Conversion of Progesterone to Estrone and Estradiol *in Vitro* by the Ovary of the Infantile Rat in Relation to the Development of Its Interstitial Tissue

STEVEN L. QUATTROPANI, AND JUDITH WEISZ

Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545

ABSTRACT. The development of the interstitial tissue and of the steroidogenic capability of the infantile rat ovary were examined by light microscopy, by histochemistry and by *in vitro* incubation. Ovaries from 5, 7, 10, 12 and 14-day-old normal rats and from rats that had received 100 μg testosterone propionate or sesame oil at 5 days of age were utilized. The structural development of the infantile rat ovary was examined in 0.5 μ sections of Epon-embedded tissues which had been fixed with glutaraldehyde and osmium. The lipid content of interstitial tissue in untreated animals was minimal at 5 days of age and then increased. The greatest change occurred between postnatal days 5 and 7. 3β-Hydroxysteroid dehydrogenase activity also became prominent at this time. The interfollicular tissue of animals treated with testosterone propionate accumulated very little lipid between postnatal days 5 and 7 and exhibited very little 3β-hydroxysteroid dehydrogenase activity. Fibroblasts rather than interstitial cells were the predominant cell type in these ovaries.

In incubations of ovaries of untreated rats with 7-^{3}H-progesterone, little change was seen in the yield of tritiated estrone between 5 and 7 days of age (0.21–0.48%) and only a slight decrease at 14 days. In contrast, the conversion to estradiol changed markedly with age. On day 5, it was comparable to that of estrone (0.21 and 0.59%), increased about 3-fold by day 7 and reached a peak (1.83 and 1.79%) by day 10. After day 10 the amounts of labeled estradiol formed decreased and by day 14 were comparable to those found on day 5.

Neonatal testosterone treatment reduced by about 90% the production of both tritiated estradiol and estrone by ovaries of 7 and 10-day-old rats. The amounts of both steroids increased slightly thereafter so that by day 14 the values were similar to those of untreated rats. The neonatal testosterone treatment eliminated the peak of labeled estradiol production found in normal rats at 10 days of age. The findings in rats injected at 5 days of age with sesame oil differed from those of untreated rats at two ages. At 7 days the yields of both labeled estradiol and estrone were increased more than 2-fold while at 12 days tritiated estrone production was reduced by about 50%. (*Endocrinology* **53**: 1269, 1973)

IN THE ovary of the rat the interstitial tissue represents a clearly recognizable structural component. The cells that make up this tissue, the interstitial cells, are characterized by a high concentration of lipids (1–4) and by 3β-hydroxysteroid dehydrogenase [3βOHD] activity (5–7). They are generally assumed to be steroidogenic primarily because of these histochemical features.

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Present address: Departments of Anatomy and Obstetrics and Gynecology, Division of Reproductive Biology, The Milton S. Hershey Medical Center, The Pennsylvania State University Hershey, Pennsylvania, 17033.

In the rat the interstitial tissue is recognizable by the end of the first week of life and, till about the third week of life, is the only ovarian component with characteristics that are generally associated with steroidogenic cells (2,3,7). Thus, for a well-defined period of time, the interstitial cells may be assumed to be solely responsible for steroids produced by the ovary. During this period the ovary of the rat may serve as a useful model for studying structural and functional correlations in the developing steroid-producing cell.

In the present study, the development of the interstitial tissue and its steroidogenic potential were examined in ovaries from 5–14-day-old rats using three approaches: light microscopy, histochemistry for the localization of lipids and 3βOHD activity, and *in*

vitro incubations to determine estrogen production from tritiated progesterone [^{3}HP]. Because testosterone propionate (TP) injected on the fifth day of life has been found to disrupt the development of interstitial cell (8) and to lower circulating FSH (unpublished observations) and LH levels (9), ovaries from rats so treated were studied in parallel with uninjected rats to determine the correlation between these changes and ovarian steroidogenesis *in vitro*. The experimental treatment of the animals and incubation conditions were identical to those used in a previous study in which the production of labeled estradiol (E_2) and estrone (E_1) from ^{3}HP were studied in normal and neonatally androgen-treated rats between 21 and 350 days of age (10).

Materials and Methods

The rats used in this study were of the Sprague-Dawley strain (Charles River CD). Ten female pups with foster mothers were received on postnatal day 3 (day of birth = day 0). Three groups of animals were studied: 1) uninjected, 2) injected sc with 100 μg TP in 0.05 ml sesame oil on day 5, and 3) injected sc with 0.05 ml sesame oil on day 5. Ovaries were obtained from the uninjected animals at 5, 7, 10, 12 and 14 days of age and from the injected animals at 7, 10, 12 and 14 days of age. The rats were killed by decapitation in the morning.

Incubation. Each 1 ml of incubation medium (pH 7.4) contained 0.91 ml Hanks TC medium (Difco), 0.09 ml of 1.3% $NaHCO_3$, 0.3 mg NADP (Sigma), 1.5 mg glucose-6-phosphate. The precursor, 50 μCi 7-^{3}H-progesterone (16 Ci/mmole, New England Nuclear) was added to this in 0.01 ml ethanol.

Ovaries were cleaned under a dissecting microscope of all nonovarian tissue and kept in Hanks TC medium at 4 C until sufficient tissue had been accumulated. Each incubation contained ovaries from no less than 5 animals. The ovaries were minced with a razor blade and placed in vials containing an aliquot of incubation medium. A second aliquot of incubation medium containing the appropriate amount of the precursor was then added to each vial. Each incubation of ovaries from 5, 7, and 10-day-old rats contained 10 mg of tissue and 1 ml of medium with 50 μCi ^{3}HP (16 Ci/mmole). Incubations of ovaries of 12 and 14-day-old rats contained 20 mg tissue and 2 ml medium with 100 μCi ^{3}HP. Vials were sealed with rubber serum stoppers and then flushed with 95% O_2: 5% CO_2 mixture through hypodermic needles. The incubation was carried out for 3 hr at 37–38 C in a Dubnoff metabolic shaking incubator, terminated by freezing on Dry Ice and stored at −20 C. Two incubations were prepared for each experimental group at each age.

Incubations were extracted by gentle horizontal shaking using 5 vol ethyl acetate saturated with water. The first volume of ethyl acetate added contained known amounts of ^{14}C-estrone, ^{14}C-estradiol and ^{14}C-progesterone tracers, and was allowed to equilibrate with the incubation media for 30 min before being removed. In Experiment I ^{14}C-labeled progesterone and testosterone were also added. The sequence of procedures used for the isolation and purification of the steroids from the extract is listed below.

1. Two-dimensional thin-layer chromatography (Brinkman, Silplate F-22) Hexane: ethyl acetate 5:2, 1st direction Dichloromethane:ethyl ether 5:3, 2nd direction Cyclohexane:ethyl acetate 1:1, 1st direction
2. Paper chromatography Cyclohexane/90% acetic acid (Bush)
3. Acetylation of E_2 and E_1 and also of testosterone in selected samples with pyridine:acetic anhydride 2:1 overnight at room temperature
4. Thin layer chromatography of the acetates Cyclohexane:ethyl acetate 6:4
5. Crystallization of the acetates from methanol/water after the addition of approximately 15 mg of carrier steroid

Three successive ^{3}H/^{14}C ratios which did not deviate more than ±5% from the mean were accepted as proof of identity and of radiochemical purity. Quantification of the steroids isolated was based on the final ^{3}H/^{14}C ratio.

Progesterone was quantified only in the extracts obtained from incubations of ovaries of untreated rats in Experiment I. Identification and quantification of testosterone was abandoned when we found that in samples from 10-day-old rats the radioactivity associated with

the testosterone acetate fell to levels below background on repeated crystallizations.

Light microscopy. Ovaries were fixed first in 3% glutaraldehyde and then in 1% osmium tetraoxide. Both fixatives were prepared with 0.1M phosphate buffer (pH 7.4). Following alcohol dehydration the tissues were embedded in Epon 812 (11). Sections approximately 0.5μ in thickness were stained with a 1:1 mixture of 1% methylene blue and 1% azure A in deionized water. Some of the sections were pretreated with 1% periodic acid.

Histochemistry. For the demonstration of 3βOHD activity, frozen sections were prepared as previously reported (12) and incubated for 60 min at 37 C in 8 ml medium containing 8 mg nitro blue tetrazolium, 16 mg EDTA, 12 mg NAD (Sigma), 4 mg dehydroepiandrosterone dissolved in 1.6 ml dimethylformamide (Sigma) and 6.4 ml Tris-HCl buffer (pH 7.3 at 25 C). After incubation the sections were fixed with 10% neutral formalin for 10 min and mounted on slides with glycerine jelly. Control incubations were made with media lacking substrate or cofactor.

Results

Morphology and histochemistry. In sections of glutaraldehyde and osmium fixed ovaries from 5-day-old animals, the inter-follicular region contained only capillaries and fibroblast-like cells. There were no identifiable interstitial cells. By postnatal day 7 (Fig. 1), islands of hypertrophied cells were seen in the interfollicular regions. These cells contained conspicuous accumulations of lipid granules and were identified as interstitial cells. In these sections from Epon-embedded tissue, the lipid granules were seen as unstained spheres due to the fact that the inner core had been extracted during tissue preparation. Removal of the osmium with periodic acid allowed intense staining of the unextracted outer rim of the granule. At successive stages in maturation, 10, 12 and 14 days of age, the islands of interstitial cells increased in size and number.

In the frozen sections a similar pattern of development was seen. Lipids, stained with oil red-O, were restricted to the interfollicular regions and their amount increased with successive stages of maturation. In sections adjacent to those stained with oil red-O and incubated for 3βHSD, the sites of enzyme activity were found to be located in the same cell groups as the lipids. The granules of tetrazolium blue, indicating 3βHSD activity, were first seen on postnatal day 4. The reaction product was sparse at this time and limited to a few areas in the interfollicular region. The amount of reaction product increased at subsequent ages in parallel to the amount of lipid.

The interstitial cells, characterized by their lipid content and 3βOHD activity, were first restricted to the central portion of the interfollicular areas. By day 12, however, they were apparent in the more peripheral areas of the interfollicular region, that is, closer to the follicle.

The effects of testosterone treatment were readily apparent in the interfollicular region on postnatal day 7 (Fig. 3). The cells remained predominantly fibroblastic, the aggregations of lipid granules were virtually absent, and the 3βHSD activity was greatly diminished. By day 10 (Fig. 4) there was also an increase in the intercellular matrix. No changes were observed in the follicles of the treated animals.

Steroid conversion. The potential to convert progesterone to E_1 and E_2 was present at all ages (Table 1, Fig. 5). There was a definite pattern of change with age in conversion of progesterone to E_2 by the ovaries characterized by an initial increase to a peak followed by a decrease after day 10. The yield of labeled E_2 increased 3-fold between days 5 and 7 and more than 4-fold between days 5 and 10 (from 0.59% and 0.21% at 5 days of age to 1.83% and 1.79% at 10 days of age). By 12 days the amount of tritiated E_2 was only twice that obtained from 5 day olds, while the yield at 14 days was about the same as that on day 5. In con-

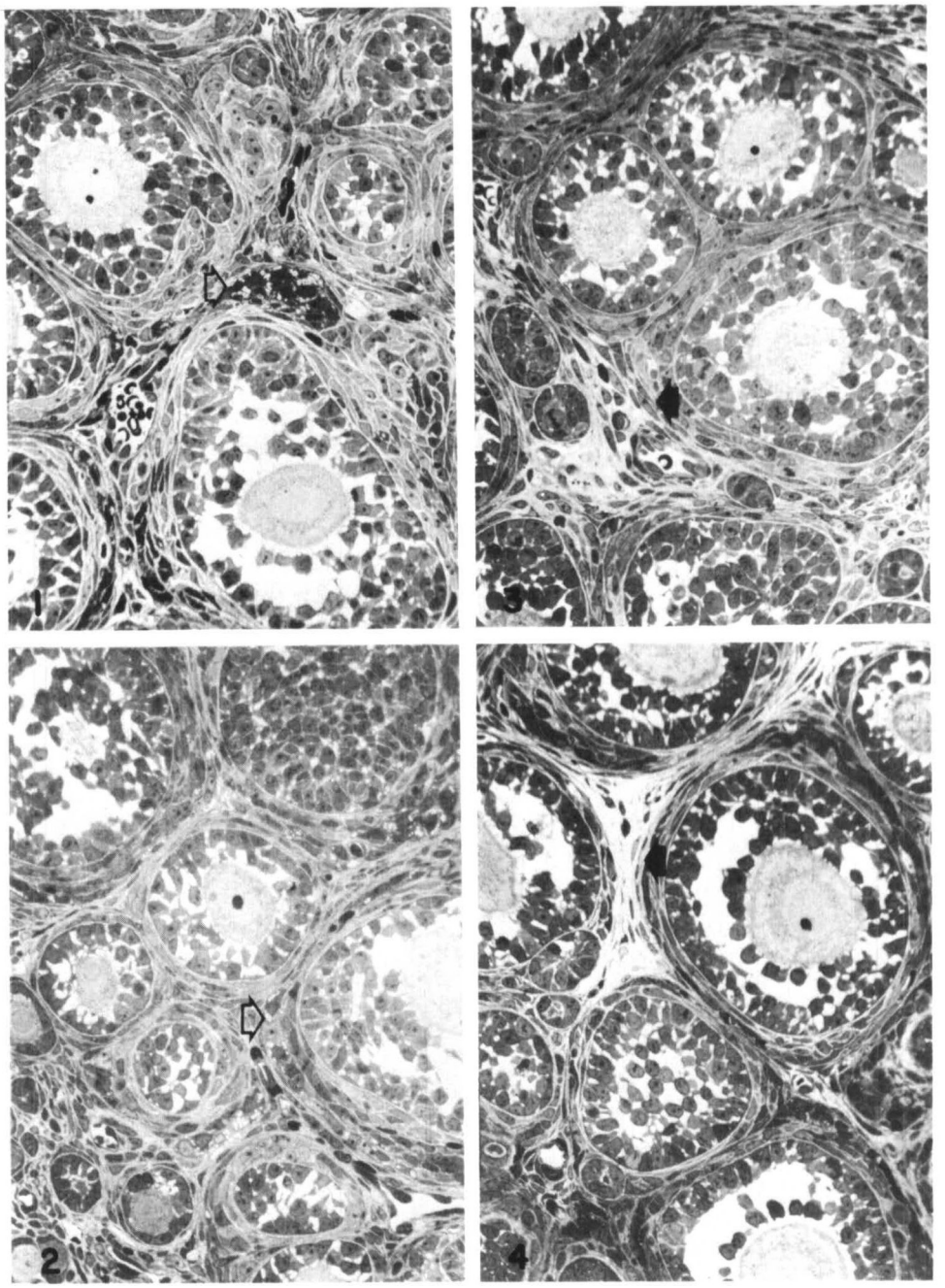

FIG. 1. Section of ovary from 7-day-old rat showing islands of interstitial cells with extracted lipid granules (open arrow). ×350 FIG. 2. Section of ovary from 10-day-old rat. Some interstitial cells are seen close to the basement lamina (thecal region) of the follicle (open arrow). ×350 FIG. 3. Ovarian section from a 7-day-old rat treated on postnatal day 5 with 100 μg testosterone propionate. Lipid is not prominant in the interfollicular tissue (closed arrow). ×350 FIG. 4. Ovarian section from 10-day-old rat treated with 100 μg testosterone propionate on postnatal day 5. Fibroblast-like cells predominate the interfollicular tissue (closed arrow). ×350

TABLE 1. Percent of 7-^{3}H-progesterone converted to estradiol (E_2) and estrone (E_1) in incubations (I, II) of minced ovarian tissue from infantile female rats[a]

Age in days	Average ovarian wt (mg)	No. ovaries/ incubation	Treatment	% Conversion E_2 I	% Conversion E_2 II	% Conversion E_1 I	% Conversion E_1 II	Ratio E_2/E_1 I	Ratio E_2/E_1 II
5	0.4	24	—	0.59	0.21	0.48	0.32	1.2	0.6
7	0.7	15	—	1.56	0.92	0.39	0.21	4.0	4.5
			Sesame[b]	3.54	2.61	0.75	0.68	4.7	3.8
			TP[c]	0.10	0.90	0.03	0.02	3.3	45.0
10	1.0	10	—	1.83	1.79	0.31	0.32	5.9	5.7
			Sesame	1.36	1.47	0.29	0.31	4.7	4.7
			TP	0.17	0.12	0.03	0.03	5.7	4.0
12	2.0	10	—	1.07	0.45	0.34	0.21	3.2	21.4
			Sesame	1.04	1.31	0.13	0.13	8.0	10.1
			TP	0.26	0.29	0.07	0.05	3.7	5.8
14	2.0	10	—	0.25	0.30	0.08	0.09	3.2	3.3
			Sesame	0.33	0.36	0.10	0.09	3.3	4.0
			TP	0.35	0.54	0.08	0.11	4.4	4.9

[a] 50 μCi 7-^{3}H-progesterone (16 Ci/mmole was used for each 10 mg of tissue.
[b] Injected with 0.05 ml sesame oil sc at 5 days of age.
[c] Injected with 100 μg testosterone propionate (TP) in 0.05 ml sesame oil sc at 5 days of age.

trast, the conversion of progesterone to E_1 varied only between 0.2 and 0.5% in incubations of ovaries from 5, 7, 10 and 12 day olds, and then decreased slightly to 0.1% at 14 days of age. Since the rise in conversion of progesterone to labeled E_2 between day 5 and 10 was not accompanied by any corresponding increase in the conversion to E_1, the ratios of E_2/E_1 changed with age rising from close to unity at day 5 to maximum of nearly 6:1 at 10 days of age (Table 1, Fig. 5). The ratios in incubations from 12 day olds were lower than at day 10 but did not decrease further at 14 days.

The amount of residual progesterone decreased from 43.2% on day 5 to 0.025% on day 14 (Table 2).

Neonatal treatment with testosterone de-

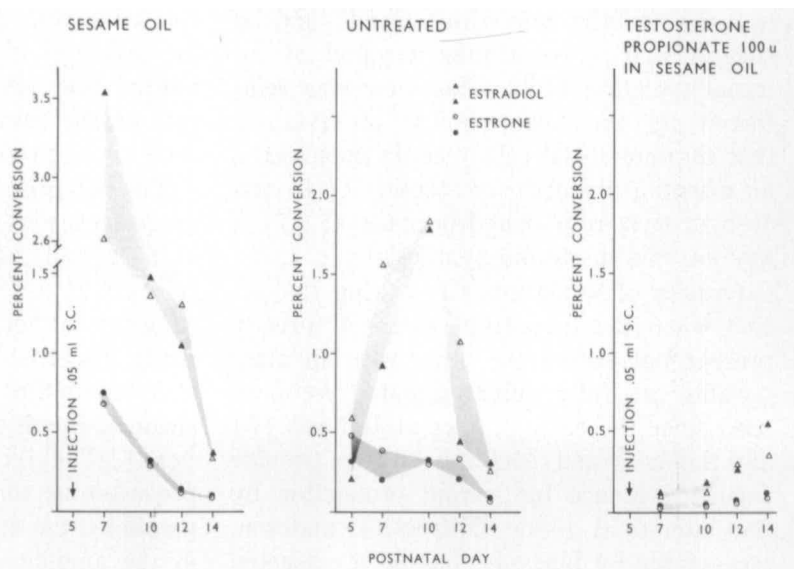

FIG. 5. Percents of 7-^{3}H-progesterone converted to estradiol and estrone in incubations (○△ and ●▲) of minced ovarian tissue from infantile female rats (incubation conditions and treatments as stated in Table 1).

TABLE 2. Percent of precursor (7-^{3}H-progesterone) remaining at the end of the incubation of minced ovarian tissue from untreated infantile female rats (Incubation I)

	Age in days				
	5	7	10	12	14
Residual Progesterone (%)	43.2	26.2	5.3	0.25	0.027

pressed the conversion of progesterone to E_2 and E_1. In incubations of ovaries from 7 and 10 day olds the yields of labeled E_2 and E_1 were about 10% of those from untreated animals. On day 12, though still depressed, the amounts approximated more closely those of untreated animals. By the 14th day, the values from the two groups were similar.

Incubations of ovaries from animals treated on day 5 with sesame oil differed from those of untreated rats at 2 ages. On day 7 the conversion of progesterone to both E_2 and E_1 was increased more than 2-fold while on day 12 the conversion to E_1 was reduced by half to two-thirds.

Discussion

The supposition that the interstitial tissue of the ovary produces steroids is supported by a great deal of indirect evidence. The high lipid content of the tissue led Bouin to name it "the interstitial gland" and to suggest that it constitutes a gland of internal secretion (13). This view was reinforced by the histochemical observations that the interstitial cells contain cholesterol, an essential precursor of steroids (1,4) and 3β-hydroxysteroid dehydrogenase (5,6,7) a key enzyme in steroid synthesis.

Ovaries of immature rats during the period when the interstitial tissue is already present but before the other ovarian components are fully differentiated, have been used since the early studies of Rennels (2) and Dawson and McCabe (3) to provide further evidence for steroid production by the interstitial tissue. Estrogenic material, measurable by bioassay, has been extracted from ovaries of immature rats. The maximum yield of this material was found around 14 days of age (14). Secretion of estrogens by the ovaries of animals of this age is suggested by the uterine growth and associated histological changes that occur during the second week of life and the demonstration that these changes depend on the presence of the ovaries (15). The finding of the present study that the ovaries of infantile rats contain the biosynthetic machinery necessary to convert a C_{21} precursor to E_2 and E_1 provides further evidence, though still only circumstantial, that the ovary of the immature rat and therefore, by inference, interstitial tissue has a steroidogenic function.

The yield of labeled estrogens produced from the ^{3}HP *in vitro* varied with age. The variations were much greater for E_2, the more potent estrogen (16), than for E_1 and appeared to follow a definite pattern probably reflecting a systematic sequence of changes in the biosynthetic machinery of the ovary.

The rise in labeled E_2 production *in vitro* correlated well with the morphological and histochemical observations on the development of the interstitial tissue. Additional correlations became evident when these data on ovarian structure and function were compared with data from this laboratory on the levels of circulating gonadotrophins in normal and neonatally testosterone-treated rats of the same strain and ages as those used in the present study (9).

The potential to synthesize estrogens from progesterone was already present in ovaries of 5-day-old rats. At this time, interstitial cells could not be recognized by light microscopy, although 3βOHD activity was already discernable histochemically. We have also found that circulating FSH levels are elevated by this age (unpublished observations). The increase in the conversion of progesterone to E_2 between days 5 and 10 paralleled the appearance and rapid increase in the amount of interstitial tissue, associ-

ated lipid and 3βOHD activity. It is during this time that the circulating FSH (unpublished observations) and LH (9) are also rising.

In contrast, there were no morphological or histochemical findings that could be correlated with the precipitous decrease in the amount of labeled E_2 produced in the ovarian incubations after 10 days of age. There was no obvious decrease in the absolute amount of interstitial tissue, and the decrease in the relative mass of this tissue due to the progressive increase in follicular size would not seem to be of sufficient magnitude to explain the findings. The gonadotropin levels, though tending to fall after 12 days of age, are still relatively high during this period reaching adult nonproestrus levels only after 21 days of age (9, and unpublished observations). These high levels of gonadotropins did not, however, cause a depletion of interstitial cell lipids as is seen in the adult proestrus female rat (1,4) after intense gonadotropic stimulation.

When the data from this study are compared with those from a previous one in which ovaries of older rats were incubated (10), it can be seen that the yield of labeled estrogens continues to fall after day 14. The lowest values were found at 21 and 28 days of age. The data from these two studies are directly comparable as the conditions of the incubation were identical.

An explanation of the lack of morphological correlation with the decrease in the yield of E_1 and E_2 is suggested by the observation of increased progesterone metabolism during this period. Based on the decrease in the amount of residual ^{3}HP, it would appear that subsequent to day 10 there is a shift in the steroid metabolism of the ovary.

The limitations of data from *in vitro* studies such as these are well recognized. At best they can only demonstrate the biosynthetic potential of the tissue. In addition, when the quantitative aspects of this study are considered, a number of factors need to be taken into account. First, we only measured the conversion of added labeled precursor to estrogens, not that produced from endogenous precursors. Therefore, the extent to which the results were effected by variations in the endogenous steroid pools cannot be estimated. Second, the tissue: substrate ratios were based on ovarian weight. Therefore, the results were most likely influenced by changes in the relative amount of steroidogenic tissue. Third, the choice of a more immediate metabolic precursor, *e.g.*, labeled testosterone might yield a greater amount of estrogen. However, the present experiment was designed as a continuation of a previous investigation (10) and for this reason ^{3}HP was used.

Nevertheless, the data from the incubations in combination with the morphological and histochemical observations and the previously cited evidence for the production (14) and secretion (15) of estrogenic material by ovaries of rats of this age strongly suggest that increasing amounts of E_2 are secreted in the rat during the second week of life. What the actual level of secretion might be and whether it declines after 10 days of age remains to be determined. An attempt was made in another study to obtain more direct information on estrogen secretion by the ovaries of rats of these ages by measuring, by radioimmunoassay, circulating E_2 and E_1 levels *in vivo* (17). These efforts were frustrated because it was found that the substantial amounts of E_1 in the blood of rats of this age were attributable to the presence of the adrenals. In addition, the high values obtained for E_2 were found to be due mainly to materials of adrenal origin, other than E_2, which cross-reacted with the antibody.

The observed patterns of change in the different parameters of ovarian development and in circulating gonadotropins must be the result of a complex sequence of interactions between the various components of the hypothalamico-pituitary-ovarian circuit. At the present time, it is not possible to establish cause and effect relationships be-

tween the various phenomena. The exact role of FSH and LH in initiating the differentiation of interstitial tissue is not known. The development of this tissue, however, appears to be linked to gonadotropins. This is suggested by our data on testosterone-treated rats and by a study in which anti-gonadotropins were used (18). Neonatal treatment with testosterone, which we have previously found causes an immediate suppression of circulating gonadotropins (9, and unpublished observations) impaired the development of the interstitial tissue and diminished the conversion of progesterone to estrogens by the ovary. The appearance of the interstitial tissue in rats treated with anti-LH and anti-FSH from 5 days of age was quite similar to that of our rats treated with testosterone propionate (18).

The prompt and substantial increase in the conversions to E_2 and E_1 following an injection of sesame oil alone was a consistent finding. At this time we have no explanation for how this effect might be mediated. The finding, however, emphasizes the labile state of the developing hypothalamico-pituitary-ovarian circuit.

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