HISTOCHEMICAL CHANGES IN THE OVARIES OF NORMAL AND EXPERIMENTALLY TREATED RATS

By

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ABSTRACT

Results from histochemical staining of ovaries of normal and experimentally treated rats using a method allegedly specific for 3β-ol steroids, together with blood progestin, progesterone (pregn-4-ene-3,20-dione) and urine pregnanediol (5β-pregnane-3α-20α-diol) determinations suggested that:

1. Thecal and interstitial tissue as well as luteal tissue of adult rat ovaries all produced progesterone and consequently the production of progesterone was not limited to a specific anatomic unit (e.g. corpus luteum).
2. The progesterone thus produced was secreted in part into the bloodstream, for with each significant increase in the intensity of the histochemical reaction there was an associated rise in blood progestin, progesterone and urine pregnanediol. This did not quantitate the amount of progesterone involved in intermediary metabolism (e.g. of allopregnane-3β-ol-20-one, pregn-4-ene-3β-ol-20-one).
3. The ovarian tissue of immature rats also produced progesterone.

In recent years the identification of the cells which elaborate the steroids of the ovary has been the object of certain histochemical investigations. Advance in this field, however, has been slow because there have been no techniques available for demonstration of specific steroids (e.g. oestrogens and progesterone). Therefore, because of the lack of more specific techniques and because of the complexity of cell types and cyclic changes in the ovary, conclusions as to the site of production of any single steroid have been limited. More precise information was needed so that the behaviour of the ovary in relation to the rest of the endocrine system and target organs could be better understood and studied.

Among the first and simplest studies in this field were those of Corner (1932),
who demonstrated sudanophilic lipids in the corpus luteum, theca interna, and interstitial tissue of the ovary. Later Dempsey & Bassett (1943) first applied a battery of indirect tests for presumptive steroids in rat ovaries. Taken together, these tests were considered to give reliable evidence of the presence of a more specific class of lipids, the steroids. This same approach, with additions and improvements in methods, was applied to human ovaries by McKay & Robinson (1947), White et al. (1952), to sow ovaries by Barker (1951), to monkey ovaries by Rossman (1942), and to rat ovarian tissue by Deane (1952). Taking into account species differences, particularly in regard to interstitial tissue Harrison (1948), most of the workers in this field agreed that the »presumptive steroids« were limited to the corpora lutea, theca interna, interstitial tissue and the cells of the degenerating corpora lutea and Graafian follicles. The trend of opinion was that the theca interna and corpus luteum were limited to production of oestrogen and progesterone respectively, though no direct evidence was at hand.

A significant contribution to the methods available was that of Wattenberg (1958). The reduction of neotetrazolium salts into purple formazans subsequent to the dehydrogenation of \(3\beta\)-ol steroids, such as occurs in the formation of progesterone from pregnenolone \((3\beta\)-hydroxy-pregn-5-en-20-one\), was developed as a histochemical technique. With proper parallel studies it was thought that this technique might allow demonstration of the site of production of a specific steroid (progesterone).

This paper describes the application of this allegedly specific technique for \(3\beta\)-ol steroid dehydrogenation in a study of the ovarian tissue of immature, cyclic adult, pregnant as well as untreated and chorionic gonadotrophin (HCG) treated hypophysectomized rats. Alkaline phosphatase, haematoxylin and eosin sections together with urine pregnanediol and blood progesterin and progesterone studies were carried out on control and experimentally treated material for comparison.

**METHODS**

**General Methods**

Immature, mature and pregnant white Chester Beatty female rats were used. They were kept in groups of four to six in wire mesh cages and were supplied with water and 14
\%\ dried skimmed milk rat cake. On all but the immature animals vaginal smears were obtained each morning for one to two weeks before each study. All the normal animals used exhibited four day oestrous cycles.

Preceding each tissue examination, twenty four hour urine samples were collected from each experimental group for pregnanediol determinations (Eberlein & Bongiovanni 1958), by means of cages with glass funnels attached. Each sample was stored with 0.5 ml of toluene added, at 4°C. The chromatographic technique was modified only in that the rapid chromatographic step described was omitted.

Each animal was then anaesthetized with ether (between 8:00 and 10:00 a.m.) and
2 ml of blood was drawn by cardiac puncture. This was placed in 0.1 ml of 0.5 \% sodium citrate and centrifuged. The plasma was immediately lyophilized prior to progestosterone determination (Woolover, in press), and progestin bioassay (Hooker & Forbes 1947, 1949; Forbes & Hooker 1957). The progestin bioassay technique differed only in that A strain Texas Inbred mice were used which (in our hands) were sensitive to 0.50 units of progestin. Only progestin in non-hydrolyzed plasma was assayed.

Following intracardiac puncture the ovarian tissue was removed and quickly divided. One half of each ovary was placed immediately in powdered dry ice for steroid 3\(^\beta\)-ol dehydrogenase (3\(^\beta\)-ol DH ase) (Wattenberg 1958), and the other half was placed in 80 \% alcohol for alkaline phosphatase determination (Gomori 1943) and for routine histologic studies. Modification of the 3\(^\beta\)-ol DH ase technique included cutting of all sections at ten microns using a freezing microtome. The intensity of the 3\(^\beta\)-ol DH ase reactions were graded in the following manner: pink, 1 plus; red, 2 plus; purple-red, 3 plus; purple, 4 plus; black, 5 plus. The alkaline phosphatase reaction intensity was graded: brown, 1 plus; black, 2 plus.

The standard incubation periods for the 3\(^\beta\)-ol DH ase and alkaline phosphatase methods were thirty and sixty minutes respectively. The controls were such that those groups which were compared were processed and examined together. Blanks consisting of diphosphopyridinenucleotide (DPN) and neotetrazolium minus pregnenolone substrate were run with each group.

The chorionic gonadotrophin (Gonan, B.D.H.) was standardized at 320 IU per cubic centimeter using the ovarian hyperaemia method of Albert & Berkson (1951).

Application of Methods in Treatment of Animals

Eighteen immature rats were divided into two groups. The first control group consisted of six animals six weeks old, and a second experimental group consisted of twelve animals of the same age. Following unilateral ovariectomy and examination, six of the experimental animals received 320 IU of HCG daily for two days and six received the same amount of HCG daily for seven days. There was a third group of immature animals consisting of three two-week and three four-week-old animals which was also examined.

Sixteen normal 200–250 g female rats were divided into four groups of four. After collection of urine and blood samples, the ovaries of groups of four at each of the four phases of the oestrous cycle were examined. Four additional animals were examined in late oestrus.

Eight pregnant rats, four each at fourteen and eighteen days' gestation were examined.

Eight normal 200–250 g female rats were hypophysectomized using the parapharyngeal approach of Thompson (1932). These animals were allowed to stabilize over three months. The adequacy of the operation was confirmed by a weight loss of not less than 30 g, vaginal smear evidence of atrophic epithelium and negative gross sella examination upon conclusion of the experiment. After three months a unilateral ovariectomy was performed and the tissues were examined. Following this, each animal was given 320 IU of HCG daily for ten days. During treatment urine collections were made and the remaining ovary was examined and compared with its previously removed opposite member. Four additional hypophysectomized animals were subjected to unilateral ovariectomy without subsequent hormone treatment. There were no compensatory changes noted in the remaining ovary.

Four additional hypophysectomized animals were adrenalectomized at the time of unilateral ovariectomy prior to treatment with HCG.
RESULTS

Immature Untreated Animals

The 3β-ol DHase reaction in the two- and four-week-old animals (Fig. 1) was confined to the thecal and interstitial tissue. Its intensity was 4 to 5 plus, while in the six-week-old animals the reaction was only 1 plus (Fig. 2). There was no reaction in the stroma or granulosa. The alkaline phosphatase reaction (not illustrated) was confined to those capillaries supplying the thecal and interstitial tissue, and was 2 plus in all immature groups.

Immature Treated Animals

The first group, treated with HCG for two days, was noted to have at least twice the amount of thecal tissue as the control group. The reaction intensity of this first group was 5 plus as compared to the 1 plus reaction of the control group.

The second group, treated for seven days, was characterized by the development of numerous luteinized follicles in which there was a 3 plus 3β-ol DHase reaction (Fig. 3). The alkaline phosphatase reaction was 2 plus throughout and was confined to the areas positive for the 3β-ol DHase reaction.

Normal Adult Animals

Thecal and interstitial cell reactions fluctuated between a 2 and 3 plus intensity for 3β-ol DHase throughout all phases of the cycle (Fig. 4). No trend in respect to the phases of the cycle could be appreciated in the number of animals used. The reaction intensity of the corpora lutea, however, was noted to drop abruptly from 3 plus to 1 plus upon completion of the vascular stage of development (Fig. 5). The alkaline phosphatase reactions were consistently 2 plus in thecal and interstitial tissues but only 1 plus in the corpora lutea except for a 2 plus reaction during the vascular stage of its development.

Second generation corpora lutea and the theca and granulosa of regressing Graaffian follicles gave a consistent 1 to 2 plus 3β-ol DHase reaction.

Plate 1.

Histochemical demonstration of 3β-ol DHase activity in the ovaries of normal immature, HCG treated immature and normal adult rats.
All figures are magnified ten times.

Fig. 1. Ovary of a two week old rat. Observe the activity of the thecal and interstitial tissue. Compare with Figs. 2 and 3.

Fig. 2. Ovary of a six week old rat. Observe the negligible activity throughout.

Fig. 3. Ovary of a six week old rat following seven days treatment with 320 IU of HCG daily. Compare with Fig. 2.

Fig. 4. Ovary of a mature rat at oestrus. Observe the enlarged follicles and attenuated, but reactive thecal and interstitial tissues.

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Pregnant Animals

The 3β-ol DHase reaction in the enlarged corpora lutea at both the fourteenth and eighteenth day of gestation was 4 plus (Fig. 6). However, in the corpora lutea of the animals pregnant eighteen days there was pooling and irregular distribution of the reactive material. The alkaline phosphatase reaction was 2 plus.

Untreated Hypophysectomized Animals

The 3β-ol DHase and alkaline phosphatase reactions were negative throughout all sections of ovarian tissue, which in every case consisted largely of connective tissue and partially collapsed non-reactive follicular and luteal elements (Fig. 7).

Treated Hypophysectomized Animals

In all cases an expanded interstitial tissue showed a 3 plus 3β-ol DHase reaction whereas that of the corpora lutea was 1 to 2 plus (Fig. 8). The alkaline phosphatase reaction was 2 and 1 plus respectively in the interstitial and luteal tissues.

Pregnanediol, Progestin and Progesterone Determinations

The values for pregnanediol, progestin and progesterone are presented in Table 1. In all experiments it will be evident that the blood and urine levels of these substances are significantly elevated in those animals either pregnant or treated with HCG as compared with those which were non-pregnant or untreated. The ranges quoted in the table for the various observations indicate the grounds on which we judged the significance of the results. More detailed statistical evaluation yielded little additional insight in an experiment in which the difference between comparative sets of data is so large.

Plate II.

Histochemical demonstration of 3β-ol DHase activity in ovaries of normal adult, pregnant, untreated and HCG treated hypophysectomized rats.

All figures are magnified forty times.

Fig. 5. Corpus luteum of a mature rat at the conclusion of the stage of vascularization.

Fig. 6. Corpus luteum of a rat pregnant fourteen days.

Fig. 7. A portion of the ovary of a rat hypophysectomized three months previously. Compare with Fig. 8.

Fig. 8. Opposite ovary of that shown in Fig. 7, following seven days treatment with 320 IU HCG daily. Compare the activity of the interstitial tissue with that of the corpus luteum.
Table 1.
Experimental results on untreated immature, mature, hypophysectomized mature and pregnant female rats, and on HCG treated immature and hypophysectomized mature female rats.

<table>
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<th>Experimental group</th>
<th>No. of animals</th>
<th>Reactive tissue</th>
<th>3β-ol DHase reaction</th>
<th>Alkaline phosphatase reaction</th>
<th>Urine pregnanediol μg/24 h*</th>
<th>Plasma progestin P. E. units/ml</th>
<th>Plasma progesterone μg/ml</th>
<th>Ovarian weights mg/100 g body weight</th>
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<td>1+</td>
<td>2.0**</td>
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<td>,</td>
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<tr>
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<td>5.1</td>
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<td>0.2–0.8</td>
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<td>7.4</td>
<td>3.5</td>
<td>6.2</td>
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* Pooled samples.
** Range of values.

I. T. = Interstitial tissue.
C. L. = Corpus luteum.
DISCUSSION

Techniques

1. Histochemical

The steroid 3\(\beta\)-ol DH ase reaction consists of a series of reduction reactions by the hydrogen ion released from the 3 position of the steroid nucleus (e.g. pregnenolone to progesterone, epiandrosterone to androst-4-ene-3,17-dione). This free hydrogen ion using DPN as an intermediate hydrogen ion acceptor, reduces neotetrazolium to form the purple formazan seen in the histochemical reaction (Wattenberg 1958).

It seemed possible, however, for false positive reactions to occur; for in addition to steroid 3\(\beta\)-ol DH ase and other DPN linked dehydrogenases (lactic and malic), there are other hydrogen ion sources such as the TPN linked enzymes (Farber et al. 1958) and the protein sulfhydryl groups (Zimmerman & Pearse 1959; Seligman et al. 1959). Controls consisting of neotetrazolium or neotetrazolium plus DPN did not give positive reaction. It appeared that hydrogen ion from these sources was ordinarily insufficient in quantity to produce a colour reaction. If, however, sufficient substrate susceptible to the action of a DPN linked dehydrogenase was added to the incubation media, sufficient hydrogen ion was produced to give a positive reaction. Thus, lactic acid substrate, when added to the incubate of a tissue enzyme source, plus DPN, and neotetrazolium, gave a positive reaction in every tissue examined except red blood cells (Seligman et al. 1959). The addition of a 3\(\beta\)-ol steroid substrate (pregnenolone, epiandrosterone) to such an incubate gave a position reaction only when steroid-producing tissue (e.g. testis, ovary, adrenal and placenta) was used.

The possibility of diffusion artifacts was ruled out by showing the failure of enzyme transference from ovarian tissue when incubated in contact with kidney tissue which had been shown not to contain 3\(\beta\)-ol DH ase. Thus, it seemed that it was truly a dehydrogenation of 3\(\beta\)-ol steroids that was being observed; and that there was no evidence of diffusion artifact.

2. Biochemical

Then there arose the question of which 3\(\beta\)-ol steroid was being metabolized in the ovary, pregnenolone or epiandrosterone. Because of the fact that androgens have yet to be isolated from rat ovarian tissue, it could be assumed that pregnenolone was the major 3\(\beta\)-ol steroid being metabolized in the ovary. On the other hand, the possibility remained that the progesterone produced was not an end product but rather an intermediate substance involved in the formation of other steroids such as allopregnane-3\(\beta\)-ol-20-one, pregn-4-ene-3\(\beta\)-ol-20-one (Dorfman & Ungar 1953), or even oestrogens (Ryan 1958, 1959). Therefore, blood progesterone, progestin, and urinary pregnanediol determinations were made along with the histochemical studies.
In an attempt to obviate the possibility that the urine pregnanediol was derived largely from progesterone of adrenal origin (Balfour & Comline 1957), or deoxycorticosterone (Dorfman & Ungar 1953) adrenalectomy was performed on a number of the hypophysectomized animals prior to HCG administration. Atherden & Grant (1957), using the extraction method of Klopper et al. (1955), were unable to extract more than 6 micrograms of »apparent« pregnanediol from a pooled urine (about 80 ml of four rats following injections of progesterone. These low values were explained in part by the finding of Riegel et al. (1950) of 50 % of the administered radioactive progesterone-21-14C in the unsaponifiable fat fraction of the faeces. However, in this study, using the method of Eberlein, and about 150 ml of urine in each determination, obtained from fasting rats which were either pregnant or treated with HCG, a definite rise in urine pregnanediol was observed. These rises were accompanied by rises in blood progesterone and progestin (progesterone, 20α-hydroxy-pregn-4-en-3-one and 20β-hydroxy-pregn-4-en-3-one).

These direct biochemical and bioassay determinations have been used in conjunction with the 3β-ol DH ase histochemical technique to show the relationship between the 3β-ol DH ase histochemical changes in the ovary and changes of the levels of biologically active, biochemically measured progesterone and related substances in blood and urine.

SIGNIFICANCE OF RESULTS

Immature Animals

The sites of presumptive progesterone production were examined in immature animals because it is necessary that information about ovarian histochemical-biochemical as well as ovarian anatomic maturation be gained. Understanding of the inter-relationships between theca, interstitial tissue and corpora lutea in mature animals is dependent on what is learned in studies of the immature forms. Our studies suggest that the functional thecal tissue of immature rats gave rise to functional interstitial tissue, and that under HCG stimulation this same thecal tissue gave rise to functional luteinized follicles (Dawson & McCabe 1951). Both primary and secondary interstitial tissue (derived from granulosal and thecal tissue respectively) as well as the thecal tissue itself were active histochemically for 3β-ol DH ase.

That this histochemical reaction intensity began to fall after the sixth week of life suggested that there might have been a source of luteinizing hormone other than that which have been gained from the mother in utero.

According to Dawson & McCabe (1951), and Rennels (1951), primary interstitial tissue has completed its formation from granulosa by the eighteenth day; and secondary interstitial tissue has completed its formation from the theca of atretic follicles by the twenty-fifth day. Rennels (1951) considered the
possibility that primary interstitial tissue produced oestrogen. In the light of our observations, it may produce progesterone. Possibly it produces oestrogen as well.

After two days' administration of HCG there was an increase in $3\beta$-ol DH ase reaction intensity along with increases in pregnanediol, progesterone and progestin levels. Similar increases in $3\beta$-ol DH ase were reported by Samuels & Helmreich (1956) in tissue homogenate studies. However, after seven days' administration of HCG, there was a moderate fall of the above-mentioned levels from the two day high. This raises the question of possible exhaustion of available substrate.

**Cyclic Adult Animals**

In the cyclic rat the area of greatest $3\beta$-ol DH ase activity was the interstitial and thecal tissues. The mature corpus luteum appeared, by comparison, less active. Only during the developmental stage of vascularization did the intensity of the reaction approach that of the thecal and interstitial tissue. This period of increased activity in the corpus luteum may coincide with the increase in blood progesterone which Hisaw (1947) suggested occurred around the period of ovulation. It may also coincide with Claesson & Hillarp's (1947) and Deane's (1952) observation of simultaneous lipid depletion of thecal, interstitial tissue and older corpora lutea during and just following ovulation. This was considered to suggest mobilization of steroid from cells to blood stream; perhaps, in part, "ovulatory" progesterone. Deane also noted an intense alkaline phosphatase reaction in atretic follicles during the time of ovulation. This increased $3\beta$-ol DH ase activity of the corpus luteum does coincide with Deane's observation of increased basophilia of the granulosa-luteal cells of the vascular stage, a phenomenon indicative of increased protein synthesis and cell growth.

Whether and how these phenomena are related is unanswered; but, in conjunction with the findings of $3\beta$-ol DH ase activity in thecal, interstitial and luteal tissues, it is possible that all these structures elaborate progesterone at all phases of the cycle, and consequently that they all may contribute to the rise in progesterone during ovulation. Such a suggestion that all steroid-bearing tissues of the ovary may produce progesterone is supported in part by Hooker & Forbes' (1947) extraction of progestin from the Graaffian follicles of mice, and by Corner & Allen's (1929) isolation of progesterone from the corpora lutea of larger animals. No $3\beta$-ol DH ase reaction was observed in the atretic follicles present at ovulation. The increased alkaline phosphatase reaction and lipid accumulation in these structures still remains to be explained.

In regard to sites of oestrogen metabolism, the observations and suggestions of Aldman et al. (1949), Rennels (1951), Deane (1952) and others taken together suggest that a similar situation involving multiple sites of production may well apply to oestrogen as it seems to apply to progesterone.
Pregnant Animals

The corpus luteum of the pregnant rat is active through the sixteenth day of gestation. Both the progestin, progesterone and pregnanediol levels of the pregnant rat as well as the intensity of the 3β-ol DHase reaction were increased. There was, however, little evidence of lipid pooling up to day sixteen. 3β-ol DHase activity began to decline after day sixteen. The marked intensity of the reaction remained, but there was pooling and irregular distribution of lipid material. Claesson & Hillarp (1948) noted a similar finding on the sixteenth and seventeenth day of pregnancy. Forbes & Hooker (1957) noted that blood progestin levels in pregnant mice dropped during the last third of the gestation period; our observations for the rat parallels his in this respect.

Hypophysectomized Animals

The apparent 3β-ol DHase inactivity of the corpora lutea relative to the activity of the interstitial tissue found in the normal animal was again noticed in the response of the HCG treated hypophysectomized animal. The interstitial tissue responded with a twofold increase in volume, with an increase in reaction intensity from 0 to 3 plus, and with approximately an eightfold rise in urinary pregnanediol, blood progestin and progesterone levels. All this occurred while the corpora lutea remained relatively inactive (Evans et al. 1941). Upon adrenalectomy and subsequent HCG treatment of these hypophysectomized animals the pregnanediol progestin and progesterone levels rose only sixfold above the untreated state. The range of variation and small number of animals allows only the conclusion that adrenalectomy did not change the results significantly.

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