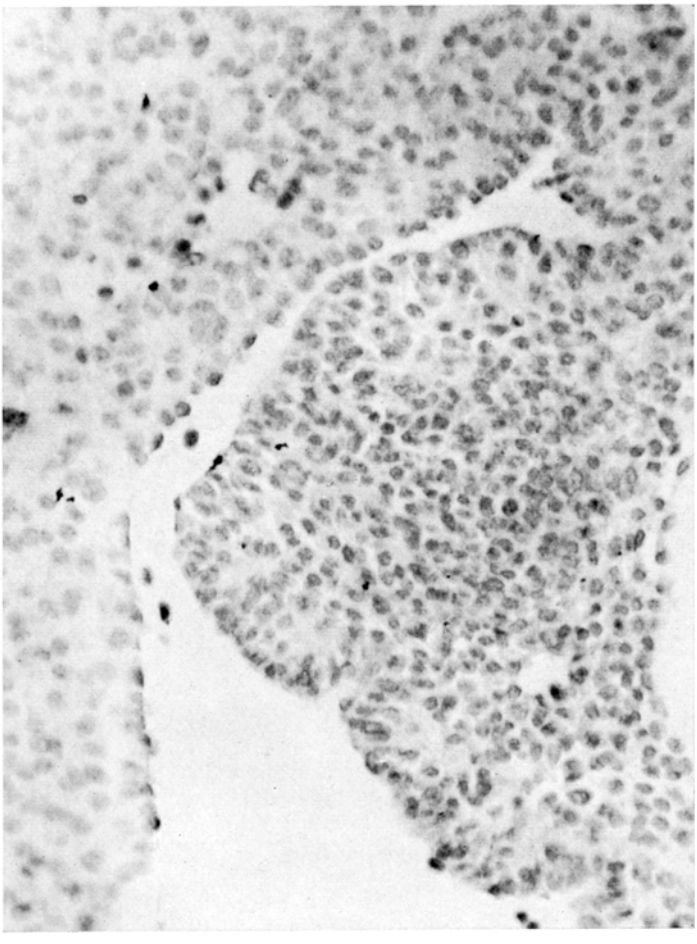

Fig. 1 b

Cytogenetic Analysis. In order to determine the sex of the embryos of Group I and II, a small piece of liver tissue was cultivated four hours in a tissue culture medium (60 per cent TMC 858 Difco + 40 per cent fetal calf serum). Colchicine was added two hours before harvesting the cells. After hypotonic treatment in 0.95 per cent sodium citrate solution for 15 min, cells were fixed in Carnoy's fluid or 50 per cent acetic acid. Air-dried preparations were stained with Giemsa. In well spread metaphase figures, sex chromosomes can be easily identified since they are the only metacentric chromosomes in the complement; all others are acrocentric. The X-chromosome is one of the largest, and the Y-chromosome is one of the smallest of the diploid set of 60 chromosomes (OHNO et al., 1962).

The more elaborate determination of the sex by chromosomal analysis could not be replaced by an evaluation of the sex chromatin, since in cattle most nuclei except those of cerebral ganglion cells (MOORE et al., 1957) contain several chromocenters in both sexes. Thus unequivocal identification of the sex chromatin bodies in the female embryonic organs is nearly impossible.

Observations

Group I, Embryos No 56 to 40 (s. Tab.)

This group includes embryos of both sexes. They represent the developmental stages from the mere gonadal ridges, the indifferent gonads, to the earliest phases

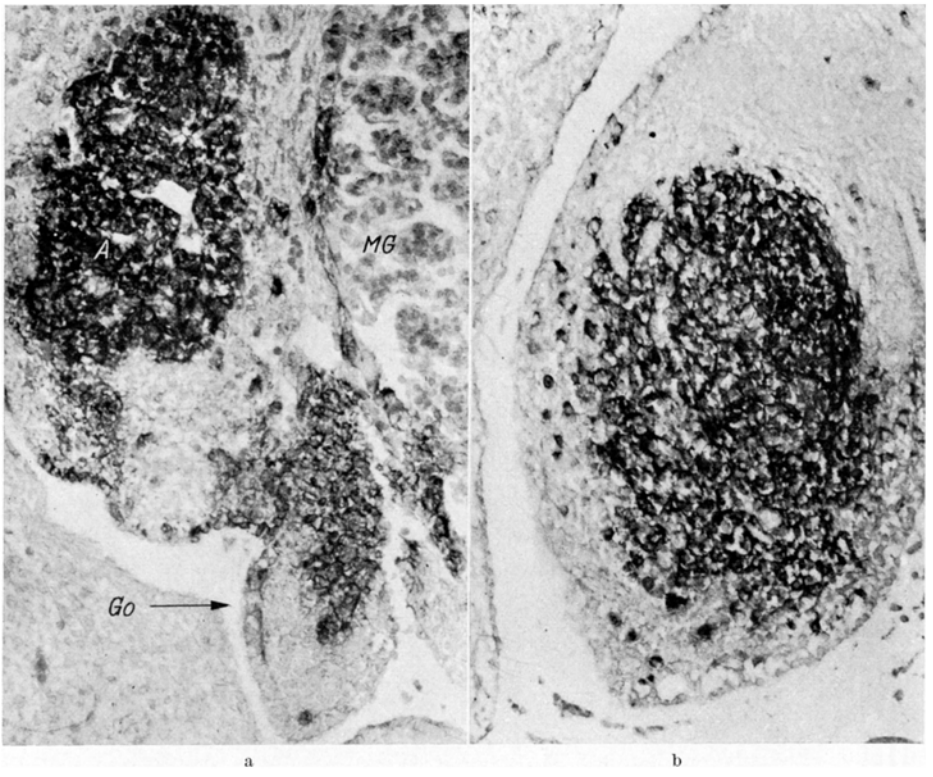

Fig. 2a and b. Gonad of embryo No 39, ♂, 27 mm. Alkaline-phosphatase reaction. (a) (low magnification 60 ×) transverse section at a cranial level; the enzyme-positive gonadal blastema extending deep in the perimesonephric tissue. *Go* Gonad, *MG* Mesonephric glomerulum, *A* Adrenal cortical anlage. (b) (high magnification 150 ×) a more caudally located part of the gonadal fold in comparison to (a). Gonadal blastema and germ cells alkaline-phosphatase-positive

of sex differentiation. In the smallest embryo of this group (No 56, 15 mm long), a distinct gonadal fold has already developed (Fig. 1). Its surface lining seems to be slightly thicker than the lining of adjacent areas of the coelomic wall. There is no sharp separation between the surface layer and the cell mass beneath (Fig. 1b) which are both alkaline-phosphatase-positive (Fig. 1a). This stage is also characterized by the fact that primordial germ cells are still in migration (OHNO and GROPP, 1965).

Shortly later, in a stage corresponding to embryos No 43 and 39 (20—27 mm long), a change in the distribution of alkaline phosphataseactivity appears. This enzyme reaction remains strongly positive in cells beneath the coelomic lining, while the lining itself from now on is phosphatase-negative (Figs. 2, 3, 11). The alkaline-phosphatase-positive blastema occupies a large area extending longitudinally from a deep perimesonephric region at the level of the adrenal cortical anlage (Fig. 2a) along the medial wall of the large mesonephric glomerular tuft and reaching far downward within the gonadal fold. It constitutes the main central part (Fig. 2b) of this fold surrounded by a narrow outer zone which contains alkaline-phosphatase-positive germ cells.

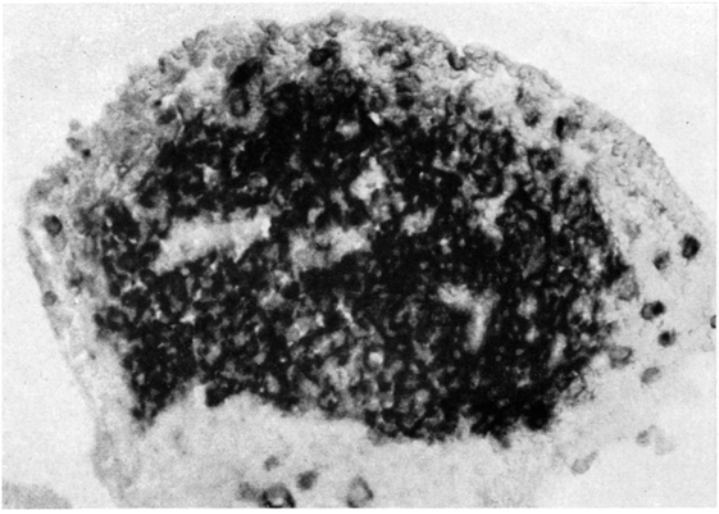

Fig. 3

Fig. 3 and 4. Gonad of embryo No 27, ♂, 30 mm (Fig. 3, Magnification 150 ×); and No 41, ♂, 30 mm (Fig. 4, Magnification, 150 ×). Gonadal blastema, and germ cells in peripheral zone, alkaline-phosphatase-positive. No augmentation of germ cells in the periphery.

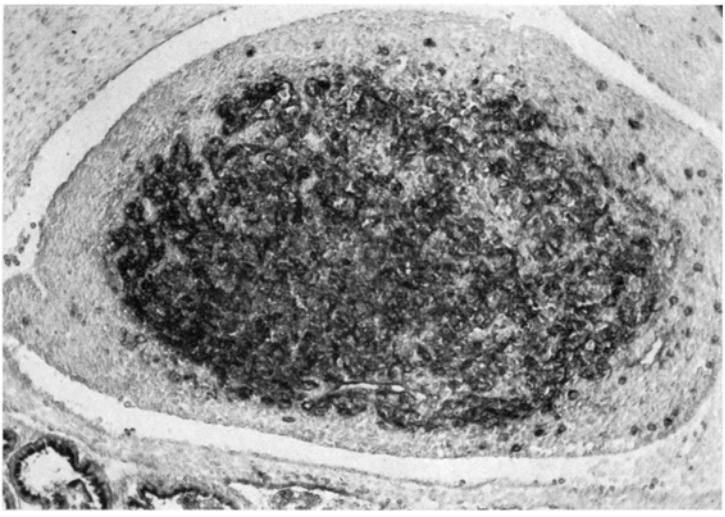

Fig. 4

In embryos from 30 to 40 mm long (Figs. 3, 4, 7, 8), the coelomic lining of the gonadal fold consists of one sharply delineated layer of cubocylindrical cells; their nuclei are arranged perpendicular to the surface. The occasional presence of a small space beneath this lining due to the influence of the fixative is one more indication of a basic morphologic difference of coelomic epithelium and underlying gonadal mesenchyme. The bulk of this mesenchymal body is formed by large, densely-packed round cells which show high alkaline phosphatase activity. At the cellular level the enzyme is localized in the cytoplasm and on the cell borders. This phosphatase-positive central blastema is sharply separated from an enzyme-

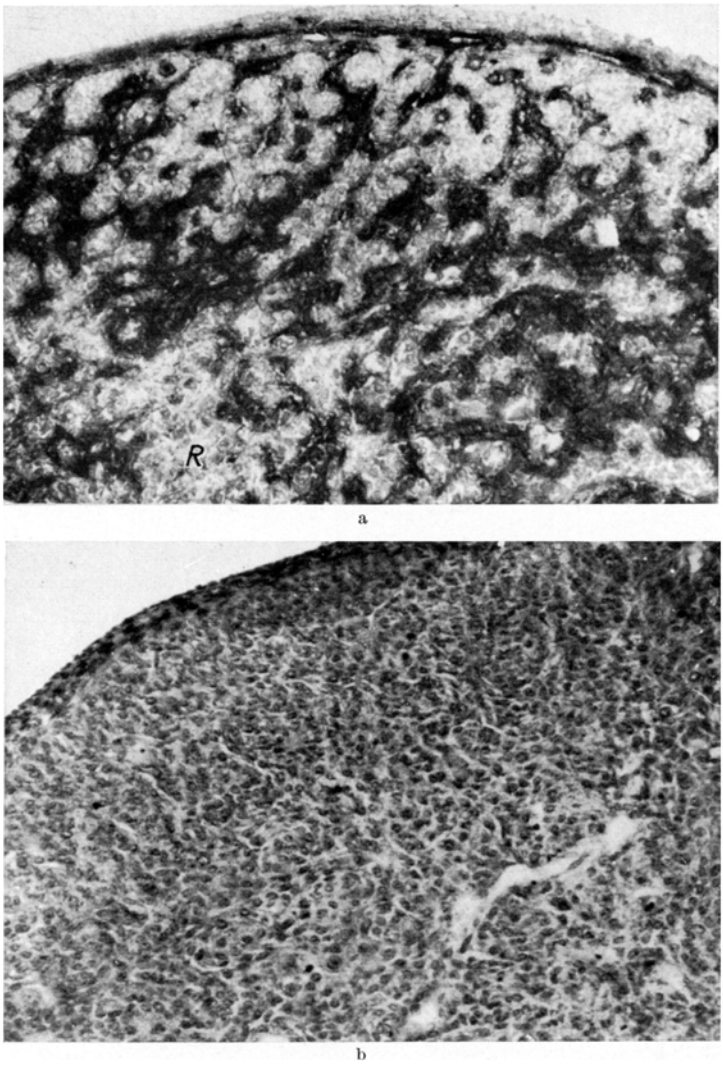

Fig. 5a and b. Gonad of embryo No. 26, ♂, 45 mm. Alkaline-phosphatase reaction (a, Magnification 125 ×) and Hematoxylin-Eosin (b, Magnification 150 ×). Enzyme-negative tubular structures and positive interstitial cells. Formation of tunica albuginea. *R* part of rete anlage

negative peripheral mesenchymal zone and the equally negative surface lining. The germ cells which by this time have practically completed migration to the gonadal anlage are at first loosely scattered throughout the peripheral phosphatase-negative area of the gonad (Figs. 3, 4, 7, 8). The identity of the individual primordial germ cells which had migrated deep into the central blastema, on the other hand, is completely lost, as blastema cells are as strongly alkaline-phosphatase-positive as primordial germ cells.

When alkaline phosphatase activity is used as a marker, the gonadal blastema is easily recognized in its entirety. Accordingly, its fate can be followed in subsequent developmental stages of early differentiation where the use of ordinary

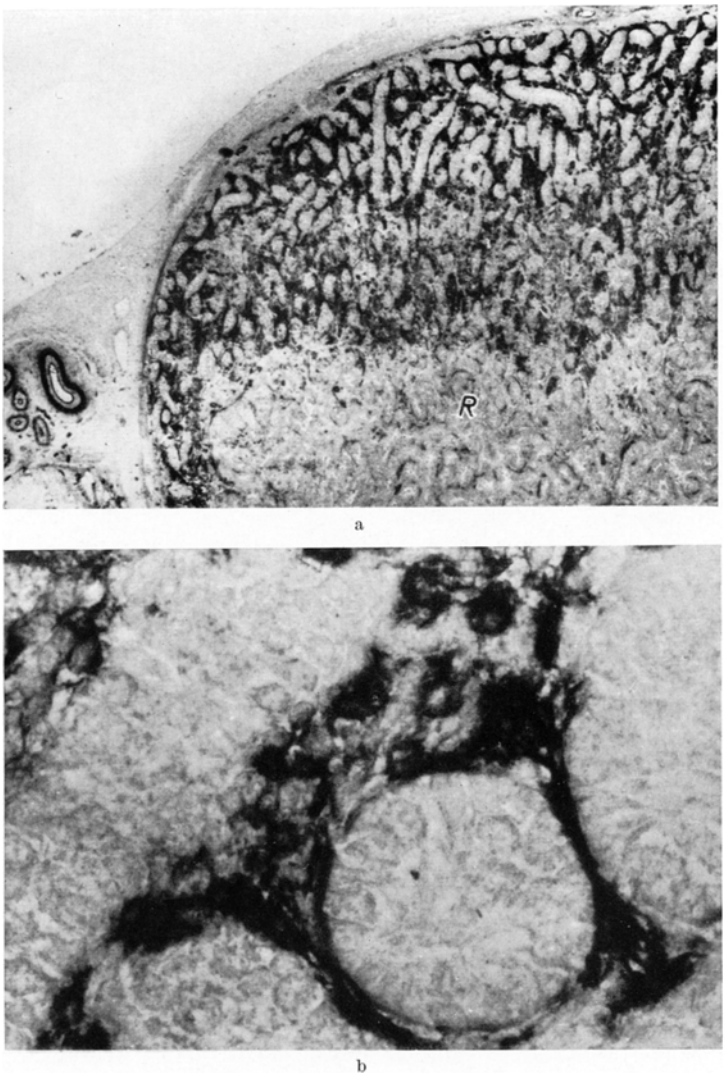

Fig. 6a and b. Gonad of fetus No. 24a, ♂, 300 mm. Alkaline-phosphatase reaction. a) (low magnification, 100 ×): abundant enzyme-positive interstitial cells. *R* rete testis. b) (high magnification 550 ×): enzyme-positive interstitial cells. Large negative cells within the tubules represent spermatogonia

staining only is of no help. Indeed, in the gonad of the 30—35 mm fetus, no morphological differences between male or female can be detected in ordinary histological sections, yet the first signs of differentiation are already apparent on the histochemically stained slides. The number of germ cells increases considerably in the peripheral zone of the femal gonad (Figs. 7, 8, 9), while in the male gonad (Figs. 3, 4) in the same peripheral zone the number of germ cells appears to be decreasing; in the male, they obviously move to the deeper areas. The alkaline-phosphatase positive blastema itself also shows slight changes. In the female its outer cell layers start to arrange in cordlike columns (Fig. 8), while in the male, conspicuously negative spots begin to emerge (Fig. 4).

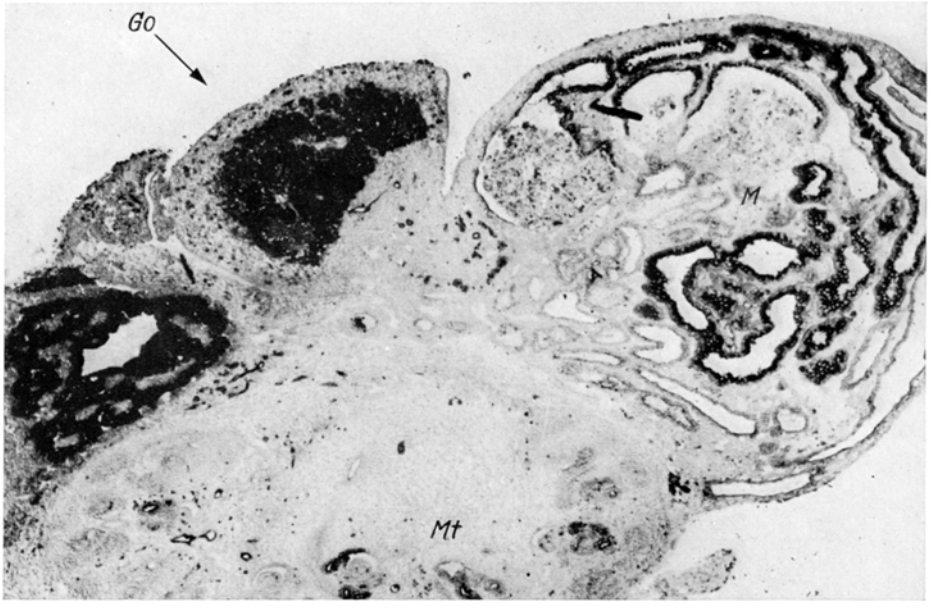

Fig. 7. Gonad of embryo No. 40, ♀, 35 mm. Alkaline-phosphatase reaction. Histologically undifferentiated stage with enzyme-positive gonadal blastema and equally positive germ cells in a peripheral zone. Positive alkaline phosphatase reaction also in the adrenal cortex and in tubular structures of the mesonephros and metanephros. *Go* Gonad; *M* Mesonephros; *Mt* Metanephros, Magnification 36 ×

Group II, Embryos No 50 to 52, Male; No 36 to 30, Female (s. Tab.)

In the *male*, the formation of a tunica albuginea and cellular segregation within the gonadal blastema proceeds rapidly (Fig. 5a). The mesenchymal cells immediately beneath the coelomic lining are arranged into a densely-packed layer which includes a vascular sheet located near the inner third of the thickness of this layer. Only the gonads of embryos No 50, 16, and 26 still contain few alkaline-phosphatase-positive germ cells in this zone which then shows further condensation and fibrous transformation of the layered cells giving rise finally to the tunica albuginea (Figs. 5, 6a). Continuing the aforementioned separation of the blastema into alkaline-phosphatase-positive strands and negative spots, these spots rapidly enlarge and emerge as tubules which are sharply outlined by the surrounding strands of alkaline-phosphatase-positive interstitial cells (Figs. 5a, 6b). After this stage, the differentiation processes also can be followed in sections with ordinary staining (Fig.s 5a, b). In fact, ordinary staining is better suited to reveal the appearance and progressive incorporation of germ cells into the tubules. Germ cells are now alkaline-phosphatase-negative or only slightly positive. At the same time they have enlarged distinctly and contain a vesicular nucleus. Mitoses within the tubules seem to represent mainly proliferation of germ cells which correspond now to spermatogonial precursor cells. No such segregation processes nor any germ cells are observed in a rounded-up and only slightly alkaline-phosphatase-positive hilar area (Fig. 5a) of the gonad. This area later on gives rise to the large rete testis (Fig. 6a).

In the *female*, gonadal differentiation is characterized by vigorous germ cell proliferation in the peripheral zone and also by a progressive transformation of the

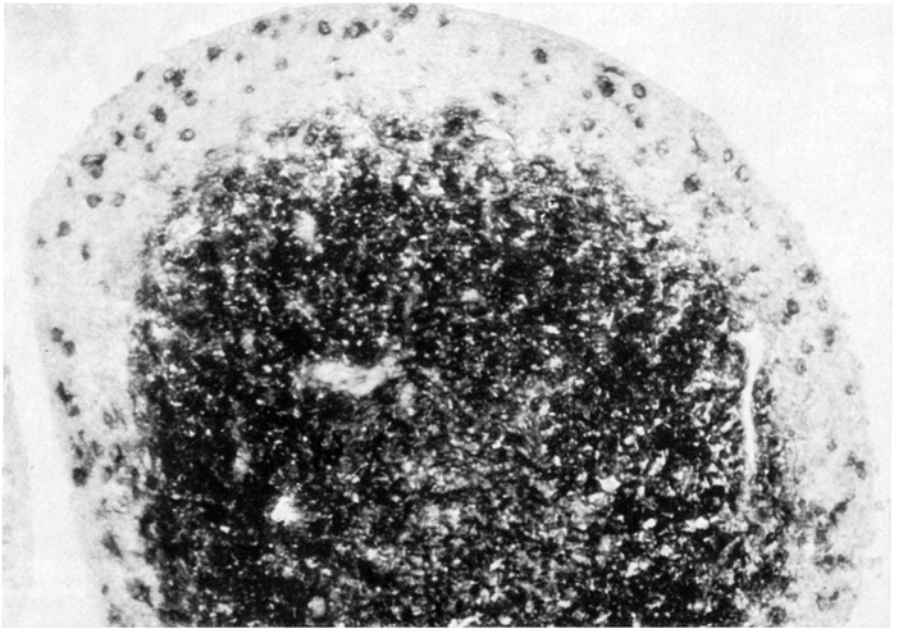

Fig. 8. Gonad of embryo No. 36, ♀, 40 mm. Alkaline-phosphatase reaction. Beginning of organization of follicular cell cords within the gonadal blastema. Slight augmentation of oögonia in the peripheral area. Magnification 130 ×

alkaline-phosphatase-positive blastema into cords which will retain this enzyme activity: Fig. 8 shows the beginning of these processes which at these and still later stages remain entirely hidden in sections studied only with ordinary stains. Subsequently germ cells multiply considerably and build up a broad peripheral layer (Figs. 9, 10) of oögonia. Germ cells in these stages are not distinctly larger than somatic cells. Occasionally they may be located within the coelomic "germinal" epithelium which itself takes no part in any further changes and remains always alkaline-phosphatase-negative. For a certain period (Figs. 8, 10), the former gonadal blastema and its cordlike derivatives seem to be separated from the layer of germ cells by a sheet of arching vascular spaces which could have a formative influence on the developmental processes. As in the male gonad, a round area of densely packed cells with less phosphatase activity appears in a central region of the blastema (Fig. 10). With the continuing growth and differentiation of the ovary, this area moves gradually towards the hilar region (Fig. 11). There it finally occupies a very small area containing some tubular structures corresponding to the rete ovarii. In the most advanced stages (50—55 mm) of this group, the cords differentiating from the gonadal blastema are well defined and clearly recognizable by their high alkaline phosphatase activity. They are broader in the deep areas of the ovary and branch out towards the periphery. Thus they seem to display a gradient of growth and differentiation which finally brings these cords in direct contact with the peripheral layer of germ cells. Fig. 9 marks this event in an ovary of a 50 mm embryo (No 31) showing on one side the cords still separated from the oögonial layer while the other side reveals the junction of these two elements. The cords correspond beyond doubt to the primordia of the follicular or granulosa cell system and should be called follicular cell cords.

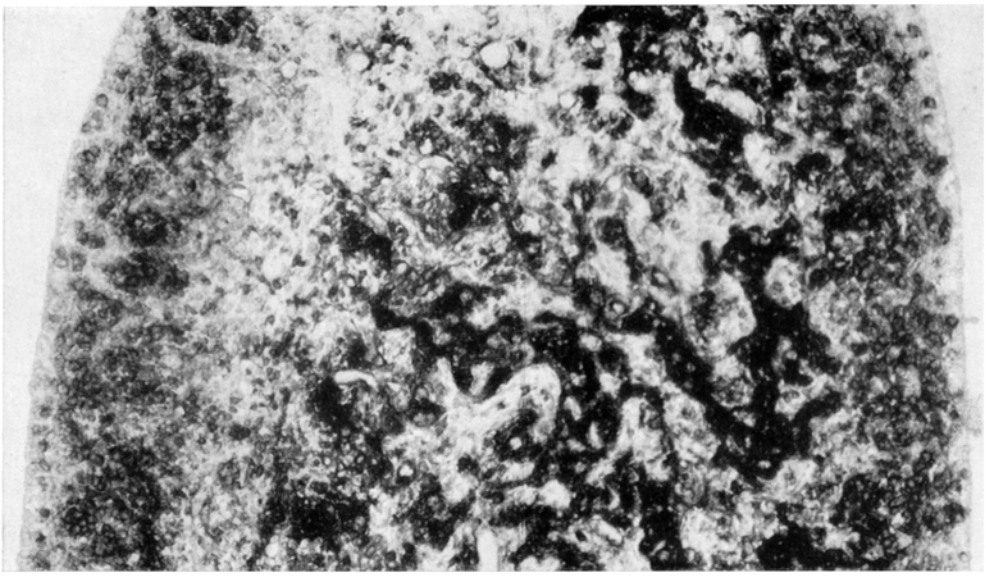

Fig. 9. Gonad of embryo No. 31, ♀, 50 mm. Alkaline-phosphatase reaction. Well-developed alkaline-phosphatase-positive follicular cell cords in central areas of the ovary. Considerably augmented number of oögonia forming a broad peripheral zone. At right, vanguards of cords have already reached the layer of oögonia. Magnification 120×

Group III, Embryos No 29, 38, 25, 22 (s. Tab.)

The developmental changes in this group of *female* embryos (males of this size range have not been investigated) ranging in size from 70 to 95 mm crown-rump length, are characterized by the progressive junction and beginning of interaction of follicular cell primordia and germ cells.

Together with a few strands of perivascular connective tissue the widely-branched follicular cell cords invade the space between the clusters and layers of the germ cells, still alkaline-phosphatase-positive. The aforementioned junction of follicular cell cords and germ cells gives rise in this developmental stage to an intimate contact between these two elements. This phenomenon becomes most conspicuous in the gonads of the larger embryos in this group (Fig. 12). The process can be compared to a stuffing of stockings (follicular cell cords) with oögonia. First, oögonia are engulfed by the peripheral branchings of the cords. Then they move downward deeper and deeper. Finally, only the most remote part of these cords remain unstuffed (Fig. 12a). Thus the cords hitherto termed follicular cell cords should now be called ovigerous cords. Whereas the follicular cells keep their alkaline phosphatase activity throughout this whole process, the germ cells rapidly lose any demonstrable activity of this enzyme after incorporation in the cords. At the same time the germ cells become enlarged in size and show voluminous clear nuclei (Fig. 12b). This change may represent a maturation of oögonia preparing for transition into oöcytes. Mitotic figures occur among them in fair numbers. Meiotic prophase has not yet begun.

As a result of these changes, the cortical parenchymatous part and the central stromatous part become two distinct entities. The stuffed follicular cell cords (ovigerous cords) comprise the cortical parenchyma. A broad intermediate zone

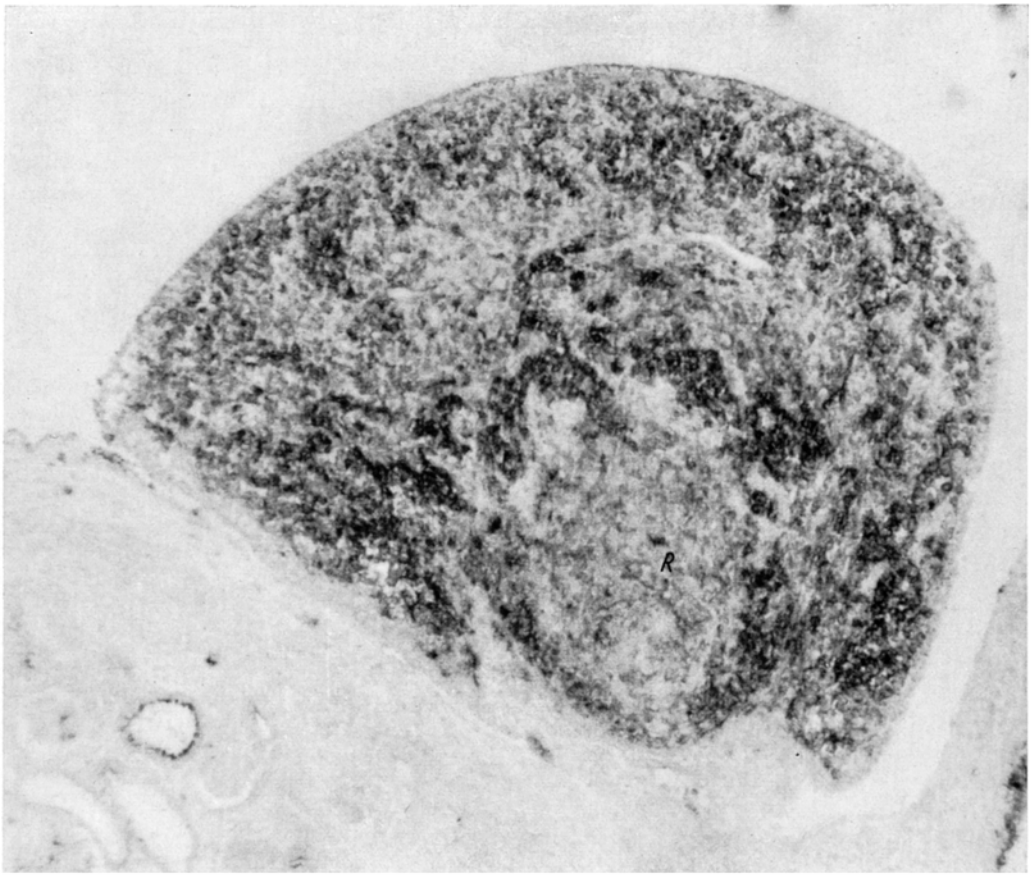

Fig. 10

Figs. 10 and 11. Gonad of embryo No. 51, ♀, 47 mm (Fig. 10); and No. 29, ♀, 70 mm (Fig. 11). Rounded body of rete ovarii (*R*) in an early stage of differentiation within the gonadal blastema (Fig. 10) and close to the hilus after completion of differentiation of follicular cell cords in the later stage (Fig. 11). Note negative "germinal epithelium." Magnification Fig. 10: 125: ×; Fig. 11: 190 ×

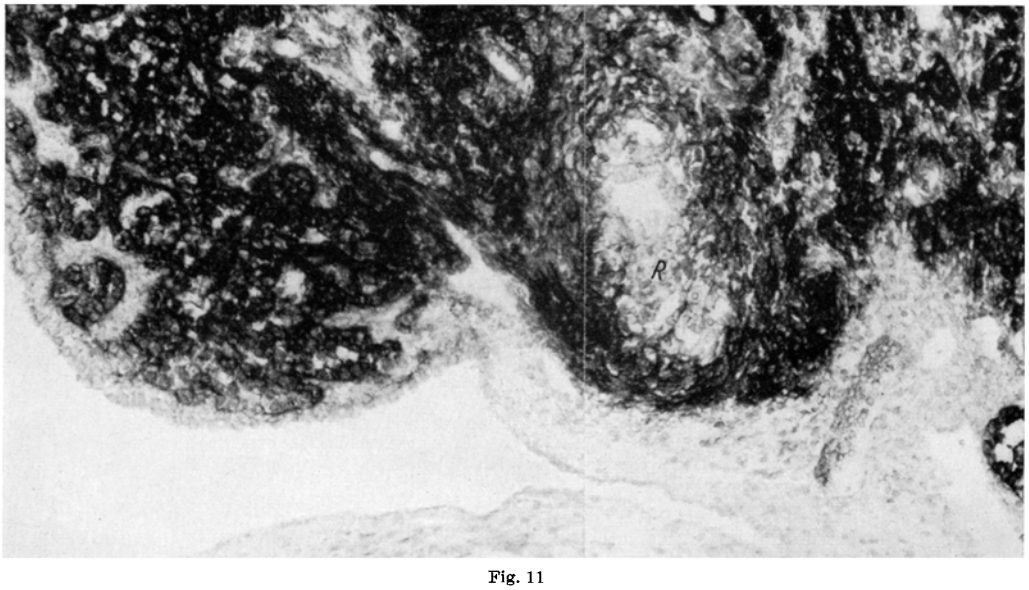

Fig. 11

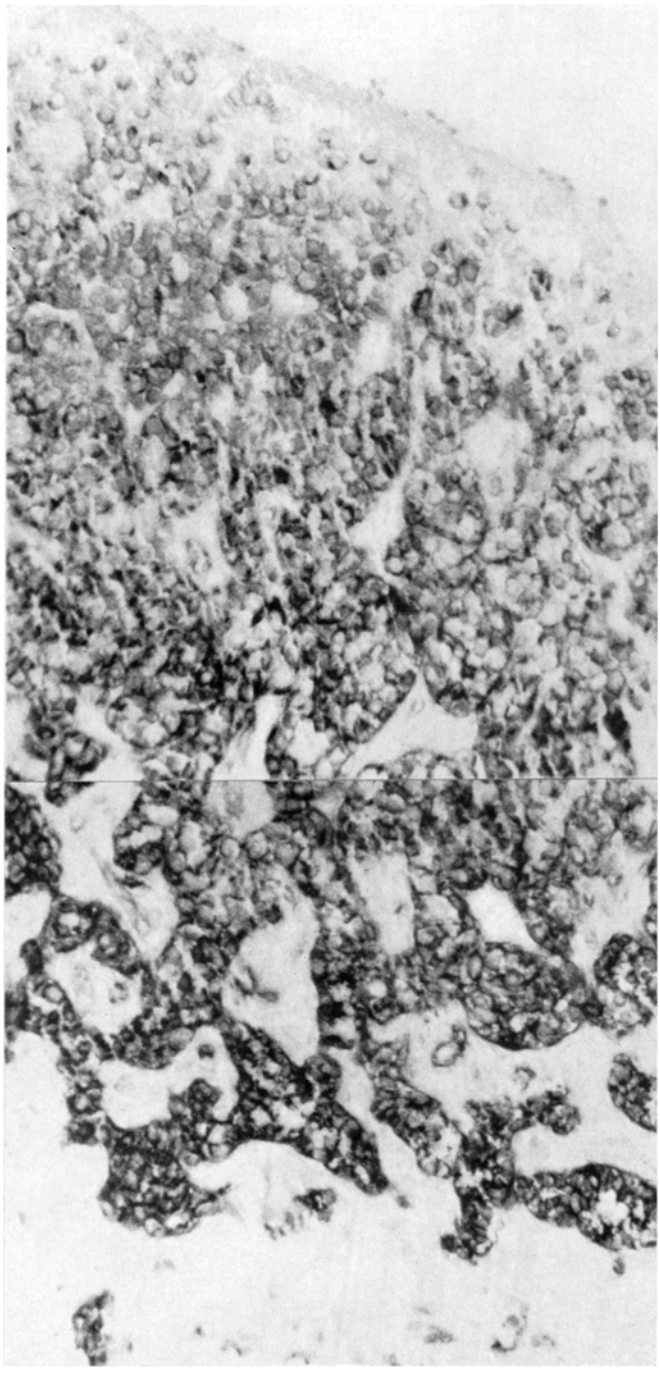

Fig. 12a

Fig. 12a and b. Ovary of fetus No. 22, ♀, 95 mm. Alkaline-phosphatase reaction (a) and Hematoxylin-Eosin (b). Large and weakly phosphatase-positive or negative oögonia with vesicular nuclei (b) entering the phosphatase-positive follicular cell cords (a). Meiosis has not yet begun. Magnification 350 ×

between the cortical area and the central region is marked by the presence of numerous unstuffed follicular cell cords which are seen either as compact strands or

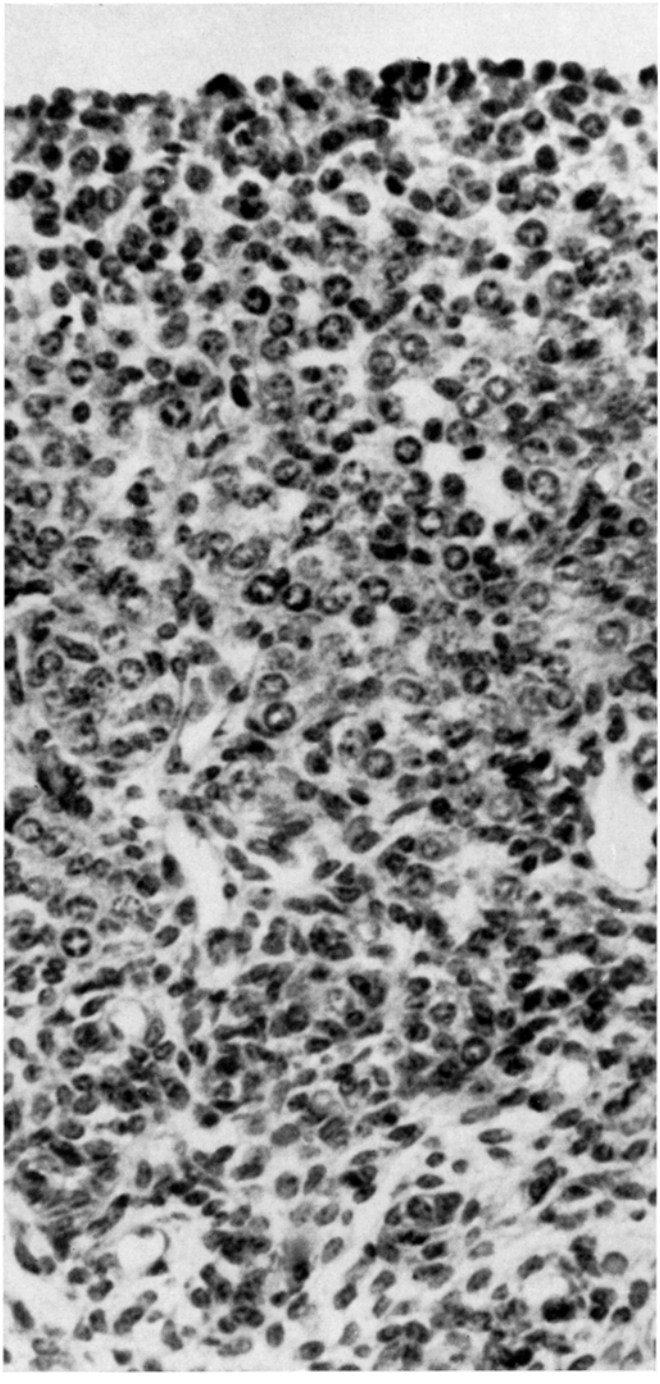

Fig. 12 b

as follicular cell remnants. They can be identified by their high alkaline phosphatase activity. At the same time, the central or medullar stromatous region becomes

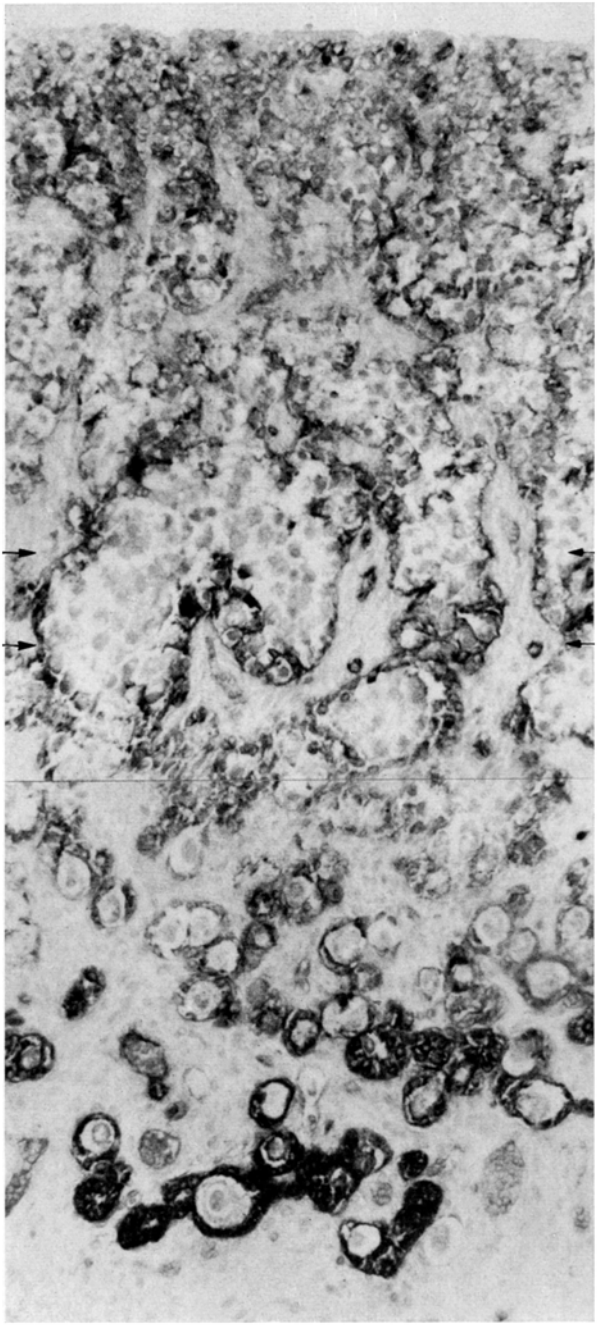

Fig. 13a

Fig. 13a and b. Ovary of fetus No. 8, ♀, 260 mm. Alkaline-phosphatase reaction (a) and Hematoxylin-Eosin (b). Maturation of oöcytes within ovigerous cords leading to the formation of primordial follicles. In an intermediate zone (arrows) "congestion"-phenomenon of oöcytes in pouch-like enlargements of the ovigerous cell cords. Blind buds of follicular cell cords in the deepest cortical layer. Magnification 170 ×

enlarged by the increase of connective tissue pushing the "rete" body which had been located in the center of the gonadal blastema entirely in a hilar position.

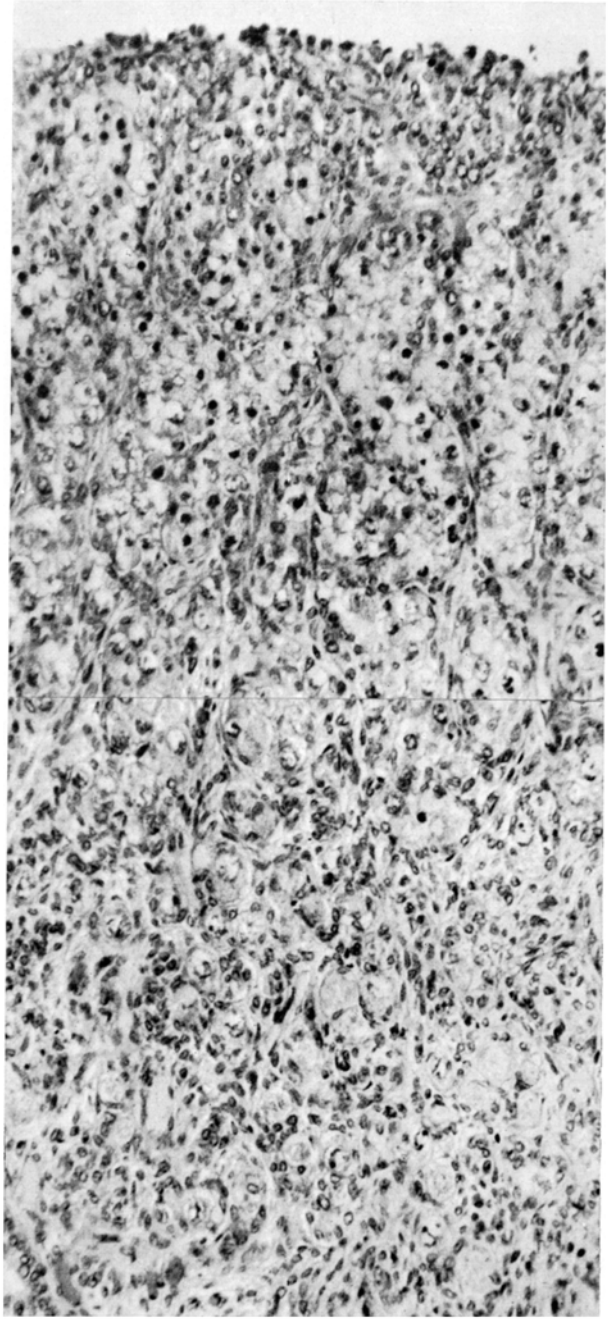

Fig. 13 b

Group IV, Embryos No 32, Male; No 42, 14, 23, 15a, 15b, 34, Female
(s. Tab.)

The only *male* in this group of embryos ranging in size from 110 to 130 mm crown-rump length shows further differentiation of the testis. This process of

differentiation seems to be a gradual course of cellular and histological maturation (Figs. 5, 6). In the *female* gonad, developmental changes in this group of embryos are marked by the onset of meiotic prophase. Most germ cells are now incorporated within the ovigerous cords. In the periphery of the more clearly defined ovarian cortex they correspond still to oögonia, displaying a large size, vesicular nuclei, and a negative-phosphatase reaction. But in an intermediate zone numerous meiotic figures can be seen. Though the oöcytes show mainly stages of early meiotic prophase, figures corresponding to diakinesis were frequently found. A similar finding was described earlier (OHNO and SMITH, 1964).

Group V, Embryos No 13a to 5, Male; No 17 to 7, Female (s. Tab.)

Further changes occurring in the *male* gonad still consist of steadily advancing differentiation of the tubular structures and the abundant interstitial cells. Germ cells within the tubules can be recognized by their large size (Fig. 6b).

The findings in the younger stages of *female* fetuses of this group are not sharply distinct from those in the later stages of the former group. The most outstanding new feature is the appearance of primordial follicles in the deepest layers of the cortical zone which now attains its maximal thickness (Fig. 13). Proceeding from the periphery to the deeper layers we find beneath the "germinal epithelium" a zone of densely packed oögonia, followed by a broad layer of oöcytes in meiosis. The deepest layer contains numerous primordial follicles. After entering meiosis the oöcytes show considerable increase of total volume with a very clear cytoplasm. All stages of meiotic prophase are present but pachytene figures are most frequent. Diakinesis figures can be seen in this group with even greater frequency than in the foregoing group. The sequence of these layers can be demonstrated best in sections stained by the alkaline phosphatase reaction (Fig. 13a). Again oögonia at the surface layer show that they have lost most of their phosphatase activity during transformation from primordial germ cells. The intermediate zone of meiotic activity is characterized by a phenomenon already present in the foregoing stages but now becoming most evident. That is ovigerous cords are almost completely transformed to large pouchlike structures. The walls of these sacs consist of a single layer of more or less flattened alkaline-phosphatase-positive follicular cells. The sacs are stuffed with dense clusters of the enlarged phosphatase-negative oöcytes.

In the deeper layers, however, the ovigerous or former follicular cell cords contain single oöcytes and, like a string of pearls, each is in close contact with the follicular cells. Apparently a centrally-directed gradient of maturation of these oöcytes leads finally to dictyotene stages. During this course of maturation they become enclosed in tied-off buds of the former follicular cell cords. Thus primordial follicles (Fig. 13a) are formed. A high alkaline phosphatase activity remains present in the deeper parts of the ovigerous or follicular cell cords and tubes, and also in the follicular cells of the still deeper layers of primordial follicles. In addition, in the border area of the cortex and stromatous medulla, numerous blind buds of the former follicular cell cords persist. They too give a strong positive reaction.

The embryos of the more advanced stages of this group show developmental changes of the ovary characterized mainly by a decrease and disappearance of the

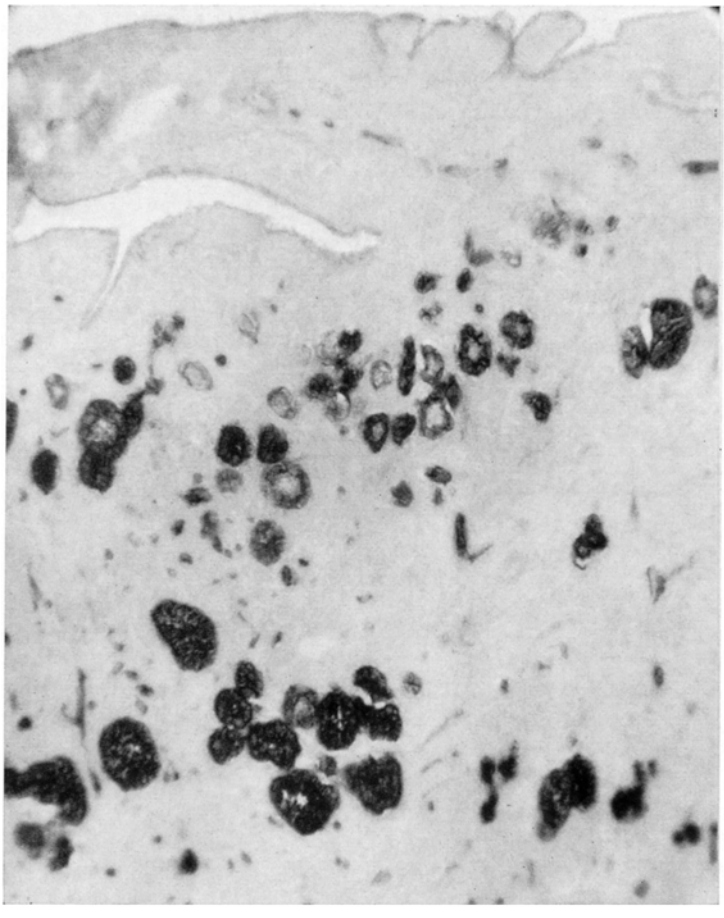

Fig. 14. Ovary near term, fetus No. 65, ♀, 880 mm. Alkaline-phosphatase reaction. Primordial follicles (outer zone of cortex) weakly positive or negative. Persistent remnants of unstuffed follicular cell cords in deeper layers strongly positive. Magnification 150 ×

described "congestion" phenomenon and simultaneously by a considerable increase in the number of primordial follicles. These processes of maturation which are accompanied by degeneration of a great number of oöcytes lead to a reduction in thickness of the ovarian cortex. The oöcyte degeneration seems to be a direct consequence of oöcyte accumulation in the "congestion zone" where they are prone to proceed precociously to diakinesis (Ohno and Smith, 1965).

Group VI, Embryos No 28a and 64, Male; No 4a and 65, Female
(s. Tab.)

In the *male* gonad, the continuous growth and differentiation of tubules, interstitial cells, and rete testis take place without dramatic structural changes. The great amount of interstitial tissue and its unchanged intense alkaline phosphatase activity commends special interest. The rather large rete testis on the other hand contains only a few alkaline-phosphatase-positive cells between the rete tubules (Fig. 6a).

In the gonads of *female* fetuses of this group, ranging in size from 620 to 880 mm, alkaline phosphatase activity becomes more confined to the deeper primordial follicles and to the solid buds and strands of the former follicular cell cords. This is shown in the ovary of embryo No 65 (880 mm, near birth) in Fig. 14. Some of the more superficially located primordial follicles now demonstrate only slight phosphatase activity in their follicular cells, or are even devoid of any demonstrable activity of this enzyme. Primordial follicles in ovaries of sexually mature cows were also mostly phosphatase-negative. These ovaries, however, disclosed a high activity of this enzyme in the theca externa of developing Graafian follicles and in theca cells during the early luteinization period of postovulation follicles.

Conclusions and Discussion

The alkaline phosphatase reaction test is a valuable means of elucidating the origin and behavior of cellular systems or blastemas in embryology. The aim of the present investigation is not to contribute to understanding the more general problems of distribution of this enzyme in embryonic organs nor to the question of its significance in development (Rossi et al., 1953; Jakoby, 1962). Rather it reveals the importance of the alkaline phosphatase technique as a specific label of developmental cell systems. McKay et al. (1953) used this method to prove conclusively the migrational path of primordial germ cells in man, which, however, had already been anticipated by Witschi (1948) with ordinary staining methods. The present results confirm previous findings (Ohno and Gropp, 1965) which established that in cattle also, the primordial germ cells give a positive alkaline phosphatase reaction, as they do in man and other species (Chiquoine, 1954; Mintz, 1957). But unlike the findings in other mammals, in cattle the primordial gonadal blastema and its derivatives also show a strongly positive enzyme reaction. This observation thus provides an appropriate tool for a close tracing of the developmental fate of the gonadal blastema and of the processes of sexual differentiation in which it becomes involved.

Alkaline phosphatase activity is already present in stages of early formation of the gonadal fold. In the youngest embryos studied (15 mm in length; see also Ohno and Gropp, 1965), no sharp separation exists between the epithelial coelomic lining and the bulk of the gonadal blastema. The present study did not reveal whether this finding indicates a temporary functional unit of the surface lining and the deeper layers or if it is to be explained by a proliferative wave and downgrowth of the surface epithelia which later form the gonadal blastema (Politzer, 1933; Watzka, 1961; Franchi et al., 1962). In a slightly later stage, the surface epithelium becomes alkaline-phosphatase-negative and separates rather clearly from the blastema. The surface lining then remains enzyme-negative throughout further development. The somatic parts of the indifferent gonad consist of an enzyme-negative peripheral zone and a large enzyme-positive central area. The latter seem to represent the intrinsic part of the gonadal blastema.

Two main developmental processes observed by the histochemical labelling technique are outstanding in preparing the basis for further sexual differentiation in the gonad, one of which concerns the germ cells, the second occuring within the blastema. In the male, the number of the alkaline-phosphatase-positive germ cells of the outer zone of the gonadal anlage remains constant or diminishes slightly.

This is apparently the result of their migration to the central gonadal blastema. On the contrary, in the female the germ cells remain in the periphery and multip y until they form a thick layer.

The alkaline-phosphatase-positive gonadal blastema simultaneously shows a specific type of segregation depending upon the sex of the embryo.

Both testicular and ovarian differentiation take place *in situ* within the gonadal blastema. Testicular development, however, is characterized by the appearance of phosphatase-negative strands and cords within the blastema which finally transform into seminiferous tubules. The process of segregation causes those members of the blastema, which retain their alkaline phosphatase activity to come to lie between the tubular primordia. Now they must be designated as interstitial cells. The tubular primordia progressively incorporate germ cells which enlarge while becoming alkaline-phosphatase-negative. These changes probably mark the transition from primordial germ cells to the more immediate precursors of spermatogonia. The enzyme-negative peripheral zone surrounding the gonadal blastema is transformed to a fibrous layer and narrows after the germ cells have left this zone. Thus the tunica albuginea forms.

An analogous segregation process in the female gonad leads to the appearance of alkaline-phosphatase-positive cords, representing the follicular cell primordia. The cords and their derivatives maintain their enzyme activity. They begin to spread out between enzyme-negative interstitial stromatous tissueand grow out towards the periphery. By a process of continuous incorporation of germ cells from the periphery, the former follicular cell cords transform into ovigerous cords. Before they are engulfed by the ovigerous cords, the germ cells begin to enlarge, and there is a progressive decrease of alkaline phosphatase activity. These changes in the female gonad probably indicate the transition from primordial germ cells to oögonia. But unlike the situation in the male gonad, the downward movement of the germ cells within the ovigerous cords is accompanied by further transition to oöcytes, as shown by a considerable cytoplasmic swelling and by changes in the prophase nucleus.

The origin of the enlargement and pouchlike widening of the ovigerous cords is in part explained by this cellular enlargement. Another important factor accounting for this feature is the congestion of the crowded oöcytes midway on their downward movement within the enveloping wall of the ovigerous cords. Former observations of OHNO and SMITH (1964) on the importance of intimate contact between follicular cells and oöcytes in order to guarantee the normal maturation of the oöcytes are confirmed by the present findings. The accumulation of germ cells within the pouchlike enlargements of the ovigerous cords apparently leads to a quantitative and structural imbalance between the enveloping follicular cells and the crowded oöcytes enclosed. Thus an imperfect control of oöcyte maturation results which is probably responsible for the occurrence of premature diakinesis and subsequent degeneration. Only in a deeper layer of ovigerous cords there are enough follicular cells to envelope individual oöcytes permitting the suspension of the meiotic process at diplotene and the formation of primordial follicles.

With the maturation of the primordial follicle the alkaline phosphatase activity of the follicular cells decreases and finally disappears. In the postnatal ovary, both, the primordial follicles and the granulosa cells of the Graafian follicles are phosphatase-negative, in contrast to the cells of the theca interna which, however, by their origin, could belong to the same system as the follicular or granulosa cells. Interestingly enough, the high activity of alkaline phosphatase is maintained in the numerous blind buds and islets derived from the former unstuffed follicular cell cords which persist in the deeper cortical layer of the adult ovary. MOSS et al. (1954) recorded alkaline-phosphatase-positive clumps in the bovine ovary and interpreted them as atretic ova. The present findings are likely to prove that at least a part of these clumps originate directly from the follicular cell cords.

There is some evidence that these persistent buds correspond to cell remnants which could give rise to gonadal stromal tumors (HERTIG and GORE, 1961) in man and other mammals. The assumption of the importance of such primitive cell vestiges is supported by the discussion of the so-called "Granulosa-cell-ballen" (R. MEYER, 1915). The present findings sustain the opinion of NOVAK (1952) who states that the origin of these "Granulosa-cell-ballen" should be traceable to an earlier progranulosal and prothecal phase of development.

In interspecific crosses between remotely related members of the horse family *Equidae*, such as the mule, all the germ elements of the female degenerate at the zygotene stage of first

meiotic prophase. Thus, the postnatal ovaries of these hybrids are completely devoid of oöcytes. Yet, sexually mature female hybrids have a regular estrus cycle and mature Graafian follicles as well as corpora lutea are present in the ovaries (Ewert, 1899; Benirschke and Sullivan, 1966). There is little doubt that persisting unstuffed follicular cell cords are responsible for the maintenance of the normal estrus cycle by these hybrids.

The results on cattle described herein do not favor the theory of the importance of a cortico-medullary antagonism (Witschi, 1951, 1962) in the differentiation of the somatic cell systems of male and female gonads in mammals. The analysis of the morphogenetic events leads convincingly to the assumption of an *in situ*-differentiation within a common gonadal blastema of all somatic elements in both sexes; i.e. of interstitial cells and tubules in the male, and follicular cell primordia in the female. In the cattle, these processes are easily traceable since they display a separation into phosphatase-positive and phosphatase-negative constituents. The problem of homology of the different somatic cell systems of the gonads is more difficult to solve. Nevertheless it can be presumed that the interstitial cells in the male and the follicular cell primordia in the female represent homologous systems. As far as the origin and the interrelationship of tubular cell primordia and the interstitial cells of the testis are concerned, it seems possible that they both develop from the phosphatase-positive cells of the gonadal blastema. The early steps of segregation seem to become obvious only by the loss of enzyme activity in the presumptive tubular cells of the common blastema. The alternative possibility of a preferential proliferation of pre-existing enzyme-negative cells enclosed within the blastema cannot be ruled out, but this appears less likely. The homology of the rete structures in both sexes is highly probable. While they remain small and do not take part in further development in the female, they exhibit a very important development in the male gonad.

There is no evidence for the existence of secondary sex cords (Felix, 1911; Gillman, 1948; Watzka, 1961; Franchi et al., 1962; McEntee, 1962). A "second proliferation wave" of the so-called "germinal epithelium" or of a subepithelial germinal layer (Gruenwald, 1934) does not play any role in development of the cattle ovary. The presence of secondary sex cords in the female gonad may be an illusion created by the peculiar arrangement of germ cells in the outer zone surrounding the gonadal blastema. In ordinary stained sections, the impression of a cord formation is easily produced by the tendency of these germ cells to queue up before being engulfed by follicular cell cords.

It has to be emphasized that during the later stages of the development of the ovary and in the definitive ovary there is reason to demarcate a cortex ovarii from the mesenchymatous central or medullar part. This cortex which defines itself later should not be confused with a hypothetical primordial cortex which has its place only in the theory of a bipartite cortico-medullary anlage. In reality, it merely represents the superficial area where follicular cell cords are stuffed with germ cells. The central or medullary part of the definitive ovary is formed by an increase of connective tissue containing vascular and nervous structures. The broadening of this mesenchymal central part accounts mainly for the cutting-off of the rete ovarii from the derivatives of the gonadal blastema.

The development of the male gonad also showed no sign of cortico-medullary antagonism. In cattle, the formation of the tunica albuginea cannot be explained by a transformation of a cortical anlage but by a simple fibrous conversion of the peripheral mesenchymal zone of the gonad after the germ cells migrated from this zone deeper towards the differentiating tubules within the blastema.

According to Witschi (1951, 1962), in his theory of a cortico-medullary antagonism in sex differentiation of the gonads, the medullary part arises from an extra-gonadal mesonephric

blastema. Though this opinion cannot be supported by these present results in cattle, one particular facet of it could hold true for this species as well as for mammals in general: in the very beginning, the alkaline-phosphatase-positive gonadal blastema might well be of perimesonephric mesenchymal origin. It may belong to the perimesonephric streak which in its cranio-anterior region comprises also the adrenal cortical anlage (Fig. 2a). Both the gonadal and the adrenal cortical blastema have in common an alkaline phosphatase activity and both give rise to cellular systems which later produce steroid hormones.

The present results favor strongly the concept of the development of the gonads by differentiation processes *in situ* without the participation of secondary sex cords. This view was expressed in a study on the human ovary by FISCHEL (1930) but his contribution was not fully accepted and is often not even cited. Moreover, his theory and the results of our work are in full agreement with the bulk of information contained in the work of PINKERTON et al. (1962) on the ovary of the human embryo. This extensive and important study treats most accurately the fate of the germ cells in the female gonad, while in the absence of a specific labelling technique, the results on the origin and behavior of the somatic elements, particularly of the follicular cells, remain necessarily incomplete. The object of the present study is to fill this gap so far as the ovary is concerned, and to extend the knowledge of the development of male and female gonads by the concept of a common somatic gonadal blastema and of a specific inter-relationship of the germ and somatic cells. There seems to be no reason to assume that the developmental processes in bovine male and female gonads deviate basically from those in other mammals, although particular details may vary from one species to another. There is evidence, for instance, that the phenomenon of a "congestion" of oöcytes within pouchlike sacs of follicular cells is restricted to ovaries of species with long gestation periods and does not occur in small rodents. Only the fact that in the cattle embryo, unlike conditions in other mammals, the primordia of the somatic gonadal parenchyma and their derivatives are alkaline-phosphatase-positive, represents an appropriate tool which makes it possible to trace their fate during the developmental processes. Thus the observations made on the development of the gonads in the cattle embryo should apply to other mammals as well as man.

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