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Histochemical Semi-Quantitative Analyses of the Ovary of the Albino Rat During the Estrous Cycle, Pregnancy and Two Types of Pseudopregnancy

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HISTOCHEMICAL SEMI-QUANTITATIVE ANALYSES
OF THE OVARY OF THE ALBINO RAT
DURING THE ESTROUS CYCLE, PREGNANCY
AND TWO TYPES OF PSEUDOPREGNANCY

by

Frances A. Kovarik

A Dissertation Submitted to the Faculty
of the Graduate School
of Loyola University of Chicago
in Partial Fulfillment of the Requirements
for the Degree
of
Doctor of Philosophy

1972

LIFE

Frances Ann Kovarik was born in Chicago, Illinois on July 25, 1939.

She graduated from St. Mary's High School in Chicago in June, 1957 and obtained the degree of Bachelor of Science from Loyola University of Chicago in June, 1961.

In September, 1961, she began her graduate studies in the Department of Anatomy, Loyola University of Chicago, Stritch School of Medicine and was awarded the degree of Master of Science in June, 1964. From 1965 to 1969, she continued her scientific studies under the direction of Professor H. Wang. More recently she developed the doctoral dissertation and performed her research under the supervision of Professor J. T. Velardo, Chairman, Department of Anatomy.

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She recently participated in the IIIrd Pan American Congress of Anatomists' Symposium on the Biology of Reproduction held in New Orleans, Louisiana, March 28-April 2, 1972, where she presented the first part of her dissertation "Localization of ovarian dehydroepiandrosterone- and pregnenolone-3 β -hydroxysteroid dehydrogenases in the rat during the estrous cycle" from the platform.

Abstract

This study involves detailed cyto- and histochemical observations and evaluations regarding the morphological components of the ovary of the albino rat which are involved in potential progestational and estrogenic hormonal biosynthesis during four physiological conditions: 1) the normal estrous cycle which was subdivided into six distinctive stages, 2) normal (ordinary-induced) pseudopregnancy, 3) prolonged pseudopregnancy (pseudopregnancy with decidual tissue), and 4) pregnancy. 3β -Hydroxysteroid:NAD oxidoreductase (3β -HSD) activity was studied using pregnenolone (P-one) and dehydroepiandrosterone (DHA) substrates in order to localize the sites of potential progestational and estrogenic biosynthesis, respectively. Ovarian NADH_2 diaphorase (lipoamide oxidoreductase) activity was also assessed. Enzymatic activity was rated on a 0 to +4 scale for each substrate.

It was possible to observe and assess both the sites of and alterations in P-one- 3β -HSD and DHA- 3β -HSD activities in the different components of the ovary, *i. e.* granulosa cells and the theca interna of secondary-, tertiary- and atretic follicles, interstitial tissue and corpora lutea in each of the physiological conditions studied. Likewise, it has been possible to observe characteristic substrate reactivity in particular ovarian components which occurred during each of the physiological conditions studied.

Ova, primary follicles and the ovarian hilus appear to be sites of negligible P-one- and DHA- 3β -HSD activities.

Secondary follicles appear to differ in degrees of and localizations for each of the substrates involved in potential estrogenic and progestational biosynthesis.

The granulosa cells of secondary-, tertiary- and atretic follicles change in their potential for steroidal production but they appear to be minor sites of potential progestational and estrogenic production.

The theca interna of tertiary and atretic follicles show a marked reactivity for P-one and DHA substrates.

The interstitial tissue consistently manifests a high potential for localization for both P-one- 3β -HSD and DHA- 3β -HSD activities.

Cells of corpora lutea were found to be capable of synthesizing both progesterone and estrogens with marked differences in times of maxima and minima, this being due to the physiological requirements of the conditions. Luteal steroidal enzymatic activity is greatest during pregnancy > prolonged pseudopregnancy > normal pseudopregnancy and lowest during the estrous cycle.

From the studies of animals having superimposed physiological requirements, it was observed that additional steroidal biosynthesis appears to be achieved by heightened enzymatic activity in all of the ovarian components, with the exception of ova, primary follicles and the ovarian hilus.

The results contained in this dissertation provide cyto- and histochemical information which can be used as a foundation or a guide by which biochemical determinations and physiological conditions can be directly related to the morphological functions of the ovary, thus re-iterating the basic form-function relationships occurring in biomorphological reactions.

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General Introduction

The ovary has been and continues to be extensively studied because of its cardinal role in the formation of all new, possible life via the storage and release of female gametes (ova), and the biosynthesis and secretion of ovarian sex steroid hormones.

A fundamental concept in the biological sciences is the form-function relationships which demonstrate the sites from which the physiological and biochemical manifestations originate. This is especially true of ovarian anatomo-physiologic experimentation. Morphological studies in the early 1900's have roundly indicated the suspected roles of the ovary in steroid production as manifested by the physiological states produced in the female (Loeb, 1907 and 1908; Allen and Doisy, 1923; Corner, 1928; Allen and Corner, 1929; Corner and Allen, 1929). More recently, studies of different dimensions concerned with the sites of ovarian biosynthesis during particular stages of the estrous cycle have been initiated (Pupkin et al., 1966; Motta and Bourneva, 1970a and b) and extended in part to certain ovarian components, i. e., corpora lutea, interstitial tissue and theca interna cells (Levy et al., 1959; Deane et al., 1962; Pupkin et al., 1966; Motta and Bourneva, 1970a and b). No one to date, however, has intensively demonstrated and critically documented the sites of progestational and estrogenic production during each of the six distinctive stages of the estrous cycle and in the different cyto- and histomorphological components of the ovary, namely, the female sex cells, the primary-, secondary- and tertiary follicles, atretic follicles, interstitial tissue, corpora lutea, and the ovarian hilus. This cyto-histochemical study, therefore, was performed in order to provide detailed information regarding the ovarian loci of estrogenic and progestational hormonal production during the six distinct and separable stages of the estrous cycle.

Ovarian morphology has been shown to be influenced by 1) alterations in blood titers of adenohipophyseal hormones, 2) the ovarian histophysiological sequences associated with and including the process of ovulation, and 3) the formation, maintenance and regression of corpora lutea. The following ovarian components, i. e., the follicles, corpora lutea and interstitial tissue, function to synthesize sex steroids. The ovarian sex hormones in turn influence the functions of the uterus, oviducts, vagina, the higher centers in the cerebral cortex, hypothalamus, adenohipophysis, and possibly the pineal gland. These changing functional interrelationships between and among numerous structures, including the cerebral cortex, pineal, hypothalamus, adenohipophysis, ovary, adrenal gland and uterus, result in the reproductive function of the female, i. e., the onset of puberty, the normal estrous or menstrual cycles, pseudopregnancy (pseudocycesis), pregnancy, aging, and senescence.

Four physiological conditions: 1) the normal estrous cycle, 2) ordinary-induced (i. e., that of normal length) pseudopregnancy, 3) pseudopregnancy with decidual tissue (prolonged pseudopregnancy), and 4) pregnancy, which can occur in the female albino rat at one time or another, were studied to obtain more critical lines of information regarding the role(s) of the different cellular components of the functional ovary. Each of these physiological conditions is presented in a separate chapter with its corresponding sections of the introduction of the problem and the pertinent background literature, experimental procedures, observations and results, discussion, and summary and conclusions. Thus, the organization of the dissertation includes, in addition to the separate aforementioned chapters, a general introduction, a general discussion, summary and conclusions, literature cited, appendices, and an abstract.

Since this dissertation involves detailed cyto- and histochemical

observations and evaluations regarding the morphological components of the ovary of the albino rat, it is advantageous to begin with a presentation of the basic methodology utilized in this study.

Cyto- and histochemical studies utilize techniques which attempt to present another aspect of the internal working of cells and tissues specifically with regard to the occurrence and location of certain substances in cellular and tissue preparations. Claesson (1947) in a number of sharp perspectives was forthright to delineate the proper scope of histochemistry, and properly elucidated a multidimensional view, with care and attention given to quality, topography and quantification. The importance of some of the histochemical techniques which are utilized in ovarian steroidogenesis has been reviewed and discussed by Velardo (1958), Jacoby (1962), Velardo and Rosa (1963), and Baillie *et al.* (1966). The histochemical methods for the localizations of steroids include: 1) staining the tissue sections with Sudan dyes in order to localize the sites of neutral lipids which might be steroidal presursors, 2) utilizing polarized light to demonstrate the sites of steroids due to the symmetry of the steroidal molecule, 3) employing the Schultz technique which localizes the sites of cholesterol and its esters, and 4) analyzing for cellular and tissue localization of 3β -hydroxysteroid:NAD oxidoreductase activity.

The enzyme 3β -hydroxysteroid:NAD oxidoreductase (3β -hydroxysteroid dehydrogenase, 3β -HSD, classified numerically as 1:1:1:51) catalyzes the oxidation of the hydroxyl group on the third carbon position in the A ring of steroids to a ketone. Using biochemical incubation techniques, Samuels *et al.* (1951) were the first to observe α , β unsaturated ketone structures as well as the enzyme which oxidizes this reaction in the presence of the co-enzyme, nicotinamide adenine dinucleotide (NAD) in mammalian organs which synthesize steroids. In 1958, Wattenberg united this oxidation reaction to the reduction of a tetrazolium salt and made the histological localization of

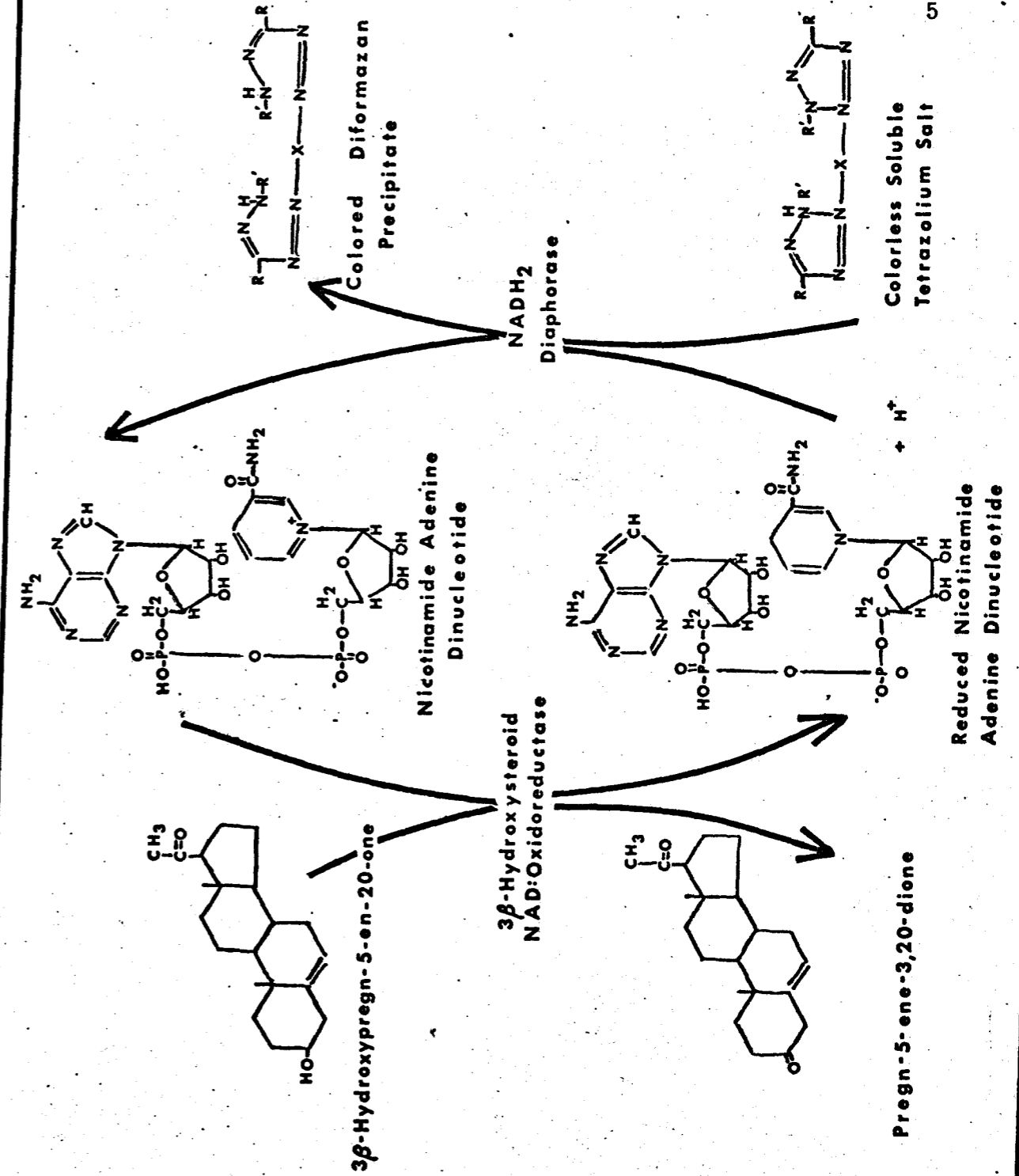
the reaction possible in tissue sections. This localization is dependent upon the transfer of the hydrogens removed from the steroid molecule at carbon 3 via NAD to the colorless, soluble tetrazolium salt which is then reduced to a colored, insoluble diformazan precipitate; this precipitate is deposited at the site of the reaction in the tissue section. This histochemical technique assumes that the enzyme activity is located at the site of the reaction in the tissue section. Text figure 1 illustrates this chemical transfer, using 3β -hydroxypregn-5-en-20-one (pregnenolone) as the precursor substrate. Wattenberg's technique has been modified and used extensively by other research workers to demonstrate enzyme activity in many diverse organ systems using different substrates.

Studies concerning 3β -hydroxysteroid dehydrogenase activity in ovaries, adrenal glands, testes, and epithelial cells of oviducts of rats, using 3β -hydroxyandrost-5-en-17-one (dehydroepiandrosterone, DHA) as the substrate for the cytochemical localization of the cells involved in the synthesis of estrogens have been initiated by Levy et al. (1959). A gradation in the activity of this enzyme was observed in the different components of the ovary of the rat. From an in vitro biochemical investigation of incubated ovarian tissues, Kalvert and Bloch (1968) found that DHA was converted to estradiol- 17β and estrone by the ovaries of rats; further, there was a greater conversion of DHA to estrogens during estrus than during diestrus. Seemingly, there is a variation in the synthesis rate of estrogens during the estrous cycle of the rat. Thus, these initial observations give added credence to the importance of ascertaining the critical changes (loci of estrogen production) during the six different, separable stages of the estrous cycle. Owing to the importance of such information, it is most desirable to incorporate this base-line study as the point of departure for this dissertation.

The steroidal precursor, pregnenolone, has been used as the substrate

Text Figure 1

Diagrammatic representation of the transfer of hydrogens in the oxidation-reduction reactions required for the visualization of the histochemical localization sites of 3 β -hydroxysteroid:NAD oxidoreductase activity in tissue sections, utilizing the substrate 3 β -hydroxypregn-5-en-20-one (pregnenolone).



Text Figure 1

when following progesterone synthesis, whereas dehydroepiandrosterone has been used when following estrogen synthesis. Crisp (1969) reported biochemical data indicating ovarian vesicular follicles of rats are capable of converting: 1) DHA to androst-4-ene-3, 17- dione and 2) pregnenolone to progesterone. Isolated theca or granulosa cells were incubated separately, to attempt to follow the sites of steroid metabolism in human ovaries (Ryan, 1965); both cellular types appear able to convert progesterone to estrogens, and pregnenolone showed a 60% conversion to progesterone, mainly by the granulosa cells of these follicles. McDonald et al. (1969) studied progesterone synthesis in the cycling rat at different stages of the estrous cycle by measuring progesterone titers in the ovarian venous blood, and by observing alterations in the Golgi apparatus of luteal tissue of fixed ovaries; they reported that: 1) measurable plasma levels of progesterone were secreted by the rat ovaries during each stage of the estrous cycle, being highest the night of proestrus (4.85 $\mu\text{g}/\text{ovary}/\text{hr}$) and lowest the morning of proestrus (0.09 $\mu\text{g}/\text{ovary}/\text{hr}$) and 2) granulosa lutein cells were probably the source of progesterone during estrus and diestrus.

These experiments in the main are principally concerned with the identification of the sites of potential estrogenic and progestational biosynthesis in the ovary and the semi-quantitative assessments of enzymatic activity associated therewith. These cyto- and histochemical studies rate enzymatic activity on a 0 (zero) to +4 scale by determining the maximal intensity of the diformazan deposited in ovarian tissue sections in a medium containing an excess of given substrate. For clarity, the semi-quantitative histochemical assessments were evaluated as:

0 = negligible or negative reaction

± = trace activity, only monoformazan material present

+1 = weakly positive activity

+2 = moderate activity

+3 = strong activity

+4 = maximal activity

Monoformazan is the diffuse pink color of the partially reduced tetrazolium indicator (Eadie et al., 1970); the partially reduced tetrazolium molecule is more soluble than the complete reduction product, diformazan, which is the dark purple granular end-point precipitate. Appendices A and B consist of a pictorial representation of the histochemical semi-quantitative scales used in this study for the substrates pregnenolone and dehydroepiandrosterone, respectively. The scales are shown in both color and black and white photomicrographs since the pictorial data of this study will be presented as black and white prints.

Thus, the major purposes of this cyto- and histochemical study were to investigate the following questions:

1) Is there an alteration in potential steroidal biosynthesis in the different components of: (a) the follicle, during folliculogenesis, i.e., the granulosa cells, theca interna and theca externa; (b) the corpora lutea during luteal development, i.e. during the pre-ovulatory structures and post-ovulatory, re-developed structures; and (c) the interstitial tissue. If so, which of the follicular components appear to be the most enzymatically active and during which physiological periods?

2) Are there alterations in the potential progestational and estrogenic biosynthesis in the different ovarian components during the six distinctive stages of the estrous cycle? If so, during which stage is potential progesterone production highest and when lowest and, similarly, when is

potential estrogenic production highest and when lowest?

3) Is there any observable alteration(s) in the biosynthesis of estrogens and progesterones by the different ovarian components during the two types of pseudopregnancy (normally-induced and prolonged) as well as during pregnancy? If so, in which ovarian component(s) is the alteration in potential steroidal biosynthetic capacity most changed and which steroid is most dominant?

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Localization of Ovarian Dehydroepiandrosterone- and Pregnenolone-
3 β -Hydroxysteroid Dehydrogenases in the Rat
During the Estrous Cycle

Introduction

Reproductive mechanisms when dissected into their component parts present an ever increasing number of directions for future study, notably areas involving form-function relationships. Perhaps one of the most involved- and yet incompletely understood - areas of reproductive biology issues forth from one of the most basic areas of endocrinology, i. e. the cellular and tissue sources of the ovarian steroid hormones. Thus, the current impetus for the present arbeit. The literature is beginning to record a significant number of research efforts in this area, but very little information is available to show the actual cellular and tissue sources of the ovarian steroid hormones throughout the sex cycles of laboratory animals and man. Therefore, the present work attempts to present a detailed analysis of the cytochemical and histochemical localization of estrogenic and progestational hormones throughout the varied and different populations of ovarian structures in each of the six different stages of the estrous cycle of the rat. The area of ovarian steroidogenesis has been investigated utilizing the techniques of in vitro incubation, tissue culture, histology, cytology, histochemistry, and electron microscopy. Of particular significance, the tests for 3 β -hydroxysteroid:NAD oxidoreductase (3 β -hydroxysteroid dehydrogenase, 3 β -HSD) have been developed and pursued extensively owing chiefly to the biologic importance of this enzyme in steroidogenesis. It has been roundly stated that 3 β -HSD is an important enzyme which is involved in the synthesis of the steroid hormones.

Enzymatic activity of 3 β -HSD has been investigated using 3 β -hydroxypregn-5-en-20-one (pregnenolone, P-one) and 3 β -hydroxyandrost-

5-en-17-one (dehydroepiandrosterone, DHA) as substrates for the histochemical localization of the cells involved in the biosynthesis of progestational and estrogenic hormones respectively. Previous reports have shown that there is a gradation of 3β -HSD activity in particular ovarian components, *i.e.* Levy *et al.* (1959a, 1959b) reported DHA reactive cells in the granulosa of atretic follicles, the theca interna, interstitial tissue, and corpora lutea of rat ovaries. Pupkin *et al.* (1966), also using the substrate DHA, localized 3β -HSD activity in corpora lutea which increased in intensity during the first cycle following luteal formation and decreased in activity thereafter, as well as in granulosa cells of tertiary follicles of ovaries of 120 to 140 day old rats. The changes in ovarian sites of steroidogenesis using P-one as the substrate, however, was not as well clarified as those elicited and manifested with DHA by Pupkin *et al.* More recently, alterations in 3β -HSD activity was followed during certain stages of the estrous cycle in the sow (Bjersing, 1967), dog (Fowler and Feldman, 1970), rat (Motta and Bourneva, 1970a, 1970b), hamster (Blaha and Leavitt, 1970), and guinea pig (Motta and Takeva, 1971). No one to date, however, has undertaken detailed stage by stage analyses of cellular and tissue sources of estrogenic and progestational hormones within the ovaries of rats of known reproductive history. Accordingly, it was of interest to thoroughly assess the 3β -HSD activity throughout the estrous cycle of the rat.

EXPERIMENTAL PROCEDURES

Female albino rats were purchased from the Charles River Breeding Laboratories and were housed in animal rooms having a temperature of 72°F and a 12 hour light-dark cycle. The rats had Purina Rat Chow and water available *ad libitum*. The animals were of two age groups: 41 to 65 days (designated as Y) and 75 to 120 days old (designated R).

The Y-designated adult rats were checked daily for the time of vaginal opening, so that the onset of puberty could be readily detected. Immediately after normal vaginal canalization, coincident with the initial spurt of ovarian activity, vaginal smears were taken daily. After establishing the regularity of the estrous cycles, the rats were pursued to obtain critical reproductive data concerning ovarian steroid biosynthesis so as to index and so characterize each of the six distinctive stages of the estrous cycle, i. e. estrus (E), metestrus (ME), early diestrus (D₁), late diestrus (D₂), preproestrus (PPE), and proestrus (PE).

The regularly cycling, non-manipulated animals of the older age group (R-, 75 to 120 days old) were followed for at least three consecutive estrous cycles prior to necropsy. The animals were similarly studied, necropsied via decapitation at each of the six aforementioned stages of the estrous cycle. The ovaries were immediately processed for these studies concerned with the detection, localization and semi-quantitation of pregnenolone-3 β -hydroxysteroid dehydrogenase (P-one-3 β -HSD), and of dehydroepiandrosterone-3 β -hydroxysteroid dehydrogenase (DHA-3 β -HSD), each presenting information regarding the biosynthesis of ovarian steroid hormones, progestational and estrogenic respectively.

Following decapitation, the left ovary was immediately removed, trimmed and frozen by submersion in cold liquid 2-methylbutane, which was chilled by being placed into a Dewar flask partially filled with liquid nitrogen. Ovaries were sectioned at eight microns and then incubated at 37°C for 2 3/4 hours in one of the following media; see tabularization, following. Since the localization of 3 β -HSD activity is dependent upon the amount of reduced nicotinamide adenine dinucleotide diaphorase (NADH₂ diaphorase) present in the tissue sections for the conversion of the colorless, soluble tetrazolium salt (Nitro blue tetrazolium chloride, Nitro-BT) to the colored, insoluble diformazan

precipitate, the localization of the NADH_2 diaphorase activity was carried out on companion, frozen sections. Suitable and meaningful histochemical controls, omitting steroidal substrates, were similarly incorporated into the experimental protocols and extensively studied, the object being the careful assessment of the reactive product so as not to incorrectly record a negative reaction.

| | Histochemical Control | Pregnenolone 3β -HSD | DHA 3β -HSD | NADH_2 Diaphorase |
|-----------------------------------|---|---|---|---|
| Propylene Glycol | 1.0 ml. | 1.0 ml. | 1.0 ml. | ----- |
| Pregnen- olone | ----- | $2.0 \times 10^{-4} \bar{M}$ 1.0 ml. | ----- | ----- |
| Dehydroepi- androsterone | ----- | ----- | $2.0 \times 10^{-4} \bar{M}$ 1.0 ml. | ----- |
| NAD | $5.0 \times 10^{-4} \bar{M}$ 1.0 ml. | $5.0 \times 10^{-4} \bar{M}$ 1.0 ml. | $5.0 \times 10^{-4} \bar{M}$ 1.0 ml. | ----- |
| NADH_2 | ----- | ----- | ----- | $1.2 \times 10^{-3} \bar{M}$ 2.0 ml. |
| Nitro-BT | $1.6 \times 10^{-4} \bar{M}$ 1.0 ml. | $1.6 \times 10^{-4} \bar{M}$ 1.0 ml. | $1.6 \times 10^{-4} \bar{M}$ 1.0 ml. | $1.6 \times 10^{-4} \bar{M}$ 1.0 ml. |
| Mixed Phosphate Buffer, pH=7.4 | 4.0 ml. | 4.0 ml. | 4.0 ml. | 4.0 ml. |

The histochemical techniques utilized in this study localize the histologic and cellular sites of P-one- 3β -HSD activity and DHA- 3β -HSD activity. P-one- 3β -HSD activity is interpreted as indicative of the biosynthesis of progesterone, whereas DHA- 3β -HSD activity is interpreted as

indicative of the biosynthesis of estrogen. Histochemical, enzymatic activity was rated on a 0 to +4 scale by determining the maximum intensity of the diformazan deposited on ovarian tissue sections in a medium containing an excess of given substrate. Monoformazan is the diffuse pink color of the partially reduced tetrazolium indicator; the partially reduced tetrazolium molecule is more soluble than the complete reduction product, diformazan, which is the dark purple granular end-point precipitate.

The right ovary, uterus and vagina were also removed from these animals, fixed and embedded in paraffin. Sections of these organs were taken at 8 μ and stained with Harris hematoxylin and eosin for morphologic confirmation of the estrous cycle staging of the animals.

For purposes of clarity, it should be emphasized that the semi-quantitative histochemical values of 0 to +4 should be interpreted as follows:

- 0 = negative reaction - no diformazan present;
- +1 = weakly positive;
- +2 = moderate activity;
- +3 = strong activity;
- +4 = maximal activity.

Also, for those reactions where there is some localizable material between 0 and +1, the conventional \pm sign is used to indicate trace activities.

OBSERVATIONS AND RESULTS

The literature is replete with attempts to ascertain and categorize certain of the cellular and tissue sites of hormonal release, but no one to date has systematically attempted to do so stage by stage step-wise. The present study has approached this aim, and the results appear to be ever so much more meaningful, especially since they form a scaffold of baselines for the superimposition of a number of extension studies, e.g. pseudopregnancy,

pseudopregnancy with decidual reactions (or prolonged pseudopregnancy) and the whole biologic career of pregnancy. Thus, the ovaries of female albino rats have been studied during the cycle so as to localize in a pin-point manner the sites of estrogenic and progestational hormones, by use of the substrates dehydroepiandrosterone (DHA) and pregnenolone (P-one), respectively. Consequently, this study assessed the intensity of mono- and diformazan deposits in the following ovarian components: primary-, secondary- and tertiary follicles, atretic follicles, interstitial tissue, corpora lutea, and the ovarian hilus. The secondary- and tertiary follicles as well as the atretic follicles were examined to determine the sites of P-one- 3β -HSD and DHA- 3β -HSD within the several components of the follicle, e. g. granulosa layers, theca interna and theca externa, and likewise the interstitial tissue, these enzymes being indicators of biosynthesis of progestational and estrogenic hormones.

For the purpose of fully orienting the reader in regard to the ovarian structures assessed and discussed in the text, Plates I and II depicting overviews of the ovaries of animals in estrus are immediately portrayed. They are comprised of photomicrographs which illustrate the general pattern of mono- and diformazan deposits in ovarian sections which have been studied histochemically. Plate I, fig. 1 is a section of a histochemical control ovary which was incubated in a medium lacking steroidal substrate and NADH_2 . Neither mono- nor diformazan materials were present in any of the histochemical controls.

The ovarian structures are quite intensely outlined in the test series using the NADH_2 substrate (Plate I, fig. 2) Both mono- and diformazan deposits are more intense in this series than in incubates using either P-one or DHA. It is to be noted that the detection of excess NADH_2 results in a blank control (Plate I, fig. 2A). Table I is a summary of the average

TABLE I

Averages of Semi-Quantitative Histochemical Estimates of Reduced Nicotinamide Adenine Dinucleotide Diaphorase (NADH₂ Diaphorase) in the Ovarian Components of Normally Cycling Non-manipulated Rats of Two Age Groups During the Six Stages of the Estrous Cycle. *

| Ovarian Components | <u>Estrus</u> | | <u>Metestrus</u> | | <u>Early Diestrus</u> | | <u>Late Diestrus</u> | | <u>Preproestrus</u> | | <u>Proestrus</u> | |
|--------------------|---------------|----|------------------|----|-----------------------|----|----------------------|----|---------------------|----|------------------|----|
| | Y | R | Y | R | Y | R | Y | R | Y | R | Y | R |
| Primary Follicle | +2 | +2 | +2 | +2 | +2 | +2 | +2 | +2 | +2 | +2 | +2 | +2 |
| Secondary Follicle | | | | | | | | | | | | |
| Granulosa Cells | +3 | +4 | +2 | +2 | +2 | +3 | +3 | +4 | +3 | +4 | +4 | +2 |
| Thecal Cells | +4 | +4 | +4 | +3 | +4 | +4 | +4 | +4 | +3 | +4 | +4 | +3 |
| Tertiary Follicle | | | | | | | | | | | | |
| Granulosa Cells | +2 | +2 | +4 | +2 | +3 | +2 | +3 | +3 | +3 | +3 | +3 | +3 |
| Thecal Cells | +4 | +4 | +4 | +4 | +4 | +4 | +4 | +4 | +4 | +4 | +4 | +4 |

*The age groups are designated:

Y- animals ranging in age from 41 to 65 days, the younger animals.

R- animals ranging in age from 75 to 120 days, those slightly more advanced in age than Y- with an established history of estrous cyclicity.

8 to 9 ovaries were used in each stage in the Y- group except proestrus which included 10 ovaries.

6 to 7 ovaries were used in each stage in the R- group except early diestrus which included 8 ovaries.

TABLE I CONTINUED

| Ovarian Components | <u>Estrus</u> | | <u>Metestrus</u> | | <u>Early Diestrus</u> | | <u>Late Diestrus</u> | | <u>Preproestrus</u> | | <u>Proestrus</u> | |
|-----------------------|---------------|----|------------------|----|-----------------------|----|----------------------|----|---------------------|----|------------------|----|
| | Y | R | Y | R | Y | R | Y | R | Y | R | Y | R |
| Atreic Follicle | | | | | | | | | | | | |
| Granulosa Cells | +2 | +2 | +2 | +2 | +2 | +2 | +2 | +2 | +2 | +2 | +4 | +2 |
| Thecal Cells | +4 | +4 | +4 | +4 | +4 | +4 | +4 | +4 | +4 | +4 | +4 | +4 |
| Interstitial Cells | +4 | +4 | +4 | +4 | +4 | +4 | +4 | +4 | +4 | +4 | +4 | +4 |
| Corpus Luteum | +4 | +4 | +4 | +4 | +4 | +4 | +4 | +4 | +4 | +4 | +4 | +4 |
| Hilar Region | +2 | +2 | +2 | +2 | +2 | +2 | +2 | +2 | +2 | +2 | +2 | +2 |

*The age groups are designated:

Y- animals ranging in age from 41 to 65 days, the younger animals.

R- animals ranging in age from 75 to 120 days, those slightly more advanced in age than Y- with an established history of estrous cyclicity.

8 to 9 ovaries were used in each stage in the Y- group except proestrus which included 10 ovaries.

6 to 7 ovaries were used in each stage in the R- groups except early diestrus which included 8 ovaries.

estimates of NADH_2 diaphorase localization in ovarian components in the Y- designated series (animals 41 - 65 days of age) and R- designated series (animals 75 - 120 days of age). In these ovarian sections, NADH_2 was in excess so that the maximal intensity of diformazan could be observed and assessed accordingly. Thus, the intensity of intrinsic NADH_2 diaphorase localization was assessed and aided in analyzing the data observed for the localization of progesterone and estrogen biosynthetic sites in the ovarian components.

Plate II, fig. 3 is a photomicrograph of an ovarian section which was incubated with P-one as substrate to localize the sites of progesterone biosynthesis in the rat during the estrous cycle. Plate II, fig. 4 is a photomicrograph of an ovarian section which was incubated with DHA substrate to localize the sites of estrogen biosynthesis in the rat during the estrous cycle. Table II (Y- series) and Table III (R- series) contain the average estimates of P-one- 3β -HSD and DHA- 3β -HSD intensities in ovarian components.

The assessments made on the intensity of the color reactions obtained by the histochemical media were based on careful inspection of the incubated ovarian sections. An intensity scale was based on a series of experiments in which the concentration of each substrate and the duration of incubation was quantitatively varied in order to obtain the maximal deposition of diformazan in the shortest incubation time interval. Maximal intensity (+4) was observed as a dense dark purple colored diformazan granular deposit which was located intracellularly while the trace color intensity (\pm) was due to a predominance of the diffuse pink color of monoformazan material. Tabular summaries which are comprised of the intensity rating for each ovarian component at each stage of the estrous cycle are found in Table I for NADH_2 diaphorase localization, Table II for P-one- 3β -HSD and DHA- 3β -HSD localization in the Y- series of

TABLE II

Averages of Semi-Quantitative Histochemical Estimates of 3β -Hydroxysteroid: NAD Oxidoreductase (3β -HSD Localization Using Two Substrates, Pregnenolone (P-one) and Dehydroepiandrosterone (DHA), in the Ovarian Components of the Young Series of Rats (41-65 Days Old) During the Six Stages of the Estrous Cycle.*

| Ovarian Components | Estrus** | | Met-estrus | | Early Diestrus | | Late Diestrus | | Prepro-estrus | | Pro-estrus | |
|---------------------------------------|----------|-----|------------|-----|----------------|-----|---------------|-----|---------------|-----|------------|-----|
| | P-one | DHA | P-one | DHA | P-one | DHA | P-one | DHA | P-one | DHA | P-one | DHA |
| Primary Follicle | ± | +1 | ± | ± | +1 | +1 | ± | +1 | +1 | +1 | ± | 0 |
| Secondary Follicle Granulosa Cells | +1 | ± | +1 | ± | +1 | +2 | +1 | +2 | +1 | +1 | +1 | +2 |
| Thecal Cells | +2 | +3 | +2 | +2 | +2 | +3 | +2 | +3 | +2 | +3 | +3 | +3 |
| Tertiary Follicle Granulosa Cells | +2 | +1 | +1 | +2 | +2 | +1 | +2 | +1 | +1 | +1 | +1 | +1 |
| Thecal Cells | +3 | +2 | +2 | +3 | +3 | +2 | +3 | +2 | +3 | +3 | +3 | +3 |

*8 to 9 ovaries were used in each stage except proestrus which included 10 ovaries.

** P-one designates the sections which were incubated with the substrate 3β -hydroxypregnen-5-en-20-one (pregnenolone).

DHA designates the sections which were incubated with the substrate 3β -hydroxyandrost-5-en-17-one (dehydroepiandrosterone).

Estimates were rated on a 0 to +4 scale and a trace amount designated '+'.

TABLE II CONTINUED

| Ovarian Components | Estrus** | | Met- estrus | | Early Diestrus | | Late Diestrus | | Prepro- estrus | | Pro- estrus | |
|-------------------------------------|-----------|----|----------------|----|-------------------|----|------------------|----|-------------------|----|----------------|----|
| | P-one DHA | | P-one DHA | | P-one DHA | | P-one DHA | | P-one DHA | | P-one DHA | |
| Atretic Follicle Granulosa Cells | \pm | +1 | +1 | +1 | +1 | +1 | +1 | +1 | +1 | +1 | +1 | +1 |
| Thecal Cells | +3 | +3 | +3 | +3 | +3 | +2 | +4 | +4 | +3 | +3 | +4 | +4 |
| Interstitial Tissue | +3 | +3 | +3 | +3 | +3 | +4 | +3 | +3 | +4 | +3 | +4 | +4 |
| Corpus Luteum | +1 | +3 | +4 | +3 | \pm | +3 | +3 | +4 | +2 | +4 | +4 | +4 |
| Hilar Region | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

* 8 to 9 ovaries were used in each stage except proestrus which included 10 ovaries.

**P-one designates the sections which were incubated with the substrate 3β -hydroxypregn-5-en-20-one (pregnenolone).

DHA designates the sections which were incubated with the substrate 3β -hydroxyandrost-5-en-17-one (dehydroepiandrosterone).

Estimates were rated on a 0 to +4 scale and a trace amount designated ' \pm '.

TABLE III

Averages of Semi-Quantitative Histochemical Estimates of 3β -Hydroxysteroid: NAD Oxidoreductase (3β -HSD) Localization Using Two Substrates, Pregnenolone (P-one) and Dehydroepiandrosterone (DHA), in the Ovarian Components of the Regularly Cycling Series of Rats (75-120 Days Old) During the Six Stages of the Estrous Cycle.*

| Ovarian Components | Estrus | | Met-estrus | | Early Diestrus | | Late Diestrus | | Prepro-estrus | | Pro-estrus | |
|---------------------------------------|--------|-------|------------|-------|----------------|-------|---------------|-------|---------------|-------|------------|-----|
| | P-one | DHA** | P-one | DHA | P-one | DHA | P-one | DHA | P-one | DHA | P-one | DHA |
| Primary Follicle | \pm | \pm | \pm | 0 | \pm | 0 | \pm | \pm | 0 | \pm | \pm | +1 |
| Secondary Follicle Granulosa Cells | +1 | +2 | \pm | \pm | 0 | +1 | +1 | +2 | \pm | \pm | \pm | +2 |
| Thecal Cells | +2 | +4 | +2 | +2 | +1 | +1 | +2 | +3 | +2 | +1 | +2 | +3 |
| Tertiary Follicle Granulosa Cells | +2 | +1 | +1 | +2 | +1 | \pm | +1 | +2 | +1 | +1 | +1 | +2 |
| Thecal Cells | +4 | +3 | +3 | +3 | +1 | +2 | +4 | +3 | +2 | +2 | +3 | +3 |

*6 to 7 ovaries were used in each stage of the cycle except early diestrus which included 8 ovaries.

**P-one designates the sections which were incubated with the substrate 3β -hydroxypregnen-5-en-20-one (pregnenolone).

DHA designates the sections which were incubated with the substrate 3β -hydroxyandrost-5-en-17-one (dehydroepiandrosterone).

Estimates were rated on a 0 to +4 scale and a trace amount designated ' \pm '.

TABLE III CONTINUED

| Ovarian Components | Estrus | | Met- estrus | | Early Diestrus | | Late Diestrus | | Prepro- estrus | | Pro- estrus | |
|-------------------------------------|-------------|----|----------------|----|-------------------|----|------------------|----|-------------------|----|----------------|----|
| | P-one DHA** | | P-one DHA | | P-one DHA | | P-one DHA | | P-one DHA | | P-one DHA | |
| Atretic Follicle Granulosa Cells | <u>±</u> | +1 | +1 | +1 | <u>±</u> | +1 | +1 | +1 | <u>±</u> | +1 | 0 | +1 |
| Thecal Cells | +4 | +4 | +3 | +3 | +2 | +2 | +3 | +3 | +2 | +3 | +3 | +4 |
| Interstitial Tissue | +4 | +4 | +3 | +3 | +2 | +2 | +4 | +3 | +2 | +3 | +3 | +4 |
| Corpus Luteum | +2 | +4 | +3 | +3 | <u>±</u> | +2 | +3 | +3 | +3 | +3 | +4 | +4 |
| Hilar Region | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

*6 to 7 ovaries were used in each stage of the cycle except early diestrus which included 8 ovaries.

**P-one designates the sections which were incubated with the substrate 3β -hydroxypregn-5-en-20-one (pregnenolone).

DHA designates the sections which were incubated with the substrate 3β -hydroxyandrost-5-en-17-one (dehydroepiandrosterone).

Estimates were rated on a 0 to +4 scale and a trace amount designated '±'.

rats, and Table III for P-one- 3β -HSD and DHA- 3β -HSD localization in the R- series of rats.

Since previous reports on the sites of P-one- 3β -HSD and DHA- 3β -HSD were mainly on large follicles, atretic follicles, interstitial tissue, and corpora lutea, the present study was designed to follow the alterations which occur in the development, maturation and regression of the follicular elements of the ovary by careful inspection of the aforementioned ovarian components during each stage of the estrous cycle.

Primary Follicles

Primary follicles were assessed at each stage of the cycle in both the Y- and R- groups of animals and were found to show only weakly positive reactions as evidenced by the presence of monoformazan material during late diestrus and preproestrus in the Y- series when incubated with P-one. The intensity of P-one- 3β -HSD was so weak that it could hardly be recorded photographically; see Plate V, fig. 23. The absence of any tetrazolium material in the remaining stages of the Y- series, *i. e.* estrus, metestrus, early diestrus, and proestrus, indicated that progesterone was probably not synthesized in the primary follicle during these stages, see Plate V, fig. 17. In the R- series, however, there was an increase in the number of stages in which the primary follicles showed the trace intensities of monoformazan material: estrus, metestrus, early diestrus, late diestrus, and proestrus; see Plate XI, fig. 72. No evidence of monoformazan nor diformazan material was observed at the stage of preproestrus in this group (Plate XI, fig. 66). Therefore, it appears that the primary follicles are not a source of progesterone production during the estrous cycle.

The alterations in the intensities and sites of DHA- 3β -HSD activity in the ovary of the rat were examined after tissue sections were incubated in a

medium containing DHA as the substrate, the enzyme serving as an indicator of estrogen biosynthesis. Intensities of diformazan reaction products, i. e. the color reaction indicator of the enzyme, ranged from 0 to +1 in both the Y- and R- series of animals during the different stages of the estrous cycle. Weak intensities of mono- and diformazan (+1) were noted in proestrus in the R- group (Plate XIV, fig. 98) as well as in the stages of estrus, early diestrus, and preproestrus in the Y- series of rats; see Plate VI, fig. 30; Plate VII, figs. 36 and 37; Plate VIII, fig. 47. Trace intensities of monoformazan reaction product were noted at estrus, late diestrus and preproestrus in the R- group and at metestrus in the Y- group (Plate VIII, fig. 41). The absence of both mono- and diformazan material in the primary follicles was found at the stage of proestrus in the Y- series of rats (Plate VIII, fig. 41) as well as during early diestrus and metestrus (Plate XII, fig. 82; Plate XIV, fig. 92) in the R- series of animals. Thus, primary follicles do not manifest any degree of positivity for either of the two substrates.

Secondary Follicles

As the primary follicles develop and mature, the number of concentrically located granulosa layers increase and thecal cells commence to appear. The secondary follicle, by definition, is a multi-laminar structure in which the ovum is enveloped by the zona pellucida, granulosa cell layers, the basement membrane, and the thecal elements.

Ova. The ova examined the primary-, secondary- and tertiary follicles always lacked both mono- and diformazan reaction products (Plate V, fig. 23; Plate X, figs. 58 and 59). Thus, using these histochemical tests, it was not possible to detect any positive indication for either estrogenic or progesterational hormone biosynthesis in the ovum during the estrous cycle.

Granulosa cells. The granulosa and thecal elements of the secondary

follicles were observed to vary in the intensity of the reaction product during the different stages of the estrous cycle. The granulosa cells, generally speaking, showed a slightly less intense histochemical reaction material for both P-one- 3β -HSD and DHA- 3β -HSD than did the thecal cells. In the Y-series of animals, the granulosa cells, which were studied for the localization of P-one- 3β -HSD were observed to consistently have a weakly positive reaction intensity (+1); Plate III, figs. 5, 8, and 9; Plate IV, figs. 10, 11, 12, and 15; Plate V, figs. 18 and 24. This consistent finding, however, was not the case in the R-series of rats. In this group, there was a gradation in the intensity of reaction products from weakly positive (+1) to totally absent (0); see Tables II and III. Low intensities of mono- and diformazan deposits were observed at estrus and late diestrus (Plate X, fig. 61; Plate XI, fig. 73); trace degrees of intensity were seen at metestrus, preproestrus and proestrus (Plate IX, fig. 57; Plate X, fig. 62) while no reaction product was noted at early diestrus (Plate X, fig. 59; Plate XI, fig. 67). Therefore, it appears that the granulosa cells of secondary follicles are only weakly positive for P-one- 3β -HSD during the estrous cycle.

In addition to our studies on P-one, we similarly examined the granulosa cells of the secondary follicles for the possible detection of DHA- 3β -HSD, the latter serving as an indication of the biosynthesis of estrogens. It was found that there was a gradation between moderate (+2) and trace (\pm) intensities in both the Y- and R-series of animals. Moderate intensities of mono- and diformazan were noted at early diestrus, late diestrus and proestrus in the Y-series of rats (Plate VII, figs. 34, 36 and 39; Plate VIII, fig. 48); moderate intensities were also found during estrus, late diestrus and proestrus in the R-series of animals (Plate XIII, fig. 90; Plate XIV, fig. 99). Low intensities of monoformazan were seen at preproestrus in the Y-group (Plate VII, fig. 37) and early diestrus in the R-group (Plate XII, fig. 84). Minimal

intensities of monoformazan were observed at estrus and metestrus in the Y-group (Plate VI, figs. 30 and 32; Plate VIII, fig. 42), as well as during metestrus and proestrus in the R- series (Plate XII, fig. 81; Plate XIII, fig. 87; and Plate XIV, fig. 93). With the ongoing development of the maturing follicle, *i.e.* from primary to secondary, it appears that the granulosa cells manifest a slight, but distinctive reaction suggestive of it being itself a cellular source of or possibly a related area for future interplay with the cellular and tissue sources of estrogenic hormone production.

Thecal cells. The thecal elements are comprised of the theca interna and theca externa which are located peripherally to the granulosa basement membrane of secondary and tertiary follicles. These layers differ in that the theca interna is highly vascular and cellular while the theca externa is predominately fibrous with a reduction in the degree of vascularity; the line of demarcation between these layers is indistinct as is that between the theca externa and the ovarian interstitial tissue. The intensity assessments reported throughout the text, recorded in the tabular data and illustrated in the pictorial plates for the thecal cells (TI) refer to the reaction products observed in the cellular aspects of the theca interna, since very minimal to negative reaction materials were seen in the cellular components of the theca externa. In the Y- series of animals, all stages of the estrous cycle except proestrus had moderate intensities (+2) of diformazan deposits using P-one substrate (Plate III, figs. 5, 8 and 9; Plate IV, figs. 10, 11 and 12; Plate V, fig. 18). At the stage of proestrus, however, a strong intensity (+3) of diformazan was noted (Plate IV, fig. 15; Plate V, fig. 24). In the R- series of animals, all stages except early diestrus showed a moderate (+2) deposition of diformazan positive granules (Plate IX, fig. 57; Plate X, figs. 61 and 62; Plate XI, fig. 73). At early diestrus, there was only a weakly positive reaction of +1 intensity (Plate X, fig. 59; Plate XI, fig. 67). Thus, the

cellular components of the theca interna of secondary follicles for the most part manifest at best a moderate index of activity for the P-one substrate.

Utilizing our second substrate, DHA, the cellular components of the theca interna of the secondary follicles were similarly pursued. Strong intensity reactions (+3) were seen in all stages of the estrous cycle in the Y- group of rats except at metestrus, at which time it was of only moderate strength (+2); see Plate VI, figs. 30 and 32; Plate VII, figs. 34, 36 and 39; Plate VIII, figs. 42 and 48. The gradation of intensities in the R- series ranged from maximally (+4) down to weakly positive (+1). The maximal deposition of diformazan material, which was finely granular in nature, appeared at estrus (Plate XIV, fig. 99). Thereafter, the reaction steadily decreased in 25% decrements, i. e. during metestrus a +2, and during early diestrus a +1 (Plate XII, figs. 81 and 84). Interestingly enough, the substrate for the estrogen precursor, DHA, became strongly intensified (+3) during late diestrus after which time it fell precipitously, just prior to proestrus (Plate XIII, fig. 87) and with the advent of proestrus rose to a strong (+3) reaction (Plate XIII, fig. 90). These data give added credence to the fact of the cellular components of the theca interna being importantly concerned with estrogenic production.

Tertiary Follicles

As folliculogenesis continues, the secondary follicles show additional growth and maturation. Fluid (liquor folliculi) begins to accumulate at this time and separates the granulosa cells; antrum formation begins; and we now begin to recognize the tertiary follicle. Those follicles possessing an antrum are truly designated as tertiary or vesicular follicles.

Granulosa cells. In the granulosa cells of tertiary follicles, using P-one as substrate, the intensity of the reaction ranged from moderate (+2)

to weakly positive (+1) in both the Y- and R- series. Moderate degrees of diformazan deposition were noted during estrus, early diestrus and late diestrus in the Y- group of animals (Plate III, figs. 6 and 9; Plate IV, figs. 10 and 11). Weakly positive reactions were observed during metestrus, preproestrus and proestrus in the Y- series of rats (Plate III, fig. 8; Plate IV, figs. 12 and 14; Plate V, figs. 19 and 25). Similarly, weakly positive reactions were seen during metestrus, early diestrus, late diestrus, preproestrus, and proestrus in the R- series (Plate IX, fig. 56; Plate X, figs. 58, 60 and 63; Plate XI, fig. 68). During estrus a moderate degree of activity (+2) was found (Plate IX, figs. 53 and 54; Plate XI, fig. 74). The granulosa cells of the tertiary follicles, therefore, seem to indicate that the components of the progestational hormonal biosynthetic machinery come into a more highly consolidated phase of activity at this stage of folliculogenesis.

Utilizing DHA, as a possible indicator of estrogen production, we similarly pursued the granulosa cells of the tertiary follicles. Accordingly, the results were only of moderate (+2) to weak (+1) intensities in the Y- series of rats, and moderate (+2) to minimal traces (\pm) in the R- series of rats. Moderate intensities of diformazan granular deposits were observed during estrus in the Y- series (Plate VI, figs. 32 and 33; Plate VIII, fig. 49). The remaining stages, i.e. estrus, early diestrus, late diestrus, preproestrus, and proestrus showed weakly intense reactions of a +1 estimate (Plate VI, figs. 29 and 30; Plate VII, figs. 34, 36, 37, and 40; Plate VIII, fig. 43). In the R- series, moderate intensities (+2) of diformazan were observed during metestrus, late diestrus and proestrus (Plate XII, fig. 80; Plate XIII, figs. 86 and 89; Plate XIV, fig. 100). A rise in activity from +1 to +2 was observed in the sequential phases between preproestrus, proestrus and estrus in the R- group of animals (Plate XII, fig. 78; Plate XIII, fig. 88). Phase-wise, a plateau of activity (+1) was observed throughout the metestrus and the

diestrus (Plate XII, figs. 82 and 84; Plate XIV, fig. 94). Thus, the granulosa cells of the tertiary follicles reveal a sustained activation of the several components associated with the biosynthesis of estrogenic hormones.

Thecal cells. In conformity with the protocols of these studies, the cellular components of the theca interna of the tertiary follicles were histochemically analyzed for the reaction(s) to the P-one substrate. Strong intensities (+3) of diformazan material were found in all stages of the estrous cycle in the Y- group of rats except during metestrus; metestrus manifested a moderate (+2) degree of diformazan deposition (Plate III, figs. 6, 8 and 9; Plate IV, figs. 10, 11, 12, and 14; Plate V, figs. 19 and 25). In the R- series of animals, there was an intensity range of reaction material from maximal (+4) to weakly positive (+1). Interestingly enough, the two maxima were observed: one during estrus, the other during late diestrus (Plate IX, figs. 53 and 54; Plate X, fig. 60; Plate XI, fig. 74). A gradual decline in diformazan intensity from a maximum peak during estrus was observed first to a +3 during metestrus (Plate IX, fig. 56), thence precipitously to a +1 during early diestrus (Plate X, fig. 58; Plate XI, fig. 68). Following the second maxima, at late diestrus, the response to the P-one substrate showed a marked drop in intensity to a +2 during preproestrus (Plate X, fig. 63), thereafter becoming intensified to a +3, i. e. during proestrus (Plate X, fig. 64). These data concerned with an elucidation of the thecal response to P-one, are quite remarkable in at least two ways: (1) the response to P-one in the Y- group shows an almost continued, strong response with the exception of metestrus; (2) the response to P-one in the R- group appears somewhat variable, although for the most part moderate to strong intensities being seen.

The response of the cellular components of the theca interna of the tertiary follicles to the precursor of estrogenic hormones, namely DHA, provides additional confirmatory data that this region is importantly concerned

with estrogen production. Strong intensities of diformazan material (+3) were seen during metestrus, preproestrus and proestrus in the Y- group of animals (Plate VI, figs. 32 and 33; Plate VII, figs. 37 and 40; Plate VIII, fig. 49). Strong degrees of diformazan deposition were noted during metestrus, late diestrus and proestrus in the R- series (Plate XII, figs. 78 and 80; Plate XIII, figs. 86 and 89; Plate XIV, fig. 100). A definitive reduction in diformazan intensity was noted during estrus, early diestrus and late diestrus in the Y- group of animals, i. e. from a +3 during preproestrus and proestrus to a +2 during estrus (Plate VI, figs. 29 and 30; Plate VII, figs. 34 and 36; Plate VIII, fig. 43) and one of similar magnitude from the +3 phases of estrus, metestrus, late diestrus and proestrus to the +2 phases of early diestrus and preproestrus in the R- group of rats (Plate XII, figs. 82 and 84; Plate XIII, fig. 88; Plate XIV, fig. 94). As indicated in the prefatory, opening remarks, it does appear that this region is definitely concerned with estrogen production.

Atretic Follicles

The process of follicular atresia or degeneration occurs throughout the estrous cycle of the rat, usually somewhat more often just prior to ovulation. Generally, atresia is first manifested by alterations in the ovum and then in the granulosa cells. As the granulosa cells continue to degenerate, the follicle collapses and the granulosa cells become resorbed by wandering cells. At the same time, the thecal cell layers increase in size (hypertrophy) and become the predominant cellular type in the atretic follicle.

Granulosa cells. The granulosa cells of atretic follicles were examined and observed to show considerably less mono- and diformazan deposits than the cells of the theca interna when incubated with both precursor substrates, P-one and DHA. Using P-one, to follow progestational hormonal production, all stages of the estrous cycle except estrus in the Y- series of

animals were noted as having weak (+1) intensities of mono- and diformazan (Plate III, figs. 8 and 9; Plate IV, figs. 10 and 15; Plate V, fig. 26). Trace reactions of diffuse pink monoformazan (\pm) were noted during estrus (Plate III, fig. 5; Plate V, fig. 20). In the R- series of animals, intensity gradients ranging from weakly positive (+1) to negative (0) were ubiquitous. Interestingly enough, a negative reaction was observed during proestrus (Plate X, fig. 64). A slight, inappreciable increase in the intensity of the reaction to monoformazan material (\pm) was seen during the stages of estrus, early diestrus and preproestrus, the best being a consolidated trace intensity (Plate IX, figs. 53 and 54; Plate XI, fig. 69). Weakly positive reactions (+1) were noted during metestrus and late diestrus (Plate IX, figs. 56 and 57; Plate X, fig. 64; Plate XI, fig. 75). These findings suggest that the granulosa cells of atretic follicles display little if any activity in regard to progesterational hormone production.

The response of the granulosa cells of atretic follicles to DHA was found to be uniform during all stages of the estrous cycle in both the Y- and R- series of rats, intensity assessments for mono- and diformazan being weakly positive (+1) (Plate VI, figs. 29 and 32; Plate VII, figs. 34, 37 and 39; Plate VIII, figs. 44 and 50; Plate XII, figs. 79, 81 and 84; Plate XIII, figs. 85 and 90; Plate XIV, figs. 95 and 101). The preceding data obtained using both P-one and DHA substrates indicate that the granulosa cells of atretic follicles are not too responsive to the precursors of progesterational and estrogenic hormones.

Thecal cells. Previously it was mentioned that the thecal cells of those follicles which become atretic hypertrophy and become the predominant type of cells in atretic follicles. In these studies using the P-one substrate, an intensity range for diformazan deposits ranged, in the Y- series of animals, from maximal to strong with the maxima at proestrus and late diestrus (Plate III,

figs. 5, 8 and 9; Plate IV, figs. 10 and 15; Plate V, figs. 20 and 26).

Diformazan deposits were maximal during estrus in the R- series of animals (Plate IX, fig. 53; Plate XI, fig. 75). Strong intensities (+3) of diformazan material were noted during metestrus, late diestrus and proestrus in the R- series (Plate IX, figs. 56 and 57; Plate X, figs. 61 and 64). Only two stages were of reduced moderate intensities, in the R- series, early diestrus and preproestrus (Plate XI, fig. 69). These findings indicate that the remnants of the theca interna of the atretic follicles are strongly reactive to the progestational hormone substrate during the estrous cycle.

Utilizing DHA, it was determined that the diformazan intensities in the thecal components of the atretic follicles varied between maximal peaks (+4) and moderate levels (+2). Maximal strengths were observed during late diestrus and proestrus in the Y- series of animals (Plate VII, fig. 39; Plate VIII, fig. 50) as well as during proestrus and estrus in the R- series of animals (Plate XII, fig. 79; Plate XIII, fig. 90; Plate XIV, fig. 101). In the Y- series, the phases of estrus, metestrus and preproestrus were seen to be strongly reactive to DHA (Plate VI, figs. 29 and 32; Plate VII, fig. 37; Plate VIII, fig. 44). Moderate intensities of diformazan granules were noted in both the Y- and R- groups of rats during early diestrus (Plate VII, fig. 34; Plate XII, fig. 84; Plate XIV, fig. 95). During metestrus, late diestrus and preproestrus in the R- group of animals, strong intensities were observed (Plate XII, fig. 81; Plate XIII, fig. 85).

These histochemical studies, performed on ovaries taken during the six stages of the estrous cycle clearly indicate that the cellular components of the theca interna of atretic follicles are strongly reactive to the precursor substrates for progestational and estrogenic hormones.

Interstitial Tissue

The ovarian interstitial tissue is composed of those cells which are

located between, and separate, the other ovarian components. In the rat and other rodents, the interstitial tissue is extensive. These cells appear to arise from the thecal cells of atretic follicles as well as from the theca lutein cells of corpora lutea undergoing resorption. Interestingly enough, the interstitial tissue appears to react the strongest of all the ovarian components studied. Utilizing P-one, the interstitial tissue cells were found to contain maximal (+4) to moderate (+2) intensities of diformazan material. Maximal peak color intensities were noted during preproestrus and proestrus in the Y- group of animals (Plate IV, figs. 12, 14 and 15; Plate V, fig. 27) as well as during estrus and late diestrus in the R- group of rats (Plate IX, figs. 53 and 54; Plate X, fig. 60; Plate XI, fig. 76). Strong intensities (+3) were observed during the remaining stages of the estrous cycle in the Y- series of animals, i. e. estrus, metestrus, early diestrus, and late diestrus (Plate III, figs. 5, 6, 8, and 9; Plate IV, fig. 11; Plate V, fig. 21). In the R- series of rats, strongly intense (+3) reaction material was noted during metestrus and proestrus (Plate IX, fig. 57; Plate X, figs. 64 and 65) while moderately intense (+2) deposits of diformazan granules were observed during early diestrus and preproestrus (Plate X, figs. 59 and 62; Plate XI, fig. 70). These data demonstrate quite remarkably that the interstitial tissue is quite responsive to the precursor substrate of the progestational hormones.

In the companion study, utilizing the DHA substrate, the cells of the interstitial tissue were observed to contain maximal (+4) to moderate (+2) intensities of diformazan deposits. Maximal intensities occurred during early diestrus and preproestrus in the Y- series (Plate VII, figs. 34, 39 and 40; Plate VIII, fig. 51) as well as during proestrus and estrus in the R- series of rats (Plate XII, figs. 78 and 79; Plate XIII, fig. 89; Plate XIV, fig. 102). The cells of the interstitial tissue in the remaining stages of the estrous cycle in the Y- series of animals appeared as strongly intense loci of

diformazan positive reaction granules, *i. e.* estrus, metestrus, late diestrus, and proestrus (Plate VI, figs. 29, 32 and 33; Plate VII, figs. 36 and 37; Plate VIII, fig. 45). Strongly intense diformazan granules were noted in the cells of the interstitial tissue during metestrus, late diestrus and proestrus in the R- group of rats (Plate XII, fig. 81; Plate XIII, figs. 86 and 88). The only degree of moderate activity was observed during early diestrus in the R- series of animals (Plate XII, figs. 82 and 84; Plate XIV, fig. 96).

Of all the ovarian components thus far studied, it does appear that the interstitial tissue is the most reactive to each of the two substrates, P-one and DHA. Furthermore, these data reveal that the interstitial tissue is a most remarkable site for the biosynthetic production of estrogenic and progestational hormones.

Corpus Luteum

Immediately following ovulation, the re-organized and luteinized components of the ovulated follicles now appear as prominent solid masses of luteinized granulosa and thecal cells - the corpus luteum.

As in the studies on the three classes of viable ovarian follicles, these studies included a number of tests utilizing P-one and DHA substrates on the corpora lutea during the six different stages of the estrous cycle. In testing P-one, it was observed that the intensities of mono- and diformazan deposits ranged from maximum (+4) to trace (†) in both the Y- and R- series of animals. Maximum peaks were noted during metestrus and proestrus in the Y- group (Plate III, fig. 8; Plate IV, fig. 16; Plate V, fig. 28) and during proestrus in the R- group (Plate X, fig. 65; Plate XI, fig. 77). During early diestrus in both the Y- and R- series of animals, the luteal cells showed trace intensities of monoformazan materials (Plate III, fig. 9; Plate V, fig. 22; Plate X, figs. 58 and 59; Plate XI, fig. 71). Following early diestrus, the intensity of the reaction product increased to strong (+3) at late diestrus in the Y- series

(Plate IV, fig. 11); identical degrees of intensity (+3) were observed during late diestrus, preproestrus and metestrus in the R- group of rats (Plate X, fig. 63). In the Y- group of animals, there was a slight reduction in the intensity of diformazan precipitate (+2) during preproestrus (Plate IV, fig. 13). During estrus in the Y- series, mono- and diformazan material was weakly intense (+2) during this same stage in the R- series (Plate III, fig. 7; Plate IX, figs. 53 and 55). Thus, these data obtained after utilizing the progestational precursor, P-one, strongly indicate that the biosynthetic pathway for progesterone production within the corpora lutea of recently ovulated follicles is operable during the estrous cycle in both age groups of animals.

Procedurally, DHA was used similarly to investigate the possible estrogenic sites in the estrous cycle in both groups of animals. In the Y-series, maximal intensity (+4) of diformazan was noted during late diestrus, preproestrus and proestrus (Plate VII, figs. 38 and 40; Plate VIII, fig. 52) which declined to strong intensities (+3) during estrus, metestrus and early diestrus (Plate VI, figs. 31 and 33, Plate VII, fig. 35; Plate VIII, fig. 46). The fluctuations in diformazan intensities were between maximal (+4) to moderate (+2) in the R- series of rats, with only one stage showing moderate activity, i. e. early diestrus. Maximal intensities were noted during proestrus and estrus (Plate XII, figs. 78 and 79; Plate XIII, fig. 91; Plate XIV, fig. 103); all other stages had uniformly strong reactions (+3) with the notable exception of early diestrus (Plate XII, figs. 80, 81 and 83; Plate XIII, figs. 86 and 87; Plate XIV, fig. 97). From the aforementioned data on the corpora lutea using progestational and estrogenic biosynthetic precursors, it appears that both substrates are quite active throughout the estrous cycle with but one exception, i. e. early diestrus.

Ovarian Hilus

The region of the ovary where the mesovarium attaches and the blood vessels, lymphatics and nerves enter and leave the ovary is called the hilus. This region of the ovary was examined following incubation with both P-one and DHA substrates, in order to determine if there are any steroidogenic properties of the cells in this region. It was found that this area consistently showed negative reaction products in all of the stages studied in both Y- and R- series of animals, cf. Tables II and III. The only positive reactions were seen to occur in the interstitial tissue cells which had wandered inwardly from the cortical region of the ovary by the physiological accommodation which occurs in the cortex with the development, growth and regression of the follicular and luteal components. Therefore, the consistent absence of any mono- or diformazan deposits in the hilar region suggests that this area is not principally concerned with estrogenic and progesterational hormonal production.

Comparative Analyses of Total Ovarian Composites vs Individual Ovarian Entities: An Overview

In an effort to consolidate myriad observations into a full view of comprehensible recognition, it appeared of interest to ascertain and project the arithmetic averages of the data obtained for each ovarian component studied. The vertical averages for each of the two ages groups (Y- and R-) are exhibited in Table IV. The cyclical arrangement of the data, for ready recognition, is portrayed in two major text figures, each of which is followed-up by a series of nine component wheels, each representing specific ovarian morphological entities, cf. Text figures 2, 2a-2i; 3, 3a-3i.

TABLE IV

Generalized and Summarized Data Based on all Ovarian Components
Presented as Averages during the Estrous Cycle. ¹

| Enzymes* | <u>Estrus**</u> | | <u>Metestrus</u> | | <u>Early Diestrus</u> | | <u>Late Diestrus</u> | | <u>Prepro-estrus</u> | | <u>Pro-estrus</u> | |
|--------------------|----------------------|-----|------------------|-----|-----------------------|-----|----------------------|-----|----------------------|-----|-------------------|-----|
| | Y | R | Y | R | Y | R | Y | R | Y | R | Y | R |
| | P-one-3 β -HSD | 1.7 | 2.1 | 1.9 | 1.8 | 1.8 | 0.8 | 2.1 | 2.1 | 2.0 | 1.3 | 2.3 |
| DHA-3 β -HSD | 1.9 | 2.6 | 1.9 | 1.9 | 2.1 | 1.2 | 2.3 | 2.2 | 2.2 | 1.6 | 2.4 | 2.7 |

¹Arithmetic mean semi-quantitative histochemical estimates of 3 β -hydroxysteroid: NAD oxidoreductase (3 β -HSD) reactivity which were derived from the follicular, luteal and interstitial components of the ovaries in the young series (41-65 days old) and older animals with regularly established reproductive history (75-120 days) as observed during the six stages of the estrous cycle.

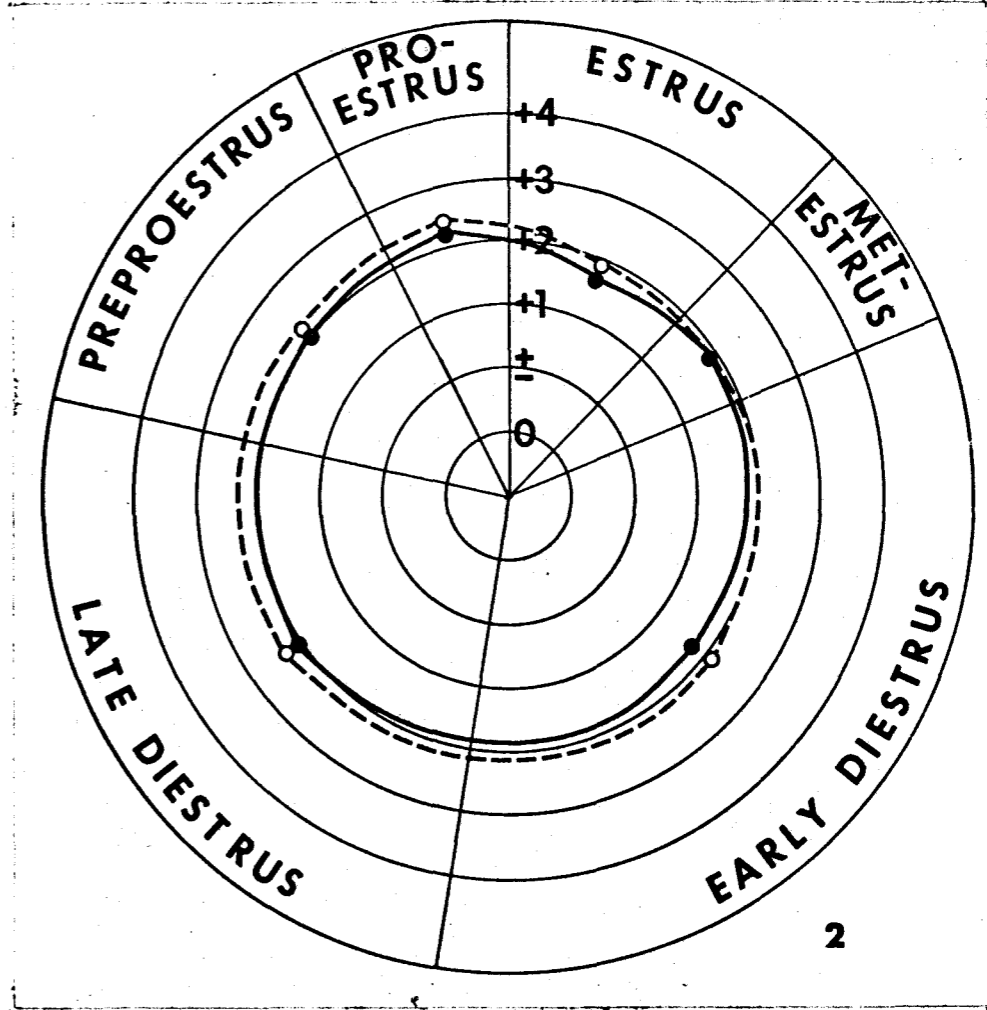
*P-one-3 β -HSD denotes 3 β -HSD intensities using pregnenolone (P-one) as the incubation substrate.
DHA-3 β -HSD denotes 3 β -HSD intensities using dehydroepiandrosterone (DHA) as the incubation substrate.

**Y-signifies the young series of animals (41-65 days old).

R-signifies the slightly older age group of animals with established reproductive history (75-120 days old).

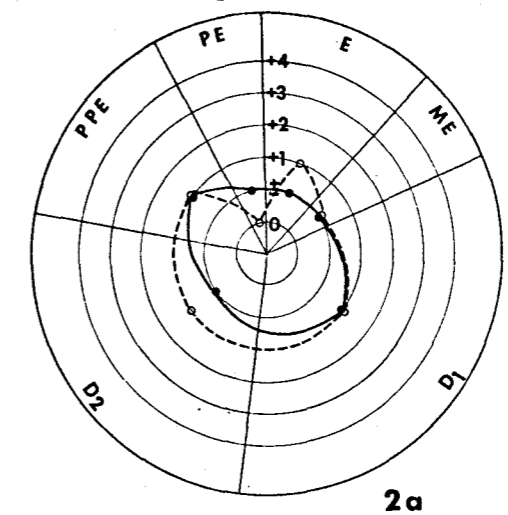
Estimates were rated on a 0 to a +4 and a trace amount designated '+±'.

Text Figure 2. A cyclical arrangement of the averages of semi-quantitative histochemical estimates of P-one-3 β -HSD (solid line) and DHA-3 β -HSD (dotted line) of all ovarian components of the younger series of rats (Y- series: 41-65 days of age) throughout the estrous cycle. Individual components are presented in similar, but individual activity wheels, in accompanying figures 2a-2i.

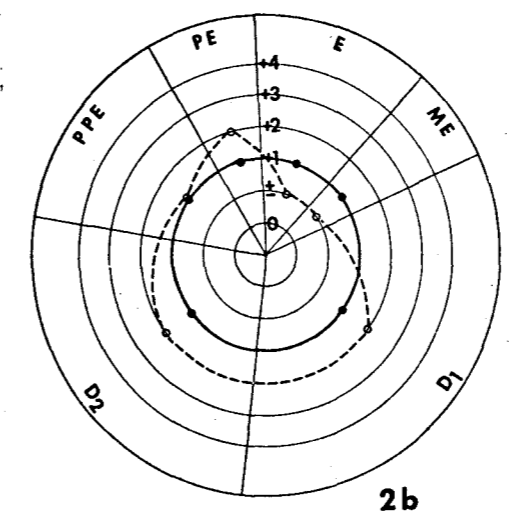


Text Figures 2a-2e. Averages of semi-quantitative histochemical estimates of P-one-3 β -HSD (solid line) and DHA-3 β -HSD (dotted line) of individual ovarian components throughout the estrous cycle of the Y-series of rats:

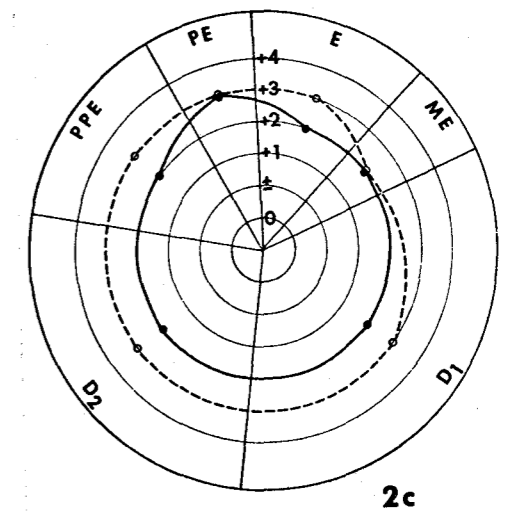
- 2a. Activities of P-one-3 β -HSD and DHA-3 β -HSD in primary follicles.
- 2b. Activities of P-one-3 β -HSD and DHA-3 β -HSD in granulosa cells of secondary follicles.
- 2c. Activities of P-one-3 β -HSD and DHA-3 β -HSD in theca interna of secondary follicles.
- 2d. Activities of P-one-3 β -HSD and DHA-3 β -HSD in granulosa cells of tertiary follicles.
- 2e. Activities of P-one-3 β -HSD and DHA-3 β -HSD in theca interna of tertiary follicles.



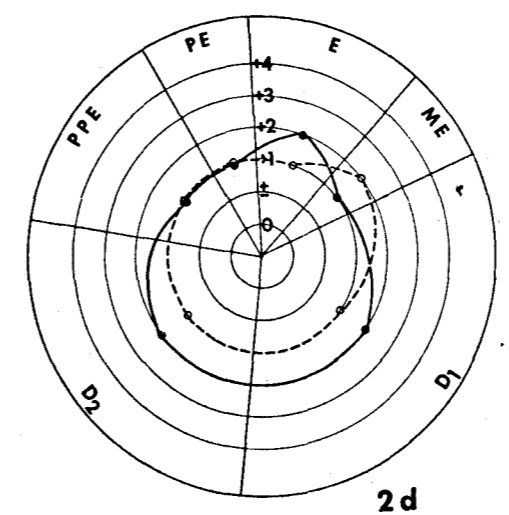
2a



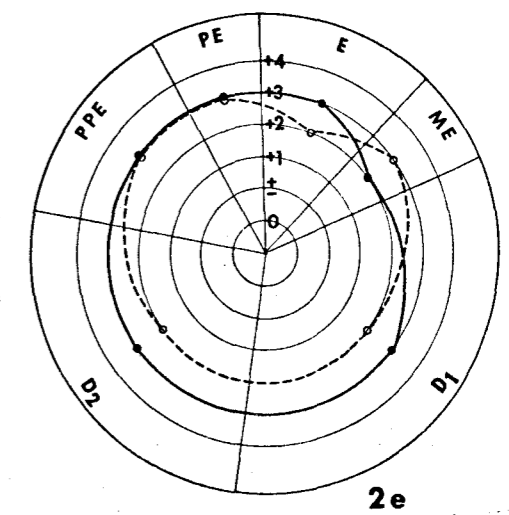
2b



2c



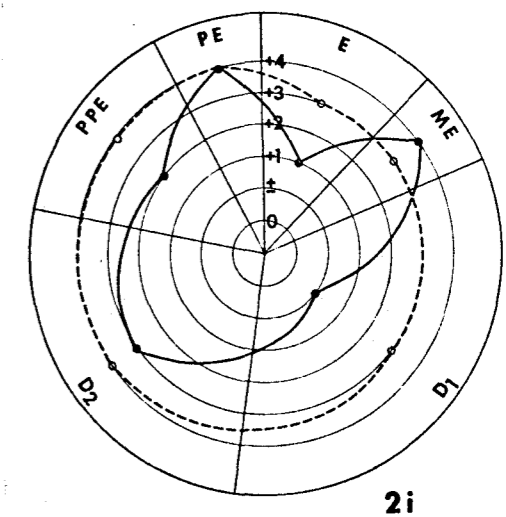
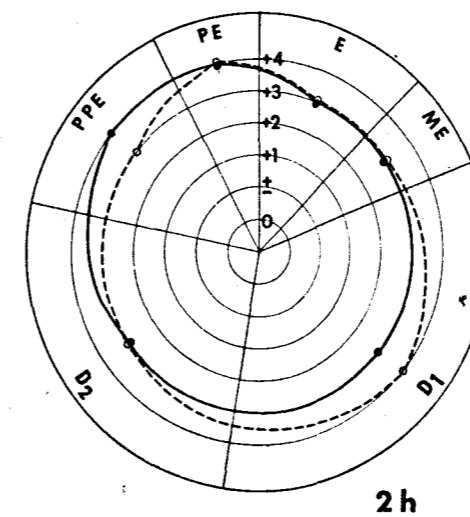
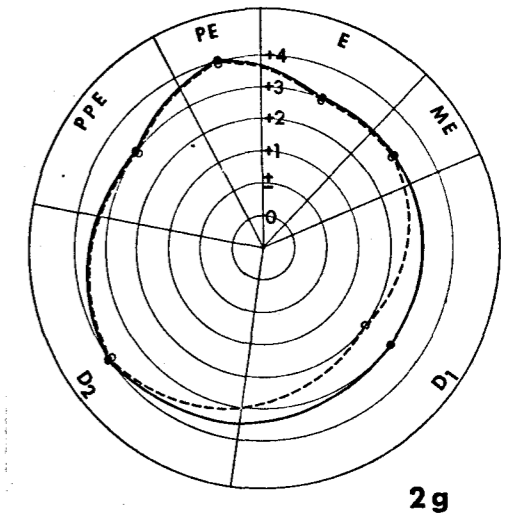
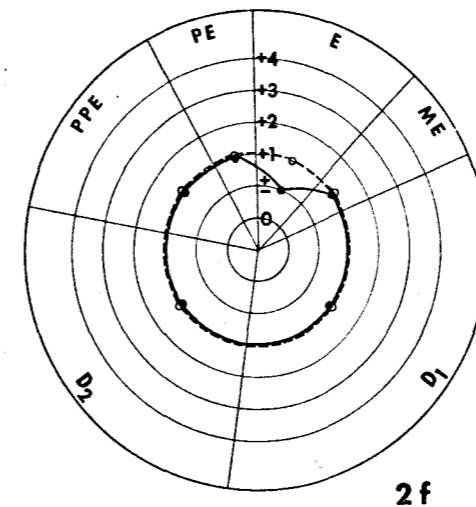
2d



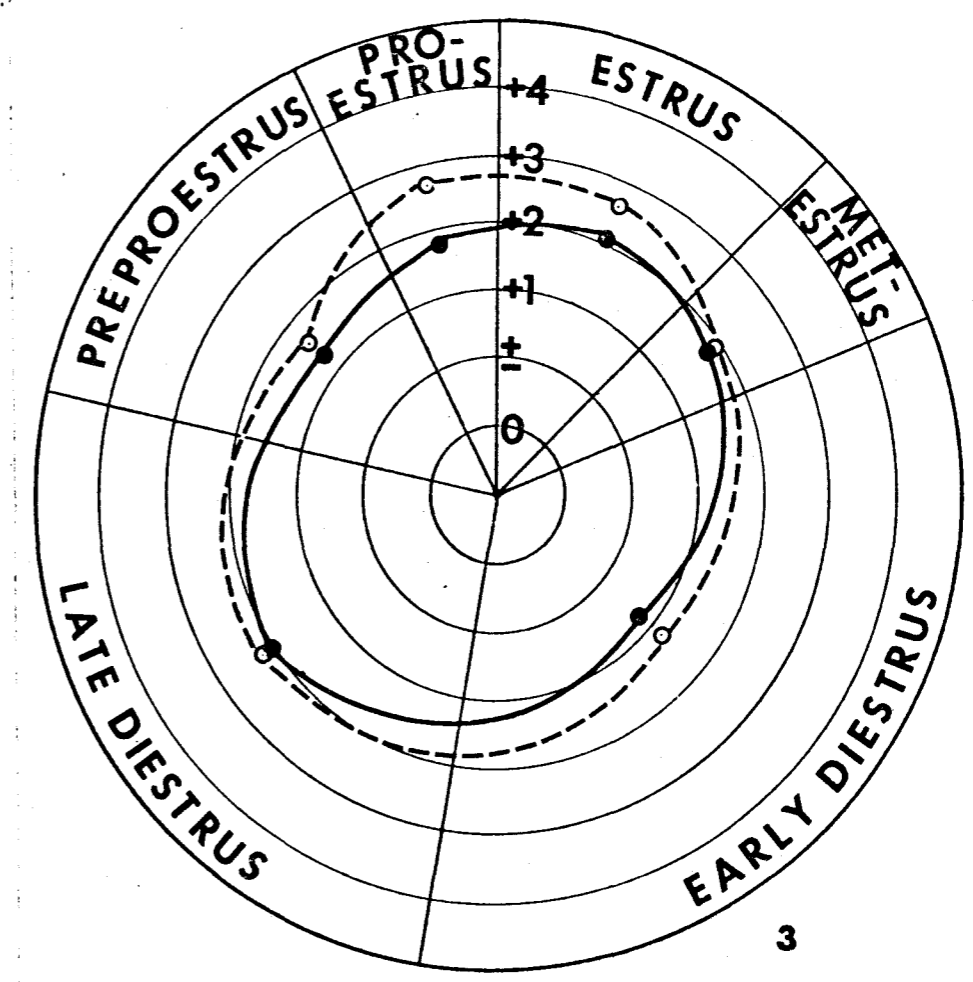
2e

Text Figures 2f-2i. Averages of semi-quantitative histochemical estimates of P-one- 3β -HSD (solid line) and DHA- 3β -HSD (dotted line) of individual ovarian components throughout the estrous cycle in the Y- series of rats:

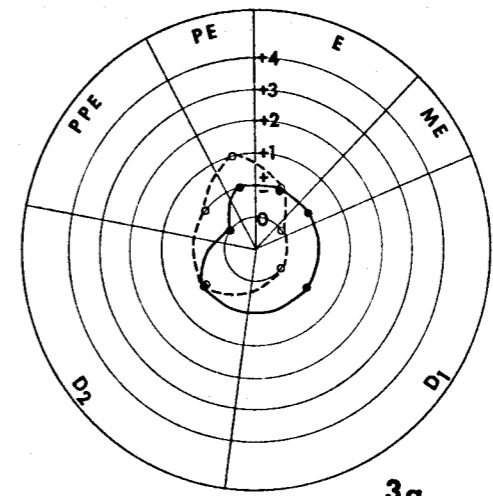
- 2f. Activities of P-one- 3β -HSD and DHA- 3β -HSD in granulosa cells of atretic follicles.
- 2g. Activities of P-one- 3β -HSD and DHA- 3β -HSD in theca interna of atretic follicles.
- 2h. Activities of P-one- 3β -HSD and DHA- 3β -HSD in ovarian interstitial tissue.
- 2i. Activities of P-one- 3β -HSD and DHA- 3β -HSD in corpora lutea.



Text Figure 3. A cyclical arrangement of the averages of semi-quantitative histochemical estimates of P-one- 3β -HSD (solid line) and DHA- 3β -HSD (dotted line) of all ovarian components of the older series of rats (R-series: 75-120 days of age) throughout the estrous cycle. Individual components are presented in similar, but individual activity wheels, in accompanying figures 3a-3i.



Text Figures 3a-3e. Averages of semi-quantitative histochemical estimates of P-one- 3β -HSD (solid line) and DHA- 3β -HSD (dotted line) of individual ovarian components throughout the estrous cycle of the R- series of rats:



3a

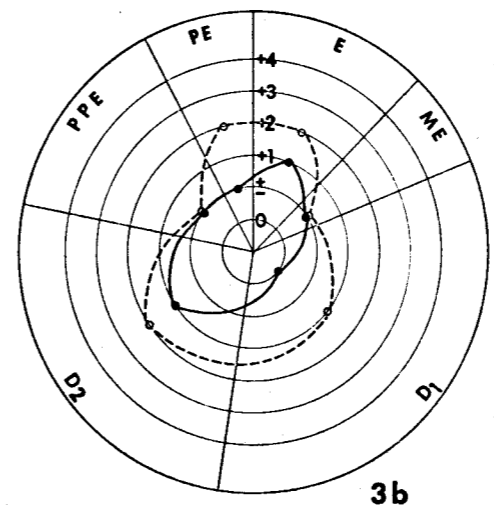
3a. Activities of P-one- 3β -HSD and DHA- 3β -HSD in primary follicles.

3b. Activities of P-one- 3β -HSD and DHA- 3β -HSD in granulosa cells of secondary follicles.

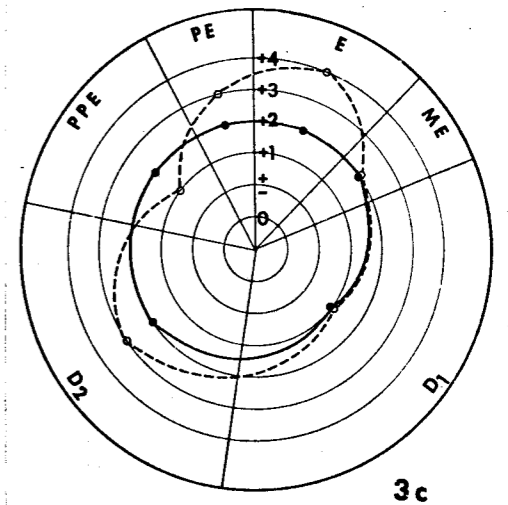
3c. Activities of P-one- 3β -HSD and DHA- 3β -HSD in theca interna of secondary follicles.

3d. Activities of P-one- 3β -HSD and DHA- 3β -HSD in granulosa cells of tertiary follicles.

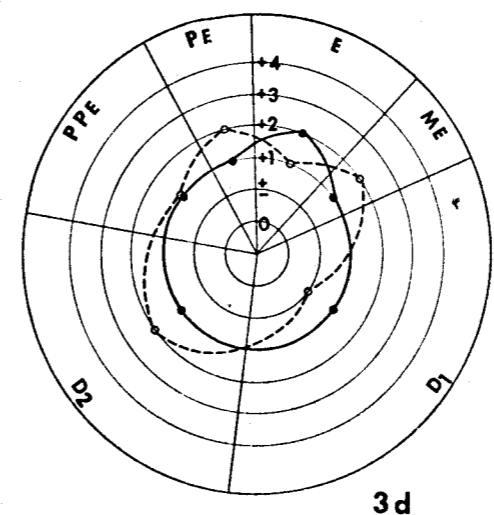
3e. Activities of P-one- 3β -HSD and DHA- 3β -HSD in theca interna of tertiary follicles.



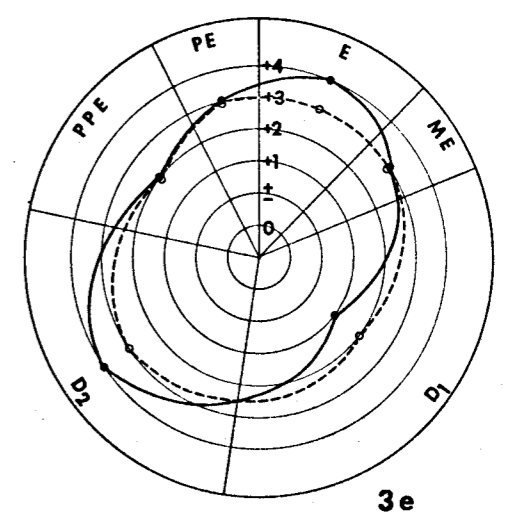
3b



3c



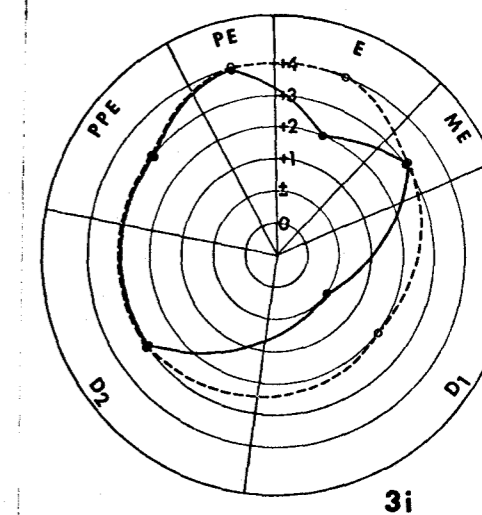
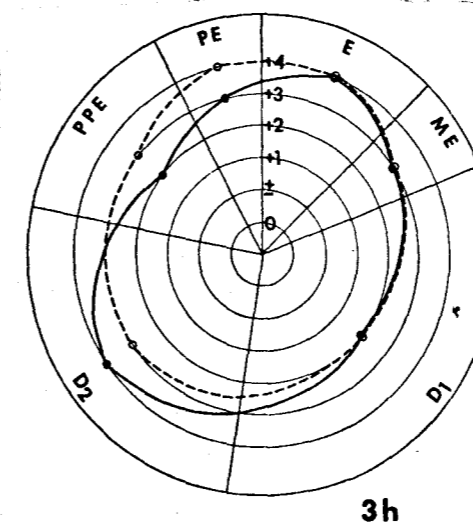
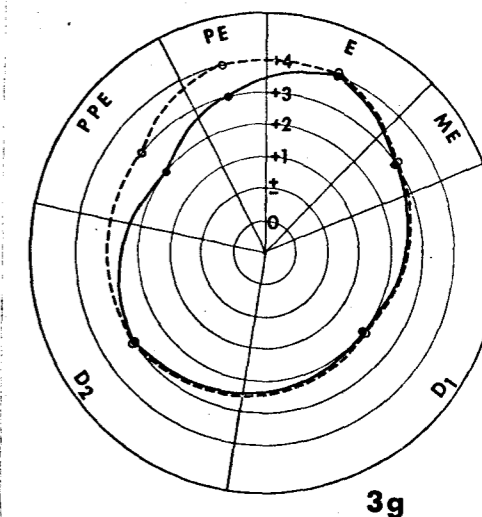
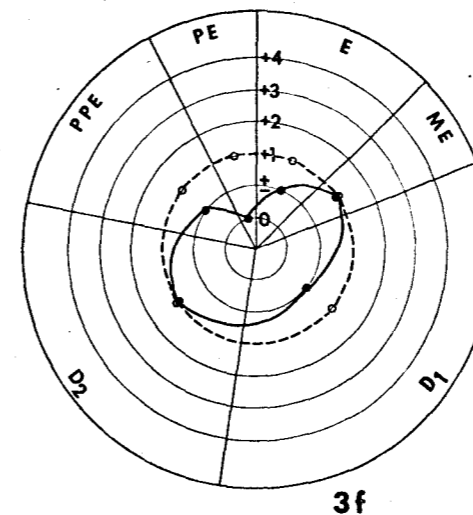
3d



3e

Text Figures 3f-3i. Averages of semi-quantitative histochemical estimates of P-one- 3β -HSD (solid line) and DHA- 3β -HSD (dotted line) of individual ovarian components throughout the estrous cycle of the R- series of rats:

- 3f. Activities of P-one- 3β -HSD and DHA- 3β -HSD in granulosa cells of atretic follicles.
- 3g. Activities of P-one- 3β -HSD and DHA- 3β -HSD in theca interna of atretic follicles.
- 3h. Activities of P-one- 3β -HSD and DHA- 3β -HSD in ovarian interstitial tissue.
- 3i. Activities of P-one- 3β -HSD and DHA- 3β -HSD in corpora lutea.



Discussion

The literature abounds with observations pertaining to steroidogenesis, with a superabundance of reports focusing on in vitro studies. These reports cover a time span of two plus decades. The advances by the numerous schools of cytochemistry and histochemistry have opened-up a new pathway within the field of in vitro studies, so much so that it now provides a means whereby one could purposefully obtain particulate data at the cellular and tissue sources of steroidogenesis. Thus, it now becomes possible to attempt a sequential analysis of the several components of the ever-changing population of ovarian structures in regard to involvement in steroidogenesis.

The histochemical techniques which attempted to localize the sites of steroid production in tissues have progressed from indirect inferences, using Sudan black B staining for lipids, through detection via phenylhydrazine reaction (Bennet, 1940) to the coupling of 3β -hydroxysteroid dehydrogenase activity with NADH_2 diaphorase activity, the latter utilizing an insoluble tetrazolium deposit at the reaction site to indicate the end-point of the reaction (Wattenberg, 1958). Wattenberg's method is based on the transfer of hydrogens from a substrate via the co-enzyme NAD to the reduction of an aqueous soluble, colorless tetrazolium salt to a colored, insoluble end-point indicator which is precipitated at the tissue site of the initial oxidation reaction. Using modifications of Wattenberg's technique for the cellular localization of steroids via 3β -hydroxysteroid:NAD oxidoreductase (3β -hydroxysteroid dehydrogenase, 3β -HSD) activity, numerous investigations in animals and man, have attempted to localize the sites of steroid hormonal production. Both pregnenolone and dehydroepiandrosterone, as precursors of progesterone and estrogens, have been utilized in a number of different conditions in the experimental animal, i. e. gonadotropic hormonal stimulation, adrenalectomy, hypophysectomy, pregnancy, etc., with a view

toward ascertaining some distinctive lines of evidence pointing up the role of precursor substances in (a) optimal physiologic conditions, (b) less than optimal conditions, and (c) super-imposed physiologic expressions. Unfortunately, it is not possible to decode such information owing chiefly to our hitherto lack of solid, base-line control data. Thus, the present study attempted to determine in a sequential manner the initial appearance, the changing intensities, and the minima and maxima of each of the steroids investigated by these cytochemical means. Giving impetus to these studies is the general lack of consolidated information pertaining to the ovarian localizations of appearances and intensities as a function of each distinctive phase of the estrous cycle in the albino rat.

Initial histochemical reports on steroid production indicated localization principally and gradation secondarily of DHA-3 β -HSD activity in the different ovarian structures of the rat (Levy et al., 1959). Confirmation of the role of DHA as a precursor of estrogens was borne out in specific double isotopic dilution experiments of Kalvert and Bloch in 1968. These workers reported that ovarian tissue of rats in estrus appeared more active than tissue in rats during diestrus, thus indicating a decreasing potential for ovarian estrogenic synthesis as the cycle proceeds from estrus to diestrus. From those studies which combined the isolation and culture of the follicular components of the ovary with in vitro biosynthesis of ovarian steroids, it was found that: (a) DHA is not converted to estradiol-17 β by isolated granulosa cells which were removed from equine ovaries (Ryan et al., 1968); (b) the isolated granulosa cells can synthesize progesterone and DHA from acetate and cholesterol precursors in the equine ovary (Channing, 1969a and 1969b; Channing and Grieves, 1969); and (c) both the thecal components as well as the granulosa cells can synthesize estrogens from the precursors pregnenolone and progesterone (Ryan and Short, 1965). Our findings on the sites of

progesterone and estrogen production in the several distinctive components of the primary-, secondary- and tertiary follicles indicate that the thecal- and granulosa-follicular components have the potential for synthesizing these hormones. Of the two components, the theca interna appears to be more responsive than the granulosa cells to both of the precursors used, i. e. P-one and DHA. Further, these data show an increase in the potential for steroidal production as the secondary follicles grow and mature to the tertiary stage.

Significant is the direct relation of these observations with the currently held concept that both the granulosa and thecal cells are required for the synthesis of estrogens. The studies of Falck (1959) and Bjersing and Carstensen (1967) have given direct confirmatory reports which indicate the contiguity of granulosa and thecal cells is required for estrogenic hormonal production. The latter workers (Bjersing and Carstensen, 1967) suggested that certain necessary steroidal intermediates are synthesized by the granulosa cells, while other such intermediates are synthesized by the thecal cells, thus hypothesizing that both intermediates are needed for estrogenic production by the follicles. These data from the current study, when viewed in light of this theory, indicate that the granulosa cells, as well as the cellular components of the theca interna have the potential to synthesize estrogenic and progestational hormones in the in vivo condition.

As the follicles degenerate and become atretic, there arises a question concerning the function of the cellular components of the granulosa and theca interna layers of such follicles. Moderate intensities of diformazan material were observed in DHA- 3β -HSD incubates in the granulosa cells of atretic follicles in the ovary of the rat (Levy et al., 1959). Motta and Bourneva (1970a and 1970b), however, reported from histochemical work on the rat ovary, a weak or negative reaction in the granulosa cells as well as the

theca interna cells of atretic follicles using P-one and DHA precursors. These findings indicate that both the cellular components of the granulosa and the theca interna are capable of synthesizing estrogens as well as progesterone using DHA and P-one precursors respectively. In the degenerating granulosa cells, the intensity of the responsiveness to DHA and P-one ranges between negative to weakly active. Thus, the granulosa cells appear to be relatively non-responsive to both steroidal precursors.

Regarding the cellular components of the theca interna of follicles undergoing different degrees of atresia, these findings indicate a marked potential for both DHA- and P-one- 3β -HSD activity. There is a difference between the two age groups of rats used in this study with respect to the stages in the cycle when the diformazan intensity appears to be moderate and maximal. P-one- 3β -HSD activity seems to be more active during late diestrus and proestrus in those animals between 41-65 days of age. The potential for P-one- 3β -HSD activity was maximal during estrus in rats 75-120 days old (R- series). Only a moderate potential for P-one- 3β -HSD activity was observed during early diestrus and preproestrus in the R- series of animals.

Chatterton, Chatterton and Greep (1969), using pooled ovarian slices which were incubated with labeled DHA, reported that the conversion of DHA- ^3H to estradiol- 17β was greatest in ovaries from rats in proestrus, less from those in estrus, and lowest in those in metestrus and diestrus. Interestingly enough, the localization studies, when summarized and assessed (a) as a function of the entire ovarian population, and (b) as separate ovarian morphological entities throughout the cycle, herein reported, reveal that: (a) the results for DHA are quite dramatic, the peak results being manifested in ovaries of proestrous animals: (1.) in the Y- series, it was determined that $\text{PE} > \text{D}_2 > \text{PPE} > \text{D}_1 > \text{E} > \text{ME}$; (2.) in the R- series, it was ascertained that $\text{PE} > \text{E} > \text{D}_2 > \text{ME} > \text{PPE} > \text{D}_1$; (b) similarly, the DHA-activation-localization studies

and analyses, when summarized as a function of separable ovarian components, present rather decisive manifestations, with prominent maximal activity being found in the cellular components that are either frank interstitial tissue or destined to become interstitial tissue: (1.) in the Y- series, the interstitial tissue was clearly the most intense and the thecal cells of atretic follicles, likewise were of maximal intensity; (2.) in the R- series, again it was observed that the interstitial tissue and the thecal cells both manifested maximal DHA activities. Although not entirely surprising, it was quite noticeable, too, that the first generation corpora lutea were on occasion maximal in DHA-expression, a fact shared in common with the earlier studies of Dawson and Velardo (1955) in which it was shown that cholesterol and its esters were positive in the corpora lutea of rats.

Rennels (1951) utilizing a number of basic techniques including Sudanophilia and birefringence, reported that the interstitial tissue of ovaries which were removed from infantile and juvenile rats contain histochemically reactive substances indicative of steroidal compounds. Pupkin *et al.* (1966) histochemically observed that the interstitial tissue is reactive when incubated with DHA, thus indicating estrogenic hormonal production. More recently, Motta and Bourneva (1970a and 1970b; and Motta, Takeva and Bourneva, 1970), following a histochemical study on the ovary of the rat using both DHA and P-one substrates, reported that the cells of the interstitial tissue show a strong reactivity to P-one- 3β -HSD during estrus and metestrus, whereas DHA- 3β -HSD remains at a constant level of moderate activity during estrus, metestrus, diestrus, and proestrus. These data, from the present study, involving DHA and P-one indicate that the interstitial tissue for the most part manifests a strong to maximal intensities, notable exceptions appearing in the R- group, wherein moderate reactions were observed for DHA in early diestrus and preproestrus, and for P-one in preproestrus.

Savard et al. (1965) reviewed the relationship between the gonadotropic hormones and ovarian steroidogenesis. They emphasized that steroid synthesis occurs in the ovarian follicle, luteal cells and the interstitial tissue, and that steroid production in the ovary is markedly influenced by the gonadotropic hormones. Utilizing gas-liquid chromatographic techniques involving electron capture detection, Feder et al. (1968) reported that plasma levels of progesterone removed from cycling rats were maximal ($3.1 \pm 0.41 \mu\text{g}/100 \text{ ml. plasma}$) during the pre-ovulatory stage of the cycle and fell to levels below $1.18 \pm 0.24 \mu\text{g}/100 \text{ ml. plasma}$ until the afternoon of proestrus. Thus, it appears from these findings that there is a continual secretion of progesterone from the ovaries during the estrous cycle as has been demonstrated following the administration of adenohipophyseal FSH and LH and ovarian progestational hormones.

A relatively recent development with regard to quantitation, purification and isolation of gonadotropic hormones and steroids is the application of immunochemical techniques to endocrinological investigations. From an immunofluorescence study on the localization of LH in the rat, Monroe and Midgley (1969) reported specific positive fluorescence in the cytoplasm of cells situated both within and peripherally located in the pars distalis of the hypophysis, but, unfortunately, this gonadotropic hormone was not so localized within the ovary, uterus and adrenal glands. Schneider et al. (1970) followed the plasma titers of LH, progesterone and 20α -hydroxypregn-4-en-20-one in female rats between 12:00 - 6:00 in the afternoon in proestrous animals; they reported that the highest mean plasma concentration of LH was $359 \pm 95 \text{ ng./ml. plasma}$ and occurred at 5:00 whereas progesterone titers began to rise only subsequent to increases in plasma LH levels. They concluded that no increase in progesterone secretion occurs prior to LH release on the afternoon of proestrus. Likewise, Eder and Lipner (1970), using the techniques of radioimmunoassay and metabolic clearance with labeled

Iodinated LH, on cycling female rats, found that the concentration of LH in the systemic blood was equivalent to 6 ng. equivalent/ml. during diestrus and metestrus and rose to a high of 300 ng. equivalent/ml. on the afternoon of proestrus. Further they reported that the secretory rate for plasma LH was 1 ng. equivalent/minute during diestrus and 30 ng. equivalent/minute during the ovulatory surge. These recent findings, utilizing the techniques of immunochemistry, have shown the relatively small quantities of plasma hormonal concentrations which are required in the in vivo state to maintain normal cycling of the adult female rat.

The application of histochemistry to the ovarian steroid localization studies have shown that the luteal cells which were removed from the ovaries of normally cycling rats were observed to increase in lipid material in the 4 to 8 day old corpora lutea (Lobel et al., 1961). From a histochemical study on the changes which occur in the ovaries of rats of advancing chronological ages (22-120 days old), Rubin et al. (1963) reported potential DHA-3 β -HSD activity was first observed in the luteal cells of animals 36 days of age. Following an electron microscopic study on ovarian corpora luteal cells of the rat, the organelles which are associated with steroidal production, i. e. tubulo-vesicular internal membranes of the mitochondria and an abundant granular endoplasmic reticulum, were found in luteal cells (Flerko et al., 1967). McDonald et al. (1969) combined biochemical and ultrastructural techniques in an experiment to find the sites of production and the amount of secreted progesterone from the ovaries of rats during the estrous cycle. Using biochemical means they observed two peaks of progesterone secretion by the ovary during the stages of estrus and diestrus. At this time the Golgi apparatus was highly developed in the luteal cells. During proestrus, there was detectable progesterone in the ovarian venous blood ; however, the Golgi apparatus in the luteal cells was beginning to fragment and degenerate. The data

obtained from this current study indicates a cyclicity in both potential DHA- and P-one- 3β -HSD activity during the estrous cycle of the rat. The maximal levels for estrogenic production seems to occur during late diestrus, pre-proestrus and proestrus in the younger rats whereas, in the slightly older series of animals, maximal potential DHA- 3β -HSD activity was observed during proestrus and estrus. Our data on progesterone synthesis appears to corroborate those of McDonald *et al.*, and the recent immunochemical studies on the progesterone plasma levels during the estrous cycle of the rat that maximal levels of P-one- 3β -HSD activity are manifested during estrus and late diestrus in the R- series of rats, whereas in the Y- series, maxima occur during metestrus and proestrus.

In the rat, no one to date has reported any data on DHA and P-one- 3β -HSD activity in either the primary follicles or the ovarian hilus. This study indicates that minimal to no activity is visible using histochemical techniques in the primary follicles. It is of further interest to comment that the only observable sites of reactivity to DHA and P-one in the ovarian hilar area were within the interstitial tissue cells which were present in this region.

The histochemical localization of the ovarian estrogens and progesterones have provided a striking example of the basic concept that differentiation and maturation of a cell involves further specialization. These findings illustrated that both progestational as well as estrogenic hormones are synthesized in highly specialized cells of the ovary, especially since the cellular components of the primary follicles, generally, manifests very little evidence of steroidogenic activity. On the other hand, the secondary follicles do indeed show heightened activity especially in the thecal component. The tertiary follicle, however, in which the granulosa cells are further matured and appear in contiguity with theca interna cells, manifested marked steroidogenic biosynthetic capabilities, this function being highly observable in the theca

interna cells of the vesicular follicle. Likewise, the interstitial tissue and the structures destined to become interstitial tissue also show definite steroidogenic capabilities. Further, the superimposition of the activity evidenced in all of the ovarian components during a given stage of the estrous cycle has provided additional pertinent information on the relative ability of the ovary to produce both estrogens and progesterones, thus emphasizing that the rat is primarily an estrogenic animal which also has a cyclic ability to synthesize moderate amounts of P-one-associated progestational hormones. This study gives added credence to the currently held concept that the hormones act in a delicately balanced system to govern the alterations which occur during normal reproductive cycles and is consonant with the general concept that the ovarian steroidal hormones act in concert (Hisaw et al., 1954; Velardo and Kaspro, 1965; Velardo and Sturgis, 1955; Velardo, 1958, 1959, 1960, 1965, and 1970).

Summary and Conclusions

In order to critically assess the histochemical sites and alterations in ovarian biosynthetic pathways of progesterone and estrogen, the estrous cycle of rats was timed and subdivided into six distinctive stages: estrus (E), metestrus (ME), early diestrus (D_1), late diestrus (D_2), preproestrus (PPE), and proestrus (PE). Sprague-Dawley derived rats were of two age groups: young adults (Y, 41-65 days) and slightly older animals, with regularly established reproductive history (R, 75-120 days). 3β -Hydroxysteroid:NAD oxidoreductase (3β -HSD) activity was studied using pregnenolone (P-one) and dehydroepiandrosterone (DHA) substrates; ovarian $NADH_2$ diaphorase activity also was assessed. Enzymatic activity was rated on a 0 to +4 scale. Analyses of 3β -HSD revealed: 1) +4 for P-one in corpora lutea (C. L.) at E and interstitial tissue (I. T.) at D_1 and PPE in group R as well as in C. L. at

ME, D₂ and PE in group Y; 2) +4 using DHA in C.L. at PPE, D₂ and PE in group Y; 3) 0 to \pm intensities for DHA and P-one in primary follicles in E, ME, D₁, D₂, PPE, and PE in groups Y and R; 4) +1 to +3 theca interna (thecal) cells and \pm to +2 granulosa cells for DHA and P-one in all follicles excepting primary in groups Y and R; 5) +2 to +3 thecal cells and \pm to +2 granulosa cells in tertiary follicles for DHA in groups Y and R, whereas P-one in thecal cells of groups E and ME were equally moderate (+2) and \pm at PE in group Y; and 6) +4 I.T. during PE and +3 at E, ME, D₁, D₂, and PPE in Y for P-one, whereas DHA was moderately to strongly intense (+2 to +3) throughout the cycle in both Y and R. NADH₂ diaphorase activity showed: 1) +4 in C.L. of Y and I.T. of Y and R in all stages; 2) +4 in C.L. in D₁ through PE>E and ME (+3) in group R; 3) in primary follicles, \pm to +2 in E through PE in groups Y and R; and 4) in thecal cells of all follicles excepting primary, +4 in all stages in groups Y and R. The sites of steroidogenic activity for estrogen and progesterone reveal different minima and maxima, owing chiefly to the physiological status of the gonads.

The histochemical manifestations of the sites of and alterations in both estrogenic and progestational hormonal production have indicated a cyclicity in the production of these hormones by the different components of the ovary of the rat. From critical observations on the sites of steroidogenesis, utilizing DHA-3 β -HSD and P-one-3 β -HSD, it has been found that each of these precursors are quite active in ovarian steroidogenesis, but not in primary follicles. The secondary follicles with the further process of follicular maturation, reveal different degrees of intensities and localizations, for each of the two substrates especially in the theca interna. Interestingly enough, the theca interna cells of the tertiary follicles and those of the atretic follicles showed marked 3 β -HSD reactivity for the estrogenic and progestational precursor substrates. The interstitial tissue has been observed to be the most

intense source of ovarian steroidal biosynthesis during the estrous cycle. Further, the cellular components of the corpora lutea were noted to be cyclical in their potential ability to synthesize estrogens and progesterones. The ovum, primary follicles and ovarian hilus were the three sites where potential steroidal production did not appear to take place in the non-manipulated, cycling albino rat. Thus, these data in the main, present overwhelming evidence that with the ongoing process of folliculogenesis, and particularly with the differentiation and maturation of the granulosa and theca interna cells, the ovarian tertiary and vesicular follicles show marked capacity for the biosynthesis of both ovarian steroid hormones - the estrogens and progesterones.

These data also present functional control, base-line studies for a systematic analysis of the several factors involved in the transition from the intact, non-manipulated estrous cycle to the ever-changing sequelae in ovarian steroidogenesis during pseudopregnancy, prolonged pseudopregnancy (i. e. pseudopregnant rats with massive experimentally-induced decidual reactions), to the frank, super-imposition of pregnancy. With the availability of such data, it should become feasible to ascertain a deeper insight into the nature of the ovarian steroidogenesis during the biological career of reproduction in the rat.

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Localization of Ovarian Dehydroepiandrosterone- 3β -Hydroxysteroid
Dehydrogenase and Pregnenolone- 3β -Hydroxysteroid
Dehydrogenase in the Rat During
Normal Pseudopregnancy

Introduction

The biosynthesis of ovarian sex steroid hormones continues to be one of the foremost areas of endocrinology pursued, particularly the aspects of cellular and tissue sources of estrogenic and progestational hormonal production. In order to obtain a comprehensive appreciation of ovarian steroid hormonal biosynthesis, it becomes of critical importance to determine the variations and differences during such super-imposed states as pseudopregnancy, with and without decidual tissue, and during pregnancy as well. The present arbeit develops upon the central theme of ovarian biosynthetic steroid hormonal cellular and tissue localizations during pseudopregnancy.

Pseudopregnancy is that physiological state which usually occurs subsequent to sterile mating in those mammals in which copulation induces ovulation; this condition somewhat resembles that of pregnancy. The characteristics of pseudopregnancy in the rat include: 1) the presence of functional corpora lutea, 2) an interruption in the estrous cycle for an average duration of 13 days, 3) increased body weight, 4) the development and hypertrophy of mammary glands, 5) predominantly leukocytic vaginal smears, 6) mucification of the vaginal epithelium, and 7) progestational modifications of the uterine mucosa so that it is susceptible to decidual formation.

Pseudopregnancy can be induced in the rat by a number of different techniques, i. e. 1) sterile matings (Long and Evans, 1922; Shelesnyak, 1931), 2) electrical stimulation of the uterine cervix of adult female rats during estrus (Shelesnyak, 1931) and diestrus (Greep and Hisaw, 1938), 3) mechanical stimulation of the uterine cervix using a glass rod inserted into the lumen

of the vagina or uterus (Long and Evans, 1922; Meyer, Leonard and Hisaw, 1929), 4) vibratory stimulation of the uterine cervix (DeFeo, 1966), 5) a single injection of a tranquilizer, e.g. chlorpromazine (Barraclough and Sawyer, 1959), and 6) electrical stimulation of the hypothalamus (Everett and Quinn, 1966). The pseudopregnant condition is maintained by the extension of the functional life of the corpus luteum, which in the cycling rat is usually very short in duration and is categorized as having a non-functional (luteal) life.

There are two types of pseudopregnancy, one of the so-called ordinary-induced length, i.e. of 13.0 days duration and without decidual reactions, and the other of the prolonged length, i.e. beyond the normal mean of 13.0 days (i.e. up to 22 days) containing decidual tissue. In those pseudopregnant animals with decidual tissue, the length of this physiological condition has been extended so that this type of pseudopregnancy is truly designated prolonged. The pseudopregnant condition in which the animals lack decidual tissue is designated normal (ordinary-induced) (Velardo et al., 1953; Dawson and Velardo, 1955).

To date there has been only a paucity of information on the cellular and tissue sources of estrogenic and progestational hormones within the ovaries of rats during pseudopregnancy. Histochemical localization of the cells involved in the biosynthesis of ovarian estrogens and progesterone involves the assessment of the enzyme, 3β -hydroxysteroid:NAD oxidoreductase (3β -hydroxysteroid dehydrogenase, 3β -HSD), using the substrates dehydroepiandrosterone (DHA) and pregnenolone (P-one), respectively. Thus, it became of interest to histochemically assess 3β -hydroxysteroid dehydrogenase activity at specific time intervals during normal pseudopregnancy using the aforementioned steroidal precursors in order to determine which, if any, alterations occur in ovarian cellular and tissue sources of

hormonal production during this interruption of the estrous cycle, particularly in luteal tissue.

Experimental Procedures

Female albino rats were purchased from the Charles River Breeding Laboratories and were housed in animal rooms having a temperature of 72°F and a 12 hour light-dark cycle. The rats had Purina Rat Chow and water available ad libitum. The animals used in this study ranged from 88 to 95 days of age.

Daily vaginal smears were taken in order to establish the regularity of the estrous cycle. After the completion of at least three consecutive estrous cycles, pseudopregnancy was induced by vibratory stimulation of the uterine cervix with a glass rod on the afternoon of vaginal cornification (estrus). The day of vaginal cornification was designated as day 0 (zero). Day 1 (one) was characterized by the appearance of leukocytes in the vaginal smear. Two vaginal smears were taken daily for the first five days of pseudopregnancy, and thence once daily thereafter in order to maintain a continuous record of the reproductive history of these animals. Pseudopregnant animals were necropsied via decapitation on days 6, 8, 10, and 13.

The following procedures were used in handling the rats on the day of necropsy. The usual A. M. vaginal smear was taken, stained with Giemsa's solution, read, and recorded. Just prior to necropsy, the final vaginal smear was taken, the animal weighed and decapitated. Following decapitation, a ventral incision was made to expose the entire abdominal cavity and the left ovary and adrenal gland were quickly removed, trimmed of adhering adipose tissue, and frozen by submersion in cold liquid 2-methylbutane, which was chilled by being placed into a Dewar flask partially filled with liquid nitrogen. Finally, the right ovary and adrenal gland, and the uterus and vagina were

removed, cleaned and immersed in Bouin's fixative solution for histological processing. Prior to fixation, the ovary, adrenal gland and uterus were weighed on a Precision torsion balance.

The frozen ovaries were sectioned at eight microns and then incubated at 37°C for 2 3/4 hours in the media tabularized on page 15 in the section dealing with the estrous cycle. The histochemical techniques, which were utilized in this portion of the study, localize the histological and cellular sites of P-one-3 β -HSD and DHA-3 β -HSD activity. P-one-3 β -HSD activity is interpreted as being indicative of progesterone biosynthesis, whereas DHA-3 β -HSD activity is interpreted as indicative of estrogenic biosynthesis. Histochemical enzymatic activity was rated on a 0 to +4 scale, by determining the maximum intensity of diformazan deposited on ovarian tissue sections in a medium containing an excess of given substrate.

The right ovary, and the uterus and vagina which were removed from these animals, were dehydrated and embedded in paraffin. Sections of these organs were taken at 8 microns and stained with Harris hematoxylin and eosin for morphological confirmation of the physiological condition of pseudopregnancy.

Observations and Results

The literature contains numerous attempts to ascertain and categorize certain of the cellular and tissue sites of ovarian steroidal hormonal secretion during certain phases of the estrous cycle, prolonged pseudopregnancy and pregnancy. No one to date, however, has attempted to exhaustively pursue and analyze the ovaries of rats during the super-imposed physiological condition of normal pseudopregnancy. The present study approaches this aim and the results are ever so much more meaningful when they are finally assessed and compared with the six distinctive stages of the estrous cycle,

prolonged pseudopregnancy and pregnancy than when presented devoid of comparisons. Therefore, the ovaries of female albino rats have been studied on days 6, 8, 10, and 13 of normal pseudopregnancy in order to localize in a pin-point manner the sites of estrogenic and progestational hormonal biosynthesis by use of the substrates dehydroepiandrosterone (DHA) and pregnenolone (P-one), respectively. Consequently, this study assessed the intensity of mono- and diformazan deposits in the following ovarian components: primary, secondary- and tertiary follicles, atretic follicles, interstitial tissue, corpora lutea, and the ovarian hilus. In the secondary- and tertiary follicles, as well as the atretic follicles, the granulosa, theca interna and theca externa layers were examined to determine the sites of P-one- 3β -HSD and DHA- 3β -HSD activity.

Gravimetric Data

Analysis of the gravimetric data was pursued in order to obtain a better understanding of some of the possible, parallel changes which might occur in the ovary and adrenal glands (steroid secreting organs) and the uterus (target organ) during pseudopregnancy. Table V is comprised of ovarian, adrenal gland and uterine wet weights, expressed in mg. %, of the rats which were necropsied on days 6, 8, 10, and 13 of normal pseudopregnancy. Ovarian weights were heaviest on day 8 and lightest on day 10 of pseudopregnancy. The adrenal gland decreased in weight as pseudopregnancy progressed, i. e. heaviest on day 6 and lightest on day 13. Uterine weights fluctuated in descending order in the following manner: days 13 and 6 (\approx) > day 10 > day 8.

Histochemical Data

Plates I and II depict overviews of ovaries for the purpose of fully

TABLE V

Comparison of Ovarian, Adrenal Gland and Uterine Weights in Milligram Per Cent for the Normal Pseudopregnant Series of Animals at the Different Days of Necropsy

| Duration of Normal Pseudopregnancy | Item ¹ | Ovary | Adrenal Gland | Uterus |
|------------------------------------|-------------------|-------|---------------|--------|
| 6 Days | n | 9 | 9 | 9 |
| | \bar{X} | 16.02 | 13.40 | 131.06 |
| | σ_e | 0.64 | 0.45 | 5.63 |
| 8 Days | n | 6 | 6 | 6 |
| | \bar{X} | 17.83 | 11.52 | 110.30 |
| | σ_e | 0.36 | 0.30 | 6.25 |
| 10 Days | n | 7 | 7 | 7 |
| | \bar{X} | 13.84 | 11.24 | 122.57 |
| | σ_e | 0.99 | 0.42 | 1.97 |
| 13 Days | n | 7 | 7 | 7 |
| | \bar{X} | 17.26 | 11.16 | 131.24 |
| | σ_e | 0.50 | 0.66 | 8.76 |

¹n = the number of rats in the group.

\bar{X} = the arithmetic mean weight of the organ expressed in mg. %.

σ_e = the standard error of the arithmetic mean.

orienting the reader in regard to ovarian structures. On pages 17 and 20 in the section dealing with the estrous cycle, the interrelationships between the intensity of diformazan deposition and the substrates used in the incubation media were discussed. P-one - 3β -HSD sites were the loci where diformazan granulation was least intense while NADH_2 diaphorase activity was maximally depicted by the intensity of diformazan deposits.

Figure 104, Plate XV, is a low power photomicrograph of a section of a histochemical control ovary which was incubated in a medium lacking steroidal substrate and NADH_2 . Neither mono- nor diformazan materials were present in any of the histochemical controls (Plate XV, fig. 104a).

In the incubation series using NADH_2 substrate, the ovarian structures are intensely outlined (Plate XV, figs. 105-111). Figure 105, Plate XV shows a region of the ovary in the NADH_2 diaphorase series in which the majority of the ovarian components studied are depicted and figs. 106-111, Plate XV are a series of photomicrographs of each of the ovarian components taken at a higher magnification which better illustrates the sites and intensities of the diformazan deposits present in this substrate series. Both mono- and diformazan materials are more intense in this series than in incubates using P-one or DHA. Table VI contains a summary of the average semi-quantitative histochemical estimates of NADH_2 diaphorase activity in the ovarian components during normal pseudopregnancy. The primary follicles varied between moderate (+2) to weakly positive (+1) intensities of diformazan granulation (Plate XV, fig. 106). Intracellular diformazan deposition appeared moderately intense in the granulosa cells of secondary and tertiary follicles (Plate XV, figs. 106 and 107) as well as in the granulosa cells of atretic follicles (Plate XV, figs. 108 and 109). Strong (+3) NADH_2 diaphorase activity was localized in the theca interna cells of secondary follicles (Plate XV, fig. 106) while maximal (+4) intensities of diformazan deposits were noted in the theca

TABLE VI

Averages of Semi-Quantitative Histochemical Estimates of 3β -Hydroxysteroid: NAD Oxidoreductase (3β -HSD) Localization Using Two Substrates, Pregnenolone (P-one) and Dehydroepiandrosterone (DHA), and Reduced Nicotinamide Adenine Dinucleotide Diaphorase (NADH_2 Diaphorase) in the Ovarian Components of the Normal Pseudopregnant Series of Rats on Days 6, 8, 10, 13.*

| Ovarian Components | 6 Days | | | 8 Days | | | 10 Days | | | 13 Days | | |
|--------------------|--------|-----|--------------------|--------|-----|-----------------|---------|-----|-----------------|---------|-----|-----------------|
| | P-one | DHA | NADH_2 ** | P-one | DHA | NADH_2 | P-one | DHA | NADH_2 | P-one | DHA | NADH_2 |
| Primary Follicle | 0 | ± | +2 | 0 | 0 | +2 | ± | 0 | +1 | 0 | 0 | +1 |
| Secondary Follicle | | | | | | | | | | | | |
| Granulosa Cells | +2 | +1 | +2 | +1 | ± | +2 | +1 | +1 | +2 | ± | ± | +2 |
| Thecal Cells | +3 | +1 | +3 | +2 | +1 | +3 | +2 | +2 | +3 | ± | +2 | +3 |
| Tertiary Follicle | | | | | | | | | | | | |
| Granulosa Cells | +2 | +1 | +2 | +1 | +2 | +2 | +2 | +1 | +2 | +1 | +1 | +2 |
| Thecal Cells | +4 | +2 | +4 | +3 | +3 | +4 | +3 | +2 | +4 | +2 | +3 | +4 |

*6 to 8 ovaries were used in each time interval of normal pseudopregnancy except day 8 which included 5 ovaries.

**P-one designates the sections which were incubated with the substrate 3β -hydroxypregn-5-en-20-one (pregnenolone).

DHA designates the sections which were incubated with the substrate 3β -hydroxyandrost-5-en-17-one (dehydroepiandrosterone).

NADH_2 designates the sections which were incubated with the substrate reduced nicotinamide adenine dinucleotide ($\text{NADH} + \text{H}^+$).

TABLE VI CONTINUED

| Ovarian Components | 6 Days | | | 8 Days | | | 10 Days | | | 13 Days | | |
|---------------------|--------|-----|----------------------|--------|-----|-------------------|---------|-----|-------------------|---------|-----|-------------------|
| | P-one | DHA | NADH ₂ ** | P-one | DHA | NADH ₂ | P-one | DHA | NADH ₂ | P-one | DHA | NADH ₂ |
| Atretic Follicle | | | | | | | | | | | | |
| Granulosa Cells | ± | +1 | +2 | 0 | ± | +2 | ± | ± | +2 | ± | +1 | +2 |
| Thecal Cells | +4 | +2 | +4 | +3 | +3 | +4 | +3 | +3 | +4 | +2 | +3 | +4 |
| Interstitial Tissue | +4 | +2 | +4 | +3 | +3 | +4 | +3 | +2 | +4 | +3 | +4 | +4 |
| Corpus Luteum | +4 | +3 | +4 | +3 | +3 | +4 | +2 | +4 | +4 | +1 | +4 | +4 |
| Hilar Region | 0 | 0 | +1 | 0 | 0 | +2 | 0 | 0 | +1 | 0 | 0 | +1 |

*6 to 8 ovaries were used in each time interval of normal pseudopregnancy except day 8 which included 5 ovaries.

**P-one designates the sections which were incubated with the substrate 3 β -hydroxypregn-5-en-20-one (pregnenolone).

DHA designates the sections which were incubated with the substrate 3 β -hydroxyandrost-5-en-17-one (dehydroepiandrosterone).

NADH₂ designates the sections which were incubated with the substrate reduced nicotinamide adenine dinucleotide (NADH + H⁺).

interna cells of tertiary follicles (Plate XV, fig. 107) and in atretic follicles (Plate XV, figs. 108 and 109). The interstitial tissue and corpora lutea consistently contained maximal (+4) diformazan deposits (Plate XV, figs. 110 and 111). In the NADH_2 diaphorase series, the substrate NADH_2 was in excess in the incubation media so that the maximally possible intensity of diformazan could be observed and assessed in these ovarian tissue sections.

Primary Follicles

Primary follicles were assessed during normal pseudopregnancy using P-one and DHA as substrates, and were found to show, at most, trace color intensities (\pm) as evidenced by the presence of only monoformazan material on day 10 with P-one and on day 6 with DHA. Intensities of both P-one- 3β -HSD and DHA- 3β -HSD were extremely weak and could hardly be detected (Plate XVIII, fig. 128; Plate XIX, fig. 134; and Plate XX, fig. 148). The absence of any tetrazolium material during the remaining stages indicated that progesterone as well as estrogen was probably not synthesized during these times. Therefore, it appears that the primary follicles are not a source of ovarian estrogen(s) and progesterone biosynthesis as studied under the conditions of normal pseudopregnancy.

Secondary Follicles

Ova. The ova observed in primary-, secondary-, and tertiary follicles seemingly lacked both mono- and diformazan reaction products (Plate XVI, fig. 116; Plate XIX, fig. 134; Plate XX, fig. 141). Therefore, it was not possible to detect any positive indication for either estrogenic or progestational hormone production, using these histochemical techniques, during normal pseudopregnancy.

Granulosa cells. The granulosa and thecal cells of secondary follicles

were seen to vary in intensity in mono- and diformazan deposits during the advancing days of normal pseudopregnancy studied. Usually, the granulosa cells were observed to be less responsive to both P-one and DHA substrates than the thecal cells. The granulosa cells were found to have moderate P-one- 3β -HSD activity on day 6 (Plate XVI, fig. 113; Plate XVIII, fig. 129), weakly positive (+1) activity on days 8 and 10 (Plate XVI, fig. 115; Plate XVII, fig. 118), and trace (\pm) activity on day 13 (Plate XVIII, fig. 123). Thus, the granulosa cells of secondary follicles appear to be only weakly positive for P-one- 3β -HSD during normal pseudopregnancy.

Utilizing a second substrate DHA, in companion ovarian sections, the granulosa cells were examined for the possible detection of DHA- 3β -HSD which is indicative of estrogenic biosynthesis. It was found that DHA- 3β -HSD activity appeared alternately in weakly positive (+1) and trace (\pm) intensities during normal pseudopregnancy (Plate XIX, figs. 134 and 137; Plate XX, figs. 140 and 142; Plate XXI, figs. 140 and 142; Plate XXI, figs. 145 and 151).

Thecal cells. The intensity assessments reported in the text, recorded in the tables and illustrated in the pictorial plates for the thecal cells (T. I.) refer to the reaction products examined in the cellular components of the theca interna, because at best, only minimal to negative reactive materials were observed in the cellular aspects of the theca externa. P-one- 3β -HSD activity was strong (+3) on day 6 (Plate XVI, fig. 113; Plate XVIII, fig. 129), decreased to moderate (+2) on days 8 and 10 (Plate XVI, fig. 115; Plate XVII, fig. 118), and then decreased to trace (\pm) intensities on day 13 (Plate XVIII, fig. 123). Thus, these data indicate a reduction in potential progesterone biosynthesis in these cellular components with the ongoing process of normal pseudopregnancy up until the day of termination.

In addition to these studies with P-one, the theca interna cells of

secondary follicles were similarly examined for the possible detection of DHA- 3β -HSD. The intensity of diformazan deposits were found to be weakly positive (+1) on days 6 and 8 (Plate XIX, figs. 134 and 137; Plate XXI, fig. 145) and increased to moderate (+2) on days 10 and 13 of normal pseudopregnancy (Plate XX, figs. 140 and 142; Plate XXI, fig. 151). Therefore, it appears that the theca interna cells of secondary follicles show an increasing ability for estrogen production as normal pseudopregnancy progresses.

Tertiary Follicles

Granulosa cells. Using the P-one precursor as the incubation media substrate, it was ascertained that the granulosa cells of tertiary follicles variably displayed moderate (+2) to weakly positive (+1) reactions as normal pseudopregnancy advanced. On day 6, the granulosa cells of the tertiary follicles appeared to have weakly positive intensities (Plate XVI, figs. 112 and 113; Plate XVIII, fig. 130). The color intensity decreased to weakly positive (+1) on day 8 (Plate XVI, figs. 115 and 116) and increased to moderate levels (+2) on day 10 (Plate XVII, fig. 117). On the final day of normal pseudopregnancy studied, P-one- 3β -HSD appeared as weakly positive (Plate XVII, fig. 120; Plate XVIII, fig. 124). The enzymatic activity in the granulosa cells of tertiary follicles appears to be only slightly higher than in the secondary follicles.

Using DHA as the substrate, the granulosa cells were observed to show a weakly positive reaction (+1) on day 6 (Plate XIX, figs. 134 and 135; Plate XXI, fig. 146), day 10 (Plate XXI, fig. 139) and day 13 (Plate XX, fig. 142); a +2, the highest observed for this substrate in those cells in tertiary follicles during pseudopregnancy, occurred on day 8 of pseudopregnancy (Plate XIX, fig. 136; Plate XXI, fig. 152). With respect to DHA- 3β -HSD, the granulosa cells of tertiary follicles show an increase in enzyme reaction

products formed which indicates an alteration in enzymic capability for potential estrogenic production.

Thecal cells. The theca interna cells were examined for evidence of P-one- 3β -HSD. Whereas maximal (+4) intensities were observed on day 6 (Plate XVI, figs. 112 and 113; Plate XVIII, fig. 130), reactions of lower, but strong to moderate, were observed on days 8 and 10 (+3) (Plate XVI, figs. 115 and 116; Plate XVII, fig. 117), and on day 13 (+2), respectively (Plate XVII, figs. 120 and 121; Plate XVIII, fig. 124). Thus it appears that the theca interna varies between maximal and moderate intensities, indicative of rather marked progesterone production. Further, potential progesterone biosynthesis in the theca interna cells decreases as pseudopregnancy progresses, indicating that these ovarian components are capable of greatest progesterone production during the earliest days of pseudopregnancy.

With respect to DHA- 3β -HSD, the theca interna cells were observed to vary in the intensity of the reaction product formed. Moderate (+2) intensities were observed on days 6 (Plate XIX, figs. 134 and 135; Plate XXI, fig. 146) and 10 (Plate XX, fig. 139) of pseudopregnancy, whereas strong (+3) intensities were observed on days 8 (Plate XIX, fig. 136; Plate XXI, fig. 152) and 13 (Plate XX, fig. 142). These findings indicate that the thecal cells of tertiary follicles are importantly involved in estrogen production, particularly since the thecal cells studied were in contiguity with granulosa cells.

Atrretic Follicles

Granulosa cells. The granulosa cells of atrretic follicles were examined and showed less mono- and diformazan deposits than the cells of the theca interna, when incubated with both P-one and DHA substrates. Using P-one, which is indicative of possible progesterone production, trace reactions of diffuse pink monoformazan (\pm) were observed on days 6, (Plate XVI,

fig. 112; Plate XVIII, fig. 131), 10, (Plate XVII, fig. 118) and 13 (Plate XVII, fig. 120; Plate XVIII, fig. 125) of pseudopregnancy. A negative reaction was noted on day 8 in the granulosa cells of atretic follicles (Plate XVI, fig. 116). Thus, these data tend to support the idea that the granulosa cells of atretic follicles seem to have little, if any, part in progestational hormone biosynthesis.

The response of the granulosa cells to DHA also showed very little detectable activity. Weakly positive (+1) intensities were observed on day 6 (Plate XIX, fig. 134; Plate XXI, fig. 147), and day 13 (Plate XX, fig. 142; Plate XXI, fig. 153). Trace reaction (\pm) intensities were seen on days 8 (Plate XIX, figs. 136 and 137) and 10 (Plate XX, fig. 141) of pseudopregnancy. The data obtained using both P-one and DHA substrates, indicate that the granulosa cells of atretic follicles are not very responsive to the precursors of progestational and estrogenic hormones.

Thecal cells. P-one- 3β -HSD was observed to be maximal (+4) on day 6 of pseudopregnancy (Plate XVI, fig. 112; Plate XVIII, fig. 131). Strong intensities of diformazan granules were noted on days 8 (Plate XVI, fig. 116) and 10 (Plate XVII, fig. 118). On day 13, moderate (+2) intensities of reaction product were observed in the thecal cells (Plate XVII, fig. 120; Plate XVIII, fig. 125). These findings indicate that the theca interna cells of atretic follicles are strongly reactive to pregnenolone, the progestational hormonal precursor, particularly during days 6, 8 and 10 of pseudopregnancy.

In a companion study, using DHA substrate, the theca interna cells showed strongly intense (+3) diformazan reaction products on days 8 (Plate XIX, figs. 136 and 137), 10 (Plate XX, fig. 141) and 13 (Plate XX, fig. 142; Plate XXI, fig. 153). Moderate (+2) intensities of diformazan deposits were observed in thecal cells on day 6 (Plate XIX, fig. 134; Plate XXI, fig. 147). Thus, the theca interna cells appear to be highly responsive to the precursor

substrate, indicative of estrogenic hormonal production during the pseudo-pregnant condition.

Interstitial Tissue

The interstitial tissue appears to be one of the most reactive cellular components studied during pseudopregnancy. The reaction product color intensities ranged between maximal (+4) and strong (+3) when the ovarian sections were incubated with P-one. The maximal peak intensities were observed on day 6 (Plate XVI, fig. 112; Plate XVIII, fig. 132), while strong intensity reactions were seen on the remaining days studied, *i. e.* days 8 (Plate XVI, figs. 115 and 116), 10 (Plate XVII, figs. 117 and 118) and 13 (Plate XVII, fig. 120; Plate XVIII, fig. 126). These findings support the idea that the interstitial tissue is very responsive to the progesterone precursor, pregnenolone, and, thus, is involved in progesterone production.

Utilizing the precursor DHA, the cells of the interstitial tissue were observed to contain maximal (+4) to moderate (+2) intensities of diformazan deposits. Maximal intensities were identified on day 13 (Plate XX, figs. 142 and 143; Plate XXI, fig. 152). The interstitial tissue cells showed strongly intense diformazan deposits on day 8 (Plate XIX, fig. 136). Moderately intense (+2) reaction material was observed on days 6 (Plate XIX, fig. 134; Plate XXI, fig. 148) and 10 (Plate XX, fig. 139). Thus, these data indicate that the interstitial cells are involved in estrogenic hormonal biosynthesis.

Corpus Luteum

As in the previous study involving the estrous cycle, histochemical tests utilizing P-one and DHA substrates were performed on the corpora lutea during normal pseudopregnancy. Further, since pseudopregnancy is maintained in rats by eliciting functional corpora lutea, the lutein tissue was

carefully examined for sites of P-one- 3β -HSD, as well as DHA- 3β -HSD. In the P-one series, it was observed that the intensities of diformazan deposits ranged from maximum (+4) to weakly positive (+1). Maximal peak intensities were found on day 6 (Plate XVI, fig. 114; Plate XVIII, fig. 133); strong intensities were noted on day 8 (Plate XVI, figs. 115 and 116). A reduction in diformazan intensity to moderate (+2) intensities were observed on day 10 (Plate XVII, fig. 119). On day 13, the luteal cells had reaction product color intensities of weakly positive (+1) levels (Plate XVII, fig. 121; Plate XVIII, fig. 127). Thus, these observations show that luteal capability to synthesize progesterone diminishes as pseudopregnancy progresses.

In another study involving the DHA substrate, the corpora lutea were similarly analyzed for cellular and tissue sites of estrogenic hormonal production. Maximal intensities were observed on days 10 (Plate XX, figs. 139-141; Plate XXI, fig. 155) and 13 (Plate XX, fig. 143). Strong intensities of diformazan granules were seen on days 6 (Plate XIX, fig. 134; Plate XXI, fig. 149) and 8 (Plate XIX, fig. 138). From these data on the histochemical assessments of P-one- 3β -HSD and DHA- 3β -HSD of the corpora lutea, it seems that the biosynthesis of progestational and estrogenic hormones is very active during normal pseudopregnancy.

Ovarian Hilus

From examination of sections of the ovarian hilar region, it was found that this area was consistently negative for both P-one- 3β -HSD and DHA- 3β -HSD reaction products (Table VII; Plate XXI, figs. 157 and 158). The only positive sites of diformazan deposition were located in the interstitial tissue cells. Those series of ovaries which had been incubated with NADH + H⁺ were seen to contain moderate to weakly positive reaction products (Plate XXI, fig. 156). Therefore, the consistent absence of any mono- or diformazan

deposits in the hilar area indicate that this region is seemingly devoid of activity pertaining to estrogenic and progesterational hormonal biosynthesis.

Comparative Analysis of Total Ovarian Composites

vs

Individual Ovarian Entities: An Overview

For ready comparison and simplification of and an overall comprehension of the total potential biosynthetic capabilities which occur within the entire ovarian population, it was of interest to ascertain and graph the arithmetic averages of the data obtained for each ovarian component studied. The vertical averages are given in Table VII. The linear arrangement of the data is depicted in a major text figure, which is followed-up by a series of nine graphs, each representing specific ovarian morphological entities, cf. Text figures 4, 4a-4i.

Discussion

Interest in steroidogenesis has generated a wealth of information, particularly in studies using in vitro techniques. Through the development and refinement of histochemical and cytochemical methodology, data regarding the tissue and cellular sources of steroid production have become possible. Histochemical and cytochemical reports, involved in eliciting accurate observations regarding the ability of the ovary to synthesize estrogenic and progesterational hormones, are numerous and have been principally concerned with hormonal production during specific stages of the estrous cycle and on given days of prolonged pseudopregnancy and pregnancy. No one to date, however, has pursued a critical examination of ovarian estrogenic and progesterational hormonal biosynthetic sites during normal pseudopregnancy in rats of known reproductive history.

TABLE VII

Generalized and Summarized Data Based on all Ovarian Components
Presented as Averages during Normal Pseudopregnancy¹

| Enzymes* | <u>6 Days</u> ** | <u>8 Days</u> | <u>10 Days</u> | <u>13 Days</u> |
|----------------------|------------------|---------------|----------------|----------------|
| P-one-3 β -HSD | 2.6 | 1.8 | 1.8 | 1.0 |
| DHA-3 β -HSD | 1.4 | 1.7 | 1.7 | 2.0 |

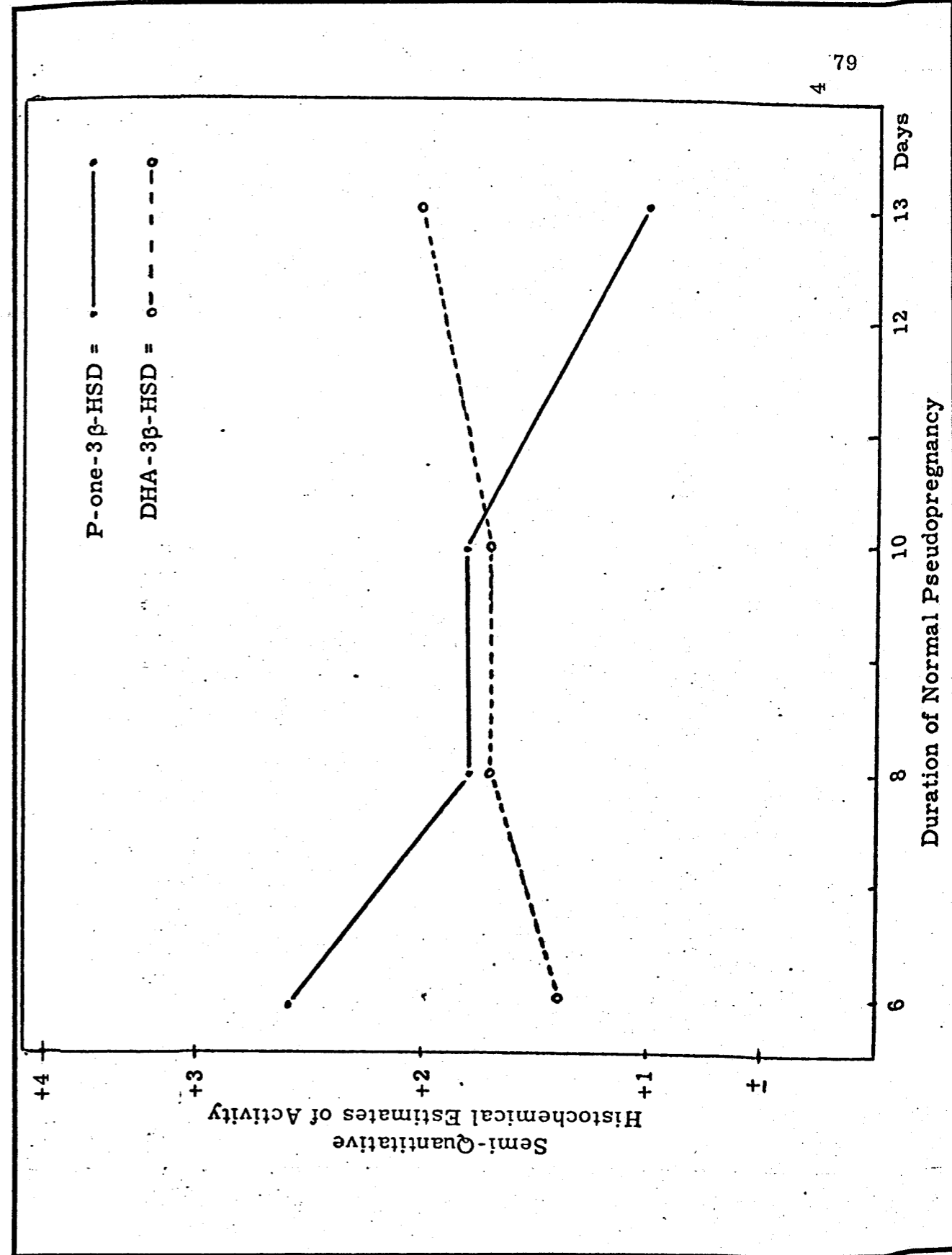
¹Arithmetic mean semi-quantitative histochemical estimates of 3 β -hydroxysteroid: NAD oxidoreductase (3 β -HSD) reactivity which were derived from the follicular, luteal and interstitial components of the ovaries in normal pseudopregnancy as observed during the four days studied.

*P-one-3 β -HSD denotes 3 β -HSD intensities using pregnenolone (P-one) as the incubation substrate.

DHA-3 β -HSD denotes 3 β -HSD intensities using dehydroepiandrosterone (DHA) as the incubation substrate.

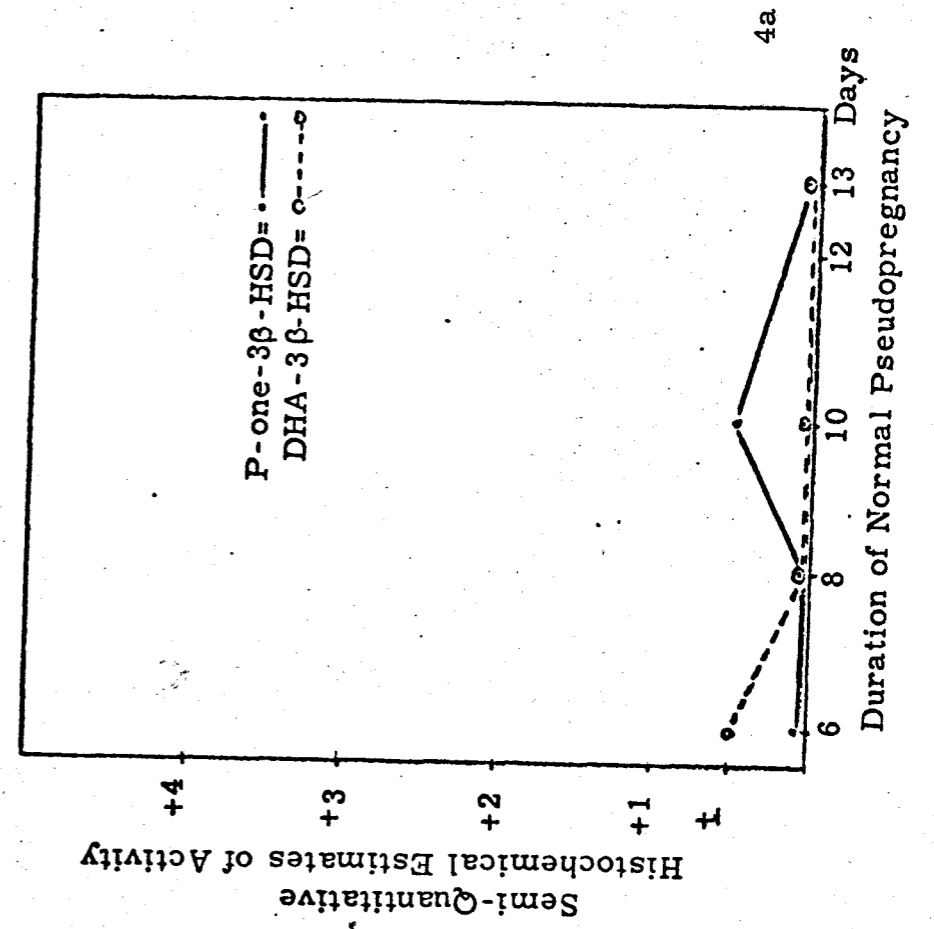
Estimates were rated on a 0 to +4 scale and a trace amount designated '+±'.

Text Figure 4. A linear arrangement of the averages of semi-quantitative histochemical estimates of P-one-3 β -HSD (solid line) and DHA-3 β -HSD (dotted line) of all ovarian components throughout the days of normal pseudopregnancy studied. Individual components are presented in similar, but individual activity graphs, in accompanying figures 4a-4i.

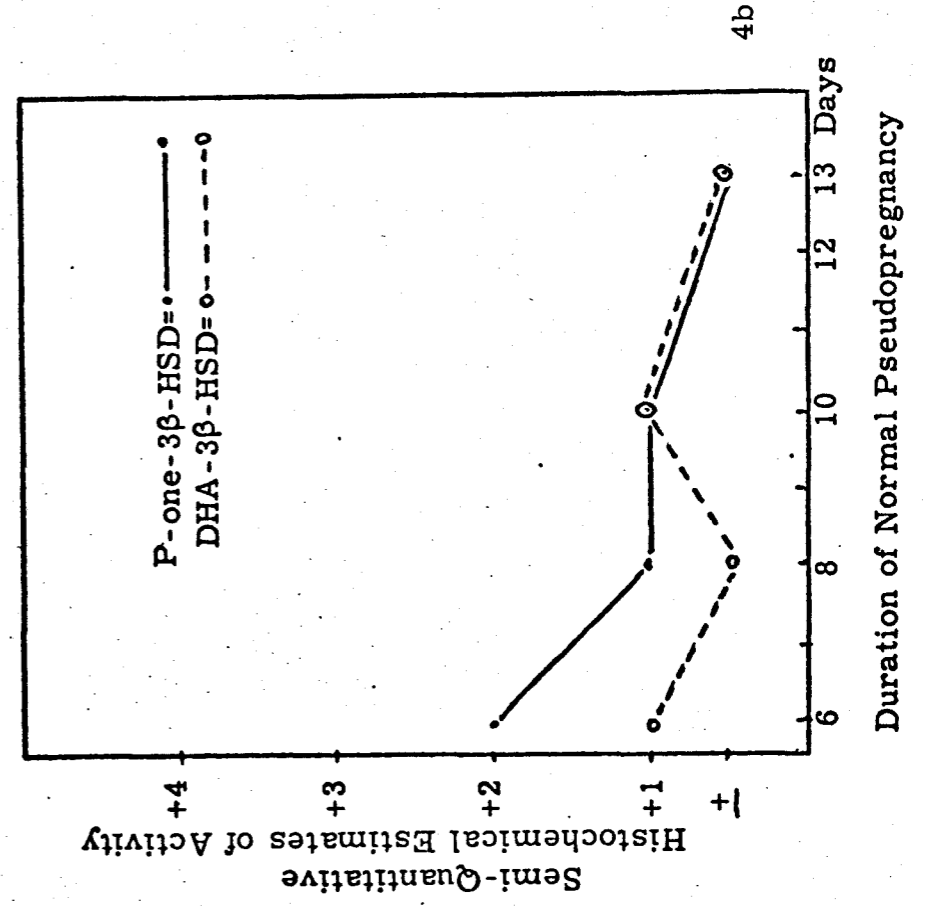


Text Figures 4a-4i. Averages of semi-quantitative histochemical estimates of P-one- 3β -HSD (solid line) and DHA- 3β -HSD (dotted line) of individual ovarian components throughout the days of normal pseudopregnancy studied:

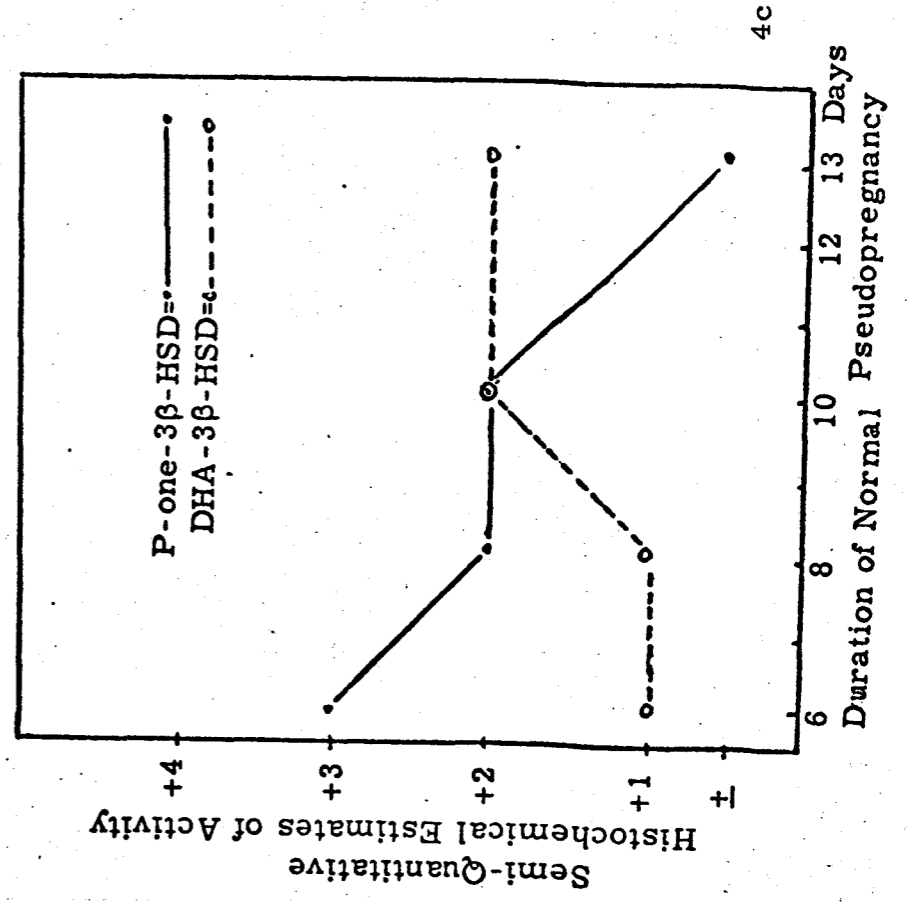
4a. Activities of P-one- 3β -HSD and DHA- 3β -HSD in primary follicles.



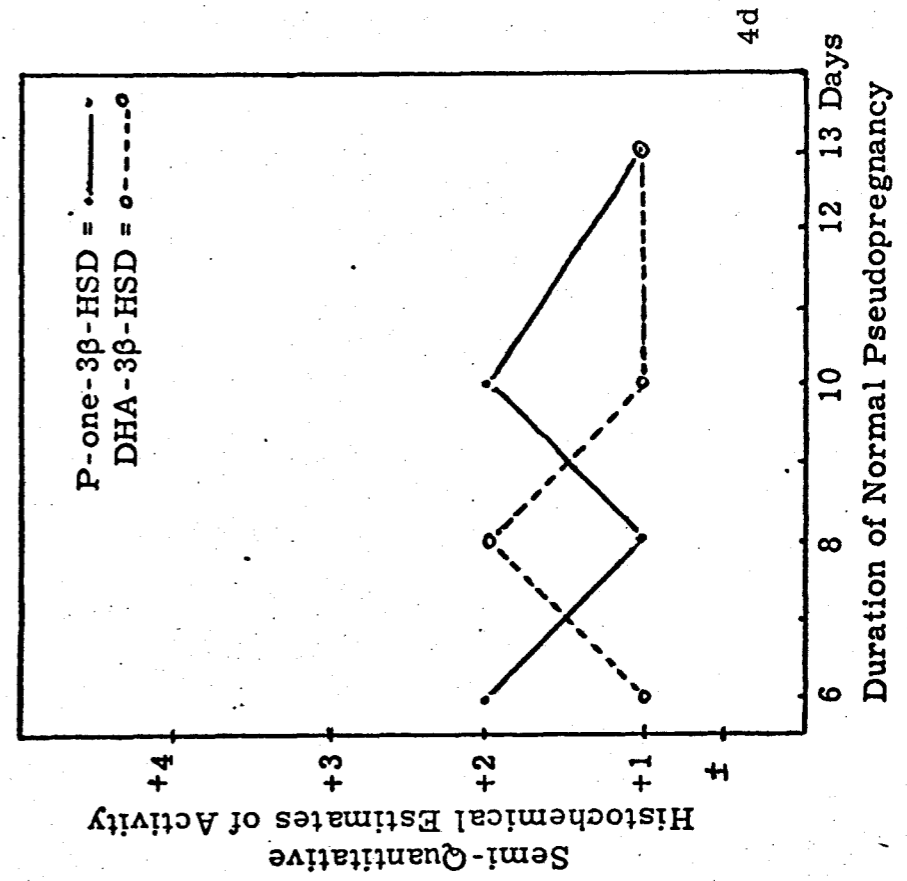
4b. Activities of P-one- 3β -HSD and DHA- 3β -HSD in granulosa cells of secondary follicles.



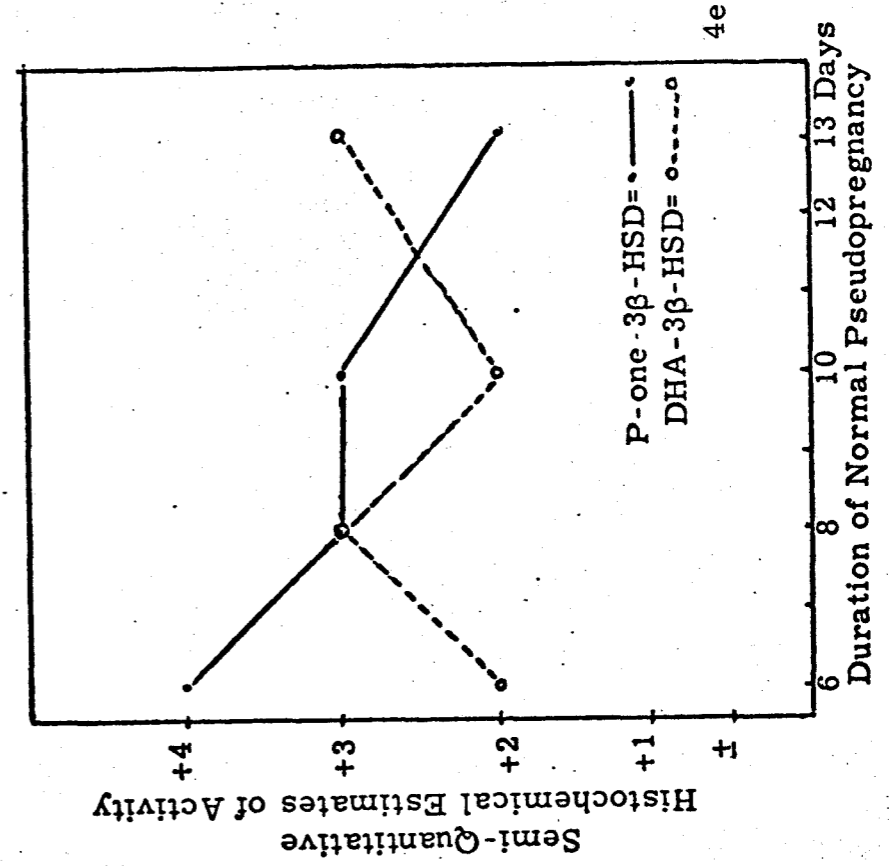
4c. Activities of P-one- 3β -HSD and DHA- 3β -HSD in theca interna of secondary follicles.



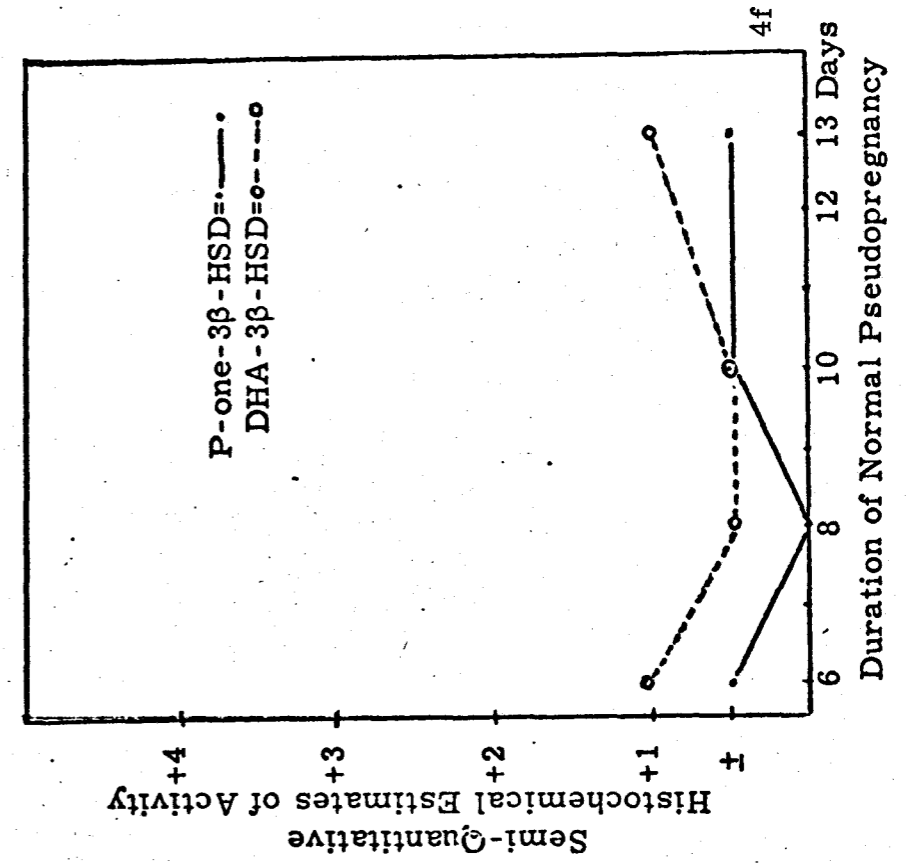
4d. Activities of P-one- 3β -HSD and DHA- 3β -HSD in granulosa cells of tertiary follicles.



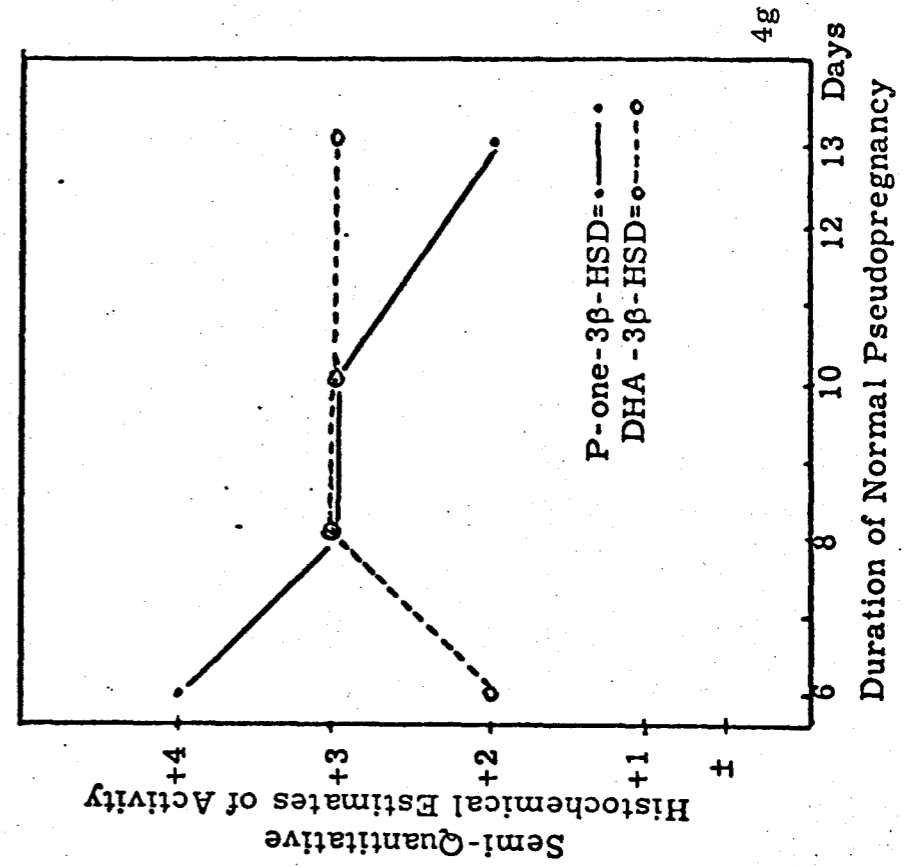
4e. Activities of P-one- 3β -HSD and DHA- 3β -HSD in theca interna of tertiary follicles.



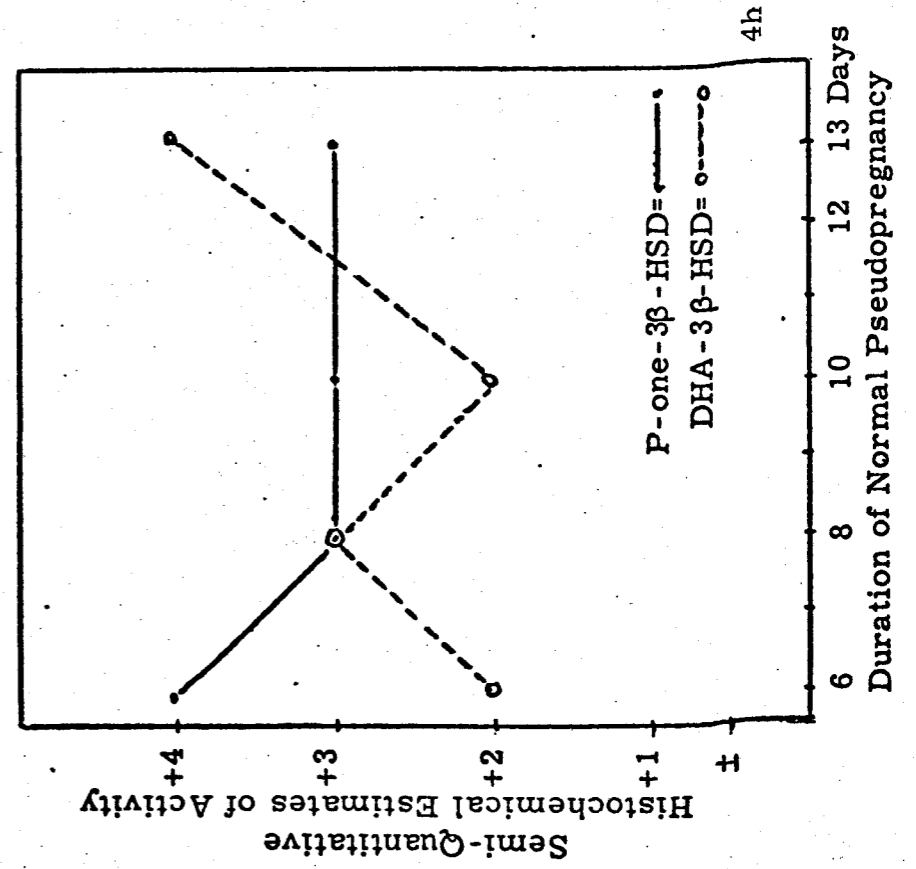
4f. Activities of P-one- 3β -HSD and DHA- 3β -HSD in granulosa cells of atretic follicles.



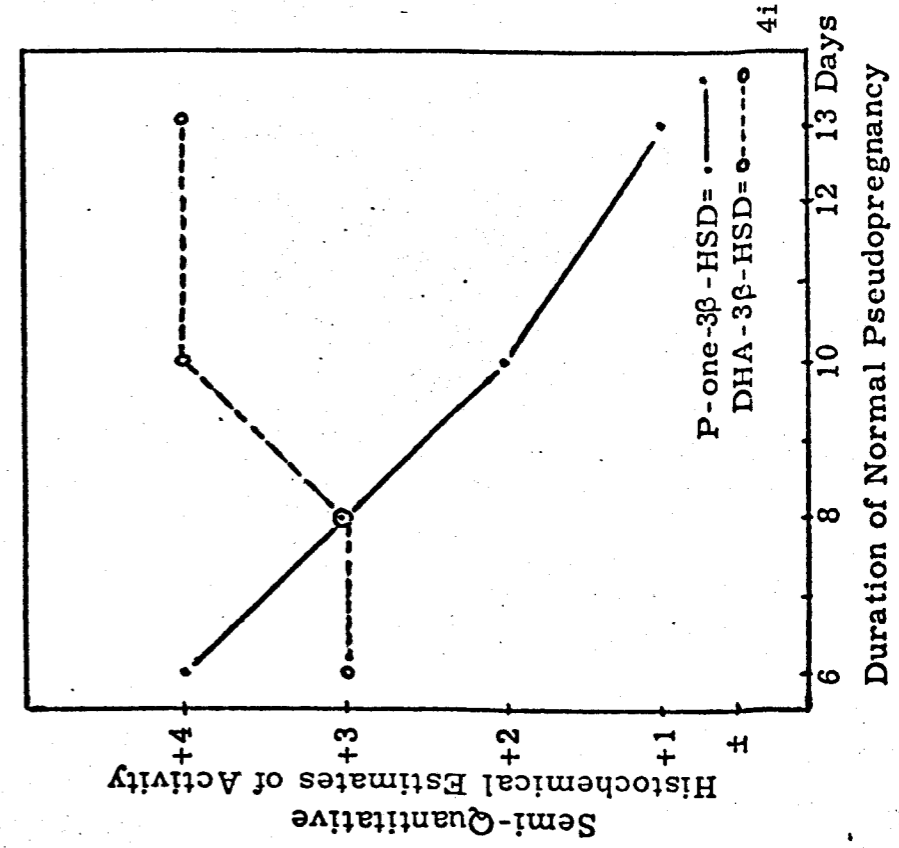
4g. Activities of P-one-3 β -HSD and DHA-3 β -HSD in theca interna of atretic follicles.



4h. Activities of P-one- 3β -HSD and DHA- 3β -HSD in ovarian interstitial tissue.



4i. Activities of P-one- 3β -HSD and DHA- 3β -HSD in corpora lutea.



The problem of steroidal biosynthesis can be studied histochemically by the application of Wattenberg's technique (1958) which localizes 3β -hydroxysteroid:NAD oxidoreductase (3β -HSD) activity in tissue sections, by incubating these sections in a medium containing a steroidal precursor, a co-enzyme which serves as an electron carrier, an end-point indicator, and an aqueous buffer of known pH strength. The substrates pregnenolone (P-one) and dehydroepiandrosterone (DHA) have been shown to be indicative of progestational and estrogenic hormonal production, respectively. Thus, the present study attempted to determine, in a sequential manner, the cellular sites and intensities of the enzyme P-one- 3β -HSD and DHA- 3β -HSD in the ovarian population, as well as the minima and maxima of each of these enzymes using a modification of Wattenberg's technique (cf. experimental procedures in the estrous cycle section).

Biochemical reports on progestational and estrogenic hormonal values in ovarian tissue and/or ovarian venous blood titers during normal pseudopregnancy are scarce. Using micro-determinations on pooled ovarian venous blood during proestrus, estrus, diestrus and mid-pseudopregnancy, progesterone concentrations were found to be highest during mid-pseudopregnancy ($15 \mu\text{g/g}$ ovarian weight) when compared with the estrous cycle values (Linder and Zmigrod, 1967). Progesterone secretion was reported to increase on days 4, 5, 7 and 9 of pseudopregnancy with maximal ovarian secretion on day 4, as determined by thin layer chromatographic and spectrophotometric measurements of pooled ovarian venous blood samples (Fajer and Barraclough, 1967). The summarized data, which presents the overall potential for progestational hormone production from this current histochemical study, is comprised of averages of semi-quantitative histochemical estimates indicating that progesterone appears to be synthesized to a higher extent on day 6 of pseudopregnancy than during any stage of the estrous

cycle. Further, potential ovarian progestational biosynthesis declines to values which are comparable to the stages of metestrus and proestrus in the R- series of animals on days 8 and 10 of normal pseudopregnancy. The lowest histochemically estimatable progesterone production occurs on day 13 which is at a level between those occurring during early diestrus and preproestrus of the estrous cycle in the R- series, otherwise known as rats manifesting matured estrous cyclicity. Thus, it is not surprising that prograavid endometrial reactions cannot be induced during the estrous cycle.

An over-view of estrogenic hormonal production by the entire ovarian population in this study showed maximal intensities of DHA- 3β -HSD on day 13 at a level comparable to values noted during metestrus and late diestrus in the R- series of animals. On day 6 DHA- 3β -HSD activity was at the lowest level observed during this physiological condition and could be compared to preproestrus during the estrous cycle of the R- series of rats.

Our findings on the sites of progestational and estrogenic production in the distinctive components of the primary-, secondary-, and tertiary follicles, indicate that the granulosa and thecal elements of follicles have a marked, albeit variable potential for synthesizing these hormones. As was the case in the estrous cycle data, the theca interna cells appear to be more responsive to the steroidal precursor substrates than are the granulosa cells. The mature tertiary follicles appear to be the most responsive of the developing follicular population in their potential for steroidal production.

With the super-imposition of normal pseudopregnancy on the base-line studies involving the estrous cycle, it is of interest to compare the histochemical intensities observed in the aforementioned follicular population with respect to P-one- 3β -HSD and DHA- 3β -HSD localizations. P-one- and DHA- 3β -HSD, as previously discussed and as found during the estrous cycle are more intensely localized in the following manner: tertiary follicles >

secondary follicles > primary follicles. Interestingly enough, potential progesterational hormonal biosynthesis in pseudopregnancy is maximal on day 6 in both secondary and tertiary follicles, and minimal on day 13. DHA- 3β -HSD results appear to be relatively stable during normal pseudopregnancy, i. e. tertiary follicles on day 8 showed an increase in estrogenic hormonal production, whereas secondary follicles on this same day decline in observable enzymatic localization intensities. Thus, the follicular elements as a group did not fluctuate appreciably with respect to estrogenic and progesterational potential.

Regarding the granulosa and thecal elements in follicles undergoing atresia, the theca interna cells were observed to have a marked potential for steroidal biosynthesis. P-one- 3β -HSD activity was maximal on day 6 and declined to moderate levels on day 13. Conversely, DHA- 3β -HSD activity was observed to be maximal on day 13 and moderate on day 6. These findings indicate that the theca interna cells of atretic follicles have the capability to synthesize both steroidal hormones and may be one of the more stable facets with respect to the steroidogenic functions of the ovarian population.

Pursuing the analysis of the alterations in the ovarian components, the interstitial tissue cells were observed to show maximal to strong intensities of P-one- 3β -HSD activity; maximal on day 6 and strong on the remaining days of pseudopregnancy. DHA- 3β -HSD activities ranged from maximal (day 13) to moderate (days 6 and 10). These data on normal pseudopregnancy, when viewed in light of the findings reported for the estrous cycle, indicate that the interstitial tissue cells, for the most part, seem to have a potential for continuous steroidal production during the functional life of the ovary i. e. during the adult stage of the life of the female.

A hallmark histochemical study on the role of the corpus luteum was

reported in the mid 1950's by Dawson and Velardo on ovaries of animals during the estrous cycle, normal pseudopregnancy and prolonged pseudopregnancy. These researchers observed that the corpora luteal cells showed increased lipid concentrations, although the tests for presence of cholesterol were negative during normal pseudopregnancy. They pointed out that:

1) cholesterol storage may be associated with corpus luteum regression and loss of function and 2) during pseudopregnancy, the newly formed corpora lutea have a lengthened and functional life span.

From the current histochemical study, the corpora luteal cells were observed to be maximally intense for P-one- 3β -HSD on day 6 and decreased in a stepwise manner to weakly positive (+1) on day 13 of normal pseudopregnancy. Further, from the studies using DHA as substrate of choice, it was found that, with the ongoing process of pseudopregnancy, the luteal cells exhibited a marked ability to synthesize estrogenic hormones. These findings indicate that the luteal cells show an extension of the enzymic potential for both estrogenic and progesterational hormonal production during the physiological condition of normal pseudopregnancy.

These data indicate that both estrogenic and progesterational hormones are synthesized by the ovary during normal pseudopregnancy. Using the criteria of a target organ reflecting the action of the hormones, it was observed that during pseudopregnancy vaginal mucification occurs (Hisaw et al., 1928; Velardo et al., 1953a). Velardo et al. (1953b) ovariectomized pseudopregnant rats and injected progesterone or progesterone and estradiol- 17β ; vaginal mucification was found to be comparable to intact pseudopregnant rats in the series of animals which were treated with both progesterone and estradiol- 17β . These data point-up the concept that the hormones act together in a delicately balanced system in order to produce the observable changes which occur during the estrous cycle, pseudopregnancy and pregnancy.

Summary and Conclusions

Normal pseudopregnancy was induced in mature estrous rats by vibratory stimulation of the uterine cervix in order to assess the histochemical and cytochemical sites and alterations in ovarian biosynthesis of progesterone and the estrogens during this physiological condition. 3β -Hydroxysteroid:NAD oxidoreductase (3β -hydroxysteroid dehydrogenase, 3β -HSD) activity was studied using pregnenolone (P-one) and dehydroepiandrosterone (DHA) substrates; ovarian NADH_2 diaphorase activity was also assessed. Enzymatic activity was rated on a 0 to +4 scale with a trace amount of activity being designated \pm . Animals were necropsied on days 6, 8, 10, and 13 of normal pseudopregnancy. Analyses of 3β -HSD revealed: 1) +4 for P-one- 3β -HSD in corpora lutea (C.L.), interstitial tissue (I.T.) and the theca interna cells of tertiary follicles (T.F.) and atretic follicles (A.F.) on day 6; 2) +1 for P-one- 3β -HSD in C.L. on day 13; 3) +4 for DHA- 3β -HSD in C.L. on days 10 and 13 and I.T. on day 13; 4) +2 for DHA- 3β -HSD in I.T. on days 6 and 10; 5) trace to +2 intensities in granulosa cells of all follicles except primary with P-one and DHA on days 6, 8, 10, and 13; 6) +1 to +3 thecal cells of all follicles with DHA- 3β -HSD on days 6, 8, 10, and 13; 7) negative to trace intensities for P-one and DHA- 3β -HSD in primary follicles on days studied. NADH_2 diaphorase activity showed: 1) maximal (+4) intensities of diformazan deposits in C.L., I.T. and theca interna cells in T.F. and A.F. on the days studied; 2) +3 in thecal cells in secondary follicles; 3) +2 intensities in granulosa cells of S.F., T.F. and A.F.; and 4) +1 to +2 in primary follicles. Neither the antra nor the ova were positive for either P-one- 3β -HSD or DHA- 3β -HSD. Concomitant controls without the addition of the steroidal substrates, P-one or DHA, or of co-enzyme, NAD, proved consistently negative. These data strongly support the physiological contention that the estrogens and progesterones act and interact in a delicate balance for the accommodation

of the physiological condition of pseudopregnancy as well as the normal estrous cycle in the adult rat.

These histochemical findings on the sites and intensities of the potential estrogenic and progestational hormonal biosynthesis in the ovaries of rats having on extended luteal phase indicate a fluctuation in the production of these hormones by the different components of the ovary of the rat. Analyses of these data indicate that the ovarian components, particularly the luteal cells, theca interna of tertiary follicles and atretic follicles as well as the interstitial tissue are importantly involved in and are most probably the main sites of progesterone biosynthesis on day 6 of normal pseudopregnancy. The granulosa cells of secondary-, tertiary- and atretic follicles change in their potential for steroidal production during the ongoing process of pseudopregnancy; their contribution however, to the steroidal biosynthetic process appears at best to be relatively minor. One main source of potential progestational and estrogenic production appears to be the interstitial tissue; the cells of the interstitial tissue consistently manifest a high potential for localization of diformazan which is indicative of not only steroidal capabilities, but likewise of high metabolic activity involving the electron transport system, thus adding credence to the theory that the interstitial cells of the ovary are an important biosynthetic locus within the ovary and not merely a non-functional site of atresia. With respect to DHA- 3β -HSD localization, these data indicate that estrogenic production is lower at the onset of normal pseudopregnancy and becomes markedly increased at the termination of this superimposed physiological condition.

From the detailed examination of the luteal cells during normal pseudopregnancy, this study concurs with findings of Dawson and Velardo (1955) that the luteal cells are functionally important during this physiological condition. The histochemical localization study using P-one and DHA as

substrates indicates that the luteal cells have the capability of synthesizing both progesterone and estrogens. From a careful analysis of the histochemical data, the luteal cells were found to secrete both estrogens and progesterone, but at markedly variable rates; progesterone biosynthesis is greater than estrogenic biosynthesis during the early stages of normal pseudopregnancy and estrogenic production than progesterone production during the later stages including the day of termination.

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Localization of Ovarian Dehydroepiandrosterone- and
Pregnenolone-3 β -Hydroxysteroid Dehydrogenases
in the Rat During Prolonged Pseudopregnancy

Introduction

Six and one-half decades have elapsed since the initial observations of Loeb (1907, 1908) showing that pseudopregnancy is related to induced changes in the uterine mucosa. The last two decades have interestingly recorded some cellular and biochemical changes in the reproductive tracts of animals made pseudopregnant. Of late, additional studies pertaining to cyto- and histochemical assessments of the ovaries and uteri of pseudopregnant animals have yielded important leads that indicate the changing steroidal hormonal requirements peculiar to normal and altered cycles and the superimposition of pregnancy. Histochemical studies on the ovary have been principally concerned with the changes which occur during the estrous cycle and pregnancy. More recently, studies have been extended to the pseudopregnant rat. The duration of prolonged pseudopregnancy was found to be proportional to the extent of endometrial traumatization and resulting decidual reaction (Masson, 1943; Peckham and Green, 1948; Velardo et al., 1953a and 1953b).

Studies concerning prolonged pseudopregnancy have centered around experiments involving: 1) induction techniques, which produce decidual reaction e. g. a) scratching the uterine mucosa with steel and glass rods (Rothschild and Meyer, 1942), b) inserting glass and wax beads into the uterine lumen (Blandau, 1947), c) electrically stimulating the uterine horn (Krehbiel, 1937), d) transverse clamping of the uterine horn (Shelesnyak, 1952), and e) injecting certain drugs and chemical irritants (Masson, 1943; Shelesnyak, 1954; Velardo, 1958); 2) massive decidual developmental effects, e. g. aging and necrosis studies on corpora luteal physiology, (Krehbiel, 1937;

Velardo et al., 1953a and 1953b), 3) hormonal, chemical mediation, modification, and inhibiting tests on the decidual cell response and in turn on pseudopregnancy (Velardo and Hisaw, 1951; Velardo, 1957a and 1957b; Chang et al., 1971; Shelesnyak, 1957; Ferrando and Nalbandov, 1968), and 4) hormonal replacement studies which can maintain prolonged pseudopregnancy in ovariectomized laboratory animals (Astwood, 1939; Velardo, 1957; Bradbury et al., 1950)

The endometrial changes induced in prolonged pseudopregnancy were shown by Allen and Corner (1929) and Corner and Allen (1929) to be similar to those which take place in early pregnancy in preparation for ovum implantation. Weichert (1928) observed that decidual changes may be due to the action of two ovarian hormones acting synergistically with the mechanical stimulation applied to the uterine endometrium. During prolonged pseudopregnancy, it was reported that the functional state of the corpus luteum is extended and maintained (Ershoff and Deuel, 1943). There were however, few studies on the changes which take place in the ovary during prolonged pseudopregnancy. Dawson and Velardo (1955) reported the alterations that take place in luteal lipid and cholesterol deposits during the estrous cycle, normal- and prolonged pseudopregnancy.

To date, only one study localized 3β -hydroxysteroid:NAD oxidoreductase activity in the ovaries or pseudopregnant rats, (Chang et al., 1971). The major purpose of the study by Chang and his colleagues was to histologically observe the effects of anti-LH serum on deciduomata in the pseudopregnant rats. Ovarian 3β -HSD and 20α -HSD activity was localized to indicate the changes in the steroidogenic potential of the ovaries of anti-LH treated rats and compare the overall activity with animals which were pregnant and animals in the diestrous stage of the estrous cycle. They reported that: 1) anti-LH serum significantly inhibited the decidual reaction in prolonged pseudopregnant

rats; 2) ovarian 20α -HSD activity was compared with the estrous cycle, and 20α -HSD activity was reported to be comparable to that observed during diestrus; and 3) 3β -HSD activity was tabularized as +2 and as strong as that observed during pregnancy and the substrate used to localize enzymatic activity was omitted. Further, ovarian histochemical data was tersely and incidentally reported in a tabular manner as either "average" or "strong" when compared with the pregnant condition. Thus, this current study, an integral part of the in-depth study on the ovary of the rat, will assess 3β -HSD activity on specific days during pseudopregnancy using dehydroepiandrosterone (DHA) and pregnenolone (P-one) as substrates in order to determine which, if any, alterations occur in ovarian cellular and tissue sources of estrogenic and progestational hormonal biosynthesis during this interruption of the estrous cycle.

Experimental Procedures

Mature, nulliparous, female, albino rats were purchased from the Charles River Breeding Laboratories and housed in animal rooms having a temperature of 72°F and a 12 hour light-dark cycle. The rats were given Purina Rat Chow ad libitum. The animals, at the time of necropsy ranged in age from 95-125 days.

Daily vaginal smears were taken in order to establish the regularity of the estrous cycle. After the completion of at least three consecutive estrous cycles, pseudopregnancy was induced by vibratory stimulation of the uterine cervix on the afternoon of vaginal cornification (estrus) using DeFeo's technique (1966). The last day of vaginal cornification was designated day 0 (zero). Day one (1) was characterized by the appearance of leukocytes in the vaginal smear. On day 5, the animals were anesthetized with a halothane-oxygen mixture and the uteri exposed via a lumbodorsal incision in the body

wall. A burr-tipped needle was inserted into the uterine lumen at the uterine-tubal junction and passed downward to the uterine cervix; then the needle was withdrawn slantwise so that the anti-mesometrial portion of the uterine cornua were traumatized. As a result, decidual reactions formed in these animals and the duration of pseudopregnancy was extended. Daily vaginal smears were taken until the termination of this study. Animals were necropsied on days 6, 8, 10, 13, 15, 18, 20, and 21 of prolonged pseudopregnancy.

Necropsy procedures utilized were the same as those described in experimental procedures in the section on the estrous cycle and normal pseudopregnancy.

Observations and Results

The literature on prolonged pseudopregnancy is principally related to the uterine changes which take place during this condition since these alterations resemble those which take place prior to implantation during early pregnancy. Studies have shown that critical ratios of ovarian estrogens and progesterone are required to maintain the decidual cell response during prolonged pseudopregnancy.

Little information is available on the ovarian cellular and tissue sites of estrogenic and progestational hormonal biosynthesis, by use of the substrates dehydroepiandrosterone (DHA) and pregnenolone (P-one), respectively. This present study has approached this goal and the results appear to be more meaningful when they are compared with the six distinctive stages of the estrous cycle, normal pseudopregnancy and pregnancy. Thus, in this study the intensity of mono- and diformazan deposits were assessed in the following ovarian components: primary-, secondary- and tertiary follicles, atretic follicles, interstitial tissue, corpora lutea, and the ovarian hilus.

Histochemical Data

The histochemical semi-quantitative assessments on the intensity of the reaction product formed were based on careful inspection of the incubated ovarian tissue sections. An intensity scale was arrived at from a series of experiments discussed on page 16 in the estrous cycle section. A tabular summary comprised of the intensity rating for each ovarian component at each day of necropsy during prolonged pseudopregnancy is found in Table VIII.

Plates I and II are comprised of overviews of ovaries in order to depict the relative intensities of the histochemical reactions for the enzymes studied. The control series, in which the steroidal substrates (P-one and DHA) and NADH_2 were lacking in the incubation media, did not contain either mono- or diformazan materials. This was also true in the studies of the estrous cycle and normal pseudopregnancy.

In the incubation media using NADH_2 as the substrate, the ovarian structures were intensely outlined as was likewise observed during the estrous cycle and normal pseudopregnancy (Plate XV) cf. Table VIII. The enzyme NADH_2 diaphorase was studied by incubating the ovarian tissue sections in a medium containing an excess of substrate (NADH_2) in order to observe and assess the maximally possible intensity of diformazan which could be deposited in these tissue sections.

Primary Follicles

Primary follicles were assessed during prolonged pseudopregnancy and were found to vary between minimal (trace) and weakly positive intensities when incubated with P-one. Weakly positive (+1) intensities of diformazan deposits were observed on days 8, 10, 13, and 15 of prolonged pseudopregnancy and were difficult to record photographically (Plate XXVII, fig. 186). Diffuse pink monoformazan material (+) intensities were noted on days 6

TABLE VIII

Averages of Semi-Quantitative Histochemical Estimates of β -Hydroxysteroid:NAD Oxidoreductase (β -HSD) Localization Using Two Substrates, Pregnenolone (P-one) and Dehydroepiandrosterone (DHA), and Reduced Nicotinamide Adenine Dinucleotide Diaphorase (NADH_2 Diaphorase) in the Ovarian Components of the Prolonged Pseudopregnant Series of Rats on Days 6, 8, 10, 13, 15, 18, 20, and 21.*

| Ovarian Components | 6 Days | | | 8 Days | | | 10 Days | | | 13 Days | | |
|----------------------|--------|-------|--------------------|--------|-------|-----------------|---------|-------|-----------------|---------|-----|-----------------|
| | P-one | DHA | NADH_2 ** | P-one | DHA | NADH_2 | P-one | DHA | NADH_2 | P-one | DHA | NADH_2 |
| Primary Follicle | \pm | \pm | +2 | +1 | +1 | +2 | +1 | \pm | +2 | +1 | +1 | +2 |
| Secondary Follicle * | | | | | | | | | | | | |
| Granulosa Cells | +1 | \pm | +2 | +1 | \pm | +2 | \pm | +1 | +2 | +1 | +1 | +2 |
| Thecal Cells | +3 | +2 | +3 | +2 | +2 | +4 | +3 | +2 | +4 | +2 | +2 | +4 |
| Tertiary Follicle | | | | | | | | | | | | |
| Granulosa Cells | +1 | +1 | +2 | +1 | +1 | +2 | +2 | +1 | +2 | +2 | +1 | +2 |
| Thecal Cells | +3 | +2 | +4 | +3 | +3 | +4 | +3 | +3 | +4 | +3 | +2 | +4 |

* 8 ovaries were used in each time interval of prolonged pseudopregnancy except days 8, 15 and 21 which included 4 ovaries.

** P-one designates the sections which were incubated with the substrate

β -hydroxypregn-5-en-20-one (pregnenolone).

DHA designates the sections which were incubated with the substrate

β -hydroxyandrost-5-en-17-one (dehydroepiandrosterone).

NADH_2 designates the sections which were incubated with the substrate reduced

nicotinamide adenine dinucleotide ($\text{NADH} + \text{H}^+$).

TABLE VIII CONTINUED

| Ovarian Components | 6 Days | | | 8 Days | | | 10 Days | | | 13 Days | | |
|---------------------|--------|-----|----------------------|--------|-----|-------------------|---------|-----|-------------------|---------|-----|-------------------|
| | P-one | DHA | NADH ₂ ** | P-one | DHA | NADH ₂ | P-one | DHA | NADH ₂ | P-one | DHA | NADH ₂ |
| Atretic Follicles | | | | | | | | | | | | |
| Granulosa Cells | +1 | +1 | +2 | +1 | ± | +2 | ± | +1 | +2 | +1 | +1 | +2 |
| Thecal Cells | +4 | +4 | +4 | +3 | +3 | +4 | +4 | +4 | +4 | +3 | +4 | +4 |
| Interstitial Tissue | +4 | +4 | +4 | +3 | +3 | +4 | +4 | +4 | +4 | +3 | +4 | +4 |
| Corpus Luteum | +4 | +4 | +4 | +4 | +3 | +4 | +4 | +3 | +4 | +4 | +4 | +4 |
| Hilar Region | 0 | 0 | +2 | 0 | 0 | +2 | 0 | 0 | +2 | 0 | 0 | +2 |

* 8 ovaries were used in each time interval of prolonged pseudopregnancy except days 8, 15 and 21 which included 4 ovaries.

** P-one designates the sections which were incubated with the substrate 3 β -hydroxypregn-5-en-20-one (pregnenolone).

DHA designates the sections which were incubated with the substrate 3 β -hydroxyandrost-5-en-17-one (dehydroepiandrosterone).

NADH₂ designates the sections which were incubated with the substrate reduced nicotinamide adenine dinucleotide (NADH + H⁺).

TABLE VIII CONTINUED

| Ovarian Components | 15 Days | | | 18 Days | | | 20 Days | | | 21 Days | | |
|--------------------|---------|-----|----------------------|---------|-----|-------------------|---------|-----|-------------------|---------|-----|-------------------|
| | P-one | DHA | NADH ₂ ** | P-one | DHA | NADH ₂ | P-one | DHA | NADH ₂ | P-one | DHA | NADH ₂ |
| Primary Follicle | +1 | +1 | +2 | ± | ± | +2 | 0 | +1 | +2 | 0 | ± | +2 |
| Secondary Follicle | | | | | | | | | | | | |
| Granulosa Cells | +1 | +1 | +2 | ± | ± | +2 | +1 | +1 | +2 | ± | ± | +2 |
| Thecal Cells | +2 | +2 | +4 | +2 | +1 | +4 | +2 | +2 | +4 | +2 | +1 | +4 |
| Tertiary Follicle | | | | | | | | | | | | |
| Granulosa Cells | +1 | +1 | +2 | ± | +1 | +2 | +1 | +2 | +2 | +1 | +2 | +2 |
| Thecal Cells | +2 | +2 | +4 | +2 | +3 | +4 | +2 | +3 | +4 | +2 | +3 | +4 |

* 8 ovaries were used in each time interval of prolonged pseudopregnancy except days 8, 15 and 21 which included 4 ovaries.

** P-one designates the sections which were incubated with the substrate 3 β -hydroxypregn-5-en-20-one (pregnenolone).

DHA designates the sections which were incubated with the substrate 3 β -hydroxyandrost-5-en-17-one (dehydroepiandrosterone).

NADH₂ designates the sections which were incubated with the substrate reduced nicotinamide adenine dinucleotide (NADH + H⁺).

TABLE VIII CONTINUED

| Ovarian Components | 15 Days | | | 18 Days | | | 20 Days | | | 21 Days | | |
|---------------------|---------|-----|----------------------|---------|-----|-------------------|---------|-----|-------------------|---------|-----|-------------------|
| | P-one | DHA | NADH ₂ ** | P-one | DHA | NADH ₂ | P-one | DHA | NADH ₂ | P-one | DHA | NADH ₂ |
| Atretic Follicle | | | | | | | | | | | | |
| Granulosa Cells | +1 | ± | +2 | +1 | ± | +2 | +1 | +1 | +2 | ± | +1 | +2 |
| Thecal Cells | +2 | +3 | +3 | +2 | +4 | +4 | +3 | +3 | +4 | +2 | +2 | +4 |
| Interstitial Tissue | +2 | +4 | +4 | +2 | +4 | +4 | +3 | +3 | +4 | +2 | +2 | +4 |
| Corpus Luteum | +2 | +3 | +4 | +3 | +3 | +4 | +2 | +2 | +4 | +1 | +2 | +4 |
| Hilar Region | 0 | 0 | +2 | 0 | 0 | +2 | 0 | 0 | +2 | 0 | 0 | +2 |

* 8 ovaries were used in each time interval of prolonged pseudopregnancy except days 8, 15 and 21 which included 4 ovaries.

** P-one designates the sections which were incubated with the substrate 3 β -hydroxypregn-5-en-20-one (pregnenolone).

DHA designates the sections which were incubated with the substrate 3 β -hydroxyandrost-5-en-17-one (dehydroepiandrosterone).

NADH₂ designates the sections which were incubated with the substrate reduced nicotinamide adenine dinucleotide (NADH + H⁺).

(Plate XXVII, fig. 180) and 18. No detectable P-one- 3β -HSD activity was observed on days 20 and 21. It, therefore, appears that the primary follicles were not a source of progesterone production during prolonged pseudo-pregnancy.

Using DHA to follow estrogenic hormonal production, the intensities of diformazan granules were at best weakly positive on days 8, 10, 13, 15, and 20; Plate XXXI, fig. 216 depicts enzymatic localization on day 8. The remaining days studied (6, 18 and 21) showed trace (monoformazan) intensities of DHA- 3β -HSD activity situated in the primary follicles (Plate XXXI, fig. 210). Thus, the primary follicles do not appear to show any capability for either estrogenic or progestational hormonal biosynthesis.

Secondary Follicles

During folliculogenesis, ovarian follicles grow and mature. The granulosa cells increase in number and multi-laminar secondary follicles develop. At this time, the theca interna and theca externa layers form around developing follicles.

Ova. The ova which were observed in primary-, secondary- and tertiary follicles showed no detectable reaction product when incubated with either P-one or DHA (Plate XXIII, fig. 161). These findings indicate that the ovum does not appear to play a role in estrogenic or progestational production during prolonged pseudopregnancy.

Granulosa cells. The cellular components of the granulosa layers of secondary follicles were found to vary between trace and weakly positive intensities of P-one- 3β -HSD activity on the days of prolonged pseudopregnancy studied. On days 6 (Plate XXIII, fig. 159; Plate XXVII, fig. 187), 8 (Plate XXIII, fig. 161), 13 (Plate XXIV, fig. 167), 15 and 20 (Plate XXVI, fig. 176), the granulosa cells, which were studied for the localization of P-one- 3β -HSD,

were observed to have only weakly positive intensities (+1). Trace intensities of monoformazan material were seen on days 10 (Plate XXIV, fig. 165), 18 (Plate XXV, fig. 172) and 21 (Plate XXVI, fig. 177; Plate XXVII, fig. 181). Thus, the granulosa cells of secondary follicles are only weakly positive for P-one- 3β -HSD activity during prolonged pseudopregnancy.

The response of the granulosa cells to DHA was similar to that noted using the other steroid substrate, i. e. varying between trace to weakly positive. Weakly positive (+1) intensities were seen on days 10 (Plate XXXI, fig. 217), 13 (Plate XXIX, fig. 198), 15 and 20 (Plate XXX, fig. 205), whereas trace intensities were seen on days 6 (Plate XXVIII, fig. 192) 8 (Plate XXVIII, fig. 194), 18 (Plate XXXI, fig. 211), and 21 (Plate XXX, fig. 209). These findings indicate that, during prolonged pseudopregnancy, the granulosa cells show an increase in potential ability to synthesize estrogenic and progestational hormones in secondary follicles as compared to primary follicles.

Thecal cells. As discussed in the section on the estrous cycle, there are two types of thecal cells: those which lie adjacent to the membrana granulosa, the theca interna and an outer concentric fibrous layer, the theca externa. P-one- 3β -HSD and DHA- 3β -HSD was localized in the cellular components of the theca interna, while minimal to negative intensities were seen in the cellular components of the theca externa. The thecal cells of secondary follicles manifested strong (+3) intensities of diformazan deposition, when incubated with the substrate P-one, on days 6 (Plate XXIII, fig. 159; Plate XXVII, fig. 187) and 10 (Plate XXIV, fig. 165) of prolonged pseudopregnancy. Moderate (+2) intensities were observed on days 8 (Plate XXIII, fig. 161), 13 (Plate XXIV, fig. 167), 15, 18 (Plate XXV, fig. 172), 20 (Plate XXVI, fig. 176), and 21 (Plate XXVI, fig. 177; Plate XXVII, fig. 181). Thus, these findings indicate that the theca interna cells of secondary follicles are, for

the majority of prolonged pseudopregnancy, moderately responsive to P-one substrate.

Utilizing the second steroidal precursor, DHA, the intensity of the diformazan deposits was seen to be moderate (+2) for the most part of prolonged pseudopregnancy, i.e. days 6 (Plate XXVIII, fig. 192), 8 (Plate XXVIII, fig. 194), 10 (Plate XXXI, fig. 217), 13 (Plate XXIX, fig. 198), 15, and 20 (Plate XXX, fig. 205). Weakly positive intensities of DHA- 3β -HSD activity were localized in the thecal cells on days 18 (Plate XXXI, fig. 211) and 21 (Plate XXX, fig. 209), of prolonged pseudopregnancy. These data suggest that estrogenic production is comparatively on-going in the theca interna cells of secondary follicles in pseudopregnant rats with decidual tissue.

Tertiary Follicles

Granulosa cells. Histochemical localization of P-one- 3β -HSD in the granulosa cells of tertiary follicles revealed moderate intensities (+2) on days 10 (Plate XXIV, fig. 163; Plate XXVII, fig. 188), and 13 (Plate XXIV, fig. 166) of prolonged pseudopregnancy. With the exception of day 18, when enzymatic intensity was of a trace (\pm) intensity (Plate XXV, fig. 172), the remaining days studied appeared to show weakly positive intensities (+1) i.e. days 6 (Plate XXIII, figs. 159 and 160), 8 (Plate XXIII, fig. 161), 15 (Plate XXV, fig. 169), 18 (Plate XXVII, fig. 182), 20 (Plate XXVI, fig. 175), and 21 (Plate XXVI, fig. 178). These data indicate quite uniformly that the granulosa cells are only weakly responsive to P-one substrate for the majority of prolonged pseudopregnancy, with a slight increase to +2 in P-one- 3β -HSD localization on days 10 and 13.

The granulosa cells of tertiary follicles varied in the localization of DHA- 3β -HSD activity between moderate and weakly positive intensities. Granular diformazan deposits of moderate intensity were observed on days 20

(Plate XXX, fig. 206) and 21 (Plate XXX, fig. 207; Plate XXXI, fig. 218). On the remaining days studied, diformazan intensities were assessed as weakly positive, *i.e.* days 6 (Plate XXVIII, figs. 192 and 193), 8 (Plate XXVIII, fig. 194), 10 (Plate XXVIII, fig. 197), 13 (Plate XXIX, figs. 198 and 199), 15 (Plate XXIX, fig. 201; Plate XXXI, fig. 212), and 18 (Plate XXIX, figs. 203 and 204). From the histochemical tests employing P-one and DHA substrates, the granulosa cells of tertiary follicles seem to contribute at best only a moderate potential ability to synthesize progesterone and estrogenic hormones.

Theca cells. As was the case in the estrous cycle and during normal pseudopregnancy, the theca interna cells appear to be more responsive than the granulosa cells to the steroidal substrates P-one and DHA. P-one- 3β -HSD was localized as strong (+3) intensity in the theca interna cells of tertiary follicles on days 6 (Plate XXIII, figs. 159 and 160), 8 (Plate XXIII, fig. 161), 10 (Plate XXIV, fig. 163; Plate XXVII, fig. 188), and 13 (Plate XXIV, fig. 166) of prolonged pseudopregnancy. Diformazan intensities were moderate on days 15 (Plate XXV, fig. 169), 18 (Plate XXV, fig. 172; Plate XXVII, fig. 182), 20 (Plate XXVI, fig. 175), and 21 (Plate XXVI, fig. 178). These findings indicate that the theca interna cells of tertiary follicles have a greater potential for synthesizing progesterone during the first 13 days of pseudopregnancy than during days 15 through 21.

Utilizing the second steroidal substrate to localize DHA- 3β -HSD activity, diformazan intensities varied between strong and moderate in the theca interna cells during prolonged pseudopregnancy. Strong intensities of diformazan deposition were observed on days 8 (Plate XXVIII, fig. 194), 10 (Plate XXVIII, fig. 197), 18 (Plate XXIX, figs. 203 and 204), 20 (Plate XXX, fig. 206), and 21 (Plate XXX, fig. 207; Plate XXXI, fig. 218). A reduction in diformazan intensities to moderate were seen on days 6 (Plate XXVIII, figs. 192 and 193), 13 (Plate XXIX, fig. 199), and 15 (Plate XXIX, fig. 201;

Plate XXXI, fig. 212). These data reveal that the theca interna ranges between strong to moderate in their potential ability to biosynthesize estrogenic and progestational hormones.

Atretic Follicles

Granulosa cells. The granulosa cells of the atretic follicles appeared to be weakly positive for P-one on days 6 (Plate XXIII, figs. 159 and 160; Plate XXVII, fig. 189), 8 (Plate XXIII, fig. 161), 13 (Plate XXIV, figs. 166 and 167), 15 (Plate XXV, fig. 170), 18 (Plate XXV, fig. 173), and 20 (Plate XXVI, fig. 176).

Monoformazan material (\pm) was found in the granulosa cells of atretic follicles on days 10 (Plate XXIV, figs. 163 and 165), and 21 (Plate XXVI, fig. 177; Plate XXVII, fig. 183). These findings indicate that the granulosa cells of atretic follicles show little, if any, observable activity with respect to progestational hormonal biosynthesis.

In the DHA- 3β -HSD series of ovarian sections, the granulosa cells varied in the intensity of reaction product formed between trace and weakly positive. Weakly positive intensities were seen on days 6 (Plate XXVIII, fig. 192; Plate XXXI, fig. 219), 10 (Plate XXVIII, fig. 196), 13 (Plate XXIX, figs. 198-200), 20 (Plate XXX, fig. 205), and 21 (Plate XXX, figs. 207 and 208; Plate XXXI, fig. 213). On the remaining days, i.e. 8 (Plate XXVIII, fig. 194), 15 (Plate XXIX, fig. 202), and 18 (Plate XXIX, fig. 204), trace reactions of diffuse pink monoformazan (\pm) were observed. The data obtained for both P-one- 3β -HSD and DHA- 3β -HSD localizations in the granulosa cells of atretic follicles suggests that these cells are not very responsive to the precursors of progestational and estrogenic hormones.

Theca cells. As discussed previously, the theca interna cells are the predominant cellular type present in atretic follicles. In the studies

using P-one, the intensity of diformazan deposition ranged between maximal (+4) and moderate (+2). Maximal diformazan deposits were found on days 6 (Plate XXIII, figs. 159 and 160; Plate XXVII, fig. 189), and 10 (Plate XXIV, figs. 163 and 165) of prolonged pseudopregnancy. Strong intensities (+3) were observed on days 8 (Plate XXIII, fig. 161), 13 (Plate XXIV, figs. 166 and 167), and 20 (Plate XXVI, fig. 176). Only three days showed moderate (+2) diformazan deposition, i. e. days 15 (Plate XXV, fig. 170), 18 (Plate XXV, fig. 173) and 21 (Plate XXVI, fig. 177; Plate XXVII, fig. 183). These data suggest that the theca interna cells of atretic follicles are, for the most part, more strongly reactive to the P-one precursor during the first 13 days of prolonged pseudopregnancy than during the latter portion of this physiological condition.

Utilizing DHA, the theca interna cells of atretic follicles were again observed to vary between maximal and moderate intensities of diformazan deposition. Maximal intensities were seen on days 6 (Plate XXVIII, fig. 192; Plate XXXI, fig. 219), 10 (Plate XXVIII, fig. 196), 13 (Plate XXIX, figs. 198 and 200) and 18 (Plate XXIX, fig. 204). On days 8 (Plate XXVIII, fig. 194), 15 (Plate XXIX, fig. 202) and 20 (Plate XXX, fig. 205), strong (+3) intensities of DHA- 3β -HSD were localized in the thecal cells of atretic follicles. Moderate (+2) DHA- 3β -HSD localization was noted on day 21 (Plate XXX, figs. 207 and 208; Plate XXXI, fig. 213). These findings indicate that the theca interna cells of atretic follicles are highly responsive to the estrogenic precursor, DHA, during prolonged pseudopregnancy.

Interstitial Tissue

Those cellular components of the ovary which are situated between the follicles and corpora lutea are called the interstitial tissue. During prolonged pseudopregnancy, the localization of P-one- 3β -HSD activity was

observed to vary between maximal (+4) to moderate (+2) intensities in the interstitial tissue. Maximal intensities of diformazan deposition were seen on days 6 (Plate XXIII, figs. 159 and 160; Plate XXVII, fig. 190) and 10 (Plate XXIV, figs. 163 and 165). Strong (+3) intensities were observed on days 8 (Plate XXIII, fig. 161), 13 (Plate XXIV, figs. 166 and 167) and 20 (Plate XXVI, figs. 175 and 176). On the remaining days of prolonged pseudopregnancy studied, the interstitial cells appeared moderately intense (+2) i. e. days 15 (Plate XXV, figs. 169 and 170), 18 (Plate XXV, figs. 172 and 173) and 21 (Plate XXVI, figs. 177 and 178; Plate XXVII, fig. 184). These data indicate that potential progesterational hormonal biosynthesis occurs to a greater extent during the first 13 days of prolonged pseudopregnancy than during the latter portion of this physiological condition.

Utilizing DHA as substrate, the cells of the interstitial tissue were observed to contain maximal (+4) to moderate (+2) intensities of diformazan deposits. Maximal intensities occurred on days 6 (Plate XXVIII, fig. 193; Plate XXXI, fig. 220), 10 (Plate XXVIII, fig. 196), 13 (Plate XXIX, figs. 198 to 200), and 18 (Plate XXIX, figs. 203 and 204). Strong (+3) intensities were seen on days 8 (Plate XXVIII, fig. 194), 15 (Plate XXIX, figs. 201 and 202) and 20 (Plate XXX, figs. 205 and 206). On day 21, only moderate intensities of diformazan material were observed (Plate XXX, figs. 207 and 208; Plate XXXI, fig. 214). These histochemical studies have demonstrated that the interstitial tissue of the rat is strongly reactive to both P-one and DHA substrates for the majority of prolonged pseudopregnancy. Further, potential progesterone synthesis by the interstitial cells appears to be reduced on days 15, 18 and 21, whereas potential estrogenic synthesis by this ovarian component appears to decline on day 21 of prolonged pseudopregnancy.

Corpus Luteum

As in the studies on the ovaries removed during the estrous cycle and normal (ordinary-induced) pseudopregnancy, this histochemical study observed the intensities of P-one- 3β -HSD and DHA- 3β -HSD localizations in the cells of corpora lutea. Using P-one as substrate, it was observed that the intensities of diformazan deposits ranged between maximal (+4) and weakly positive (+1) during pseudopregnancy with decidual tissue. Maximal (+4) intensities were seen on days 6 (Plate XXIII, fig. 159; Plate XXVII, fig. 191), 8 (Plate XXIII, fig. 162), 10 (Plate XXIV, fig. 164), and 13 (Plate XXIV, fig. 168). On day 18, strong intensities of diformazan were observed (Plate XXV, fig. 174). Moderate intensities were noted on days 15 (Plate XXV, fig. 171) and 20 (Plate XXVI, fig. 175). On the remaining day, *i. e.* 21, weakly positive P-one- 3β -HSD activities were localized in the luteal cells (Plate XXVI, fig. 179; Plate XXVII, fig. 185). Thus, these findings demonstrate that potential progesterational hormonal biosynthesis is maximal at the onset of prolonged pseudopregnancy and with the waning of the corpus luteum through day 21, the reaction decreases down to the weakly reactive index of a +1.

The response of the luteal cells to DHA were observed to vary between maximal and moderate during prolonged pseudopregnancy. On day 6, maximal (+4) intensities of diformazan deposits were seen in the luteal cells (Plate XXVIII, fig. 193; Plate XXXI, fig. 221). Strong (+3) intensities were noted on days 8 (Plate XXVIII, fig. 195), 10 (Plate XXVIII, fig. 196), 13 (Plate XXIX, fig. 200), 15 (Plate XXIX, fig. 201), and 18 (Plate XXIX, fig. 203). Moderate (+2) intensities were seen on days 20 (Plate XXX, fig. 206) and 21 (Plate XXX, fig. 208; Plate XXXI, fig. 215). From these histochemical data on the corpora lutea, it appears that both P-one and DHA are highly active throughout the major part of prolonged pseudopregnancy and decline only

during the last days of this condition.

Ovarian Hilus

The ovarian hilus was examined following incubation with both P-one and DHA substrates, in order to determine if there were any observable steroidogenic properties of the cells in this region during prolonged pseudopregnancy. It was found that this area consistently showed the absence of reaction products in animals having decidual tissue, cf. Table X. The only positive reaction products were seen in the interstitial tissue cells in the hilar region of the ovary. Thus, the consistent absence of any mono- or diformazan deposits in the ovarian hilus indicates that this region is not principally concerned with either estrogenic or progestational hormonal biosynthesis.

Comparative Analyses of Total Ovarian Composites

vs.

Individual Ovarian Entities: An Overview

In order to obtain a better understanding of the total ovarian potential biosynthesis of estrogenic and progestational hormones, the arithmetic averages of the data obtained for each ovarian component studied was computed and graphed. The vertical averages for each day studied during prolonged pseudopregnancy are shown in Table IX. The linear representation of the data is shown in a major text figure and a series of nine component graphs, each of which representing specific ovarian morphological entities, cf. Text figures 5, 5a-5i.

TABLE IX

Generalized and Summarized Data Based on all Ovarian Components Presented as
Averages during Prolonged Pseudopregnancy¹

| Enzymes* | <u>6 Days</u> | <u>8 Days</u> | <u>10 Days</u> | <u>13 Days</u> | <u>15 Days</u> | <u>18 Days</u> | <u>20 Days</u> | <u>21 Days</u> |
|----------------------|---------------|---------------|----------------|----------------|----------------|----------------|----------------|----------------|
| P-one-3 β -HSD | 2.3 | 2.1 | 2.3 | 2.2 | 1.6 | 1.3 | 1.7 | 1.1 |
| DHA-3 β -HSD | 2.0 | 1.8 | 2.1 | 2.2 | 1.9 | 1.8 | 2.0 | 1.4 |

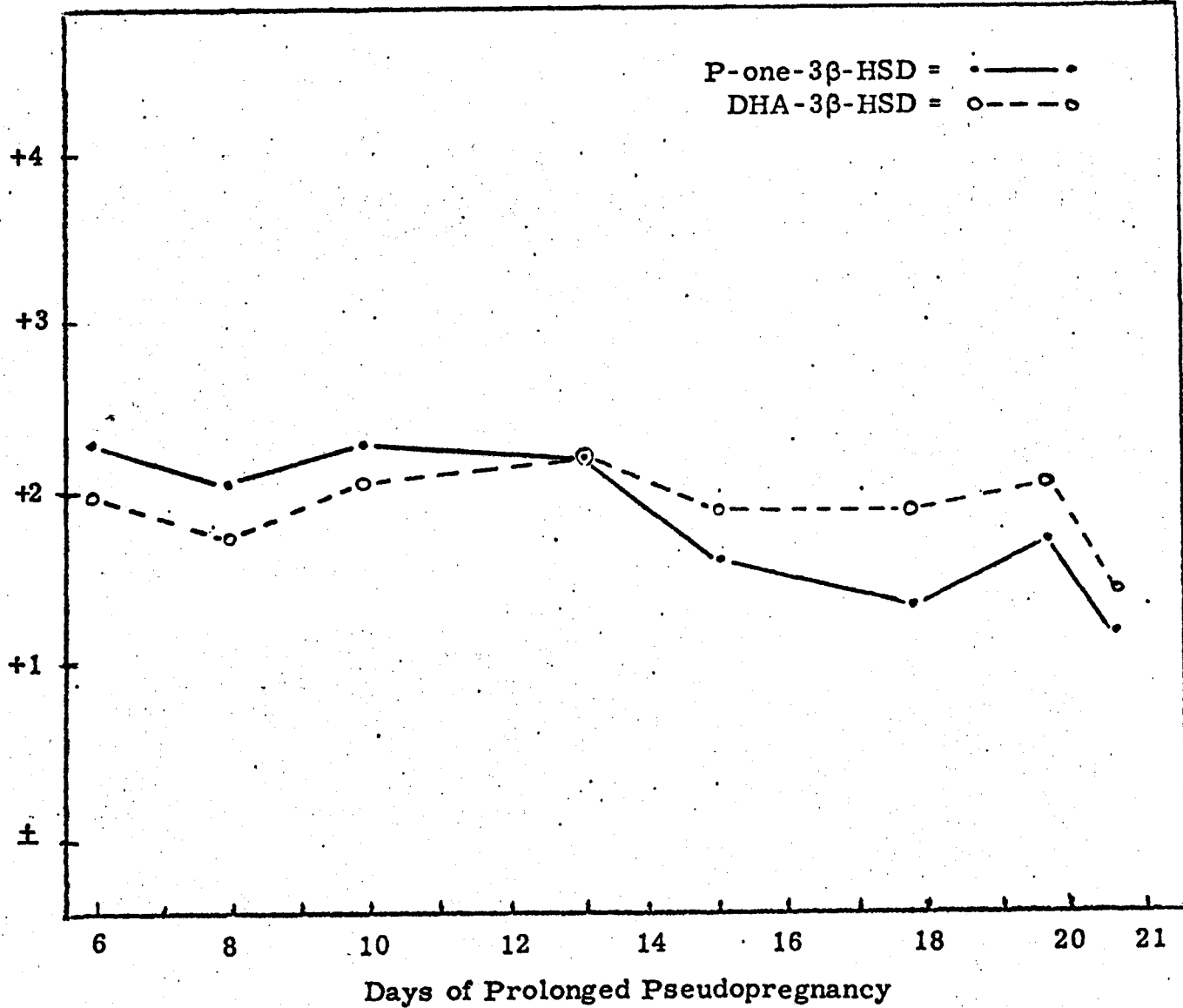
¹Arithmetic mean semi-quantitative histochemical estimates of 3 β -hydroxysteroid: NAD oxidoreductase (3 β -HSD) reactivity which were derived from the follicular, luteal and interstitial components of the ovaries in prolonged pseudopregnancy on the days studied.

*P-one-3 β -HSD denotes 3 β -HSD intensities using pregnenolone (P-one) as the incubation substrate. DHA-3 β -HSD denotes 3 β -HSD intensities using dehydroepiandrosterone (DHA) as the incubation substrate.

Estimates were rated on a 0 to +4 scale and a trace amount designated '+

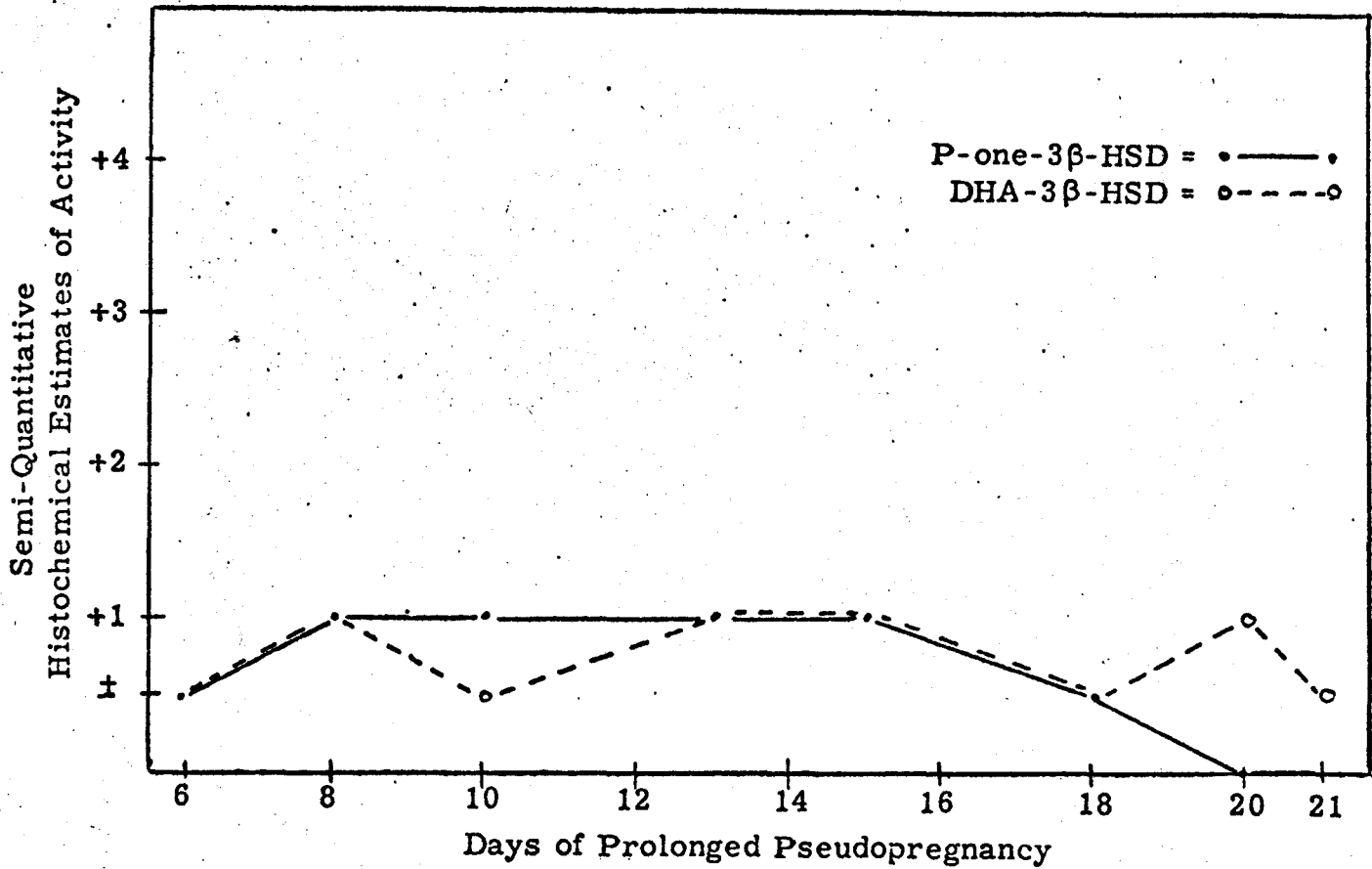
Text Figure 5. A linear representation of the averages of semi-quantitative histochemical estimates of P-one- 3β -HSD (solid line) and DHA- 3β -HSD (dotted line) of all ovarian components throughout the days of prolonged pseudopregnancy studied. Individual components are presented in similar, but individual activity graphs, in accompanying figures 5a-5i.

Semi-Quantitative
Histochemical Estimates of Activity



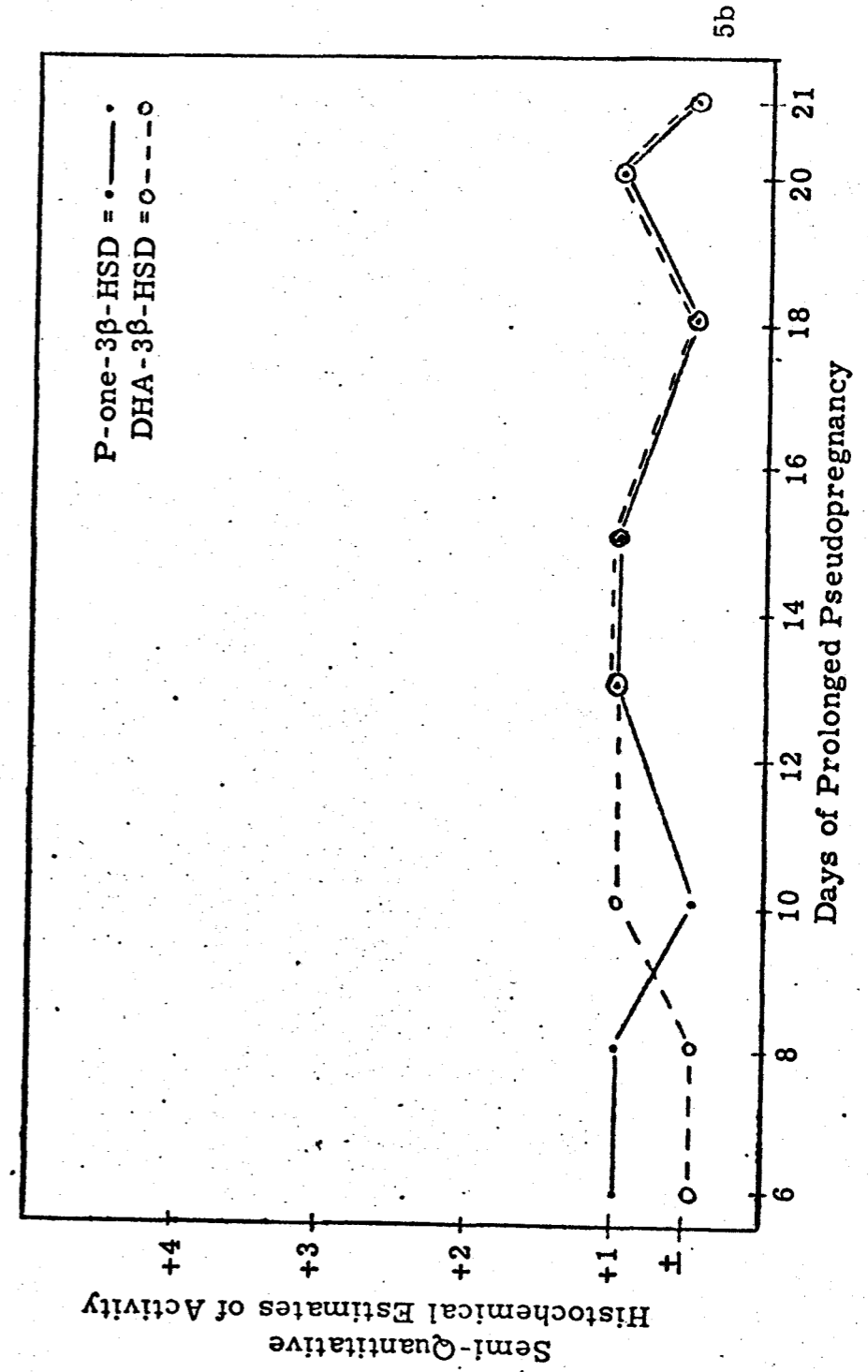
Text Figures 5a-5i. Averages of semi-quantitative histochemical estimates of P-one- 3β -HSD (solid line) and DHA- 3β -HSD (dotted line) of individual ovarian components during prolonged pseudopregnancy:

5a. Activities of P-one- 3β -HSD and DHA- 3β -HSD in primary follicles.

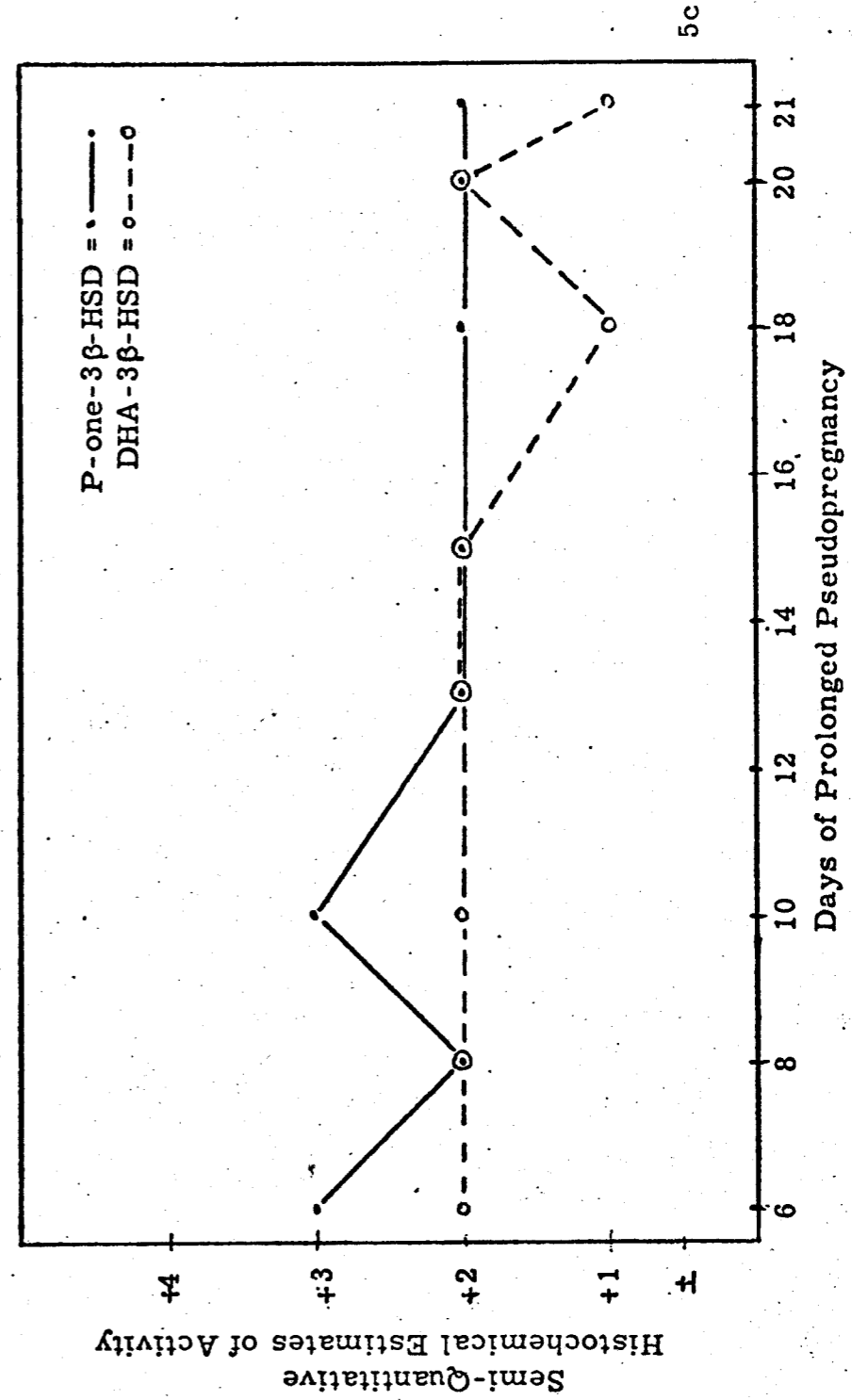


5a

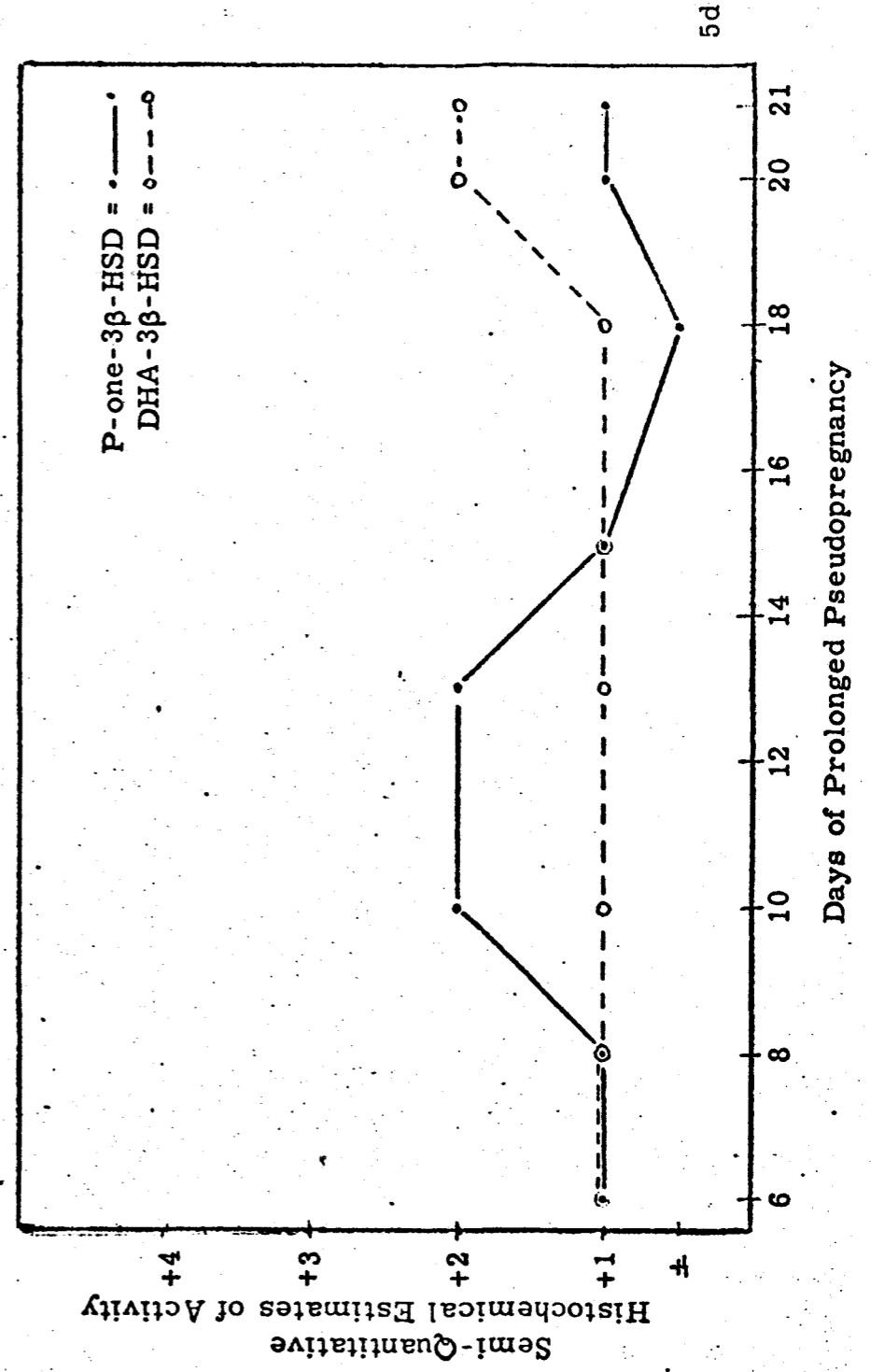
5b. Activities of P-one- 3β -HSD and DHA- 3β -HSD in granulosa cells of secondary follicles.



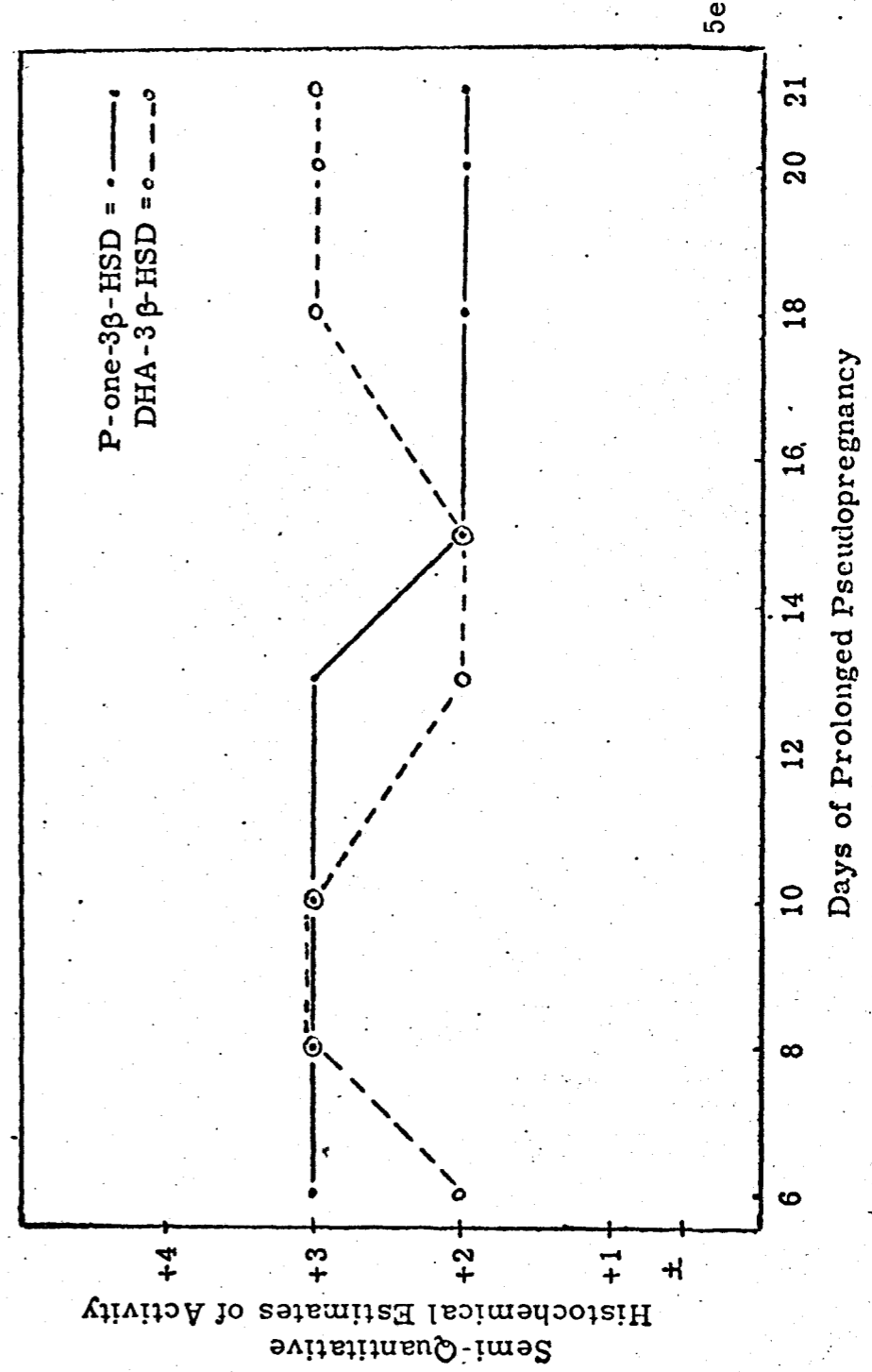
5c. Activities of P-one- 3β -HSD and DHA- 3β -HSD in theca interna of secondary follicles.



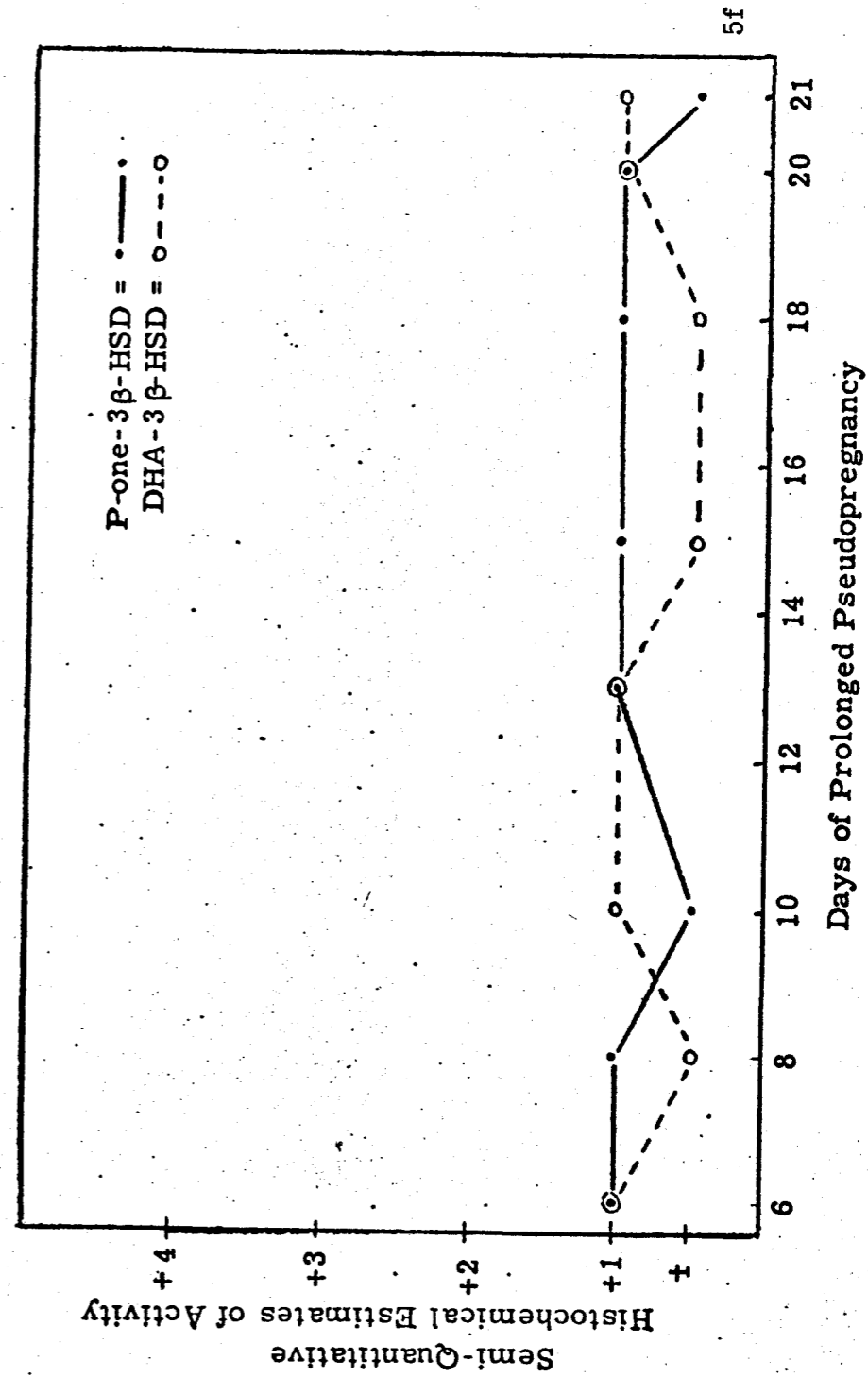
5d. Activities of P-one- 3β -HSD and DHA- 3β -HSD in granulosa cells of tertiary follicles.



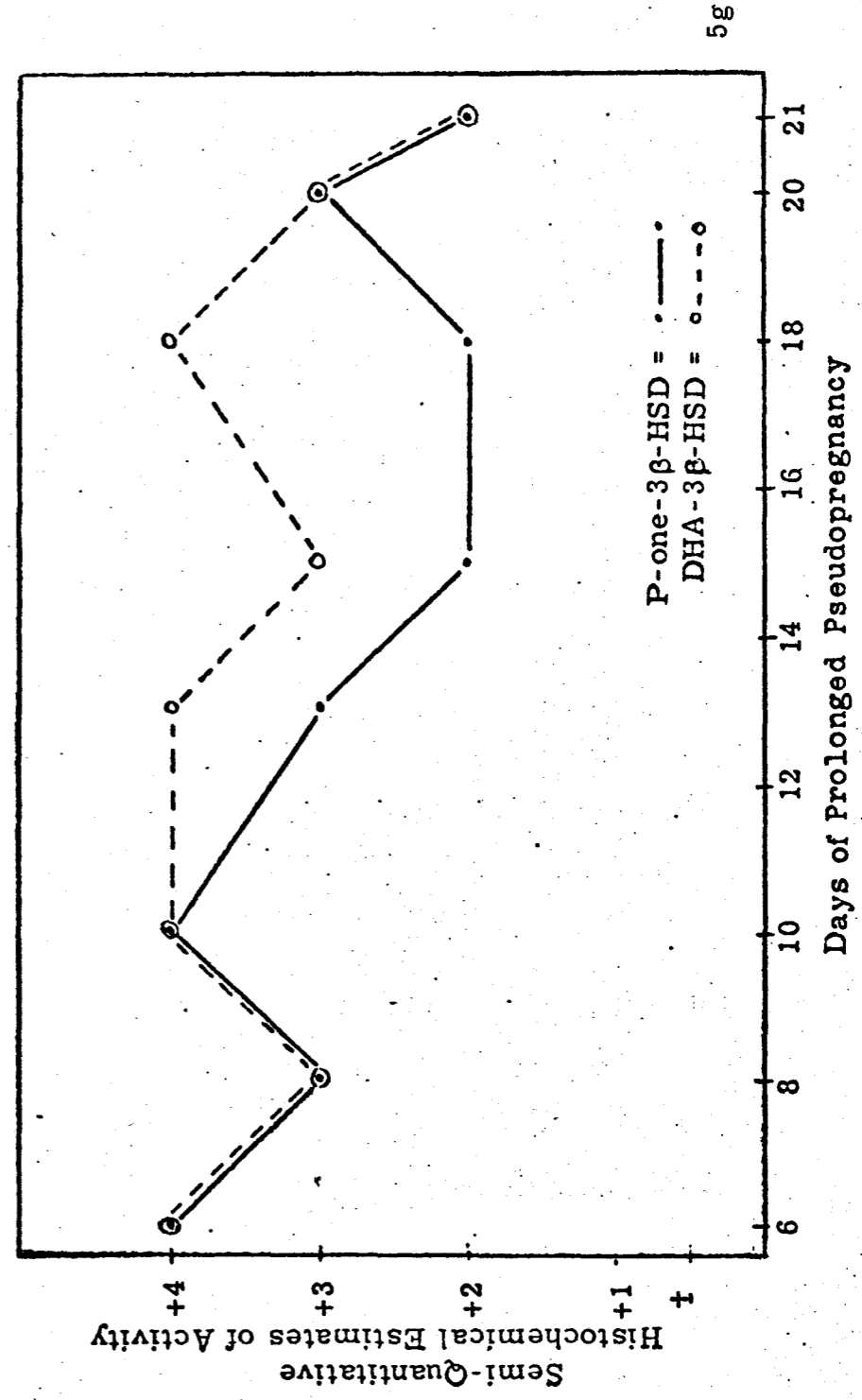
5e. Activities of P-one- 3β -HSD and DHA- 3β -HSD in theca interna of tertiary follicles.



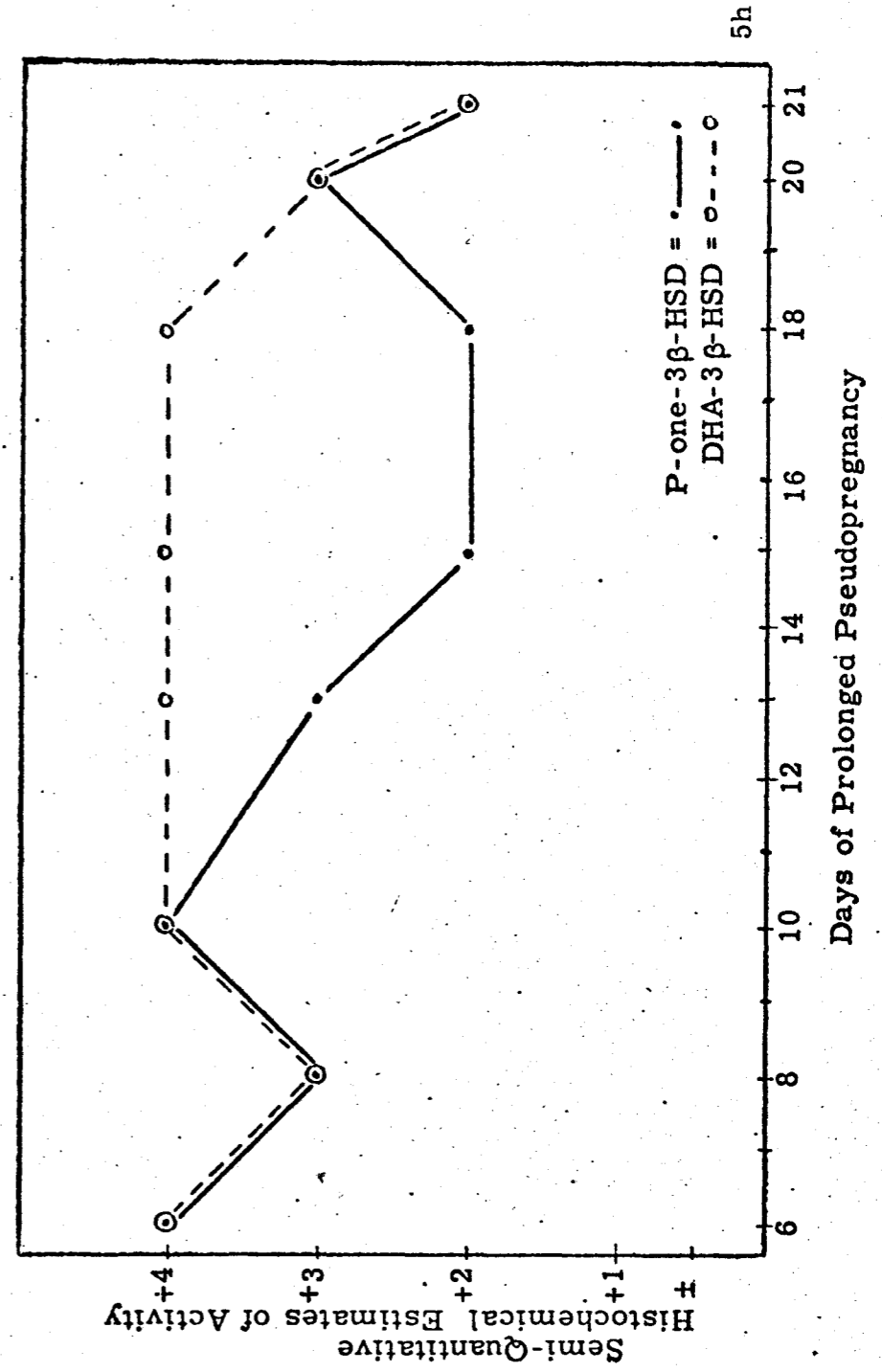
5f. Activities of P-one-3 β -HSD and DHA-3 β -HSD in granulosa cells of atretic follicles.



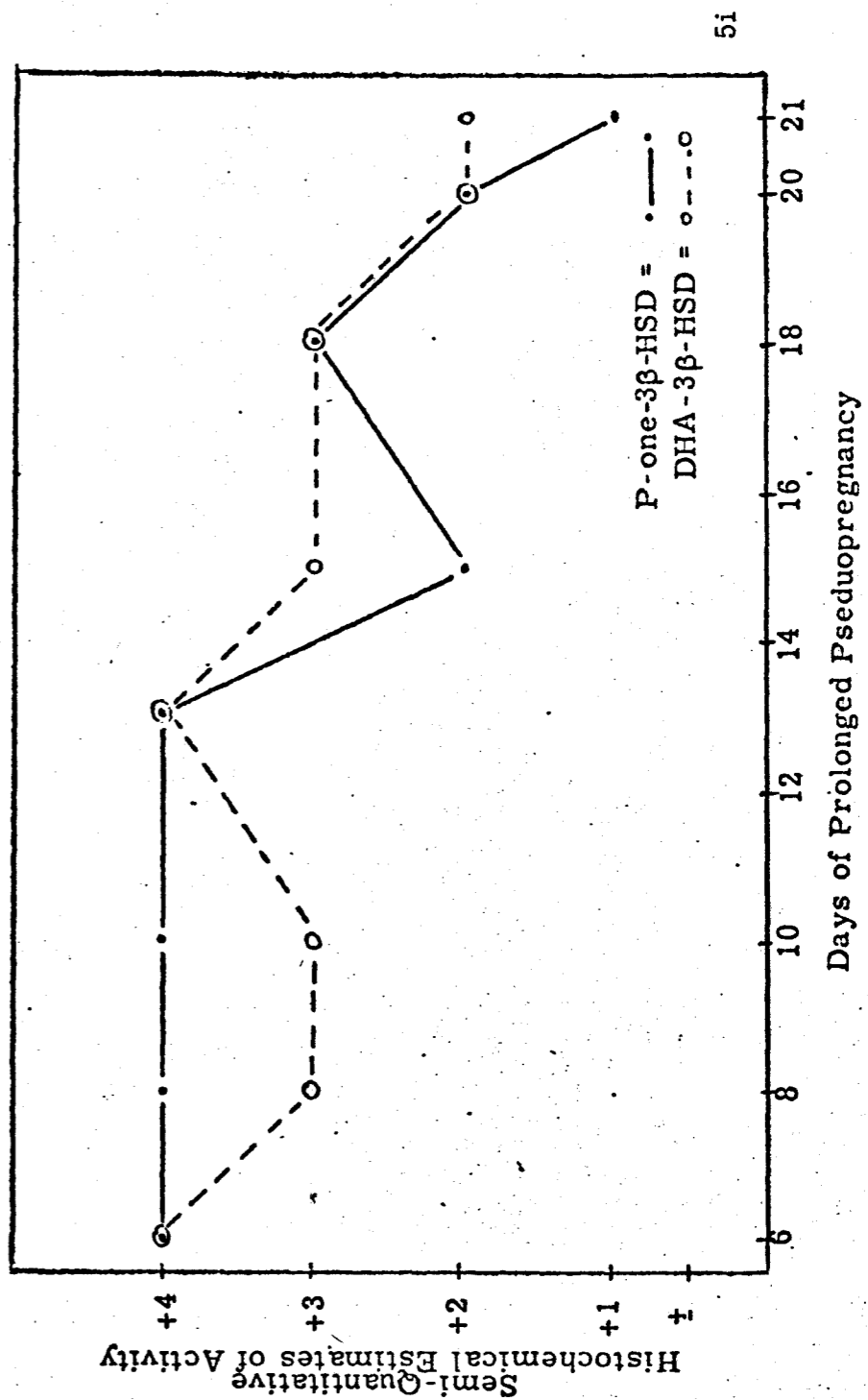
5g. Activities of P-one-3 β -HSD and DHA-3 β -HSD in theca interna of atretic follicles.



5h. Activities of P-one- 3β -HSD and DHA- 3β -HSD in ovarian interstitial tissue.



5i. Activities of P-one- 3β -HSD and DHA- 3β -HSD in corpora lutea.



Discussion

In the area of reproductive experimentation, the anatomo-physiologic concept of prolonged pseudopregnancy is highlighted due to its comparative similarities with early pre-implantation stages of pregnancy. Interest in ovarian steroidal activity dates back to the early 1900's with the studies of Loeb with his experiments which he designed to induce and maintain pseudopregnancy. Although ovarian hormones and their actions in pseudopregnancy have been documented, the aspect of the development and alterations in the morphology of the decidual tissue has been more intensely studied (Astwood, 1939; Hisaw *et al.*, 1928; Velardo *et al.*, 1953a and 1953b). Histochemical studies of ovaries removed from pseudopregnant animals are comparatively few in number (Dawson and Velardo, 1955). Wattenberg's technique which localizes the sites of 3β -HSD activity has been applied to ovaries during prolonged pseudopregnancy as corroborative evidence for the suppression of decidual tissue by anti-LH serum during gestation (Chang *et al.*, 1971).

Therefore, the present study was performed in order to determine whether the ovarian components change in their potential(s) to synthesize progesterone and estrogen(s), using the substrates P-one- 3β -HSD and DHA- 3β -HSD respectively, during prolonged pseudopregnancy. Further, these histochemical findings were assessed and then compared with data obtained regarding ovarian steroidogenesis during the estrous cycle, ordinary-induced pseudopregnancy (normal) without decidual tissue, and further, compared with the data of pregnancy.

The importance of both estrogens and progesterone in supporting decidual tissue in the uterus of rats was demonstrated by Astwood (1939) who found that, in uterine traumatized, ovariectomized rats, the administration of progesterone usually resulted in decidual tissue formation, whereas estrogen inhibited the decidual response; daily administration of estrogen and

progesterone in critical amounts and ratios in ovariectomized, pseudo-pregnant rats with uterine traumatization resulted in the formation of decidual tissue (Velardo and Hisaw, 1951). These findings agree with the histochemical data obtained in the current study, that the ovary has the ability to synthesize both estrogenic and progestational hormones during prolonged pseudopregnancy.

Initially, the function of the hormones involved in the induction of decidual tissue in pseudopregnant rats was derived from indirect evidence, i. e. hormonal administration to ovariectomized, pseudopregnant animals having uterine traumatization. Hormonal administration of estrogens (Velardo and Hisaw, 1951) and pregnanes (Velardo, 1957a) to ovariectomized pseudopregnant rats with traumatized uteri resulted in inhibition of the induction of decidual tissue, which was believed to be due to an inhibition of the expression of progesterone. When a depot progestational hormone (*Delalutin*) was administered on day 5, to ovariectomized uterine traumatized pseudopregnant rats, deciduomata formed whereas a combination of *Delalutin* and estrogens inhibited the formation of the decidual reaction (Velardo, 1957b). These findings pointed-up the importance of progesterone in the induction of deciduomata and corroborated the work of Corner and Allen (1929a and 1929b). From the aforementioned, indirect evidence on the need for estrogens and progesterone, the report of Weichert (1928) is significant since he showed that the induction of the decidual reaction is due to the synergistic action of these hormones in proper proportions. The histochemical data obtained for P-one- 3β -HSD and DHA- 3β -HSD activity of the ovarian components indicate that the potential progesterone: estrogen biosynthetic ratio favors progesterone on days 6, 8 and 10. Interestingly enough, these data tend to suggest that both the estrogenic and progestational hormonal synthetic pathways are operative to similar degrees during the terminal aspect of normal pseudo-

pregnancy and the initial continuation of the prolonged event of pseudo-pregnancy, *i. e.* on day 13. With the further progression of the super-imposed event of prolonged pseudopregnancy, on days 15 and following, the seemingly change in the biosynthetic pathway appears more toward the estrogenic phase as reflected in the enhancement of the DHA- 3β -HSD intensities.

Harper (1969) demonstrated that the uteri of intact or adrenalectomized, immature, female rats which were treated with DHA are histologically similar to those of rats treated with estradiol- 17β . Further, he suggests that DHA requires the presence of the ovary in order to attain estrogenic expression. These findings give further support to the data that DHA is a precursor for the estrogens particularly estradiol- 17β . The current studies, using DHA as the estrogenic precursor in the incubation media, are harmonious with Harper's findings, and further extend them showing that the loci of marked DHA- 3β -HSD activity during prolonged pseudopregnancy includes the theca interna cells of tertiary follicles and those of atretic follicles, as well as interstitial tissue and corpora lutea.

Companion studies, on the biochemical determinations of the ovarian blood levels of progesterone of rats, during prolonged pseudopregnancy reported in the literature yield additional lines of corroborative evidence indicating that the titers of progesterone and the highs and lows of the P-one- 3β -HSD in corpora lutea are somewhat parallel in appearance. Progesterone secretion was found to be highest on day 6 of pseudo-pregnancy when compared to values obtained during the estrous cycle (Chatterton and Greep, 1965). Hashimoto and Melampy (1967) found that progesterone blood titers rose on day 4 of prolonged pseudopregnancy and did not fall until day 21. The histochemical data with respect to potential ovarian progestational production during prolonged pseudopregnancy, indicates that P-one- 3β -HSD activity on days 6, 8, 10, and 13 are greater

than the semi-quantitative estimates for P-one- 3β -HSD made during the estrous cycle in the rats of established reproductive cyclicity (R-series). Between days 15 and 21, the progesterone production did appear to be reduced with respect to P-one- 3β -HSD diformazan intensities which were similar to those assessed during late diestrus, prooestrus and oestrus in the R-series of animals.

Ovaries of rats which were removed during prolonged pseudopregnancy and histologically examined were characterized by having many involuting corpora lutea and large tertiary follicles (Hisaw *et al.*, 1928). From a histochemical study on the ovaries of rabbits, fine birefringent intracellular granules were found in the theca interna and interstitial tissue; these granules represent stored esterified sterols and are believed to be closely related to estrogenic production (Claesson and Hillarp, 1947). Stored esterified steroids were observed to be most abundant in the theca interna and interstitial cells during the estrous cycle, but seemingly lower during pregnancy and least during pseudopregnancy. Thus, it appears that estrogenic hormonal biosynthesis is greater in these ovarian components during pseudopregnancy than during the estrous cycle. The histochemical data on prolonged pseudopregnancy indicates that the theca interna cells of both the secondary and tertiary follicles appear to localize DHA- 3β -HSD activity to a greater extent than the granulosa cells of these same follicles. Likewise, the theca interna cells of the atretic follicles seem to be significantly greater in DHA- 3β -HSD localization than are the granulosa cells of atretic follicles. It was observed that the interstitial tissue is the main ovarian component which appears to have the greatest potential to synthesize the estrogens among the ovarian components studied.

From a previous histochemical study, it was found that cholesterol is not present in corpora lutea during either normal or prolonged pseudopregnancy

whereas lipids are prominent in luteal cells from day 3 to the termination of pseudopregnancy (Dawson and Velardo, 1955). The current findings indicate that intensities of P-one- 3β -HSD activity are maximal in the luteal cells between days 6 and 13 of prolonged pseudopregnancy and then diminish to weak intensities on day 21. Likewise, the intensities of DHA- 3β -HSD are strongly to maximally present in luteal cells from days 6 to 18 and then decline to moderate activity on days 20 and 21. These data suggest that the lipoidal material which is present in the luteal cells may be the ovarian steroids, i. e. estrogens and progesterones which are being synthesized during prolonged pseudopregnancy.

Interestingly enough, other histochemical reports have indicated that 20α -hydroxysteroid dehydrogenase activity (20α -HSD) does not occur in corpora lutea during pseudopregnancy (Wiest et al., 1963). The point of interest resides in the fact that this enzyme 20α -HSD has been shown to be indicative of non-functional corpora lutea or of luteal degeneration (Wiest et al., 1968). On the other hand, these studies clearly indicate that P-one- 3β -HSD is quite active, thus pointing-up the importance of this Arbeit which is illustrated in this dissertation.

Added lines of evidence regarding the significance of both progesterone and 20α -HSD emerge from the experimental work on ergocornine. If pseudopregnant rats are treated with ergocornine on day 4, 24 hours following drug administration, the ovarian content of progesterone is reduced to 44% of control values and 20α -hydroxyprogesterone values increase markedly, thus demonstrating that there is a reduction in progesterone with a corresponding increase in 20α -hydroxyprogesterone biosynthesis (Lindner and Shelesnyak, 1967). Using histochemical techniques, Turolla et al. (1969) reported that 20α -HSD activity is not observed prior to day 9 of pseudopregnancy in corpora luteal cells. These aforementioned studies support the view that the corpora

lutea of pseudopregnancy are functional and are capable of synthesizing progesterone, and that initial luteal degeneration most likely begins on day 9 of pseudopregnancy with respect to progesterone production.

The histochemical data obtained, during the study on normal pseudopregnancy, indicate that the enzyme P-one- 3β -HSD associated with progesterone biosynthesis initially declines from strong to moderate intensities between days 8 and 10 of this condition, whereas, during the study on prolonged pseudopregnancy, luteal P-one- 3β -HSD activity declines from maximal to moderate intensities somewhat later, i. e. between days 13 and 15, a fact especially noteworthy inasmuch as decidual tissue growth and development are hormonally dependent.

Summary and Conclusions

Previously, it was shown that pseudopregnant rats with large decidual reactions manifest prolonged pseudopregnancy in which the length of this condition is extended from 13 to approximately 21-22 days. Utilizing identical procedures, ovaries of rats during prolonged pseudopregnancy were analyzed on days 6, 8, 10, 13, 15, 18, 20, and 21 for pregnenolone- 3β -hydroxysteroid dehydrogenase (P-one- 3β -HSD), dehydroepiandrosterone- 3β -hydroxysteroid dehydrogenase (DHA- 3β -HSD) and NADH₂ diaphorase. Enzymatic activity was rated on a 0 to +4 scale for each substrate. Histochemical controls without the addition of the steroidal substrates, P-one or DHA, or of NADH₂ were observed to be consistently negative. Results show: 1) for P-one- 3β -HSD: a) corpora lutea (C.L.) on days 6 through 13 maximal intensity (+4), then decrease from +3 to +1 on days 18 to 21; b) granulosa cells (G.) of primary-, secondary-, tertiary-, and atretic follicles (P.F., S.F., T.F., A.F.) vary between trace (\pm) to +2; c) theca interna cells (T.I.) vary: in S.F. strong (+3) to moderate (+2), in T.F.

likewise, in A.F. +4 to +2; and d) interstitial tissue (I.T.) on days 6-13 alternately +4 to +3, thereafter, +2 except +3 on day 20; 2) for DHA- 3β -HSD: a) C.L. on day 6 +4 then plateau to +3 on days 8-18, thereafter +2; b) the intensities of G. closely paralleled those for P-one- 3β -HSD; c) T.I. show increasing intensity; S.F. +2 for the most part, T.F. +3 generally, A.F. +4 to +3 on day 21; and d) the I.T. also paralleling P-one- 3β -HSD; and 3) for NADH₂ diaphorase: a) C.L. consistently +4; b) G. consistently +2; c) T.I. consistently +4, and d) I.T. uniformly +4. Thus, ovarian steroid dehydrogenases and NADH₂ diaphorase vary as to specific needs during prolonged pseudopregnancy.

The histochemical results on the cellular and tissue sites and sequential alterations, in both potential progesterational and estrogenic biosynthesis, suggest a fluctuation in the production of these steroidal hormones by the different components of the ovaries of rats during prolonged pseudopregnancy. Following an intensive examination on the sites of potential steroidogenesis, utilizing P-one- 3β -HSD and DHA- 3β -HSD, it was found that each of these precursor substrates are very active in many of the different ovarian components studied excepting the ovum, primary follicle and hilar region. The corpus luteum of pseudopregnant rats was observed to be maximally responsive to P-one on days 6 through 13 and then declined in enzymatic P-one- 3β -HSD activity during the remainder of the prolonged pseudopregnant state. DHA- 3β -HSD localization in the luteal cells was very strong between days 6-18 and then declined slightly on the remaining days studied. These findings suggest that both potential progesterational and estrogenic biosynthesis occurs in increasing levels and both were sustained at these increased rates during, at least, the early and middle stages of prolonged pseudopregnancy. One important site for consistently high localization of P-one- and DHA- 3β -HSD activity was the interstitial tissue. This

was also the situation in studies performed on rats in each of the six stages of the estrous cycle as well as during normal pseudopregnancy. These findings indicate that this abundant ovarian component in the rat is a main source for the potential production of both estrogenic and progestational hormones. Furthermore, the theca interna cells of tertiary- and atretic follicles appear to be another strong source of potential ovarian steroidal biosynthesis.

Thus, in summation, additional evidence was adduced to support the concept of enhanced ovarian steroidal biosynthesis during extended luteal life. These data clearly indicate that the ongoing process of prolonged pseudopregnancy is associated with an increase in the steroidal capabilities of the secondary-, tertiary- and atretic follicles as well as the interstitial tissue, and corpora lutea, thus yielding increased levels of estrogens and progesterone obviously required in this physiological condition.

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Localization of Ovarian Dehydroepiandrosterone- 3β -Hydroxysteroid
Dehydrogenase and Pregnenolone- 3β -Hydroxysteroid Dehydrogenase
Activity in the Rat during Pregnancy

Introduction

Studies of estrogenic and progestational biosynthesis are of paramount importance in the area of endocrinology. This interest pertaining to the cellular and tissue sites of ovarian hormonal production has recently been extended to the ovaries of pregnant rats. During pregnancy, there is an alteration in the cyclic hormonal biosynthesis which occurs during the normal sex cycle in order to maintain the proper environment for the developing embryo. During pregnancy, the ovaries, adrenal glands, placenta, and fetal gonads are potential sites of estrogenic and progestational hormonal production. This study involves a detailed analysis of potential estrogenic and progestational biosynthesis in the different components of the ovary during pregnancy in order to obtain a clearer understanding of the functional capacity which the ovary possesses during this condition.

Initially the sites of steroidogenesis were localized utilizing the steroidal characteristics of birefringence and the Schultz technique for cholesterol and its esters. Dempsey and Bassett (1943), utilizing a series of indirect tests for steroidal precursors, reported that the theca interna cells of secondary- and tertiary follicles, the granulosa-lutein cells of corpora lutea, atretic follicles and interstitial tissue are the sites of estrogenic production. Claesson and Hillarp (1947a and 1947b, 1948) reported that histologically observable reduction in esterified cholesterol is related to an increase in steroidal secretion, and that during gestation in rats, no significant cholesterol was observed in the interstitial tissue and corpora lutea prior to days 19 and 20. Using hypophysectomized, gonadotropin-treated

pregnant rabbits, it was determined that 24 hours after gonadotropin administration, there is a biochemically demonstrable reduction in the cholesterol content of the ovary (Claesson et al., 1948). This provides additional data that cholesterol is the precursor observed utilizing the Schultz technique, and further, cholesterol appears to be mobilized, prior to increased steroidal secretion by the ovary.

Only a paucity of information is extant regarding research involving the localization of the enzyme 3β -hydroxysteroid:NAD oxidoreductase (3β -hydroxysteroid dehydrogenase, 3β -HSD) in the ovaries of pregnant animals and women. In such studies when pregnenolone(P-one) is used as the precursor substrate, P-one- 3β -HSD activity is localized in those cells which have the potential ability to synthesize progesterone. Utilizing dehydroepiandrosterone (DHA) as the substrate of the histochemical reaction, DHA- 3β -HSD activity is localized in those cells which have the potential ability to synthesize estrogens. Ferguson (1965) and Davies et al. (1966) tested a series of substrates on the ovaries of mice, rats, guinea pigs, and rabbits in order to assess the specificity of 3β -HSD activity and reported that DHA, P-one and epiandrosterone are preferential substrates for histochemical localization of 3β -HSD activity. Deane et al. (1962) observed that there is strongly observable DHA- 3β -HSD activity in the corpora lutea in women at 6 weeks of gestation and that the theca interna cells of normal and atretic follicles are only moderately reactive. More recently, Govan (1968) reported DHA- 3β -HSD activity in the theca interna cells of developing follicles and in the granulosa and thecal cells of the atretic follicles in the human ovary.

In a histochemical study involving the localization of 35 enzymes in the ovaries of pregnant rabbits, Hadjiisky (1970) reported strong intensities of DHA- 3β -HSD activity. Strong reactions appeared first in the theca-lutein cells, and later in the granulosa-lutein cells. In another study, involving

pregnant bitches, the corpora lutea manifested strong DHA- 3β -HSD activity, whereas the interstitial tissue was only negligible to weak (Fowler and Feldman, 1970). A most recent report by Motta and Takeva, (1971) further indicated that the ovaries of the pregnant guinea pig yield much useful information. Specifically, it was determined that the ovaries of guinea pigs reveal that: 1) both P-one- 3β -HSD activity and DHA- 3β -HSD activity are highest in luteal cells in the latter stages of gestation; 2) the theca interna cells appear lowest for both enzymes throughout pregnancy; and 3) the interstitial cells are moderately intense for both enzymes in the middle stages of pregnancy and lowest during the latter portion of pregnancy. Motta and Bourneva (1970a and 1970b) studied P-one- 3β -HSD and DHA- 3β -HSD activity in the rat on days 5 to 6, 10, 15, and 19 of pregnancy, as well as during the estrous cycle; they observed that both of the enzymes appear to be strongly active in the luteal cells, moderately active in the interstitial tissue and theca interna, and weakly active in the atretic follicles. Thus, the literature is devoid of sufficient studies in this area to develop a concrete picture of the steroidal hormonal biosynthesis during pregnancy. Inasmuch as a paucity of information does not permit in depth conclusions, and inasmuch as detailed information is required to ascertain as complete a picture on ovarian steroid hormonal biosynthesis, it appeared of specific interest to extend these detailed studies to the ovaries of pregnant rats. Moreover, no one has followed P-one- and DHA- 3β -HSD activity in the different types of follicles, the interstitial tissue, and the corpora lutea of ovaries at closely spaced intervals during gestation. Thus, it was of interest to evaluate and assess 3β -HSD activity in the ovaries of rats at closely spaced intervals during pregnancy in order to more closely follow the sites of and alterations in progestational and estrogenic hormonal biosynthesis.

Experimental Procedures

Mated female albino rats were purchased from the Charles River Breeding Laboratories and were housed in animal rooms having a 12 hour light-dark cycle and a room temperature of 72°F. The rats had Purina Rat Chow and water available ad libitum.

Daily vaginal smears were taken and then the animals were necropsied via decapitation on days 6, 8, 10, 13, 14, 15, 18, 20, and 21 of pregnancy. Following decapitation, the left ovary was immediately removed, trimmed of adipose tissue and frozen by submersion in cold liquid 2-methylbutane, which was chilled by being placed into a Dewar flask partially filled with liquid nitrogen. The right ovary and adrenal gland, as well as the uterus and vagina were removed. The uterus was checked for the number of implantation or resorption sites and the feti were separated from the placentae on days 15, 18, 20, and 21 of pregnancy. The ovaries, adrenal glands and uterus from each animal were weighed on a Precision[®] torsion balance and fixed in Bouin's solution for histological processing. The feti were measured using crown-rump lengths, weighed, and then fixed by immersion into either Bouin's solution or neutral formalin solution. The feti were examined utilizing Christie's criteria (1964), see appendix C, and staged in order to establish more exactly the duration of gestation at the time of necropsy.

The frozen ovaries were sectioned at eight microns and then incubated at 37°C for 2 3/4 hours in the media tabularized in the section dealing with the estrous cycle, page 15. The histochemical techniques, which were utilized in this study, localized the histological and cellular sites of P-one-3β-HSD, DHA-3β-HSD and NADH₂ diaphorase activity. P-one-3β-HSD activity is interpreted as being indicative of progestational biosynthesis; DHA-3β-HSD activity is interpreted as being indicative of estrogenic biosynthesis; and NADH₂ diaphorase activity is indicative of the hydrogen trans-

port system. Histochemical controls, omitting the steroidal substrates and NADH_2 , were incorporated into the experimental procedures and carefully examined in order to carefully assess the reaction product and, thus, not to incorrectly record a negative reaction.

Histochemical, enzymatic activity was rated on a 0 to +4 scale by determining the maximal intensity of the diformazan deposited on ovarian tissue sections in a medium containing an excess of given substrate.

Observations and Results

Recently, histochemical studies have been performed which attempt to determine and assess the cellular and tissue loci of steroidal biosynthesis during pregnancy. However, no one to date has attempted to follow P-one and DHA- 3β -HSD activity in the different ovarian components at closely spaced intervals during gestation. This histochemical study has approached this aim and the results appear ever so much more meaningful when they are compared with the findings during the six stages of the estrous cycle, as well as during normal pseudopregnancy (ordinary-induced) and prolonged pseudopregnancy (with massive decidual induction). The following ovarian components were assessed: primary-, secondary- and tertiary follicles, atretic follicles, interstitial tissue, corpora lutea, and the ovarian hilus. The secondary and tertiary follicles, as well as the atretic follicles, were analyzed to determine the sites of dehydrogenase activity in the different cellular components, *i. e.* granulosa cells, theca interna and theca externa.

Gravimetric Data

Analysis of the gravimetric data was performed in order to obtain correlations which occur among the ovary, adrenal gland and uterus during pregnancy. Table X is comprised of ovarian, adrenal gland and uterine

TABLE X

Comparison of Ovarian, Adrenal Gland and Uterine Weights in Milligram Per Cent for the Pregnant Series of Animals at the Different Days of Necropsy

| <u>Duration of Pregnancy</u> | <u>Item¹</u> | <u>Ovary</u> | <u>Adrenal Gland</u> | <u>Uterus</u> |
|----------------------------------|-------------------------|--------------|--------------------------|---------------|
| 6 Days | n | 5 | 5 | 5 |
| | \bar{X} | 14.71 | 11.75 | 176.05 |
| | σ_e | 1.19 | 0.94 | 14.80 |
| 8 Days | n | 6 | 6 | 6 |
| | \bar{X} | 15.03 | 12.37 | 348.78 |
| | σ_e | 0.98 | 0.49 | 22.96 |
| 10 Days | n | 5 | 5 | 5 |
| | \bar{X} | 13.96 | 13.54 | 445.54 |
| | σ_e | 0.53 | 0.93 | 83.38 |
| 13 Days | n | 5 | 5 | 5 |
| | \bar{X} | 18.38 | 11.29 | 1706.80 |
| | σ_e | 0.43 | 0.41 | 141.40 |

¹n = the number of rats in the group.

\bar{X} = the arithmetic mean weight of the organ expressed in mg. %.

σ_e = the standard error of the arithmetic mean.

TABLE X CONTINUED

| <u>Duration of Pregnancy</u> | <u>Item¹</u> | <u>Ovary</u> | <u>Adrenal Gland</u> | <u>Uterus</u> |
|----------------------------------|-------------------------|--------------|--------------------------|---------------|
| 14 Days | $\frac{n}{X}$ | 4 | 4 | 4 |
| | \bar{X} | 17.03 | 11.60 | 4912.25 |
| | σ_e | 1.25 | 0.61 | 135.90 |
| 15 Days | $\frac{n}{X}$ | 6 | 6 | 6 |
| | \bar{X} | 20.87 | 12.40 | 3216.00 |
| | σ_e | 1.70 | 0.63 | 205.26 |
| 18 Days | $\frac{n}{X}$ | 7 | 7 | 7 |
| | \bar{X} | 22.84 | 13.18 | 11824.83 |
| | σ_e | 1.48 | 0.90 | 560.11 |
| 20 Days | $\frac{n}{X}$ | 5 | 5 | 5 |
| | \bar{X} | 16.68 | 11.99 | 16275.36 |
| | σ_e | 0.64 | 1.06 | 989.62 |
| 21 Days | $\frac{n}{X}$ | 6 | 6 | 6 |
| | \bar{X} | 14.65 | 8.27 | 19538.78 |
| | σ_e | 0.88 | 0.35 | 611.43 |

¹n = the number of rats in the group.

\bar{X} = the arithmetic mean weight of the organ expressed in mg. %.

σ_e = the standard error of the arithmetic mean.

wet weights expressed in mg % of the rats. Ovarian weights were heaviest on day 18 and lightest (\approx) on days 6 and 21. The adrenal glands were heaviest on day 10 and lightest on day 21. Comparisons of uterine weights showed maxima on day 21 and minima on day 6.

Histochemical Data

The semi-quantitative estimates of the histochemical reactions were based on careful examination of the incubated ovarian tissue sections. The intensity scale was arrived at from a series of experiments discussed on page in the estrous cycle chapter. A tabular summary of the histochemical semi-quantitative assessments for each ovarian component on each day of necropsy during pregnancy is found in Table XI.

Primary Follicles

Primary follicles, during pregnancy, were observed to vary between trace (\pm) and weakly positive (+1) intensities when incubated with pregnenolone and dehydroepiandrosterone. Trace (monoformazan material) intensities were noted on days 8, 10, 13, 14, 18, and 21 (Plate XXXVII, fig. 243) using the substrate P-one. Weakly positive intensities of P-one- 3β -HSD diformazan were seen on days 6 (Plate XXXII, fig. 223; Plate XXXVII, fig. 249), 15 and 20. Using DHA as the substrate, trace intensities were seen on days 8, 10, 13, 14, 18, and 21 (Plate XXXVII, fig. 243) of pregnancy and weakly positive intensities on days 6 (Plate XXXII, fig. 223; Plate XXXVII, fig. 249), 15 and 20. These findings indicate that the primary follicles appear to have little ability to synthesize either progestational or estrogenic hormones.

Secondary Follicles

Ova. The ova observed in the primary-, secondary- and tertiary

TABLE XI

Averages of Semi-Quantitative Histochemical Estimates of 3β -Hydroxysteroid:NAD Oxidoreductase (3β -HSD) Localization Using Two Substrates, Pregnenolone (P-one) and Dehydroepiandrosterone (DHA), and Reduced Nicotinamide Adenine Dinucleotide Diaphorase (NADH_2 Diaphorase) in the Ovarian Components of the Pregnant Series of Rats on Days 6, 8, 10, 13, 14, 15, 18, 20, and 21.*

| Ovarian Components | 6 Days | | | 8 Days | | | 10 Days | | |
|---------------------------------------|--------|-----|----------------------|--------|-----|-----------------|---------|-----|-----------------|
| | P-one | DHA | NADH_2^{**} | P-one | DHA | NADH_2 | P-one | DHA | NADH_2 |
| Primary Follicle | +1 | +1 | +2 | ± | ± | +2 | ± | ± | +2 |
| Secondary Follicle Granulosa Cells | +1 | +1 | +2 | +1 | +1 | +2 | ± | +1 | +2 |
| Thecal Cells | +2 | +3 | +4 | +1 | +3 | +4 | +1 | +3 | +4 |
| Tertiary Follicle Granulosa Cells | +2 | +1 | +2 | +2 | +2 | +2 | +1 | +2 | +2 |
| Thecal Cells | +3 | +2 | +4 | +3 | +3 | +4 | +3 | +3 | +4 |

* 5 to 6 ovaries were used in each time interval of pregnancy except day 14 which included 4 ovaries.

** P-one designates the sections which were incubated with the substrate 3β -hydroxypregn-5-en-20-one (pregnenolone).

DHA designates the sections which were incubated with the substrate 3β -hydroxyandrost-5-en-17-one (dehydroepiandrosterone).

NADH_2 designates the sections which were incubated with the substrate reduced nicotinamide adenine dinucleotide ($\text{NADH} + \text{H}^+$).

TABLE XI CONTINUED

| Ovarian Components | 6 Days | | | 8 Days | | | 10 Days | | |
|---------------------|--------|-----|----------------------|--------|-----|-------------------|---------|-----|-------------------|
| | P-one | DHA | NADH ₂ ** | P-one | DHA | NADH ₂ | P-one | DHA | NADH ₂ |
| Atretic Follicle | | | | | | | | | |
| Granulosa Cells | +1 | +1 | +2 | +1 | +1 | +2 | +1 | +1 | +2 |
| Thecal Cells | +4 | +4 | +4 | +3 | +3 | +4 | +3 | +3 | +4 |
| Interstitial Tissue | +4 | +4 | +4 | +3 | +3 | +4 | +3 | +3 | +4 |
| Corpus Luteum | +3 | +4 | +4 | +3 | +4 | +4 | +3 | +3 | +4 |
| Hilar Region | 0 | 0 | +2 | 0 | +0 | +1 | 0 | 0 | +1 |

* 5 to 6 ovaries were used in each time interval of pregnancy except day 14 which included 4 ovaries.

** P-one designates the sections which were incubated with the substrate 3 β -hydroxypregn-5-en-20-one (pregnenolone).

DHA designates the sections which were incubated with the substrate 3 β -hydroxyandrost-5-en-17-one (dehydroepiandrosterone).

NADH₂ designates the sections which were incubated with the substrate reduced nicotinamide adenine dinucleotide (NADH + H⁺).

TABLE XI CONTINUED

| Ovarian Components | 13 Days | | | 14 Days | | | 15 Days | | |
|--------------------|---------|-----|----------------------|---------|-----|-------------------|---------|-----|-------------------|
| | P-one | DHA | NADH ₂ ** | P-one | DHA | NADH ₂ | P-one | DHA | NADH ₂ |
| Primary Follicle | ± | ± | +2 | ± | +1 | +2 | +1 | +1 | +2 |
| Secondary Follicle | | | | | | | | | |
| Granulosa Cells | ± | +1 | +2 | +1 | +1 | +2 | +1 | +1 | +2 |
| Thecal Cells | +2 | +2 | +4 | +2 | +3 | +4 | +2 | +2 | +4 |
| Tertiary Follicle | | | | | | | | | |
| Granulosa Cells | +1 | +1 | +2 | +1 | +2 | +2 | +1 | +2 | +2 |
| Thecal Cells | +2 | +2 | +4 | +2 | +3 | +4 | +2 | +3 | +4 |

* 5 to 6 ovaries were used in each time interval of pregnancy except day 14 which included 4 ovaries.

** P-one designates the sections which were incubated with the substrate 3 β -hydroxypregn-5-en-20-one (pregnenolone).

DHA designates the sections which were incubated with the substrate 3 β -hydroxyandrost-5-en-17-one (dehydroepiandrosterone).

NADH₂ designates the sections which were incubated with the substrate reduced nicotinamide adenine dinucleotide (NADH + H⁺).

TABLE XI CONTINUED

| Ovarian Components | 13 Days | | | 14 Days | | | 15 Days | | |
|---------------------|---------|-----|----------------------|---------|-----|-------------------|---------|-----|-------------------|
| | P-one | DHA | NADH ₂ ** | P-one | DHA | NADH ₂ | P-one | DHA | NADH ₂ |
| Atretic Follicle | | | | | | | | | |
| Granulosa Cells | +1 | +1 | +2 | +1 | +1 | +2 | +1 | ± | +2 |
| Thecal Cells | +3 | +3 | +4 | +3 | +3 | +4 | +4 | +3 | +4 |
| Interstitial Tissue | +3 | +3 | +4 | +3 | +3 | +4 | +3 | +3 | +4 |
| Corpus Luteum | +3 | +4 | +4 | +4 | +4 | +4 | +3 | +4 | +4 |
| Hilar Region | 0 | 0 | +1 | 0 | 0 | +1 | 0 | 0 | +1 |

* 5 to 6 ovaries were used in each time interval of pregnancy except day 14 which included 4 ovaries.

** P-one designates the sections which were incubated with the substrate 3 β -hydroxypregn-5-en-20-one (pregnenolone).
 DHA designates the sections which were incubated with the substrate 3 β -hydroxyandrost-5-en-17-one (dehydroepiandrosterone).
 NADH₂ designates the sections which were incubated with the substrate reduced nicotinamide adenine dinucleotide (NADH + H⁺).

TABLE XI. CONTINUED

| Ovarian Components | 18 Days | | | 20 Days | | | 21 Days | | |
|---------------------------------------|---------|-----|----------------------|---------|-----|-------------------|---------|-----|-------------------|
| | P-one | DHA | NADH ₂ ** | P-one | DHA | NADH ₂ | P-one | DHA | NADH ₂ |
| Primary Follicle | ± | +1 | +2 | +1 | ± | +2 | ± | +1 | +2 |
| Secondary Follicle Granulosa Cells | +1 | +1 | +2 | ± | +1 | +2 | +1 | +1 | +2 |
| Thecal Cells | +2 | +2 | +4 | +1 | +1 | +2 | +3 | +3 | +4 |
| Tertiary Follicle Granulosa Cells | ± | +2 | +2 | ± | +1 | +2 | +1 | +1 | +2 |
| Thecal Cells | +2 | +3 | +4 | +2 | +2 | +4 | +3 | +2 | +4 |

* 5 to 6 ovaries were used in each time interval of pregnancy except day 14 which included 4 ovaries.

** P-one designates the sections which were incubated with the substrate 3β-hydroxypregn-5-en-20-one (pregnenolone).

DHA designates the sections which were incubated with the substrate 3β-hydroxyandrost-5-en-17-one (dehydroepiandrosterone).

NADH₂ designates the sections which were incubated with the substrate reduced nicotinamide adenine dinucleotide (NADH + H⁺).

TABLE XI CONTINUED

| Ovarian Component | 18 Days | | | 20 Days | | | 21 Days | | |
|---------------------|---------|-----|----------------------|---------|-----|-------------------|---------|-----|-------------------|
| | P-one | DHA | NADH ₂ ** | P-one | DHA | NADH ₂ | P-one | DHA | NADH ₂ |
| Atretic Follicle | | | | | | | | | |
| Granulosa Cells | +1 | +1 | +2 | ± | ± | +2 | ± | +1 | +2 |
| Thecal Cells | +3 | +3 | +4 | +2 | +2 | +4 | +2 | +4 | +4 |
| Interstitial Tissue | +3 | +3 | +4 | +2 | +2 | +4 | +2 | +4 | +4 |
| Corpus Luteum | +3 | +4 | +4 | +2 | +3 | +4 | +1 | +2 | +4 |
| Hilar Region | 0 | 0 | +1 | 0 | 0 | +1 | 0 | 0 | +2 |

* 5 to 6 ovaries were used in each time interval of pregnancy except day 14 which included 4 ovaries.

** P-one designates the sections which were incubated with the substrate 3 β -hydroxypregn-5-en-20-one (pregnenolone).

DHA designates the sections which were incubated with the substrate 3 β -hydroxyandrost-5-en-17-one (dehydroepiandrosterone).

NADH₂ designates the sections which were incubated with the substrate reduced nicotinamide adenine dinucleotide (NADH + H⁺).

follicles appear to have inappreciable (negative) P-one- 3β -HSD activity (Plate XXXII, fig. 224; Plate XXXV, fig. 235; Plate XXXV, fig. 239), and DHA- 3β -HSD activity (Plate XXXX, fig. 268). These data suggest that the ova are not potential sources of the ovarian steroidal hormone.

Granulosa cells. In the secondary follicles, the granulosa cells were observed to vary between trace and weakly positive intensities of P-one- 3β -HSD activity. Trace intensities were seen on days 10 (Plate XXXIII, fig. 227; Plate XXXVII, fig. 244), 13 (Plate XXXIII, fig. 229) and 18 (Plate XXXV, fig. 235). Weakly positive intensities were noted on days 6 (Plate XXXII, figs. 222 and 223), 8 (Plate XXXII, fig. 224), 14 (Plate XXXVII, fig. 250), 15 (Plate XXXIV, fig. 233), 20 (Plate XXXV, fig. 238), and 21 (Plate XXXVI, fig. 241). These findings indicate that the granulosa cells of secondary follicles are, in the main, weakly positive sites of potential P-one- 3β -HSD activity during pregnancy.

Using the substrate DHA, the granulosa cells are consistently weakly positive (+1) sites of potential estrogenic hormonal production, *i. e.* days 6 (Plate XXXVIII, fig. 255; Plate XXXXIII, fig. 285), 8 (Plate XXXVIII, fig. 259), 10 (Plate XXXIX, fig. 261), 13, 14 (Plate XXXX, fig. 266), 15 (Plate XXXX, fig. 269), 18 (Plate XXXXI, fig. 271), 20 (Plate XXXXI, fig. 273; Plate XXXXIII, fig. 279), and 21 (Plate XXXII, fig. 276). These histochemical data, therefore, suggest that the granulosa cells of secondary follicles show at best weak P-one- and DHA- 3β -HSD activity during pregnancy.

Theca cells. The layer of thecal cells which showed enzymatic activity using histochemical techniques are theca interna cells. P-one- 3β -HSD activity fluctuated between weakly positive to moderate in the theca interna cells of the secondary follicles. Weakly positive (+1) intensities were observed on days 8 (Plate XXXII, fig. 224), 10 (Plate XXXIII, fig. 227; Plate XXXVII, fig. 244) and 18 (Plate XXXV, fig. 235) whereas moderate (+2) intensities of diformazan were seen the remaining days of pregnancy *i. e.*

days 6 (Plate XXXII, figs. 222 and 223), 13 (Plate XXXIII, fig. 229), 14 (Plate XXXVII, fig. 250), 15 (Plate XXXIV, fig. 233), 20 (Plate XXXV, fig. 238), and 21 (Plate XXXVI, fig. 241). Thus, these findings indicate that potential P-one- 3β -HSD activity is generally greater from day 13 to day 21 than it is earlier in pregnancy.

The response of the cellular components of the theca interna to the substrate DHA appeared strong (+3) on days 6 (Plate XXXVIII, fig. 255; Plate XXXXIII, fig. 285), 8 (Plate XXXVIII, fig. 259), 10 (Plate XXXIX, fig. 261), and 14 (Plate XXXX, fig. 266). Moderate (+2) intensities of diformazan were seen on days 13, 15 (Plate XXXX, fig. 269), 18 (Plate XXXXI, fig. 271), and 21 (Plate XXXXII, fig. 276). On day 20, DHA- 3β -HSD activity was assessed as weakly positive (Plate XXXXI, fig. 273; Plate XXXXIII, fig. 279). These data suggest that the theca interna cells of secondary follicles are possible sources of estrogenic production from day 6 to 14 of pregnancy and of progesterational production from day 13 to 21 of pregnancy in the rat.

Tertiary Follicles

Granulosa cells. In the granulosa cells of tertiary follicles, using P-one as substrate the intensity of the reaction ranged from moderate (+2) to trace (\pm) during pregnancy. Moderate intensities of diformazan deposition were observed on days 6 (Plate XXXII, fig. 223; Plate XXXVII, fig. 251) and 8 (Plate XXXII, fig. 224). Weakly positive reactions were seen on days 10 (Plate XXXIII, fig. 226), 13 (Plate XXXIII, fig. 229), 14 (Plate XXXIV, fig. 231), 15 (Plate XXXIV, fig. 233), and 21 (Plate XXXVI, fig. 241). On days 18 (Plate XXXV, fig. 236; Plate XXXVII, fig. 245) and 20 (Plate XXXV, figs. 238 and 239), trace intensities were noted. The granulosa cells of tertiary follicles appear to have great progesterational biosynthetic capacities

on days 6 and 8 of pregnancy than on days 18 and 20.

Utilizing DHA, as an indicator of estrogenic production, the granulosa cells of tertiary follicles ranged from moderate to weakly positive in DHA- 3β -HSD activity. Moderate intensities of diformazan were seen on days 8 (Plate XXXVIII, fig. 258), 10 (Plate XXXIX, fig. 260), 14 (Plate XXXX, fig. 266), 15 (Plate XXXX, figs. 268 and 269), and 18 (Plate XXXXI, figs. 271 and 272; Plate XXXXIII, fig. 286). Weakly positive reactions were observed on the remaining days of pregnancy, i. e. days 6 (Plate XXXVIII, fig. 225; Plate XXXXIII, fig. 280), 13 (Plate XXXIX, figs. 262 and 263), 20 (Plate XXXXI, fig. 273), and 21 (Plate XXXXII, fig. 275). Thus, the granulosa cells of tertiary follicles show moderate P-one- and DHA- 3β -HSD activities during pregnancy.

Thecal cells. The cellular components of the theca interna of the tertiary follicles were incubated with P-one in order to localize P-one- 3β -HSD activity which is indicative of progesterone biosynthesis. Strong (+3) intensities of diformazan deposition were seen on days 6 (Plate XXXII, fig. 223; Plate XXXVII, fig. 251), 8 (Plate XXXII, fig. 224), 10 (Plate XXXIII, fig. 226), and 21 (Plate XXXVI, fig. 241). Moderate intensities of P-one- 3β -HSD activity were observed on days 13 (Plate XXXIII, fig. 229), 14 (Plate XXXIV, fig. 231), 15 (Plate XXXIV, fig. 233), 18 (Plate XXXV, fig. 236; Plate XXXVII, fig. 245), and 20 (Plate XXXV, figs. 238 and 239). The response of the theca interna of tertiary follicles are importantly concerned with potential progestational production during pregnancy.

Utilizing the other substrate, DHA, the theca interna cells were observed to vary between strong and weak intensities of DHA- 3β -HSD activity during pregnancy. DHA- 3β -HSD activity was seen to be strongly intense on days 8 (Plate XXXVIII, fig. 258), 10 (Plate XXXIX, fig. 260), 14 (Plate XXXX, fig. 266), 15 (Plate XXXX, figs. 268 and 269), and 18

(Plate XXXXI, figs. 271 and 272; Plate XXXXIII, fig. 286). Moderate intensities of diformazan were noted on days 6 (Plate XXXVIII, fig. 255; Plate XXXXIII, fig. 280), 13 (Plate XXXIX, figs. 262 and 263), 20 (Plate XXXXI, fig. 273), and 21 (Plate XXXXII, fig. 275). It thus appears that this region is importantly involved in both estrogenic and progestational production during pregnancy.

Atretic Follicles

Granulosa cells. The granulosa cells of atretic follicles were observed to show more markedly reduced intensities of diformazan deposits with P-one and DHA than the theca interna cells. Weakly positive P-one- 3β -HSD activity was seen on days 6 (Plate XXXII, fig. 222; Plate XXXVII, fig. 252), 8 (Plate XXXII, fig. 224), 10 (Plate XXXIII, fig. 227), 13 (Plate XXXIII, fig. 229), 14 (Plate XXXIV, fig. 231), 15 (Plate XXXIV, fig. 234), and 18 (Plate XXXV, fig. 235). On days 20 (Plate XXV, fig. 238) and 21 (Plate XXXVI, fig. 241; Plate XXXVII, fig. 246), only trace (\pm) intensities of diffuse monoformazan material were noted in the granulosa cells of atretic follicles. These findings indicate that the granulosa cells of atretic follicles display little enzymatic activity in regard to progestational biosynthesis.

The response of the granulosa cells to DHA was found to vary between weakly positive (+1) and trace (\pm). Weakly positive DHA- 3β -HSD activity was observed on days 6 (Plate XXXVIII, fig. 255; Plate XXXXIII, fig. 287), 8 (Plate XXXVIII, fig. 259), 10 (Plate XXXIX, figs. 260 and 261), 13 (Plate XXXIX, figs. 262 to 264), 14 (Plate XXXX, fig. 265), 18 (Plate XXXXI, figs. 271 and 272), and 21 (Plate XXXXII, figs. 275 and 276). Diffuse pink monoformazan material (trace intensities) was noted on days 15 (Plate XXXX, fig. 269) and 20 (Plate XXXXI, fig. 274; Plate XXXXIII, fig. 281). The preceding data obtained, utilizing both P-one and DHA substrates, indicate that the granulosa

cells of atretic follicles are not very responsive to the precursors of progestational and estrogenic hormones.

Thecal cells. In these studies using the substrate P-one, an intensity for diformazan deposits ranged from maximal (+4) to moderate (+2). Diformazan deposits were maximal on days 6 (Plate XXXII, fig. 222; Plate XXXVII, fig. 252) and 15 (Plate XXXIV, fig. 234) of pregnancy. Strong (+3) intensities were observed on days 8 (Plate XXXII, fig. 224), 10 (Plate XXXIII, fig. 227), 13 (Plate XXXIII, fig. 229), 14 (Plate XXXIV, fig. 231), and 18 (Plate XXXV, fig. 235). Only two days were of moderate (+2) P-one- 3β -HSD activities, *i. e.* days 20 (Plate XXXV, fig. 238) and 21 (Plate XXXVI, fig. 241; Plate XXXVII, fig. 246). These findings indicate that the theca interna cells of atretic follicles are, for the most part, strongly reactive to the progestational hormonal substrate during pregnancy.

Utilizing DHA, it was found that the diformazan intensities in the thecal components of the atretic follicles varied between maximal (+4) and moderate (+2). Maximal intensities were observed on days 6 (Plate XXXVIII, fig. 255; Plate XXXXIII, fig. 287) and 21 (Plate XXXXII, figs. 275 and 276) of pregnancy whereas moderate intensities occurred on day 20 (Plate XXXXI, fig. 274; Plate XXXXIII, fig. 281). The remaining days of gestation, *i. e.* days 8 (Plate XXXVIII, fig. 259), 10 (Plate XXXIX, figs. 260 and 261), 13 (Plate XXXIX, figs. 262 to 264), 14 (Plate XXXX, fig. 265), 15 (Plate XXXX, fig. 269), and 18 (Plate XXXXI, figs. 271 and 272), strong intensities were noted. These data suggest that the cells of the theca interna are, for the most part, strongly reactive to both the estrogenic and progestational precursors during pregnancy.

Interstitial Tissue

The interstitial tissue appears to be very responsive to both the P-one

and DHA substrates during pregnancy. P-one- 3β -HSD activity was observed to be maximally intense (+4) on day 6 (Plate XXXII, figs. 222 and 223; Plate XXXVII, fig. 253). Strong (+3) intensities were seen on days 8 (Plate XXXII, fig. 224), 10 (Plate XXXIII, figs. 226 and 227), 13 (Plate XXXIII, fig. 229), 14 (Plate XXXIV, fig. 231), 15 (Plate XXXIV, figs. 233 and 234), and 18 (Plate XXXV, figs. 235 and 236). On days 20 (Plate XXXV, fig. 238) and 21 (Plate XXXVI, fig. 241; Plate XXXVII, fig. 247), moderate (+2) P-one- 3β -HSD activities were noted. Thus, these findings on the whole suggest that the interstitial tissue is indeed an important site of potential progesterational hormonal biosynthesis during pregnancy.

In the companion study, utilizing the DHA substrate, the cells of the interstitial tissue were observed to contain maximal (+4) to moderate (+2) intensities of diformazan deposits. Interestingly enough, maximal intensities of diformazan deposition were noted at the early aspect of pregnancy, i. e. on day 6 (Plate XXXVIII, figs. 255 and 256; Plate XXXVIII, fig. 288). Moderate intensities were noted on day 20 (Plate XXXI, fig. 273; Plate XXXIII, fig. 282). Strong (+3) intensities of diformazan deposition were observed during the first half of pregnancy, with the exception of day 6, specifically on days 8 (Plate XXXVIII, figs. 257 and 258), and 10 (Plate XXXIX, figs. 260 and 261); thereafter on day 13 (Plate XXXIX, figs. 262 to 264), 14 (Plate XXXX, figs. 265 and 266), 15 (Plate XXXX, fig. 270), 18 (Plate XXXXI, figs. 271 and 272), and 21 (Plate XXXXII, fig. 276), strong (+3) intensities were observed. These data demonstrate that the interstitial tissue is a major site of estrogenic production throughout pregnancy in the rat.

Corpus Luteum

During pregnancy, the corpora lutea which are present in the ovary are apparently functional. Thus, this study critically evaluates the

intensities of P-one- 3β -HSD and DHA- 3β -HSD activity observable in corpora luteal cells during the days of pregnancy studied. It was found that the intensities of diformazan deposits ranged from maximal (+4) to weakly positive (+1) for both substrates. P-one- 3β -HSD activity was maximal on day 14 (Plate XXXIV, figs. 231 and 232; Plate XXXVII, fig. 254) of pregnancy. Strong (+3) intensities of diformazan were seen on days 6 (Plate XXXII, fig. 223), 8 (Plate XXXII, figs. 224 and 225), 10 (Plate XXXIII, figs. 226 and 228), 13 (Plate XXXIII, fig. 230), 15 (Plate XXXIV, fig. 234), and 18 (Plate XXXV, figs. 235 and 237). Moderate (+2) intensities were noted on day 20 (Plate XXXV, fig. 240) whereas weakly positive (+1) intensities were observed on day 21 (Plate XXXVI, fig. 242; Plate XXXVII, fig. 248). These data, therefore, indicate that the corpora luteal cells appear to be, for the most part, strongly reactive for the P-one substrate from days 6 to 18 of pregnancy.

Utilizing DHA as the steroidal precursor, corpora luteal cells appeared to vary between maximum (+4) and moderate (+2) for DHA- 3β -HSD activity. Maximal intensities of diformazan deposition were observed on days 6 (Plate XXXVIII, fig. 256; Plate XXXXIII, fig. 289), 8 (Plate XXXVIII, fig. 257), 13 (Plate XXXIX, fig. 262), 14 (Plate XXXX, figs. 265 and 267), 15 (Plate XXXX, fig. 270), and 18 (Plate XXXXI, figs. 271 and 272). On days 10 (Plate XXXIX, fig. 261) and 20 (Plate XXXXI, figs. 273 and 274), strong (+3) intensities of diformazan deposition were seen. Moderate (+2) intensities were noted on day 21 (Plate XXXXII, fig. 277; Plate XXXXIII, fig. 283). These findings indicate that potential DHA- 3β -HSD activity appears to be greater than P-one- 3β -HSD activity during pregnancy in the rat.

Ovarian Hilus

The cells in the hilar region of the ovary were observed to contain

inappreciable to negative reaction products when incubated with the substrates P-one and DHA (Table XI). Those cells which did contain diformazan deposits were the cellular components of the interstitial tissue. Thus, the consistent lack of observable P-one- 3β -HSD and DHA- 3β -HSD activity in the cells of the hilus suggest that this region of the ovary is not seemingly concerned with steroid biosynthesis.

Comparative Analyses of Total Ovarian Composites

vs

Individual Ovarian Entities: An Overview

In order to simplify the cyto- and histochemical observations, it appeared of interest to ascertain and graph the arithmetic averages of the data obtained for each ovarian component studied. The vertical averages for each enzyme, *i. e.* P-one- 3β -HSD and DHA- 3β -HSD, appear in Table XII. The linear data is depicted in a major text figure, which is followed-up by a series of nine component graphs, each representing specific ovarian morphological entities, cf. Text figures 6, 6a-6i.

Discussion

Steroidogenesis in the pregnant animal has been studied using biochemical, histochemical and histological techniques. Early reports on the role of the ovary during pregnancy involved the surgical technique of ovariectomy in order to determine whether or not the ovary was required to maintain the pregnant condition in animals (Harris, 1927; Corner, 1928). Later, ovariectomized animals were treated with exogenous estrogens and progesterone to elucidate the function of the ovarian hormones during pregnancy (Lyons, 1943; Harris and Pfiffner, 1929; Suchowsky, 1963).

Biochemical studies pertaining to the secretion of estrogens into the

TABLE XII

Generalized and Summarized Data Based on All Ovarian Components

Presented as Averages for the Pregnant Series of Animals¹

| Enzymes* | <u>6 Days</u> | <u>8 Days</u> | <u>10 Days</u> | <u>13 Days</u> | <u>14 Days</u> | <u>15 Days</u> | <u>18 Days</u> | <u>20 Days</u> | <u>21 Days</u> |
|----------------------|---------------|---------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| P-one-3 β -HSD | 2.3 | 1.9 | 1.7 | 1.7 | 1.9 | 2.0 | 1.4 | 1.3 | 1.3 |
| DHA-3 β -HSD | 2.3 | 2.2 | 2.1 | 1.9 | 2.3 | 2.1 | 2.2 | 1.3 | 1.8 |

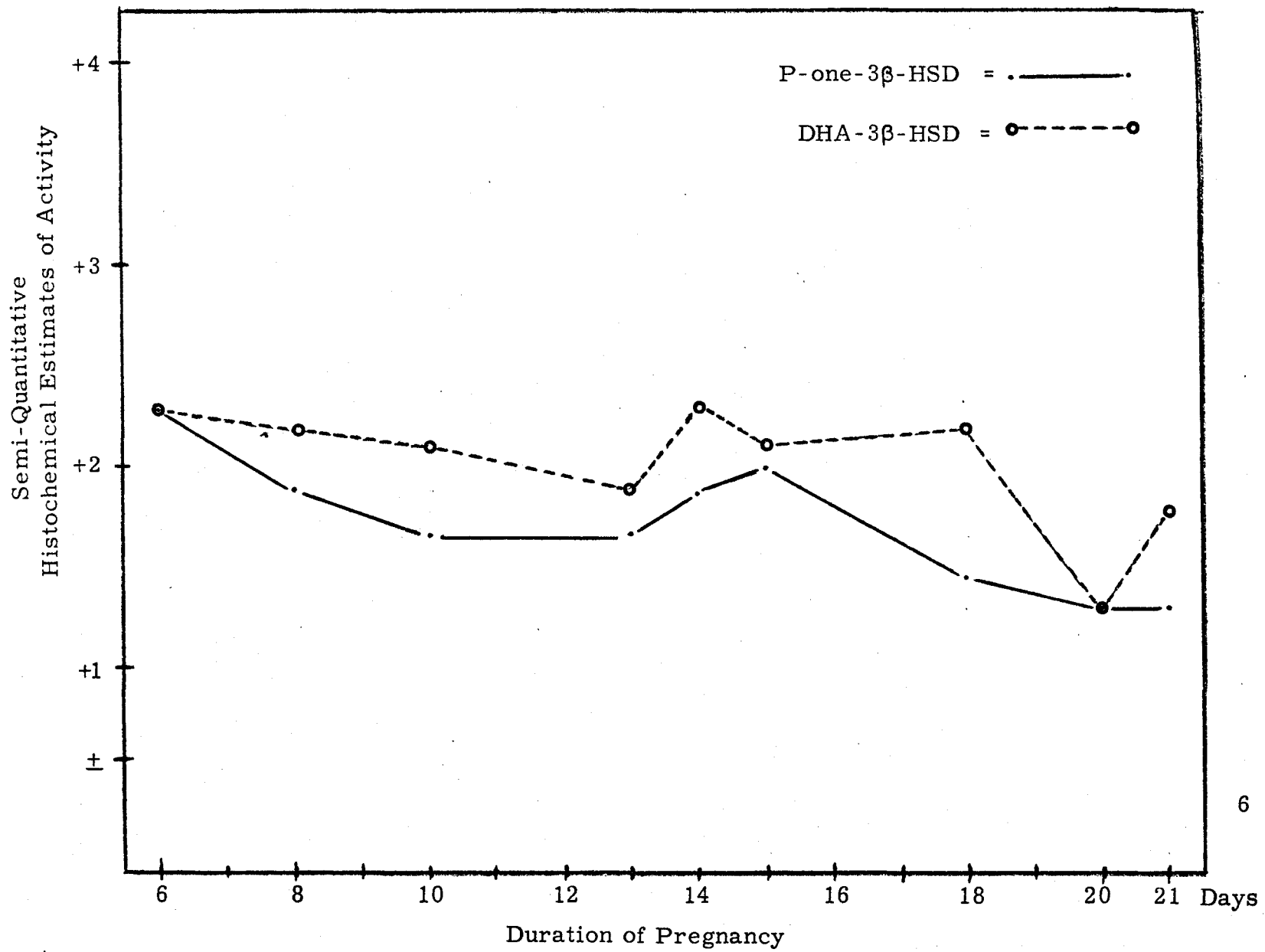
¹Arithmetic mean semi-quantitative histochemical estimates of 3 β -hydroxysteroid: NAD oxidoreductase (3 β -HSD) reactivity which were derived from the follicular, luteal and interstitial components of the ovaries in pregnancy as observed during the days studied.

*P-one-3 β -HSD denotes 3 β -HSD intensities using pregnenolone (P-one) as the incubation substrate.

DHA-3 β -HSD denotes 3 β -HSD intensities using dehydroepiandrosterone (DHA) as the incubation substrate.

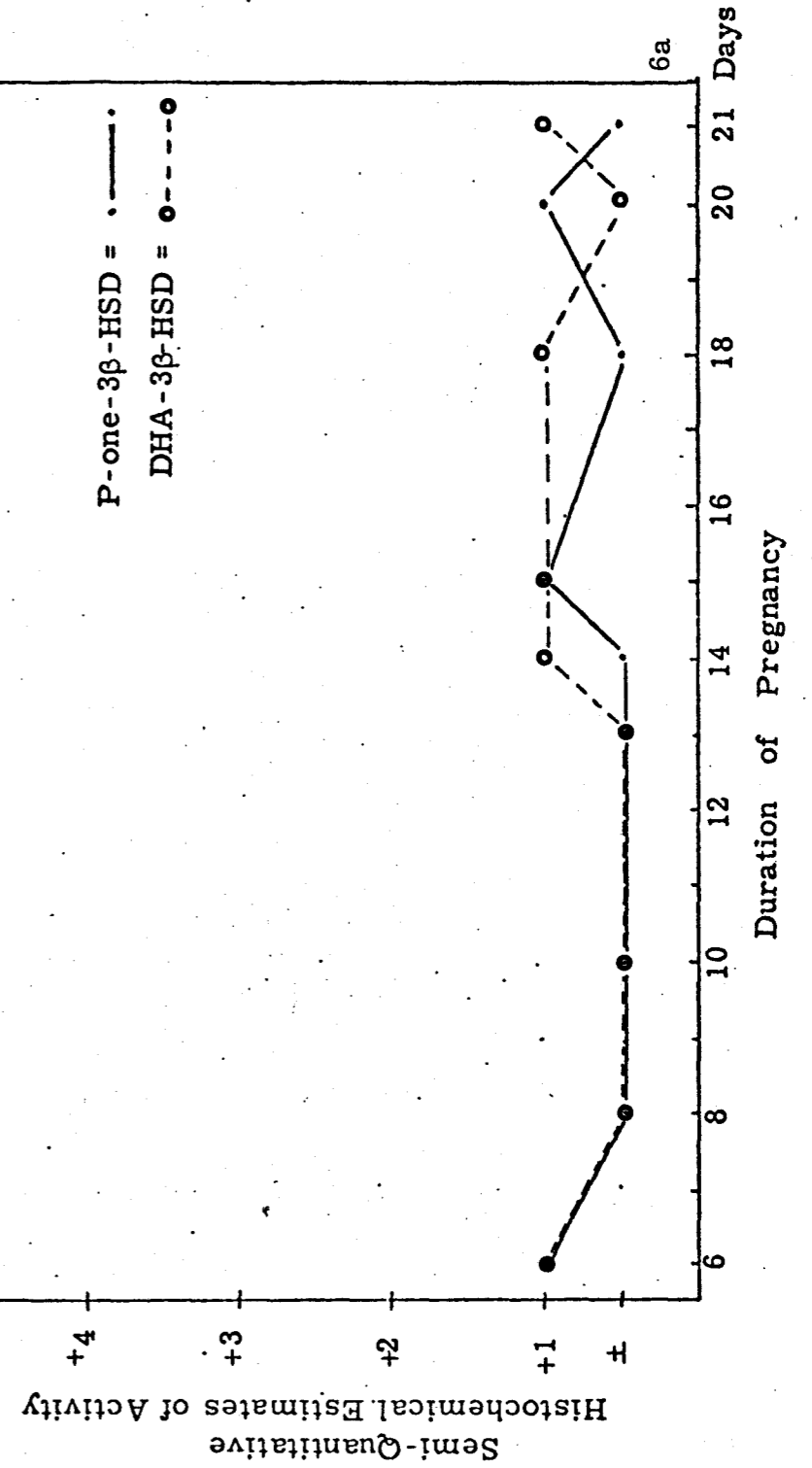
Estimates were rated on a 0 to +4 scale and a trace amount designated '+'.

Text Figure 6. A linear arrangement of the averages of semi-quantitative histochemical estimates of P-one- 3β -HSD (solid line) and DHA- 3β -HSD (dotted line) of all ovarian components on the days of pregnancy studied. Individual components are presented in similar, but individual activity graphs, in accompanying figures 6a-6i.

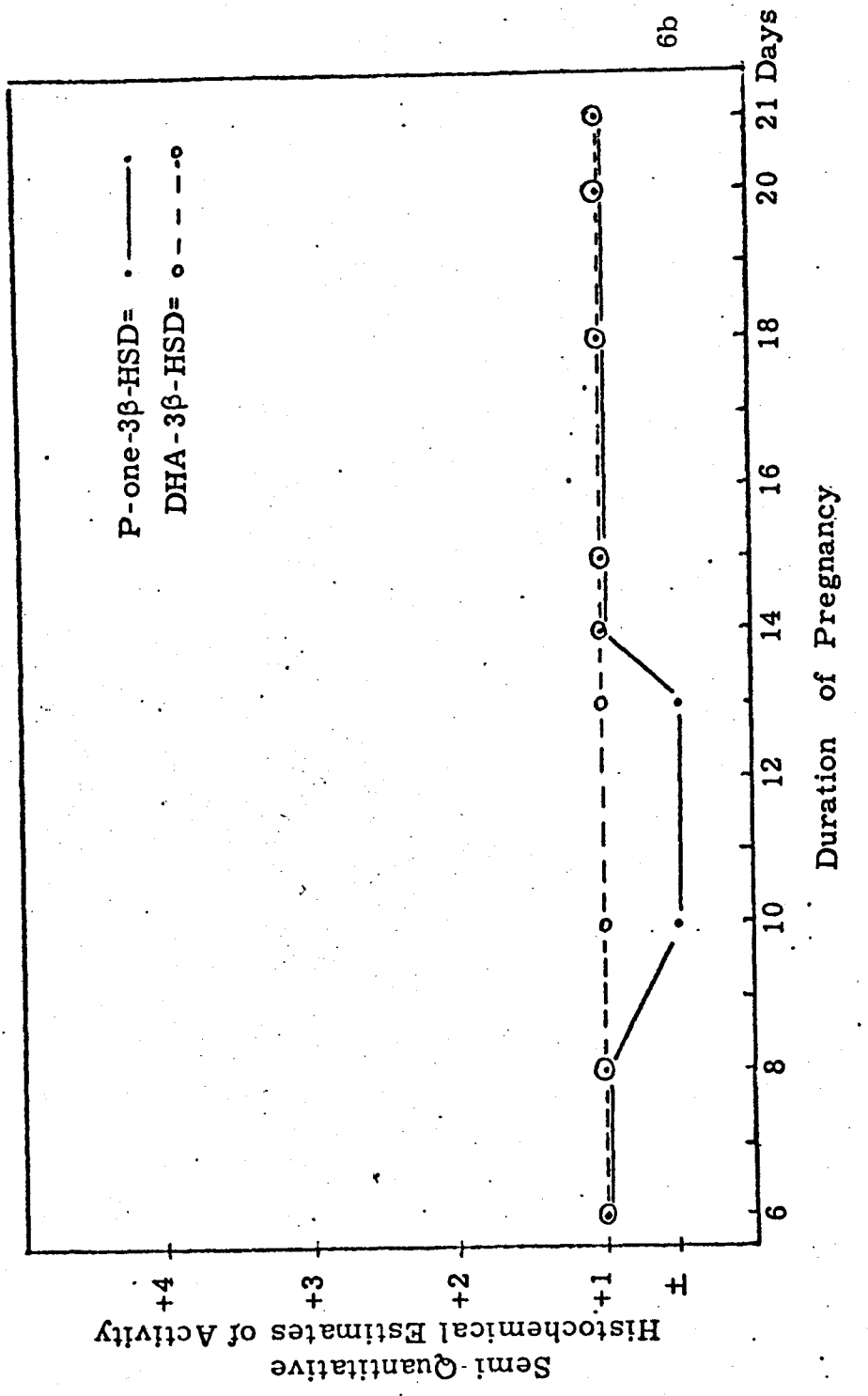


Test Figures 6a-6i. Averages of semi-quantitative histochemical estimates of P-one- 3β -HSD (solid line) and DHA- 3β -HSD (dotted line) of individual ovarian components during pregnancy:

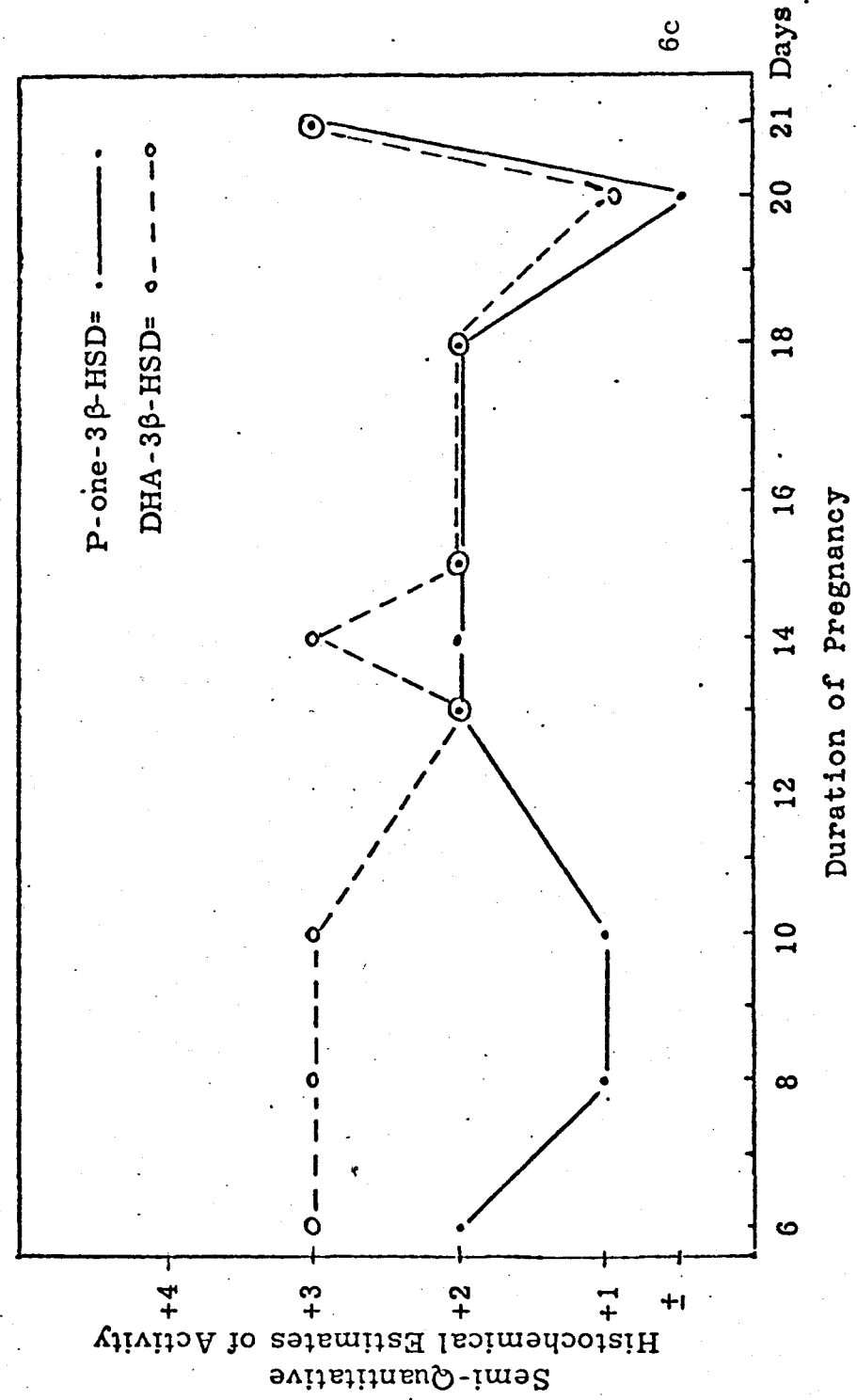
6a. Activities of P-one- 3β -HSD and DHA- 3β -HSD in primary follicles.



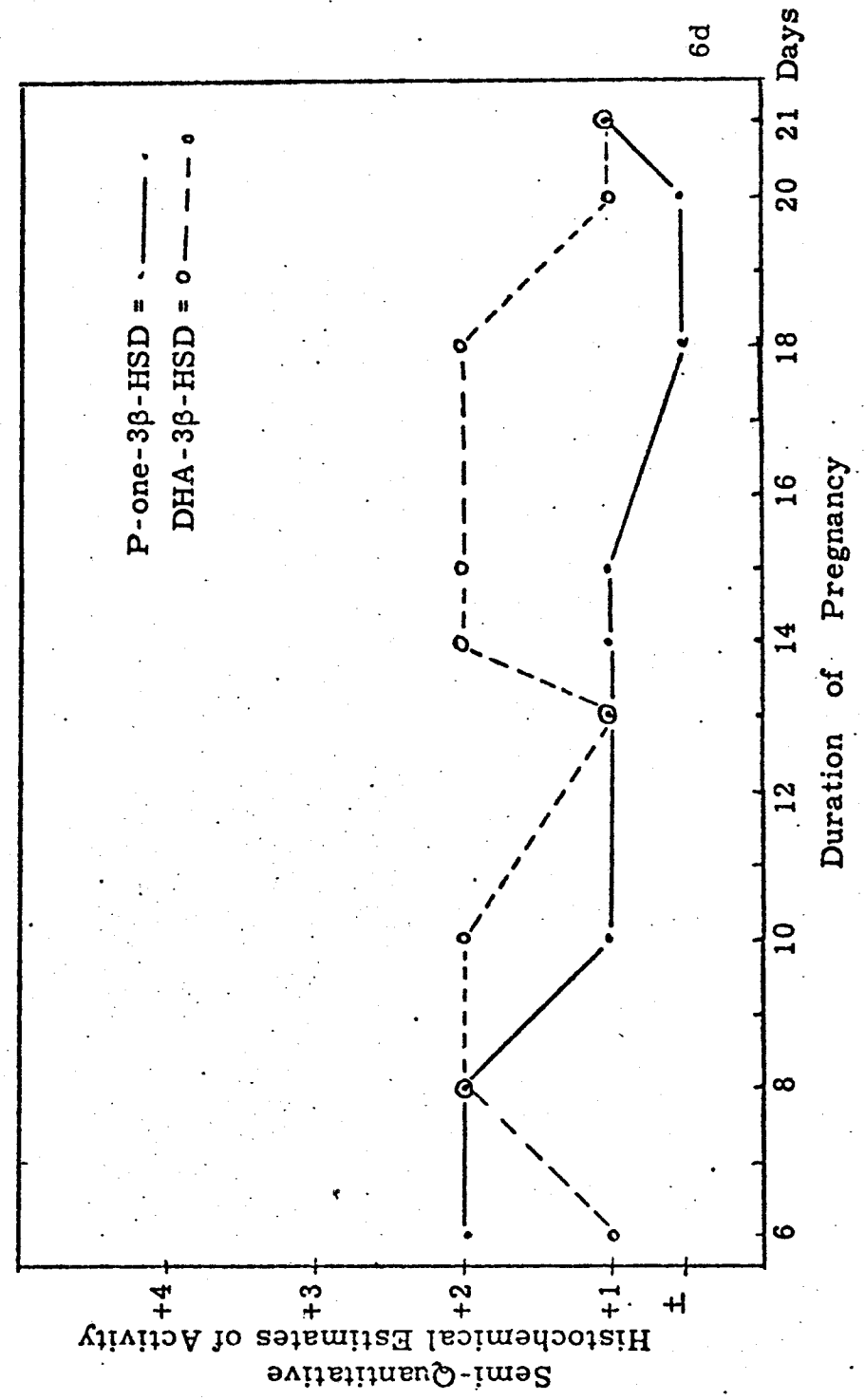
6b. Activities of P-one-3 β -HSD and DHA-3 β -HSD in granulosa cells of secondary follicles.



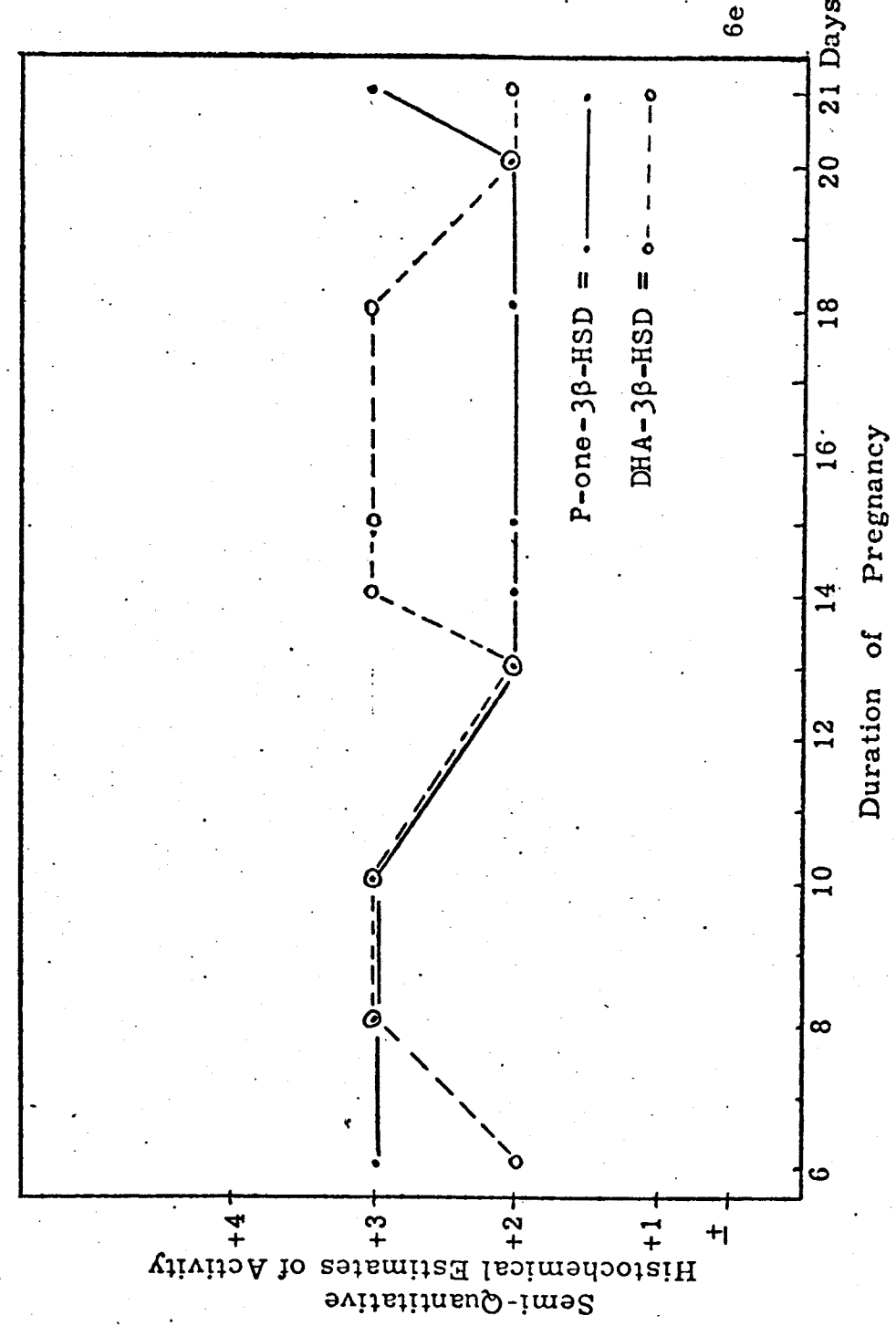
6c. Activities of P-one- 3β -HSD and DHA- 3β -HSD in theca interna of secondary follicles.



6d. Activities of P-one- 3β -HSD and DHA- 3β -HSD in granulosa cells of tertiary follicles.

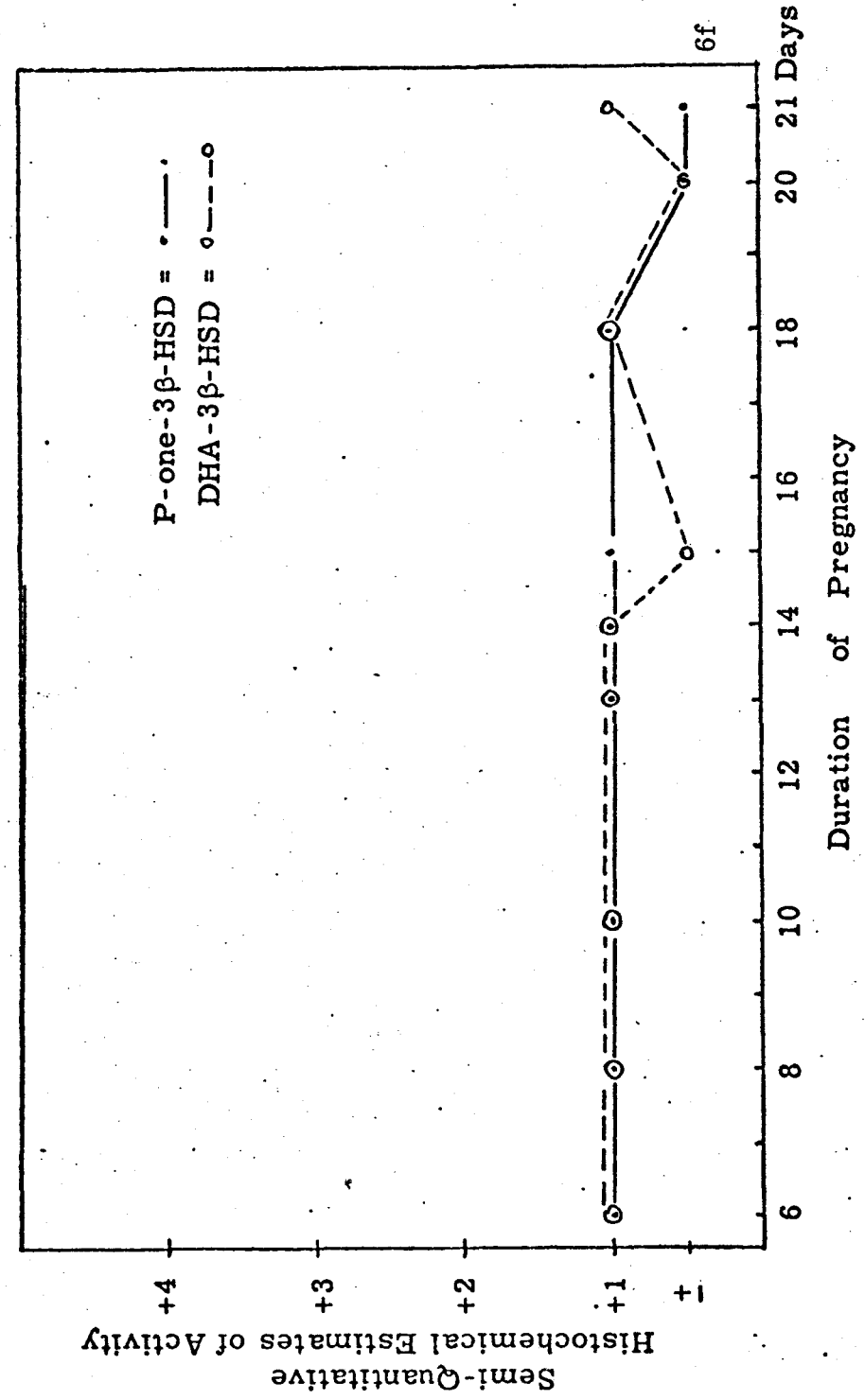


6e. Activities of P-one- 3β -HSD and DHA- 3β -HSD in theca interna of tertiary follicles.

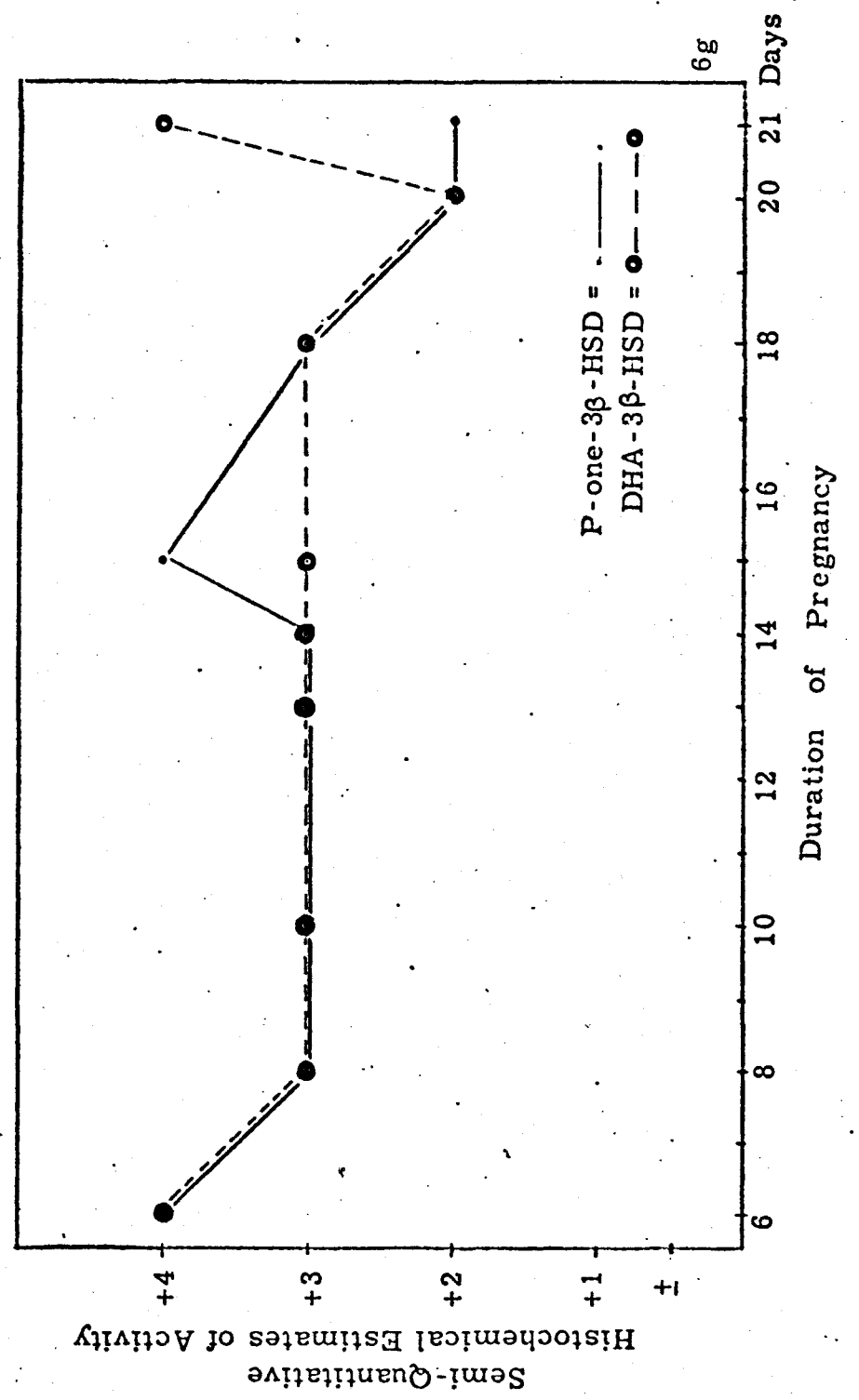


6e

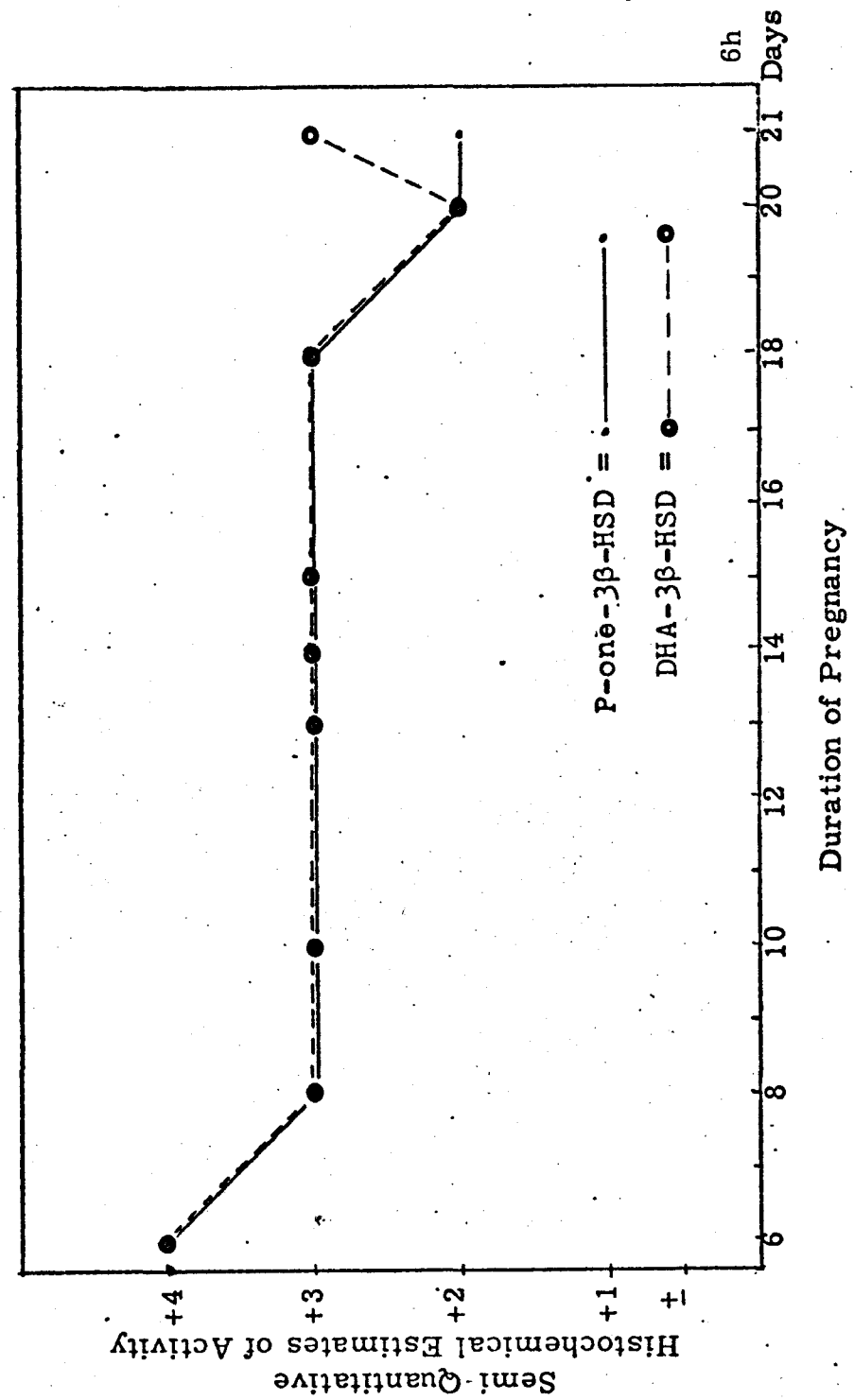
6f. Activities of P-one- 3β -HSD and DHA- 3β -HSD in granulosa cells of atretic follicles.



6g. Activities of P-one- 3β -HSD and DHA- 3β -HSD in theca interna of atretic follicles.

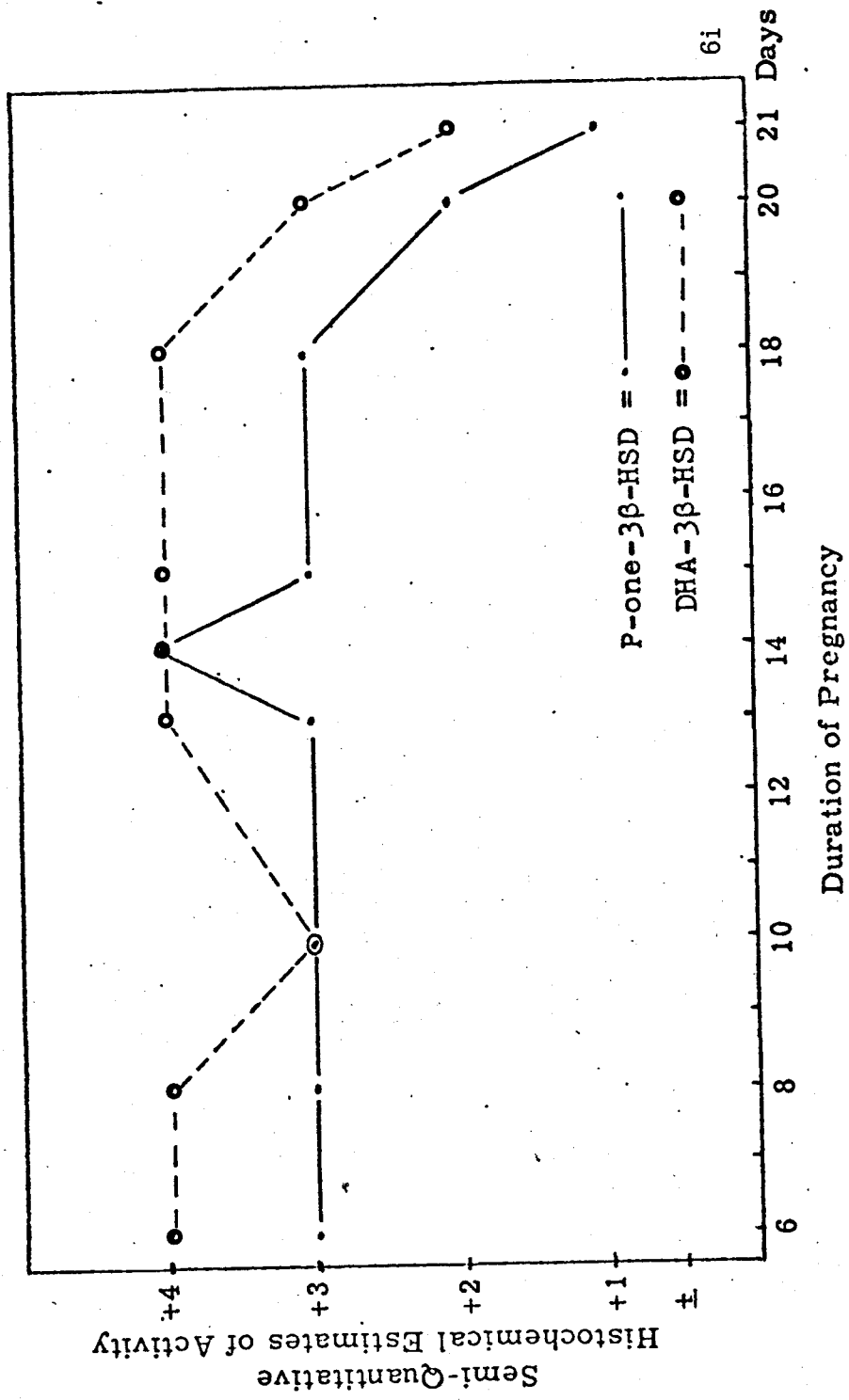


6h. Activities of P-one- 3β -HSD and DHA- 3β -HSD in ovarian interstitial tissue.



Duration of Pregnancy

6i. Activities of P-one- 3β -HSD and DHA- 3β -HSD in corpora lutea.



ovarian venous blood of pregnant rats, performed by Pope and Waynforth (1970) determined that there does not appear to be a peak of estradiol secretion on day 3, the day before implantation. Estrogen titers were approximately 100 pg./ovary/hr. on day 2, rising to approximately 500 pg./ovary/hr. on day 3, obviously low when contrasted with that of proestrus (approximately 3 ng/ovary/hr). Their findings further revealed that the estrogen titer on day 3 appears to be maintained at least to day 16, and further that the level on day 3 is quite similar to that for day 21, the time of parturition and the ensuing period of estrus. Yoshinaga et al. (1969) cannulated the ovarian vein of pregnant rats, determined the concentration of estrogens in the ovarian venous blood, and found that estrogenic secretion is low during pregnancy (6.68 ng/ovary/hr.) except near parturition (14.65 ng/ovary/hr.). The semi-quantitative histochemical averages of DHA-3 β -HSD activity in the ovary during pregnancy indicate that on days 6, 8, 10, 14, 15, and 18 potential estrogenic biosynthesis is approximately equal (+2.3 to +2.1) while on days 20 and 21 there appears to be a decrease in observable DHA-3 β -HSD activity. These histochemical findings agree in part with the studies of Pope and Waynforth.

Similarly, ovarian venous blood levels were assayed for progesterone titers during pregnancy, peak progesterone secretion being reported on day 13 of pregnancy in rats (26.9 μ g/hr./ovary), likewise high secretion rates were also found on day 15 (20.5 μ g/hr./ovary) ovarian progesterone venous blood titers were minimal on day 20 (2.7 μ g/hr./ovary) of pregnancy (Fajer and Barraclough, 1967). The semi-quantitative averages of P-one-3 β -HSD histochemical activity indicate that maximal observable activity occurs on day 6 and is high on day 15, while the lowest observable activity was seen on days 20 and 21 of pregnancy. The histochemical data indicates that potential progestational biosynthesis is higher between days 6 and 15 than

on the remaining days of pregnancy studied.

In studies on the hormonal requirements of pregnancy, Yochim and Zarrow, 1961 performed several noteworthy experiments, they ovariectomized pregnant rats on day 12 of pregnancy and administered estradiol-17 β , progesterone, relaxin or combinations of these hormones. They observed that pregnancy was maintained and there was an increase in fetal survival when estrogen and progesterone were administered twice daily to these animals. If, however, only progesterone was administered there was a marked reduction in fetal survival. This histochemical study likewise indicates that both estrogenic and progestational production occurs during pregnancy.

In a study on the effectiveness of exogenous progesterone administration in ovariectomized, de-luteinized or ergocornine injected pregnant rats, it was reported that progesterone administration (4 mg. daily) results in normally maintained pregnancy in the de-luteinized and ergocornine-treated rats, while it is not effective in ovariectomized pregnant rats (Kraicer et al., 1971), thus indicating that pregnancy is maintained by the activities of luteal and non-luteal ovarian components. This current study has shown a marked potential for both estrogenic and progestational biosynthesis by the theca interna cells of secondary-, tertiary- and atretic follicles as well as by the interstitial tissue. Further, on certain days the granulosa cells exhibited a moderate capability for the production of estrogenic and progestational hormones.

Motta and Bourneva (1970a and 1970b) reported that P-one-3 β -HSD activity appeared to be highest in the interstitial tissue on days 5 to 6 of pregnancy and was moderate on days 10 to 14. The findings of this present study show that P-one-3 β -HSD activity is maximal on day 6, declines to strong intensities between days 10 and 18, and then is moderate on days 20

and 21 of pregnancy. These data suggest that the interstitial cells are an important source of progesterone synthesis during pregnancy, and add additional corroborative evidence to the work of Kraicer et al. (1971).

In an autoradiographic study on folliculogenesis during pregnancy in the mouse, it was determined that follicles continue to grow and develop during pregnancy (Pedersen and Peters, 1971). This current study observed secondary follicles in the ovaries of rats on the days of pregnancy studied, thus confirming and strengthening the foundation of these cyto- and histochemical studies during pregnancy. Analysis of the histochemical findings indicate that the granulosa cells of secondary follicles showed little potential P-one- and DHA- 3β -HSD activity, whereas the cells of the theca interna appeared to vary between moderate and strong enzymatic activity indicating steroidal biosynthetic activity in this component during pregnancy. Since this histochemical study observed potential steroidal biosynthesis in follicles in which the granulosa and thecal components were in juxtaposition, these results suggest that the follicular components, i. e. the granulosa and theca interna cells, have the ability to synthesize both progesterone and estrogens. Further, the cells of the theca interna appear to be more responsive to the estrogenic precursor (DHA) during days 6 through 14 than to P-one.

In initial studies by Motta and Bourneva (1970a and 1970b), the granulosa cells of large follicles showed trace intensities of P-one- 3β -HSD on day 14 of pregnancy, whereas theca interna cells appeared to localize moderate intensities of both P-one- and DHA- 3β -HSD on days 5 to 6, 10 and 14 of pregnancy. Our histochemical investigation revealed only weakly positive to moderate intensities of diformazan deposition located in the granulosa cells on the days of pregnancy studied using the substrates P-one and DHA which are indicative of progestational and estrogenic hormonal

production, respectively. The cells of the theca interna were seen to vary between moderate and maximal in the tertiary follicles during pregnancy. Strong potential P-one- 3β -HSD activity was seen on days 6, 8, 10, and 21; strong potential DHA- 3β -HSD activity was observed on days 8, 10, 14, 15, and 18. Furthermore, strong intensities of diformazan were observed, in the main, during pregnancy when the ovarian sections were incubated with P-one and DHA. These histochemical findings indicate that the cellular components of tertiary follicles are able to synthesize progesterone and estrogen during pregnancy.

From a study in which daily injections of ergocornine were administered to pregnant rats, Turolla *et al.* (1969) observed that 20α -hydroxysteroid dehydrogenase (20α -HSD) activity appeared in ovarian luteal cells if ergocornine treatment was given during the first five days of pregnancy. As discussed in the section on prolonged pseudopregnancy, 20α -HSD activity in corpora luteal cells is indicative of a non-functional corpus luteum. Loewit and Lawrence (1969) administered anti-LH serum to rats on days 7-10, 7-11, 12-16 and 14-18 of pregnancy and found that the termination of pregnancy due to anti-LH treatment was the result of interference with the functioning of the corpus luteum since anti-LH serum had no effect on either feti, period of gestation or parturition when given from days 14-18. Our histochemical study observed that the corpora luteal cells have the ability to synthesize both estrogenic and progestational hormones during pregnancy. Peak potential for progesterone biosynthesis appears to occur on day 14 of pregnancy whereas maximal potential for estrogenic biosynthesis seems to take place between days 6 through 18 of pregnancy.

As indicated in the introduction, during pregnancy, the sites of potential estrogenic and progesterone production include the maternal ovaries, the placenta and the fetal gonads. Schlegel *et al.* (1967),

following a histochemical study which localized P-one- 3β -HSD and DHA- 3β -HSD in the adrenal cortex, ovaries and testes of fetal rats between 15 1/2 and 20 1/2 days of gestation reported that: 1) the fetal ovaries showed negligible histochemical enzymatic activity for both substrates; 2) the fetal adrenal cortex was observed to have a positive reaction for both P-one- and DHA- 3β -HSD on the days of gestation studied; and 3) the interstitial tissue of the testes also had positive reactions for both substrates during the fetal period. Their findings indicate that the fetal adrenal gland and testes have the potential for steroidal production even though the fetal ovaries are devoid of enzymatic activity. The required level of estrogenic and progestational hormones required to maintain the pregnant condition may, therefore, arise from all of the aforementioned organs. Our studies indicate that the maternal ovary appears to be a dominant site of potential steroid capabilities during this physiological condition.

Summary and Conclusions

The ovaries of pregnant rats were histochemically studied to determine the sites and intensities of P-one- 3β -HSD and DHA- 3β -HSD. Animals were necropsied on days 6, 8, 10, 13, 14, 15, 18, 20, and 21 of pregnancy. Enzymatic activity was rated on a 0 to +4 scale. Assessments revealed: 1) +4 for P-one- 3β -HSD in theca interna (T.I.) of atretic follicles (A.F.) and interstitial tissue (I.T.) on days 6 and 15 and +2 on days 20 and 21; 2) +4 for P-one- 3β -HSD on day 14 in corpora lutea (C.L.) and +1 on day 21; 3) +4 for DHA- 3β -HSD in C.L. on days 13, 14, 15, and 18 and +2 on day 21; 4) +4 for DHA- 3β -HSD in T.I. of A.F. and I.T. on day 21 and +2 on day 20; and, on days studied, 6-21 v. s.: 5) traces (\pm) to +1 in primary follicles (P.F.) for P-one- and DHA- 3β -HSD; 6) \pm to +2 in granulosa cells (G.) of secondary- (S.F.) and tertiary follicles (T.F.) for P-one- and DHA- 3β -HSD; 7) \pm to +1 for P-one- and DHA- 3β -HSD in G. of A.F.; and 8) +1 to +3 in

T.I. of S.F. and T.F. for both substrates. Thus, P-one- 3β -HSD and DHA- 3β -HSD activities vary at different times during the stages of pregnancy studied, owing chiefly to changing ovarian steroid hormonal requirements for a successful pregnancy.

The histochemical data pertaining to the cellular and tissue sources of potential estrogenic and progestational hormonal production indicates a variation in the enzymatic activity localized in the different components of the ovaries of rats during pregnancy. Utilizing critical observations on the sites of P-one- 3β -HSD and DHA- 3β -HSD activity, one observes that each of these enzymes is very active in the corpora lutea, interstitial tissue, and theca interna cells of secondary- and tertiary follicles as well as in atretic follicles. The granulosa cells appear to play a relatively less important role in steroidal biosynthesis, particularly in the secondary- and atretic follicles. The tertiary follicles appear to have a greater potential for progestational hormonal biosynthesis than estrogenic biosynthesis on day 6 of pregnancy; however, on days 10, 14, 15, and 18 of pregnancy, the tertiary follicles display an apparently greater potential for estrogenic production.

The interstitial tissue appears to be the one consistently strong source for both estrogenic and progestational production. In the main, the intensities of diformazan deposition in the cells of the interstitial tissue are strong. This is indicative of steroidal capabilities and the involved hydrogen transport system. These findings are consistent with the data observed during the estrous cycle, and those of normal and prolonged pseudopregnancy in the rat, as well.

Further, the cellular components of the corpus luteum are noted to be one of the major sources of potential estrogenic and progestational hormonal synthesis up to day 20 of pregnancy. These data indicate that potential

estrogenic production by the luteal cells is greater than that of potential progesterone production as observed from the intensities of diformazan deposited in the loci of DHA- and P-one- 3β -HSD activity, respectively. On day 14 of pregnancy, the luteal cells showed maximal histochemical localization of both P-one- 3β -HSD and DHA- 3β -HSD. There was a marked reduction in response in both P-one and DHA substrates on day 21 of pregnancy in the corpora lutea.

These histochemical findings indicate that in the pregnant condition there is an increase in enzymatic activity in all of the ovarian components, excepting the primary follicles, ova, and hilus, for potential estrogenic and progesterone hormonal biosynthesis. These data, when viewed in light of those of the estrous cycle, present a picture which shows that the ovarian components are capable of synthesizing greater amounts of both estrogen and progesterone by increasing the enzymatic activity in the several components of secondary-, tertiary- and atretic follicles, except the ova, and in the interstitial tissue and corpora lutea throughout 20 days of the 21-22 day event of pregnancy. The ovarian hilus, the ova and primary follicles were notably devoid of appreciable activity. Thus, in summation, these data conclusively show that the ovary of the pregnant rat is quite involved in the biosynthesis of estrogen and progesterone.

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General Discussion

The dissertation, herein presented in four distinctive units, makes a unified attempt to ascertain the localization of the actual ovarian sites involved in the production of estrogen and progesterone. Utilizing pregnenolone-3 β -hydroxysteroid dehydrogenase (P-one-3 β -HSD) and dehydroepiandrosterone-3 β -hydroxysteroid dehydrogenase (DHA-3 β -HSD) as substrates, respectively for progesterone and estrogen, it was determined that these substrates proved quite revealing, especially since both of the steroid hormones were capable of detection throughout the estrous cycle and the superimposed events of normal pseudopregnancy, prolonged pseudopregnancy, and the full biological career of pregnancy. Thus, the intensive utilization of specific cytochemical studies permits of rather detailed analyses of both the sites and semi-quantitative estimations of estrogen and progesterone. Results at hand provide an informational bridge between the early techniques and those derived from methods involving the presently used enzymatic cytochemical techniques.

It has been almost 25 years since the initial studies of Professor A. B. Dawson and his students displayed quite convincingly the relationship of cholesterol and its esters to ovarian steroid hormonal production in juvenile and immature female rats and throughout the estrous cycle and pseudopregnancy as well (Rennels, 1951; Dawson and Velardo, 1955). Of parallel interest and of more recent time, Wattenberg, 1958; Levy et al., 1959; Deane et al., 1962; Pupkin et al., 1966; Motta and Bourneva, 1970a and 1970b, determined that the utilization of tetrazolium dyes permitted the localization of steroid hormone precursors. Inasmuch as no one to date has critically demonstrated or presented extensive pictorial documentation of the cellular and tissue sites of potential progestational and estrogenic production during each of the six distinctive stages of the estrous cycle and in the

different cyto- and histomorphological components of the ovary, *i.e.* the ova, primary-, secondary- and tertiary follicles, atretic follicles, interstitial tissue, corpora lutea, and the ovarian hilus, it became of significant interest to initially ascertain the critical changes in P-one- 3β -HSD and DHA- 3β -HSD associated with reproductive cyclicity and reproductive function. Further, these aforementioned morphological components of the ovary were also assessed during normal (ordinary-induced) pseudopregnancy, pseudopregnancy with decidual tissue (prolonged pseudopregnancy) and pregnancy.

As presented in the general introduction, this cyto- and histochemical study of the ovary of the rat was performed to investigate three major questions, *i.e.* there alterations in potential progesterational and estrogenic biosynthesis: 1) in the different ovarian components, 2) during the six distinctive stages of the estrous cycle, and 3) during normal and prolonged pseudopregnancy, as well as during pregnancy (see pages 7 and 8).

Data at hand reveals that there is an increase in the capability of the granulosa cells for progesterational and estrogenic biosynthesis as reflected in P-one- 3β -HSD and DHA- 3β -HSD activity during folliculogenesis. The granulosa cells which comprise the primary follicles appear to have negligible ability to synthesize ovarian progesterone and estrogens during the estrous cycle, normal and prolonged pseudopregnancy as well as during pregnancy. As the follicles grow and mature during folliculogenesis, the granulosa cells manifest an increase in ability to synthesize ovarian estrogenic and progesterational hormones. Such changes can be seen in the secondary follicles. The granulosa cells are further matured in tertiary follicles, especially the granulosa cells which are in contiguity with the cells of the theca interna, and show further increase in observable activity for both enzymes.

There is a marked distinction between the functions of the cellular

components of the theca interna and theca externa with respect to potential steroidal biosynthesis. The theca interna cells were observed to be responsive to both steroidal precursor substrates, *i.e.* P-one and DHA, whereas the theca externa cells were observed to be non-responsive to these substrates or showed negligible activity. Therefore, it appears that the highly vascular theca interna of follicles is an important site of potential steroidal biosynthesis while the theca externa does not appear to contribute to the production of ovarian estrogens and progesterone.

As was the case for the granulosa cells, the cellular components of the theca interna do appear to have gradations in observable P-one- and DHA- 3β -HSD activity during folliculogenesis. The thecal elements of tertiary follicles, particularly in animals having experienced numerous estrous cycles, were histochemically more responsive to the steroidal substrates than was the case of thecal elements of secondary follicles. These findings pertain to follicles in which the thecal components are in contiguity with the granulosa components. The studies of Falck (1959) and Bjersing and Carstensen (1967) are consonant with the idea that for estrogenic biosynthesis to occur, the granulosa and theca interna cells are required to be in contiguity. Histochemical data presented in this dissertation actually confirms and photographically records evidence to support the hypothesis that both follicular elements have the ability to synthesize estrogenic and progestational hormones in the *in vivo* condition.

The corpora lutea were examined in order to critically assess their potential ability to synthesize both progesterone and estrogens. It was observed, as was true of the follicular components, that the luteal cells display alterations in the potential for both progestational and estrogenic production. The localization and distribution of diformazan granules in granulosa-lutein as well as theca-lutein cells indicates that both are cellular

sites of P-one- 3β -HSD and DHA- 3β -HSD activity. These histochemical findings are in agreement with the studies of Dawson and Velardo (1955) who localized cholesterol esters in luteal cells of rats during the estrous cycle, normal pseudopregnancy and prolonged pseudopregnancy.

Utilizing biochemical and ultrastructural techniques, McDonald *et al.* (1969) correlated the luteal sites of progesterone biosynthesis with the blood titers of this steroid in the rat during the estrous cycle. Our histochemical findings present additional evidence that luteal cells vary in potential P-one- 3β -HSD and DHA- 3β -HSD activities during each of the physiological conditions studied. Times of maximal P-one- 3β -HSD activity in the luteal cells occurred during metestrus and proestrus in the younger series of animals (Y-) and during proestrus in the regularly cycling series (R-), on day 6 of normal pseudopregnancy, on days 6 through 13 of prolonged pseudopregnancy, and on day 14 of pregnancy. As previously discussed, when the functional life of the corpus luteum is extended, as in pseudopregnancy and pregnancy, there is heightened activity for extended periods of time in the luteal cells as demonstrated in this cyto- and histochemical study.

Ovarian interstitial tissue and the theca interna of atretic follicles were seen to vary in the intensity of the reaction product formed. This study adds additional evidence that the interstitial tissue and those elements destined to become interstitial tissue are important sites of both P-one- and DHA- 3β -HSD activities during the estrous cycle, normal and prolonged pseudopregnancy and in pregnancy. Our data are in agreement with the findings of Rennels (1951), Pupkin *et al.* (1966), Motta and Bourneva (1970a and 1970b), and Motta and Takeva (1971) who reported that the interstitial tissue is a source of ovarian estrogenic and progestational formation. Our current histochemical study indicates that the interstitial tissue is one of the main sites of both potential estrogenic and progestational biosynthesis during the estrous cycle

and also during the conditions of extended luteal functional life, i. e. in both forms of pseudopregnancy and during pregnancy.

From the tabular data in the text which presents an average of the total estimates (i. e. total average of all estimates of all the ovarian components) for both P-one- 3β -HSD and DHA- 3β -HSD activities, it appears that the ovary does change in steroidogenic capabilities as a whole in order to maintain the proper balance and titers of estrogens and progesterone required at any one time in the rat. Although variations do appear in intensities from structure to structure, ranging from trace (\pm) to maximal (+4), an overall estimate of all structures yielding the highest estimate for P-one- 3β -HSD is (+2.3) during proestrus in the Y- series, and lowest (+1.7) during estrus. In the R- series, P-one- 3β -HSD activity was highest (+2.1) during estrus and late diestrus and quite low (+0.8) during early diestrus. These fluctuations in the activities of both steroidal enzymes correspond to the times of highest progestational and estrogenic blood titers as reported in the discussion of the estrous cycle. Our findings for DHA- 3β -HSD activity are in accord with the biochemical report by Chatterton, Chatterton and Greep (1969) in which it was shown that the conversion of DHA- to estradiol- 17β was greatest in pooled ovarian homogenates which were taken from rats in proestrus and lowest in those necropsied during metestrus and diestrus. DHA- 3β -HSD activity was highest during proestrus in both the Y- and R- series of rats and lowest during estrus and metestrus in the Y- series as well as during early diestrus in the R- series. These data indicate that there is in fact a change in the production of estrogens at different times during the estrous cycle which is reflected in the physiology of the normally cycling non-manipulated female rat as herein studied.

During normal pseudopregnancy, P-one- 3β -HSD activity was highest (+2.6) on day 6 and lowest (+1.0) on day 13 while DHA- 3β -HSD activity

was highest (+2.0) on day 13 and lowest (+1.4) on day 6. These average estimates indicate that, during early phases of normal pseudopregnancy, potential progesterational biosynthesis appears to be higher than estrogenic biosynthesis whereas the reverse appears to be the case on day 13 of normal pseudopregnancy. When compared with the summarized data obtained for the estrous cycle R- series of rats which were of comparable age, it was found that observable P-one- 3β -HSD activity was higher on day 6 of normal pseudopregnancy than at any time during the estrous cycle. DHA- 3β -HSD activity on day 13 of normal pseudopregnancy appeared to be at levels between late diestrus and metestrus in the R- series of animals. This is indicative of the apparent lesser amounts of estrogenic compounds needed during normal pseudopregnancy.

In the condition of extended luteal function during prolonged pseudopregnancy, P-one- 3β -HSD localization was approximately similar at the higher levels on days 6, 8 and 13 (+2.1 to +2.3) whereas lowest observable summarized localizations (+1.4) were noted on day 21. The estimates for P-one- 3β -HSD activity appeared to be only slightly greater than that observed during estrus in the R- series of rats. These findings are in partial agreement with the biochemical studies of Hashimoto and Melampy (1967) who reported that progesterone blood levels rose on day 4 of prolonged pseudopregnancy and did not decline until day 21. Between days 15 and 21, the current histochemical data appeared to be at levels similar to those observed during late diestrus, preproestrus and proestrus in the R-series of animals. Interestingly enough, these are the stages of the estrous cycle during which folliculogenesis occurs in preparation for the process of ovulation.

DHA- 3β -HSD activity appeared to be highest (+2.2) on day 13 of prolonged pseudopregnancy and lowest (+1.4) on day 21. The high estimate at this time is the same as that occurring during late diestrus whereas the low

estimate during prolonged pseudopregnancy is the same as the value noted on day 6 of normal pseudopregnancy. From experimental studies involving hormonal administration to ovariectomized, pseudopregnant rats having uterine traumatization (Velardo and Hisaw, 1951; Velardo, 1957a and 1957b; Corner and Allen, 1929; Allen and Corner, 1929), it was demonstrated that both estrogens and progesterone are needed to maintain prolonged pseudopregnancy. Weichert (1928) showed that the induction of the decidual reaction is due to the synergistic action of these hormones in proper proportion. It appears that the requirements for potential estrogenic production during prolonged pseudopregnancy are slightly less than those required during proestrus and estrus in the R- series of animals.

During pregnancy, the highest potential progestational biosynthesis appears to occur on day 6(+2.3) and a second high on days 14 and 15 (+1.9 to 2.0) whereas low potential for progesterone biosynthesis was assessed on day 20 and 21 (+1.3). When these histochemical generalized findings are compared to biochemical determinations of progesterone blood titers (Fajer and Barraclough, 1967), high ovarian secretion rates were reported on day 15 of pregnancy (20.5 $\mu\text{g/hr. /ovary}$) and minimal ovarian progesterone venous blood titers (2.7 $\mu\text{g/hr. /ovary}$) were noted on day 20. These biochemical results are in accord with the semi-quantitative histochemical estimates which reflect the entire average estimates of the functional capabilities of the ovary. Thus, these findings indicate that higher levels of progesterone are synthesized and detected in circulating venous blood on day 6 of pregnancy than at times close to parturition, *i. e.* days 20 and 21.

When the summarized estimates of total potential estrogenic biosynthesis are examined, days 6 through 18 (+2.1 to +2.3) with the exception of day 13 are found to be days of relatively high DHA-3 β -HSD activity, whereas day 20 (+1.3) appears to be markedly low. These changes in

potential estrogenic biosynthesis are indicative of the alterations in the steroidal requirements during pregnancy. As Yochim and Zarrow (1961) demonstrated both estrogens and progesterone are required in a proper proportion to maintain fetal survival during pregnancy. The studies of Velardo (1960 and 1961) further stress the synergistic actions of the estrogens and progesterone which are responsible for the steroidal balance necessary for maintaining normal pregnancy.

These cyto- and histomorphological studies on the localization of P-one and DHA-3 β -HSD activities in the ovaries of rats have shown that the biochemical techniques used to measure actual circulating venous blood levels of progesterone and estrogens become more meaningful when interpreted in light of the functional abilities of the ovarian components. From the studies of animals having superimposed physiological requirements, it was observed that additional steroidal biosynthesis appears to be achieved by heightened enzymatic activity in all of the ovarian components, with the exception of ova, primary follicles and the ovarian hilus. It appears that there is a coordination in all of the ovarian components, *i.e.* follicles, interstitial tissue and corpora lutea, to meet the steroidal requirements of the given situation and not increased activity in a single component. The results contained in this dissertation provide cyto- and histochemical information which can be used as a foundation or a guide by which biochemical determinations and physiological conditions can be directly related to the morphological functions, thus re-iterating the basic form-function relationships occurring in the biological sciences.

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Summary and Conclusions

This dissertation was principally concerned with a cyto- and histochemical analysis and photographic documentation of the ovarian cellular and tissue sources of potential estrogenic and progestational hormonal production during four physiological conditions: 1) the normal estrous cycle, 2) ordinary-induced (normal) pseudopregnancy, 3) pseudopregnancy with decidual tissue (prolonged pseudopregnancy), and 4) pregnancy, which can occur in the female albino rat. The enzymes 3β -hydroxypreg-5-en-20-one- 3β -hydroxysteroid:NAD oxidoreductase (pregnenolone- 3β -hydroxysteroid dehydrogenase, P-one- 3β -HSD) and 3β -hydroxyandrost-5-en-17-one- 3β -hydroxysteroid:NAD oxidoreductase (dehydroepiandrosterone- 3β -hydroxysteroid dehydrogenase, DHA- 3β -HSD) were studied since they have been shown to be indicative of potential progestational and estrogenic hormonal biosynthesis, respectively. Further, concomitant histochemical series were performed using reduced nicotinamide adenine dinucleotide (NADH_2) in order to localize NADH_2 diaphorase activity in the ovarian sections since it is a necessary requirement for the production of the end-point indicator utilized in this technique. Histochemical control series were also performed on the ovarian tissue sections and were observed to be devoid of any reaction material. A total of 223 frozen ovaries were examined in this study.

1. In order to critically assess the cyto- and histochemical sites of and alterations in potential ovarian biosynthetic pathways of progesterone and estrogen, the estrous cycle of rats was timed and subdivided into six distinctive stages, *i. e.* estrus (E), metestrus (ME), early diestrus (D_1), late diestrus (D_2), preproestrus (PPE), and proestrus (PE). The animals used in this study were of two age groups: young adults (Y-, 41-65 days) and slightly older animals, with regularly established reproductive history (R-, 75-120 days). The studies on the ovaries of rats during the six different

stages of the estrous cycle revealed:

- a) Maximal (+4) P-one- 3β -HSD activity was localized in corpora lutea during estrus in the R- series and during metestrus, late diestrus and proestrus in the Y- series of rats, whereas trace (\pm) P-one- 3β -HSD activity was observed during early diestrus in both R- and Y- series.
- b) Maximal P-one- 3β -HSD was seen in interstitial tissue during early diestrus and preproestrus in the R- series of animals and during preproestrus and proestrus in the Y- series. Moderate (+2) diformazan intensities were observed in the interstitial tissue during early diestrus and preproestrus in the R- series while strong intensities were noted during all stages except the aforementioned ones in the Y- series.
- c) Maximal DHA- 3β -HSD activity was assessed in corpora lutea during late diestrus, preproestrus and proestrus in the Y- group and during proestrus and estrus in the R- group.
- d) DHA- 3β -HSD was moderately to strongly intense in the interstitial tissue throughout the cycle in both the R- and Y- series.
- e) Strongly to weakly positive intensities were noted in theca interna cells and moderate to trace activities in granulosa cells for P-one and DHA in all follicles excepting the primary follicles in groups R- and Y- .
- f) Strong to moderate DHA- 3β -HSD activity was localized in theca interna cells and moderate to trace activity was assessed in granulosa cells of tertiary follicles in both age groups of animals.

- g) Negligible to trace intensities for P-one- and DHA-3 β -HSD were observed in primary follicles throughout the estrous cycle in groups R- and Y-.
- h) No observable P-one- or DHA-3 β -HSD localization was seen in ova and the ovarian hilus in both the R- and Y- series throughout the estrous cycle.
- i) NADH₂ diaphorase activity appeared maximal in corpora lutea, interstitial tissue and the theca interna, in the main, for secondary-, tertiary- and atretic follicles and ranged between moderate and strong in ova, the granulosa cells of all types of follicles, and the ovarian hilus.
- j) The reactions were consistently negative when the substrates were omitted.

2. Normal pseudopregnancy was induced in mature estrous rats by vibratory stimulation of the uterine cervix in order to assess the histochemical and cyto-chemical sites and alterations in potential ovarian biosynthesis of progesterone and the estrogens on days 6, 8, 10, and 13 of this physiological condition. Analysis of enzymatic localizations in the ovaries of pseudopregnant rats revealed interesting features:

- a) Maximal P-one-3 β -HSD activity was found in corpora lutea on day 6 which declined in a step-wise manner to weakly positive on day 13.
- b) The interstitial tissue was observed to be maximally intense for P-one on day 6 and strongly intense on days 8, 10 and 13.
- c) Maximal DHA-3 β -HSD activity was assessed on days 10 and 13 in corpora lutea, whereas strong activity was assessed on days 6 and 8 in this ovarian component.

- d) DHA- 3β -HSD localization in the interstitial tissue was maximal on day 13, strong on day 8 and moderate on days 6 and 10.
- e) Maximal P-one intensities were observed in theca interna of tertiary- and atretic follicles on day 6 while strong DHA intensities in these components were observed on days 8 and 13.
- f) Moderate to trace intensities appeared in granulosa cells of all follicles except primary with P-one and DHA on days 6, 8, 10, and 13.
- g) Theca interna of all follicles displayed +3 to +1 diformazan intensities with DHA on the days studied while, with P-one, intensities ranged from +4 to trace.
- h) Negative to trace intensities for P-one and DHA were consistently observed in primary follicles on the days studied.
- i) NADH₂ diaphorase localization revealed: +4 diformazan intensities in corpora lutea, interstitial tissue and in the theca interna of tertiary and atretic follicles; +3 in theca interna cells of secondary follicles; and +2 in granulosa cells of secondary-, tertiary- and atretic follicles on the days studied.
- j) The absence of either substrate produced consistently negative results.

3. Prolonged pseudopregnancy was induced in rats in order to localize the cyto- and histochemical sites of and alterations in ovarian P-one- 3β -HSD and DHA- 3β -HSD as well as NADH₂ diaphorase on days 6, 8, 10, 13, 15, 18, 20 and 21 of this lengthened pseudopregnant condition. The results for these enzymatic studies on the ovaries of prolonged pseudopregnant rats indicated a number of significant points:

- a) Maximal intensities of P-one- 3β -HSD in corpora lutea were

observed on days 6 through 13, whereas the reactions were strong on day 18, moderate on days 15 and 20, and weakly positive on day 21.

- b) Interstitial tissue varied between maximally and strongly intense on days 6 to 13, thereafter, moderately intense except on day 20 when it appeared strong.
- c) Maximal intensities of DHA- 3β -HSD were found in corpora lutea on days 6 and 13, whereas strong reactions were observed between days 8-13 and 15-18, and moderate reactions were noted on days 20 and 21.
- d) Interstitial tissue localization of DHA- 3β -HSD closely paralleled that of P-one except +4 diformazan intensities on days 15 and 18 of prolonged pseudopregnancy.
- e) Granulosa cells of primary-, secondary-, tertiary- and atretic follicles varied between moderate and trace for both P-one and DHA.
- f) Theca interna cells varied for P-one- 3β -HSD: in secondary- and tertiary follicles +3 to +2 and in atretic follicles +4 to +2.
- g) For DHA- 3β -HSD, the theca interna showed increasing intensities of diformazan deposits: +2 for the most part in secondary follicles, generally +3 in tertiary follicles, and +4 to +3 in atretic follicles.
- h) The ova and hilar regions were non-responsive to P-one and DHA.
- i) NADH₂ diaphorase activity was consistently maximal in corpora lutea, interstitial tissue, and the theca interna and moderately intense in ova, the granulosa cells and ovarian hilus.

- j) The reactions were consistently negative when the substrates were omitted.

4. Ovaries of pregnant rats were histochemically studied to determine the sites and intensities of P-one- and DHA- 3β -HSD activities on days 6, 8, 10, 13, 14, 15, 18, 20, and 21 in order to assess the progestational and estrogenic steroidal capabilities of the maternal ovary during this condition. Cytological and histochemical studies of these pregnant rats yielded a number of interesting points:

- a) Maximal intensities of P-one- 3β -HSD were observed in corpora lutea on day 14, whereas strong intensities were localized on days 6-13 and 15-18, moderate on day 20, and weakly positive on day 21.
- b) Interstitial tissue was generally strong for P-one except maximal on day 6 and moderate on days 20 and 21.
- c) Maximal intensities of DHA- 3β -HSD were generally localized in corpora lutea on days 6 through 18, whereas the reactions were strong on day 20 and moderate on day 21.
- d) In the interstitial tissue, DHA- 3β -HSD appeared maximal on day 6, strong on days 8 through 18 and also on day 21, and moderate on day 20.
- e) Weakly positive to trace intensities were the rule in primary follicles for P-one and DHA- 3β -HSD on the days studied.
- f) In the granulosa cells, moderate to trace intensities were observed in secondary- and tertiary follicles for both P-one and DHA, whereas reactions for P-one- 3β -HSD and DHA- 3β -HSD in atretic follicles appeared weakly positive to trace.
- g) The theca interna appeared strong to weakly positive in secondary and tertiary follicles for both substrates and maximal to moderate

in atretic follicles for both substrates on the days studied.

- h) Negligible to inappreciable intensities of P-one- and DHA-3 β -HSD were consistently manifested in ova and the ovarian hilus during pregnancy.
- i) NADH₂ diaphorase was observed to be consistently maximally intense in corpora lutea, interstitial tissue and the theca interna of all follicles and moderately intense in ova, granulosa cells of all follicles and the ovarian hilus.
- j) The reactions proved consistently negative with the omission of the substrates.

5. It is noteworthy at this point to re-emphasize a number of conclusions which permit of a dynamic comprehension of the major findings which appear as generalized conclusions:

- a) Secondary follicles reveal different degrees of intensities and localizations for each of the two substrates particularly in the theca interna during each of the physiological conditions studied.
- b) Theca interna cells of the tertiary and atretic follicles show marked 3 β -HSD reactivity for the estrogenic and progestational precursor substrates, particularly during prolonged pseudo-pregnancy and pregnancy when there is an increased need for higher levels of these steroids.
- c) The interstitial tissue during the estrous cycle is one of the main sources for potential progestational and estrogenic biosynthesis, and likewise during normal- and prolonged pseudo-pregnancy and pregnancy in the rat, as well.
- d) The several cellular components herein described vary in their potential ability to synthesize estrogens and progesterone during the estrous cycle and normal pseudopregnancy. There appears

to be a marked increase in biosynthesis of progesterone and estrogen during prolonged pseudopregnancy in which the corpora lutea are known to be functional. Furthermore, the highest sustained assessments for both P-one- and DHA-3 β -HSD occurs during pregnancy in which prolonged luteal activity takes place.

- e) The ova, primary follicles and ovarian hilus are the three ovarian components which do not appear to take a noticeable part in potential estrogenic and progestational production during any of the four physiological states studied in the rat.
- f) These cyto- and histochemical findings provide additional corroborative evidence supporting the concept of enhanced ovarian steroidal biosynthesis during prolonged pseudo-pregnancy and pregnancy.
- g) Finally, these results present a detailed cytochemical and histochemical analysis of the cellular and tissue sources of, as well as the alterations in, potential progestational and estrogenic biosynthesis in the ovaries of rats during the six distinctive stages of the estrous cycle, normal (ordinary-induced) pseudopregnancy, prolonged pseudopregnancy, and pregnancy.

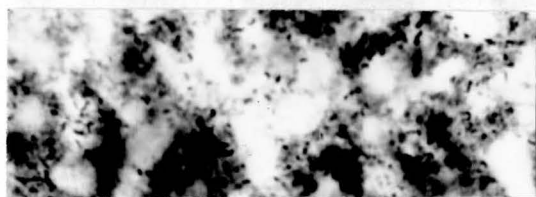
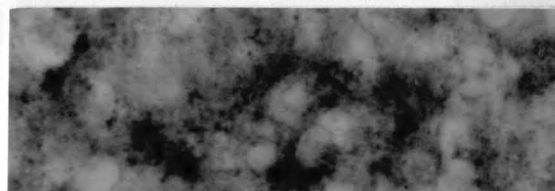
Semi-Quantitative Histochemical Scale of
Pregnenolone-3 β -Hydroxysteroid Dehydrogenase
Activity in Ovarian Sections

This appendix is comprised of portions of color and corresponding black and white photomicrographs which depict the different intensities of diformazan present in ovarian tissue sections as described in the text, pages 7 and 15. Ovarian components were assessed by comparison to this color scale in which monoformazan appears as the diffuse pink color of the partially reduced tetrazolium indicator and diformazan appears as the dark purple granular precipitate which is the complete reduction indicator. The corresponding black and white photomicrographic scale was made to serve as a standard to compare the pictorial documentation used in this dissertation. For uniformity, sections of corpora lutea were used to depict the semi-quantitative histochemical scale:

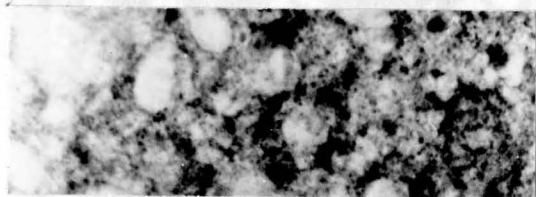
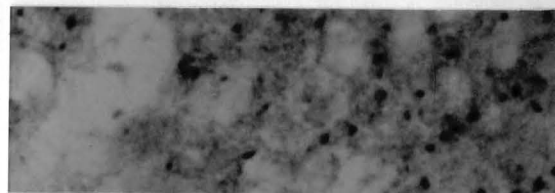
- | | | |
|--|---|---|
| +1 = weakly positive P-one-3 β -HSD activity | " | " |
| +2 = moderate | " | " |
| +3 = strong | " | " |
| +4 = maximal | " | " |



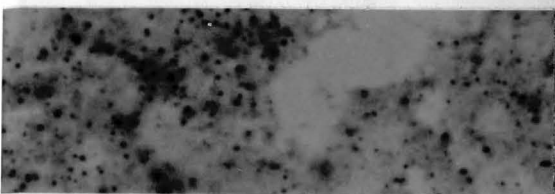
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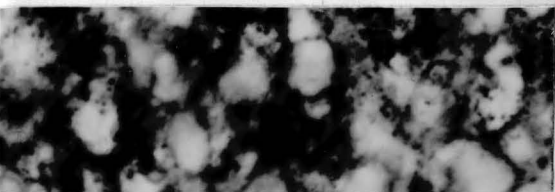
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+3



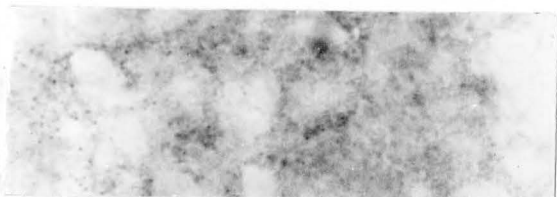
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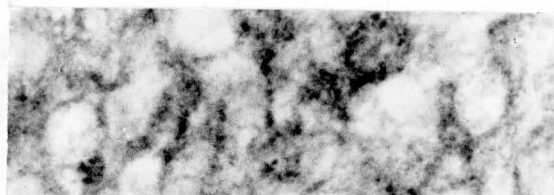
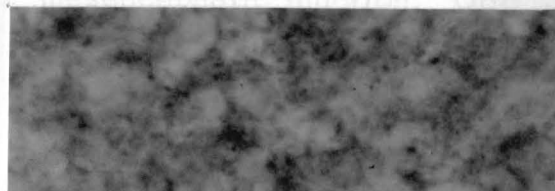
Semi-Quantitative Histochemical Scale of
Dehydroepiandrosterone- 3β -Hydroxysteroid Dehydrogenase
Activity in Ovarian Sections

This appendix is comprised of portions of color and corresponding black and white photomicrographs which depict the different intensities of diformazan present in ovarian tissue sections as described in the text, pages 7 and 15. Ovarian components were assessed by comparison to this color scale in which monoformazan appears as the diffuse pink color of the partially reduced tetrazolium indicator and diformazan appears as the dark purple granular precipitate which is the complete reduction indicator. The corresponding black and white photomicrographic scale was made to serve as a standard to compare the pictorial documentation used in this dissertation. For uniformity, sections of corpora lutea were used to depict the semi-quantitative histochemical scale:

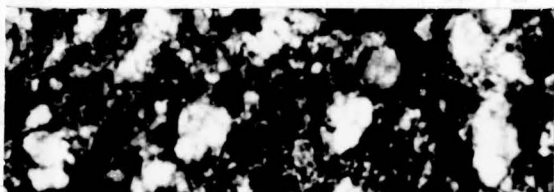
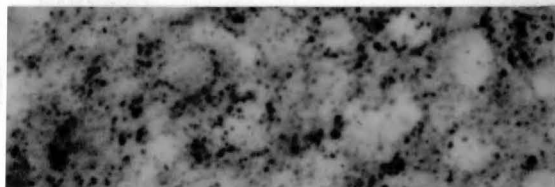
- | | | |
|--|---|---|
| +1 = weakly positive DHA- 3β -HSD activity | " | " |
| +2 = moderate | " | " |
| +3 = strong | " | " |
| +4 = maximal | " | " |



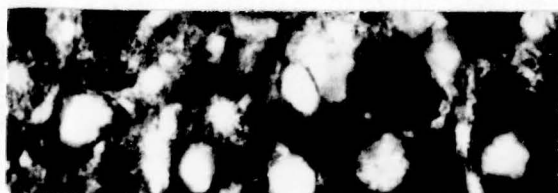
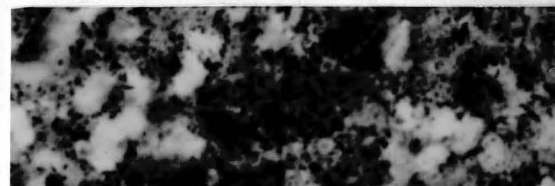
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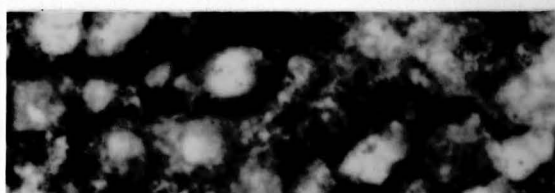
+2



+3



+4



Appendix C

Tabularization of the Morphological Criteria Used to Stage Rat Embryos Up to Day 18 of Pregnancy. Taken from Christie, G. A., 1964. J. Morph. 14: 263-286.

| Stage | Length mm | Somite Number | Age in days | Main Commencing Features |
|-------|--------------|------------------|-------------------------|---|
| 16 | | 1-3 | $9^{1/2}$ - $9^{3/4}$ | Somites have appeared. Neural folds elevating. |
| 17 | | 4-6 | $9^{3/4}$ -10 | Delimited otic (4th) rhombomere and post-otic sulcus. |
| 18 | | 7-9 | 10- $10^{1/2}$ | Neural canal closed from the level of the second to the sixth somite. |
| 19 | | 10-14 | $10^{1/2}$ - $10^{3/4}$ | Neural folds fused at diencephalic-mesencephalic junction. Otic pit forms. |
| 20 | 1.6-3.0 | 15/16-22 | $10^{3/4}$ - $11^{1/2}$ | Anterior neuropore and rhombencephalon closed. |
| 21 | 3.0-4.1 | 23/24-28 | $11^{1/2}$ -12 | Posterior neuropore and otic pit closed. |
| 22(A) | 4.1-4.6 | 29-32 | 12- $12^{1/2}$ | Endolymphatic sac appears "pinched off" from otic vesicle. Maxillary process has reached lateral nasal process. |
| 22(B) | 4.6-5.8 | 33-37 | $12^{1/2}$ -13 | Projection into roof of Rathke's pouch visible. Dorsum of posterior limb-bud flattened. |
| 23(A) | 5.8-7.1 | 38/39-41 | 13- $13^{1/2}$ | The lens vesicle is closed. Tubercles visible on contiguous sides of mandibular and hyoid arches. |
| 23(B) | 7.1-7.9 | 42-44 | $13^{1/2}$ - $13^{3/4}$ | Primitive posterior naris visible. |
| 23(C) | 7.9-9.4 | 45-47 | $13^{3/4}$ -14 | Rathke's pouch closed. |

Appendix C - Cont'd.

| Stage | Length mm | Somite Number | Age in days | Main Commencing Features |
|-------|--------------|------------------|--|---|
| 24 | 9.4-10.3 | 48- | 14-14 ¹ / ₄ | First vibrissary papilla appears on maxillary process. First traces of digital condensations in fore-paw. |
| 25 | 10.3-11.5 | | 14 ¹ / ₄ -14 ³ / ₄ | Four rows of vibrissary papillae visible, with invagination starting. |
| 26 | 11.5-12.1 | | 14 ³ / ₄ -15 ¹ / ₂ | Six rows of papillae present. Swelling of dorsal part of tongue visible. |
| 27 | 12.1-12.7 | | 15 ¹ / ₂ -16 | First trunk hair papillae present. |
| 28 | 12.7-14.5 | | 16-16 ¹ / ₂ | First set of papillae on dorsum of tongue. |
| 29 | 14.5-16.0 | | 16 ¹ / ₂ -17 | Digits fully separated on fore-paw. Ventral third of palate fused. |
| 30 | 16.0-17.6 | | 17-17 ¹ / ₂ | Vibrissae appear from maxillary follicles. Middle thirds of palate fused. |
| 31 | 17.6-19.1 | | 17 ¹ / ₂ -18 | Digits separated on hind-paw. Claw bearing area differentiated on fore-paw. |
| 32 | 19.1-22.0 | | 18-18 ¹ / ₂ | Umbilical hernia reduced. Eye and ear not yet fully closed. |

Closure of the eye and ear, at the end of stage 32, mark the final attainment of definitive foetal form.

DESCRIPTION OF PLATES

All figures are photomicrographs taken at one of three magnifications: 70x, 175x or 875x. All ovaries were frozen, sectioned at 8 microns on a cryostat, incubated for 2 3/4 hours at 37°C, specifically tested for reactions to ovarian steroidal hormonal substrates and suitable controls, fixed in neutral formalin, and cover slipped with glycerin-gelatin media.

Plates I through VIII are taken from ovaries of animals in the young adult series, ages 41-65 days (Y); whereas, Plates IX through XIV are from regularly cycling adult rats, ages 75-120 days (R), as indicated. Plates XV through XXII are taken from ovaries of animals in the normal pseudo-pregnant series; whereas, Plates XXIII through XXXI are from the prolonged pseudopregnant series and Plates XXXII through XXXXIII are taken from ovaries of animals in the pregnant series. The ovarian components are designated by the following abbreviations:

- A = antrum
- AF = atretic follicle
- CL = corpus luteum
- G = granulosa cells
- IT = interstitial tissue
- O = ovum
- TI = theca interna
- PF = primary follicle
- SF = secondary follicle
- TF = tertiary follicle

For the purpose of clarity, the following alphabetized names are here explained:

- DHA = 3 β -hydroxyandrost-5-en-17-one (dehydroepiandrosterone)
- NADH₂ = reduced nicotinamide adenine dinucleotide
- P-one = 3 β -hydroxypregn-5-en-20-one (pregnenolone)
- 3 β -HSD = 3 β -hydroxysteroid:NAD oxidoreductase;
3 β -hydroxysteroid dehydrogenase

Stages of the Estrous Cycle are indicated thusly:

- E = estrus
- ME = metestrus
- D₁ = early diestrus
- D₂ = late diestrus
- PPE = preproestrus
- PE = proestrus

PLATE I

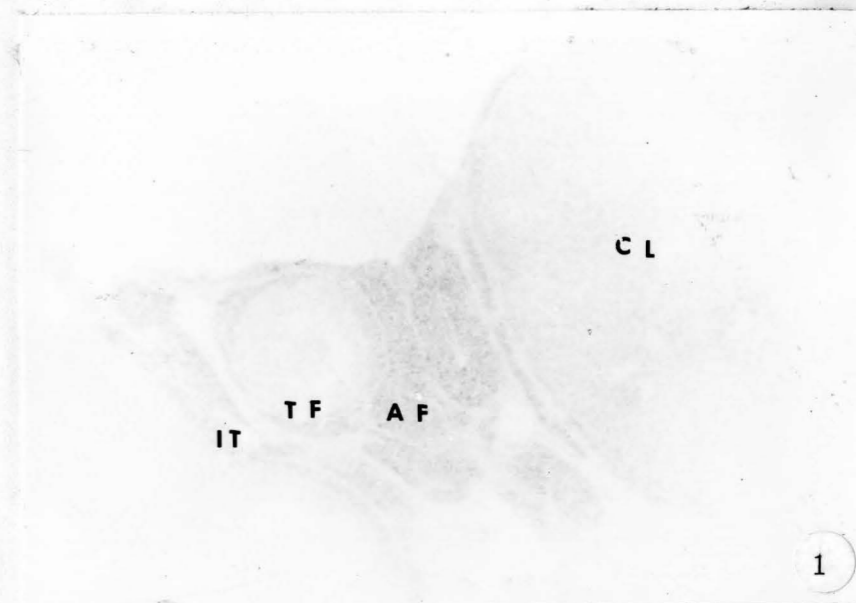
Plates I and II are overviews of the ovary of an animal in the stage of estrus of the estrous cycle. Figures 1, 3 and 4 are printed darker than they appear on the ovarian sections in order to bring out the ovarian components for orientation purposes only. Figures 1-4 have a final magnification of 70x, whereas figures 2a, 3a and 4a have a final magnification of 875x.

PLATE I

Fig. 1. The histochemical control section of an ovary in which the substrates are omitted shows A.F., C.L., I.T., and T.F. Neither mono- nor diformazan material is present in any of the histochemical control sections.

Fig. 2. A section of an ovary showing the presence of NADH_2 diaphorase localized in A.F., S.F., T.F., G., I.T., and C.L. Note the dense diformazan deposits in the ovarian sections which are indicative of the intensity of intrinsic enzymatic activity. Note A. and O. in T.F. and O. in S.F. lack diformazan material.

Fig. 2a. Control ovarian section. NADH_2 substrate omitted. Section is devoid of any noticeable diformazan, thus, the section does not show any detectable NADH_2 - diaphorase activity. 875x.



2a

PLATE II

An overview of the animal depicted in the prior exhibit, Plate I. Explanation is the same.

Fig. 3. Section of an ovary for the demonstration, localization and appearance of P-one- 3β -HSD in the several components studied: S.F., T.F., G., T.I., A.F., and C.L. Note O. and A. in T.F. lack both mono- and diformazan deposits.

Fig. 3a. Control ovarian section. The substrate, pregnenolone (P-one), was omitted; the section is devoid of any detectable P-one- 3β -HSD.

Fig. 4. Section of an ovary for the demonstration, localization and appearance of DHA- 3β -HSD in the several components studied: A.F., T.F., T.I., and C.L. Note O. and A. lack positive mono- and diformazan deposits.

Fig. 4a. Control ovarian section. The substrate dehydroepiandrosterone (DHA) was omitted; the section is devoid of any detectable DHA- 3β -HSD.

PLATE II

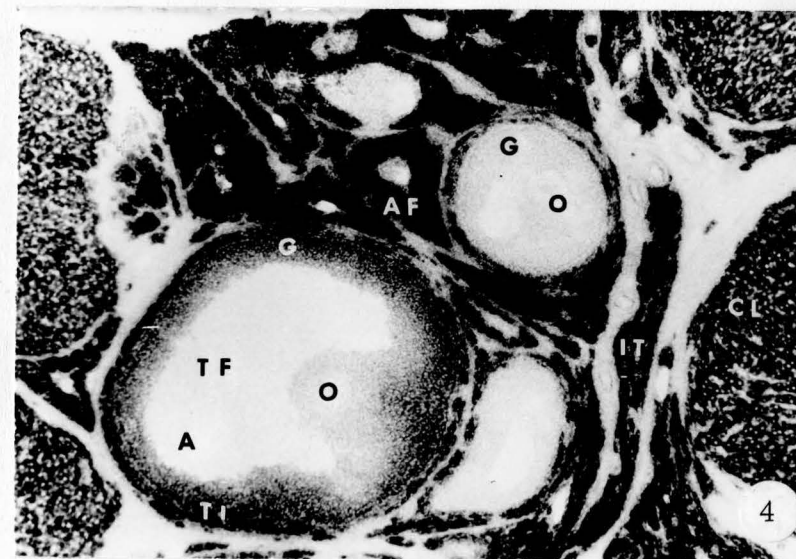
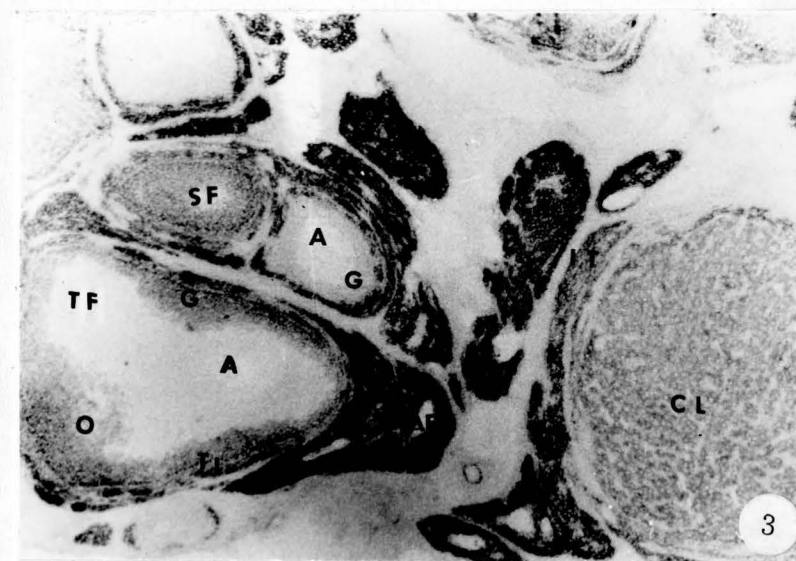


PLATE III

Plates III and IV are comprised of photomicrographs of sections taken from the ovaries removed from animals of the young adult series (Y-, 41-65 days old) which were incubated with pregnenolone (P-one) to localize the sites of the biosynthesis of progesterones. Figures 5 - 16 have a final magnification of 175x.

PLATE III

Fig. 5. An ovary removed during estrus showing weak to moderate P-one- 3β -HSD activity localized in A.F., S.F., G., and T.I.; moderate to strong activity appearing in I.T.

Fig. 6. Another section taken from ovary depicted in fig. 5, depicting moderate to strong activity in P-one- 3β -HSD localized in T.F., I.T., G., and T.I. Note that the antrum (A.) in T.F. lacks positive P-one- 3β -HSD material.

Fig. 7. A corpus luteum (C.L.) appearing in another area of the ovary depicted in fig. 5. Note the weak P-one- 3β -HSD activity in the C.L. cells.

Fig. 8. An ovary removed during metestrus depicting variable reaction sites for P-one- 3β -HSD. Monoformazan (trace intensities) located in T.F., S.F., and G. Weak to maximal diformazan present A.F., T.I., I.T., and C.L.

Fig. 9. An ovary removed during early diestrus depicting variable reaction sites for P-one- 3β -HSD. Monoformazan (trace intensities) located in S.F. and G. Weak to maximal diformazan localized in A.F., T.F., I.T., C.L., and T.I. It is to be noted that A. lacks both mono- and diformazan material.

PLATE III

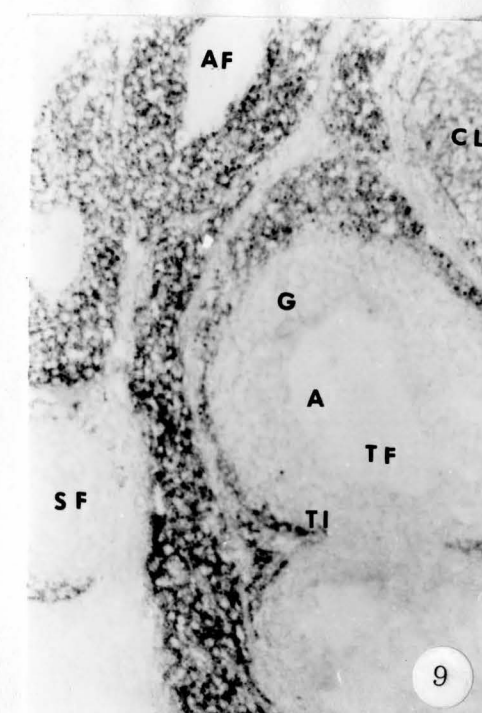
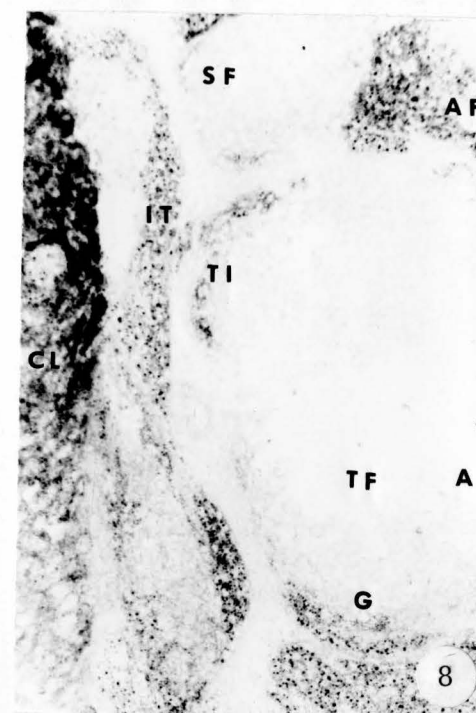
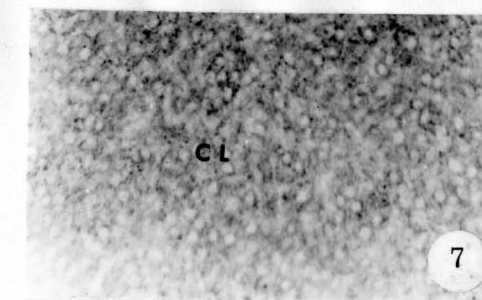
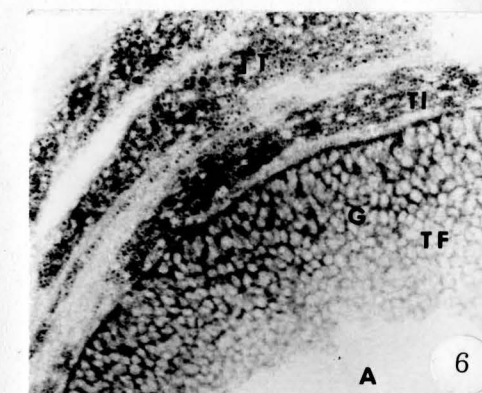


PLATE IV

Fig. 10. An ovary removed during late diestrus depicting trace to maximal diformazan material localized in A.F., S.F., T.F., G., and T.I. It is to be noted that the most intense reactive sites are thecal cellular components of the atretic follicles.

Fig. 11. Another section of ovary described in fig. 10, removed during late diestrus, and showing weak to strong P-one- 3β -HSD activity.

Fig. 12. An ovary removed during proestrus. The T.I. of the T.F. and the T.I. of the A.F. are strongly intense for P-one- 3β -HSD; the I.T. is the most intense and is maximal, whereas the other components are weak to moderate in activity excepting the A. which is again negative.

Fig. 13. A moderately active P-one- 3β -HSD deposition in a C.L. of a prooestrous ovary described in fig. 12.

Fig. 14. An ovary of a proestrous rat displaying two large T.F.'s with maximal P-one- 3β -HSD activity in the I.T., slightly less reactive, but yet strongly reactive enzymatic activity in the T.I., and only weakly appearing activity in the G. cells. The antra (A.) are typically negative.

Fig. 15. Another area of the same ovary of proestrous rat, depicted in fig. 14, showing maximally reactive T.I. of A.F. and weakly positive P-one- 3β -HSD in the G; the other elements do not reveal strongly reactive areas for this particular substrate in this section.

Fig. 16. Another area of proestrous ovary described in fig. 14, showing a corpus luteum (C.L.) with maximally reactive diformazan granules after incubation with P-one.

PLATE IV

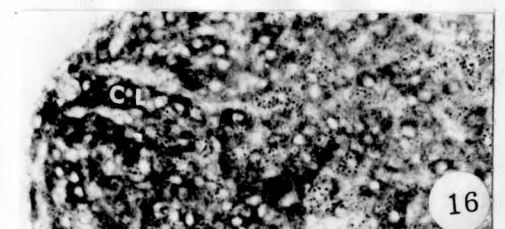
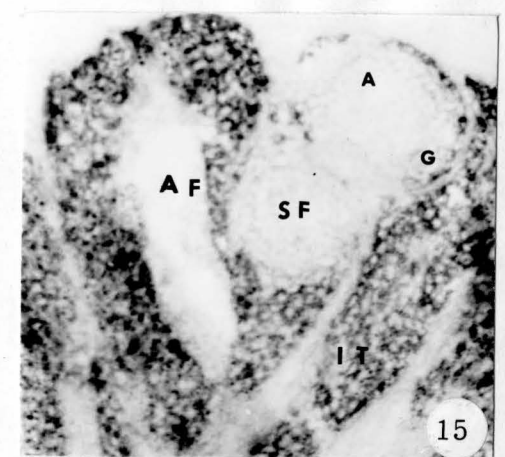
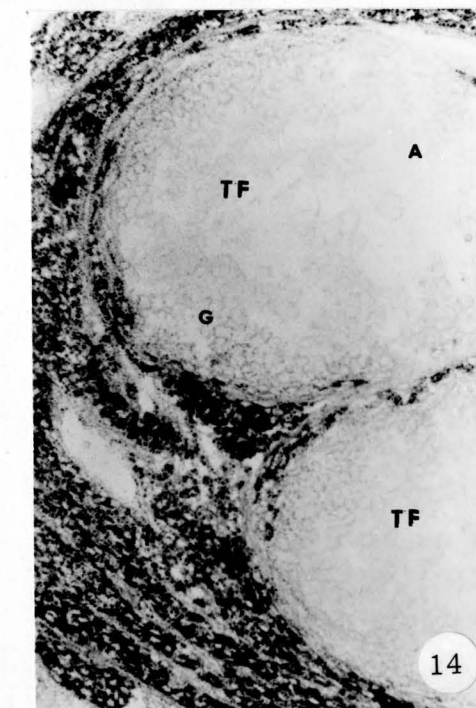
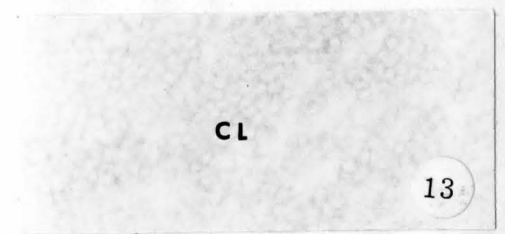
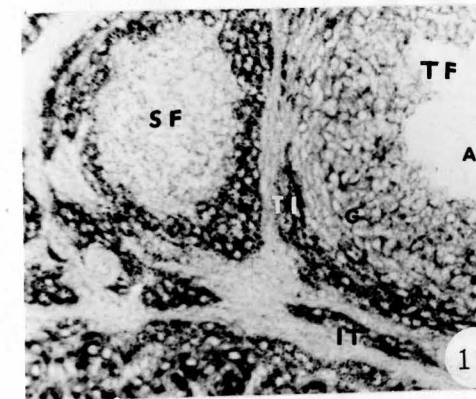
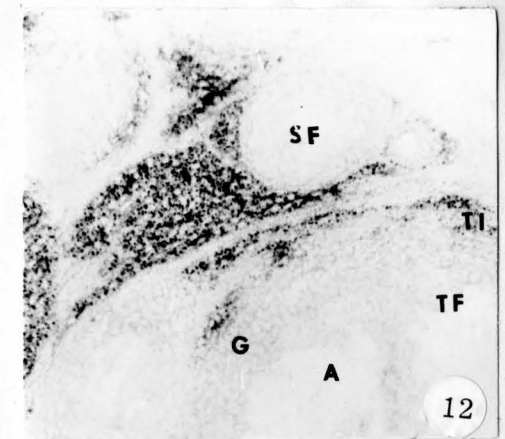
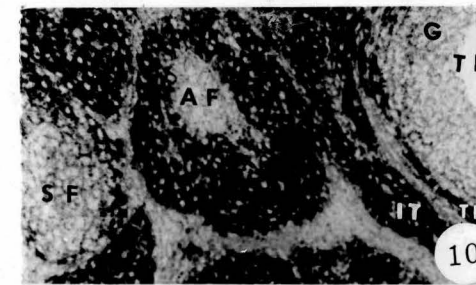


PLATE V

Plate V is comprised of high-powered photomicrographs of diformazan deposits observed in the P-one- 3β -HSD series of the Y- group of animals for each of the ovarian components studied. The left column consists of examples of photomicrographs depicting lowest observable intensities while the right column consists of examples of highest intensities in similar components, e. g. lowest P.F. (+) fig. 17, highest P.F. in fig. 23. All figures which comprise this plate have a final magnification of 875x.

Fig. 17. Primary follicle (P.F.) - lowest P-one- 3β -HSD activity in ovary of rat in estrus (E.); cf. fig. 23, highest activity observed during PPE, increasing from negative or traces to +1.

Fig. 18. Secondary follicle (S.F.) - lowest P-one- 3β -HSD activity in ovary of rat in E.; cf. fig. 24, highest activity observed during PE, increasing from +2 to +3 in T.I. with comparable activity in G.+1.

Fig. 19. Tertiary follicle (T.F.) - lowest P-one- 3β -HSD activity ovary of rat in ME; cf. fig. 25, highest activity in D_2 increasing from weakly positive to moderate in G., and moderately active to strongly active in T.I.

Fig. 20. Atretic follicle (A.F.) - lowest P-one- 3β -HSD activity in ovary of rat in E.; cf. fig. 26, highest activity in D_2 , increasing from trace to weakly positive in G., and strong to maximal (+4) in T.I.

Fig. 21. Interstitial tissue (I.T.) - strongly reactive (+3) P-one- 3β -HSD in D_1 ; maximally positive (+4) in D_2 ; cf. fig. 27, highest activity in PPE, increasing from strong to maximal.

Fig. 22. Corpus luteum (C.L.) - lowest and minimal reaction (traces, monoformazan) during D_1 ; cf. fig. 28, highest and maximal P-one- 3β -HSD activity (+4) during PE.

PLATE V - Cont'd.

Fig. 23. Primary follicle (P.F.) - highest P-one- 3β -HSD (+1) during PPE; cf. fig. 17, lowest during E.

Fig. 24. Secondary follicle (S.F.) - highest P-one- 3β -HSD (G. - +1 and T.I. - +3) during PE; cf. fig. 18, lowest during E.

Fig. 25. Tertiary follicle (T.F.) - highest P-one- 3β -HSD (G. - +2 and T.I. - +3) during D₂; cf. fig. 19, lowest during ME.

Fig. 26. Atretic follicle (A.F.) - highest P-one- 3β -HSD (G. - +1 and T.I. - +4) during D₂; cf. fig. 20, lowest during E.

Fig. 27. Interstitial tissue (I.T.) - highest P-one- 3β -HSD (+4) during PPE; cf. fig. 21, lowest during D₁.

Fig. 28. Corpus luteum (C.L.) - highest P-one- 3β -HSD (+4) during PE; cf. fig. 22, lowest during D₁.

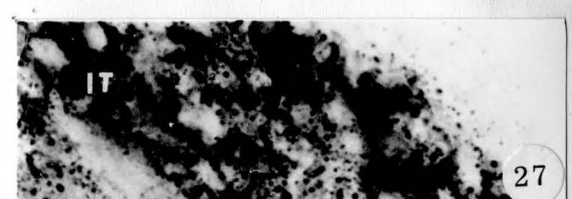
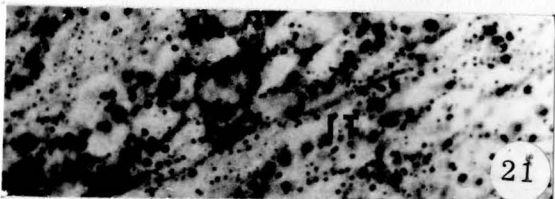
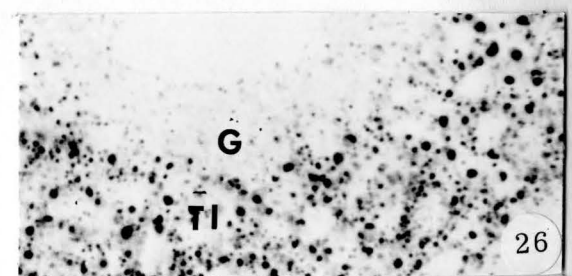
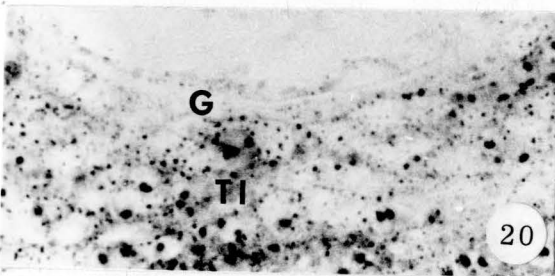
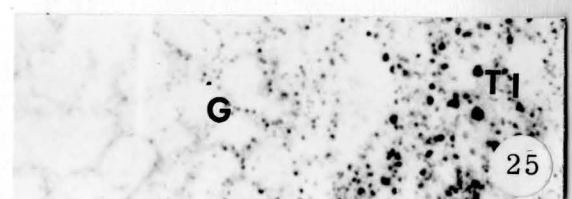
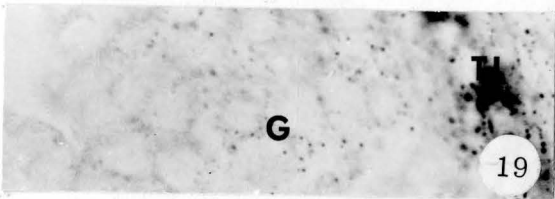
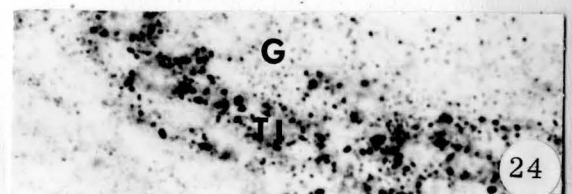
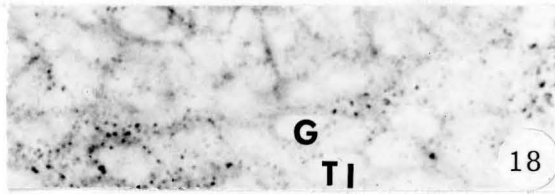
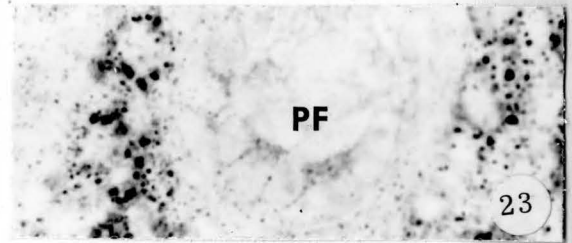
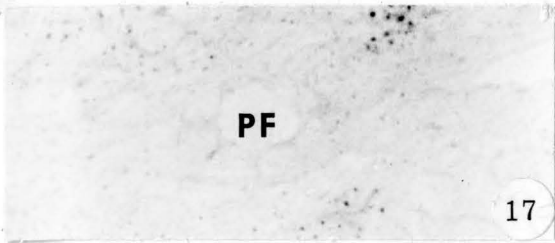


PLATE VI

Plates VI and VII are comprised of photomicrographs of sections of ovaries from rats of the Y- series (41 - 65 days of age) which were incubated with dehydroepiandrosterone (DHA) to localize the sites of the biosynthesis of estrogens. Figures 29-40 have a final magnification of 175x.

PLATE VI

Fig. 29. Section of an ovary of a rat in estrus (E) incubated with DHA. Strongest obtainable (+3) enzymatic reactions in this section are localized in theca interna cells of A. F. and in I. T. ; moderate (+2) reactions in T. I. of T. F. ; and weakest (+1) in granulosa cells of A. F. and T. F. The large appearing antrum (A.) is typically negative.

Fig. 30. Another section from the ovary of rat in E described in fig. 29. Note weakly to strongly active patches of DHA-3 β -HSD activity in T. I. of S. F. and trace intensities in G. of S. F. ; P. F. and T. F. appear weakly to moderately intense for DHA-3 β -HSD activity. The antrum (A.) of T. F. as well as the ovum (O.) of S. F. are typically negative.

Fig. 31. A corpus luteum (C. L.) appearing in another area of the ovary depicted in fig. 29. Note strong (+3) DHA-3 β -HSD activity in C. L. cells.

Fig. 32. An ovary removed during metestrus (ME.) displaying variable reaction sites for DHA-3 β -HSD activity. Strong intensities of diformazan in theca interna cells of A. F. as well as in I. T. ; weak to moderate intensities in granulosa cells (G.) of T. F. and A. F. as well as in theca interna cells of S. F. ; and trace intensities (monoformazan) in granulosa cells of P. F. and S. F. Antrum (A.) in A. F. lacks positive DHA-3 β -HSD activity.

Fig. 33. Another section of ovary described in fig. 32, removed during ME. Maximal (+4) activity of DHA-3 β -HSD in C. L. ; and moderate to strong diformazan deposition localized in G. and T. I. of T. F. and in I. T. Antrum (A.) is typically negative.

PLATE VI

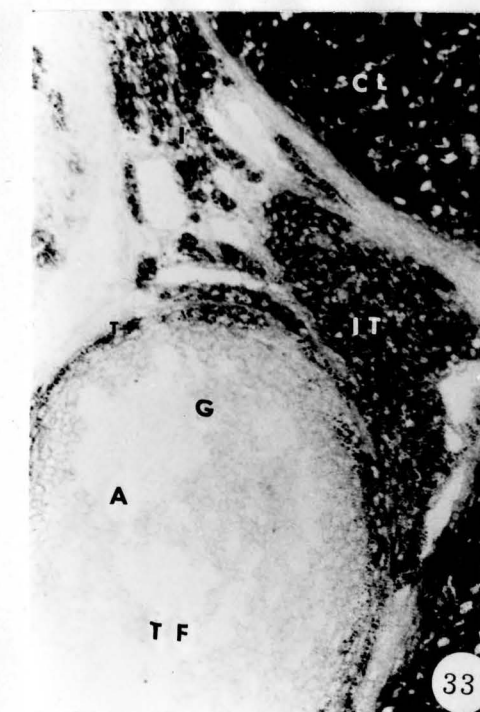
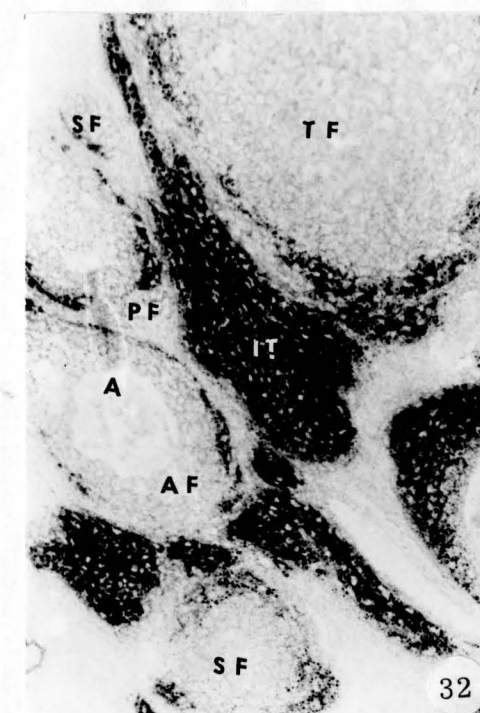
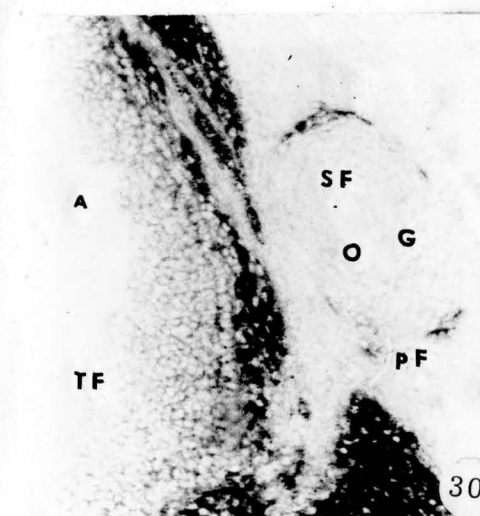
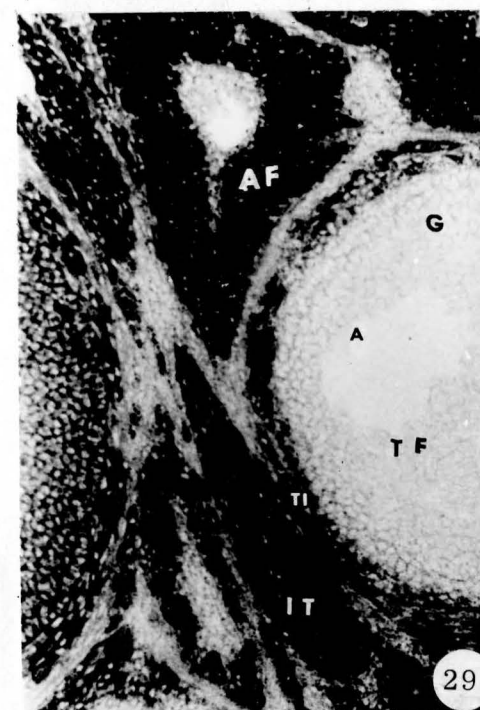


PLATE VII

Fig. 34. An ovary removed during early diestrus, depicting maximal (+4) DHA-3 β -HSD activity localized in I. T. ; strong in theca interna of A. F. ; weak to moderate S. F. and T. F. The antrum (A.) in T. F. lacks positive DHA-3 β -HSD activity.

Fig. 35. A corpus luteum (C. L.) appearing in another area of the ovary described in fig. 34. Note strong DHA-3 β -HSD activity in C. L. cells.

Fig. 36. An ovary removed during late diestrus depicting variable reaction sites for DHA-3 β -HSD activity. Maximal (+4) diformazan deposition appears in theca interna of A. F. ; strong intensities in I. T. and in theca interna of S. F. ; and weakly positive to moderate intensities in P. F. and the granulosa cells (G.) of S. F. and T. F. The ovum (O.) is uniformly negative.

Fig. 37. An ovary removed during preproestrus (PPE) displaying strongly intense DHA-3 β -HSD activity in theca interna (T. I.) of S. F., T. F., and A. F. as well as in I. T. ; weakly intense activity in granulosa cells (G.) of P. F., S. F., T. F., and A. F.

Fig. 38. Another area of ovary depicted in fig. 37 showing a maximally intense diformazan reaction in a corpus luteum (C. L.).

Fig. 39. An ovary of a proestrous (PE) animal displaying maximal (+4) DHA-3 β -HSD activity in I. T. and the theca interna cells of A. F. ; and moderate to strong intensities in isolated patches of diformazan material in S. F.

Fig. 40. Another section of ovary described in fig. 39, removed during PE and displaying maximal intensities of DHA-3 β -HSD activity in I. T. and C. L. ; weakly active in G. of T. F. ; usually strongly reactive the T. I. of the T. F. in this section is quite variable (weakly positive to strong).

PLATE VII

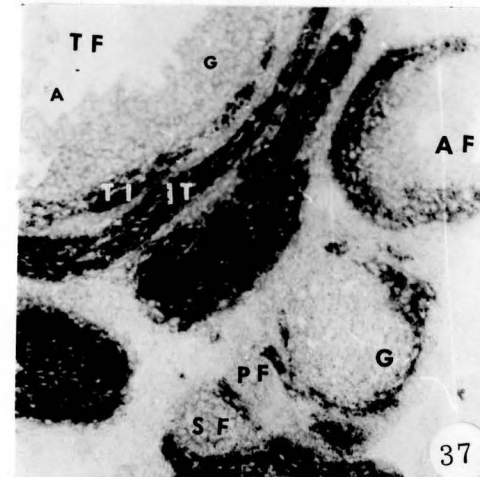
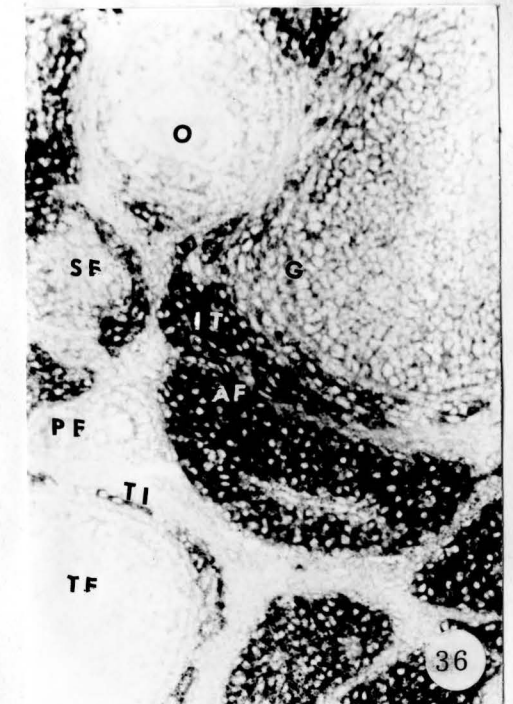
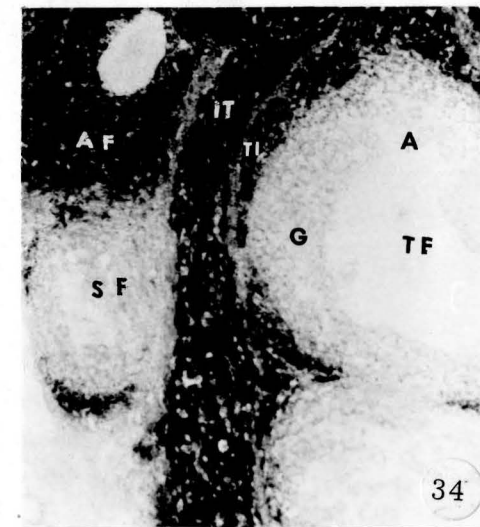


PLATE VIII

Plate VIII is comprised of high-powered photomicrographs of diformazan deposits observed in the DHA-3 β -HSD series of the Y- group of animals. The left column consists of examples of photomicrographs depicting lowest observable intensities, while the right column consists of examples of highest intensities in similar components, e.g. lowest P.F. (\pm , actually monoformazan) fig. 41, highest P.F. (+1) fig. 47. All figures which comprise this plate have a final magnification of 875x.

- Fig. 41. Primary follicle (P.F.) - lowest DHA-3 β -HSD activity in ovary of rat in metestrus (ME); cf. fig. 47, highest activity observed during PPE increasing from trace (monoformazan) to weakly positive (+1).
- Fig. 42. Secondary follicle (S.F.) - lowest DHA-3 β -HSD activity in ovary of rat in ME; cf. fig. 48, highest activity observed during D₂, increasing from moderate to strong (+3) in T.I. and from trace (\pm) to weakly positive (+1) in G.
- Fig. 43. Tertiary follicle (T.F.) - lowest DHA-3 β -HSD activity in ovary of rat in D₁; cf. fig. 49, highest activity observed during ME, increasing from moderate to strong in T.I. and weakly positive to moderate in G.
- Fig. 44. Atretic follicles (A.F.) - lowest DHA-3 β -HSD activity in ovary of rat during E; cf. fig. 50, highest activity observed during PE, increasing from strong (+3) to maximal (+4) in T.I. and trace (\pm) to weakly positive (+1) in G.
- Fig. 45. Interstitial tissue (I.T.) - lowest DHA-3 β -HSD activity in ovary of rat in E; cf. fig. 51, highest activity observed during D₁, increasing from variably strong (+3) to maximal (+4).

PLATE VIII - Cont'd.

- Fig. 46. Corpus luteum (C. L.) - lowest observable DHA-3 β -HSD activity during E; cf. fig. 52, highest observable activity during PE, increasing from moderate (+2) to maximal (+4).
- Fig. 47. Primary follicle (P. F.) - highest DHA-3 β -HSD (+1) activity during PPE; cf. fig. 41, lowest during ME.
- Fig. 48. Secondary follicle (S. F.) - highest DHA-3 β -HSD (G. - +2 and T. I. - +3) activity during D₂; cf. fig. 42, lowest during ME.
- Fig. 49. Tertiary follicle (T. F.) - highest DHA-3 β -HSD (G. - +2 and T. I. - +3) activity during ME; cf. fig. 43, lowest during D₁.
- Fig. 50. Atretic follicle (A. F.) - highest DHA-3 β -HSD (G. - +1 and T. I. - +3) activity during PE; cf. fig. 44, lowest during E.
- Fig. 51. Interstitial tissue (I. T.) - highest DHA-3 β -HSD (+4) activity during D₁; cf. fig. 45, lowest during E.
- Fig. 52. Corpus luteum (C. L.) - highest DHA-3 β -HSD (+4) activity during PE; cf. fig. 46, lowest during E.

PLATE VIII

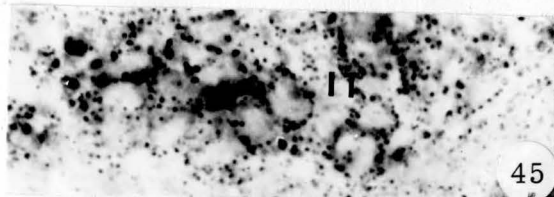
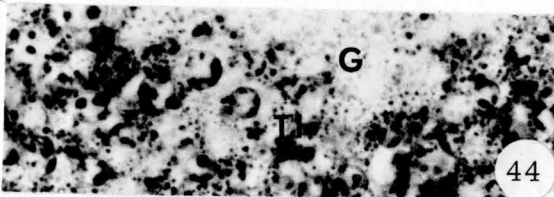
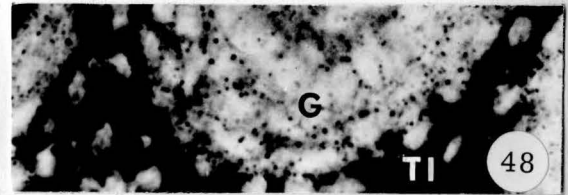
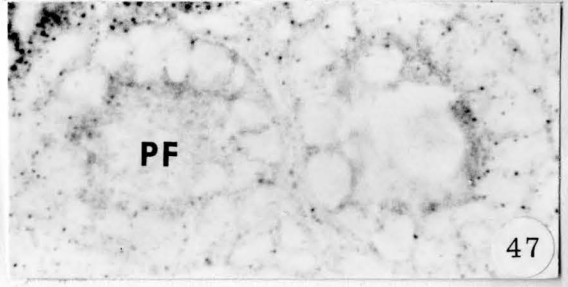
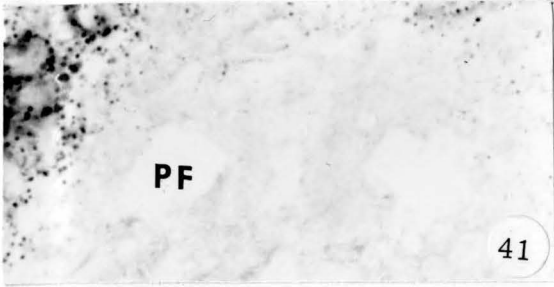


PLATE IX

Plates IX and X are comprised of sections taken from the ovaries removed from animals which were slightly more advanced in age than those of the Y- series and had an established history of estrous cyclicity (R-, 75-120 days old). The ovaries were incubated with pregnenolone (P-one) to localize the potential sites of biosynthesis of progesterone. Figures 53-65 have a final magnification of 175x.

PLATE IX

Fig. 53. An ovary removed during estrus (E) depicting variable intensities of P-one- 3β -HSD in the different components of the ovary. Maximal diformazan sites are in T. I. of A. F. and T. F. as well as in I. T.; moderate intensities in C. L. and G. of T. F.; and trace (monoformazan) intensities in granulosa cells of A. F.

Fig. 54. Another section of ovary described in fig. 53, removed during E, and showing maximal (+4) P-one- 3β -HSD activity in T. I. of T. F. and in I. T.; moderate activity in G. of T. F. Antrum (A.) is typically negative.

Fig. 55. A moderately active P-one- 3β -HSD deposition in a C. L. of an ovary described in fig. 53.

Fig. 56. An ovary removed during metestrus (ME) depicting strong P-one- 3β -HSD activity in the theca interna (T. I.) cells of A. F. and T. F. as well as in the C. L.; the granulosa cells of A. F. and T. F. are weakly positive.

Fig. 57. Another section of ovary described in fig. 56, removed during ME and showing variable reaction sites for P-one- 3β -HSD. Strong intensities are found in the theca interna cells of A. F. as well as in I. T.; trace to weakly positive reactive sites in granulosa cells of S. F. and A. F.

PLATE IX

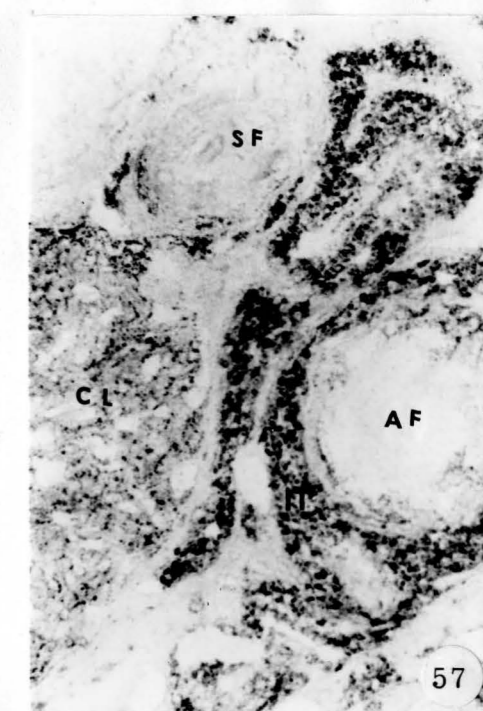
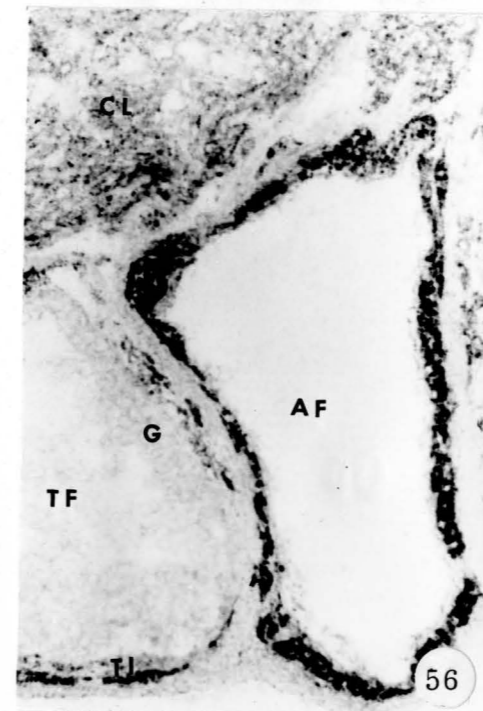
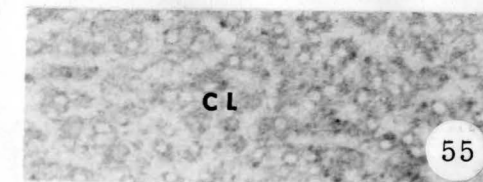
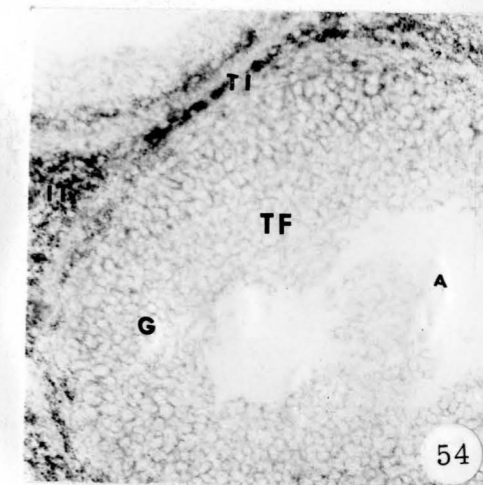
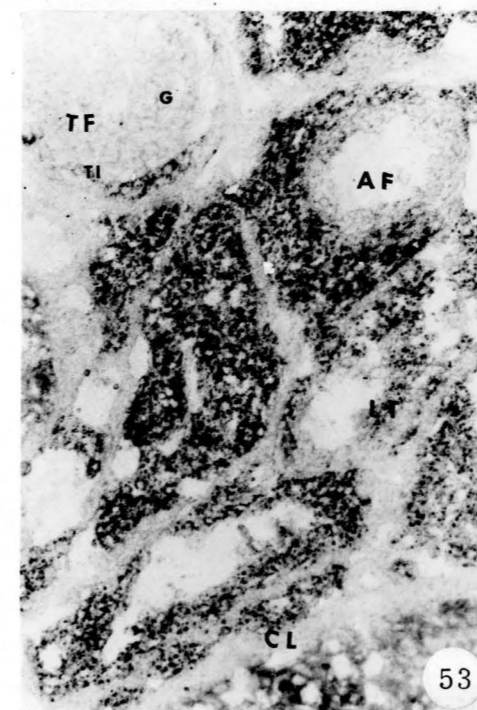


PLATE X

Fig. 58. An ovary removed during early diestrus (D_1) depicting trace to moderate activity of P-one- 3β -HSD in G. and T. I. of T. F., in I. T., and in C. L. Note ovum (O.) lacks P-one- 3β -HSD activity.

Fig. 59. Another section of ovary described in fig. 58, removed during D_1 , and showing moderate intensities of diformazan deposits in I. T.; weakly positive P-one- 3β -HSD activity in theca interna cells of S. F. whereas granulosa cells of S. F. are negative; and C. L. shows trace (monoformazan) activity. Note ovum (O.) in S. F. is negative.

Fig. 60. An ovary removed during late diestrus depicting maximal intensities of P-one- 3β -HSD activity in T. I. of T. F. and I. T.; weakly positive activity in G. of T. F. The antrum (A.) typically lacks P-one- 3β -HSD activity.

Fig. 61. Another section of ovary described in fig. 60, and showing moderate to strong activity in theca interna cells of S. F. and A. F. respectively. Granulosa cells are weakly positive in both S. F. and A. F.; the antra are again negative.

Fig. 62. An ovary removed during preproestrus (PPE), showing trace P-one- 3β -HSD in granulosa cells and moderate activity in T. I. of S. F. I. T. also has moderate activity.

Fig. 63. Another section of ovary displayed in fig. 62, removed during PPE. Strong P-one- 3β -HSD activity in C. L.; moderate in T. I. of T. F. and trace activity in G. of T. F.

Fig. 64. An ovary of a proestrous rat depicting strong P-one- 3β -HSD activity in theca interna cells of A. F. and T. F., as well as in I. T.; weakly positive activity in granulosa cells of T. F.; and negative activity in granulosa cells of A. F.

Fig. 65. Another region of the same ovary depicted in fig. 64, and showing variable P-one- 3β -HSD activity in C. L. ranging from moderate to strong and strong (+3) activity in I. T.

PLATE X

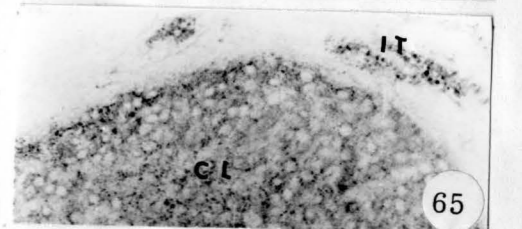
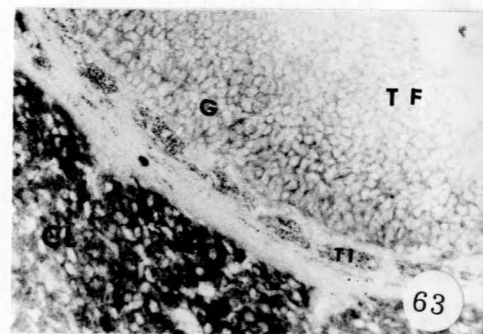
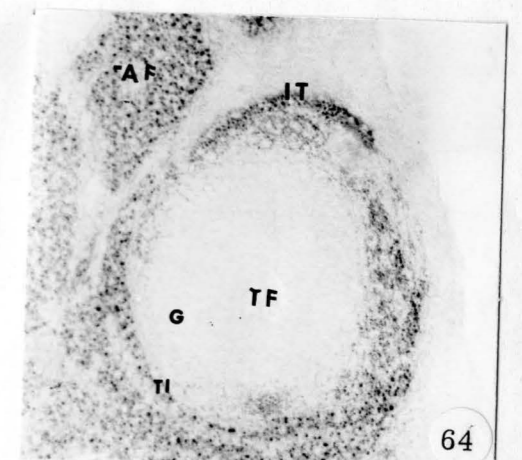
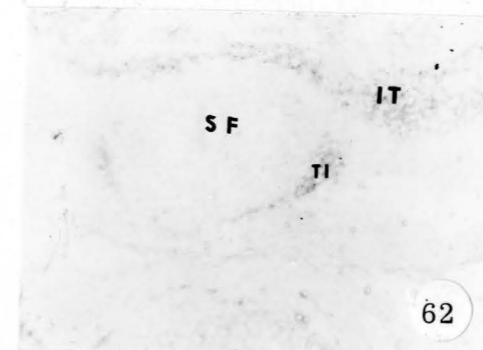
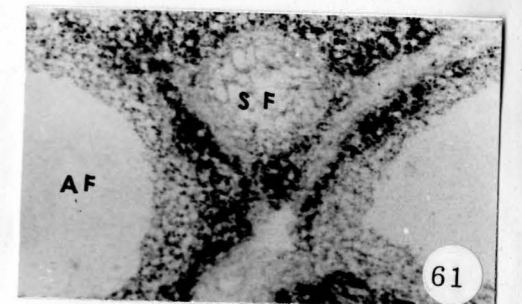
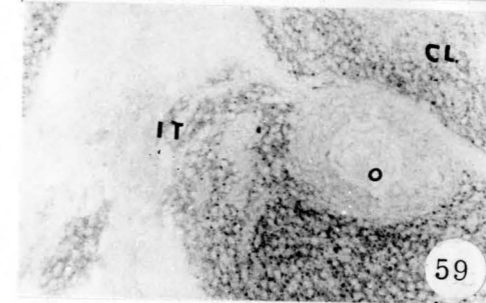
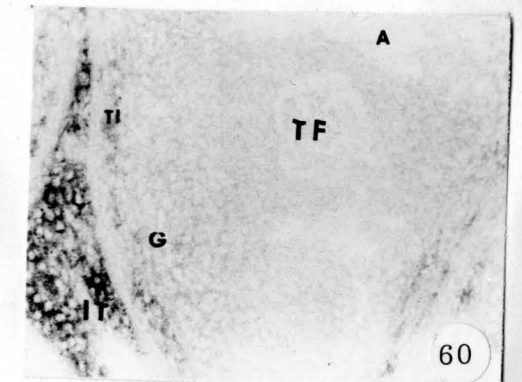
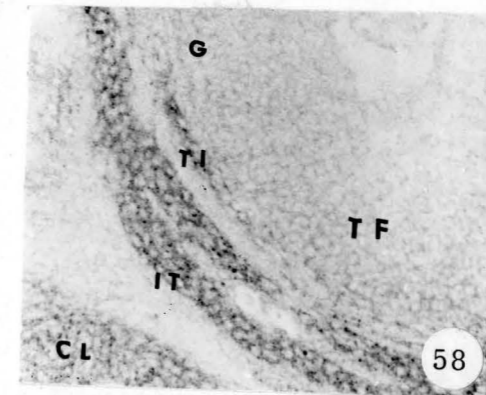


PLATE XI

Plate XI is comprised of high-powered photomicrographs of P-one- 3β -HSD appearing as diformazan deposits in the ovaries of the R- group of animals. The left column consists of examples of photomicrographs depicting lowest observable intensities while the right column consists of exhibits of highest intensities in similar ovarian components; e. g. lowest P. F. (O, i. e. zero) fig. 66, highest P. F. in fig. 72. All figures which comprise this plate have a final magnification of 875x.

- Fig. 66. Primary follicle (P. F.) - lowest P-one- 3β -HSD activity in ovary of rat in preproestrus; cf. fig. 72, highest activity observed during D_2 , increasing only slightly from negative (0) to weakly positive (+1).
- Fig. 67. Secondary follicle (S. F.) - lowest P-one- 3β -HSD activity in ovary of rat in early diestrus (D_1); cf. fig. 73, highest activity during E, increasing from negative to weakly positive in G. and weakly positive to moderate in T. I.
- Fig. 68. Tertiary follicle (T. F.) - lowest observable P-one- 3β -HSD activity in ovary of rat in D_1 ; cf. fig. 74, highest activity during E., increasing from weakly positive to moderate in G., and weakly positive to maximal (+4) in T. I.
- Fig. 69. Atretic follicle (A. F.) - lowest P-one- 3β -HSD activity in ovary of rat in PPE; cf. fig. 75, highest activity during E., increasing from moderate (+2) activity to maximal (+4) in T. I., with trace activity in G., as in fig. 69.
- Fig. 70. Interstitial tissue (I. T.) - lowest P-one- 3β -HSD activity in ovary of rat in D_1 ; cf. fig. 76, highest activity during D_2 (exhibited) and also during E., increasing from moderate to maximal (+4).

PLATE XI - Cont'd.

- Fig. 71. Corpus luteum (C.L.) - lowest and minimal P-one- 3β -HSD activity in ovary of rat in D₁; cf. fig. 77, highest activity during PE, increasing from trace to maximal (+4).
- Fig. 72. Primary follicle (P.F.) - highest P-one- 3β -HSD (\pm) activity during D₂; cf. fig. 66, lowest during PPE.
- Fig. 73. Secondary follicle (S.F.) - highest P-one- 3β -HSD (G. - +1 and T.I. - +2) activity during E; cf. fig. 67, lowest during D₁.
- Fig. 74. Tertiary follicle (T.F.) - highest P-one- 3β -HSD (G. - +2 and T.I. - +4) activity during E; cf. fig. 68, lowest during D₁.
- Fig. 75. Atretic follicle (A.F.) - highest P-one- 3β -HSD (G. - \pm and T.I. - +4) activity during E; cf. fig. 69, lowest during PPE.
- Fig. 76. Interstitial tissue (I.T.) - highest P-one- 3β -HSD (+4) activity during D₂; cf. fig. 70, lowest during D₁ (exhibited) and also during E.
- Fig. 77. Corpus luteum (C.L.) - highest P-one- 3β -HSD (+4) activity during PE; cf. fig. 71, lowest during D₁.

PLATE XI

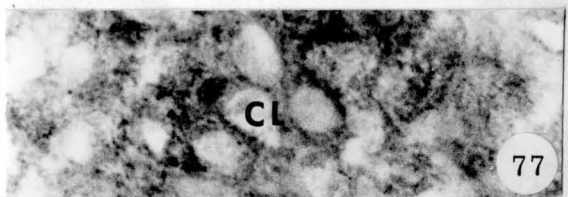
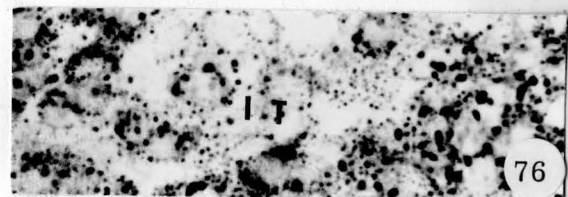
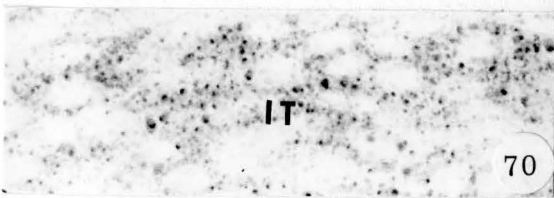
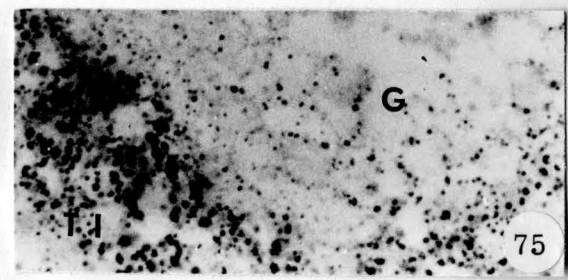
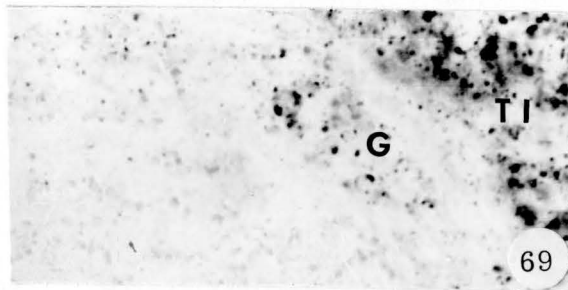
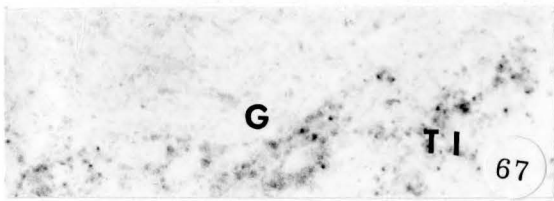
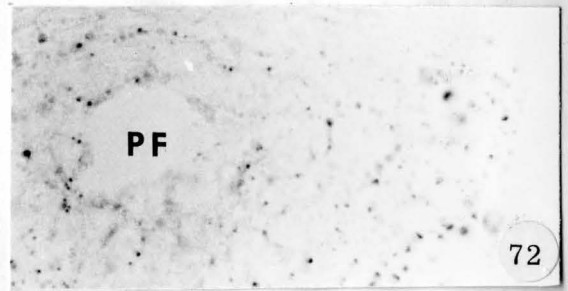
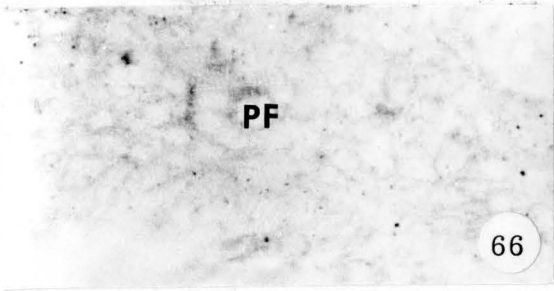


PLATE XII

Plates XII and XIII are comprised of photomicrographs of sections taken from the ovaries removed from animals which were slightly more advanced in age than those of the Y- series, and had an established history of estrous cyclicity (R-, 75 - 120 days old). The ovarian sections were incubated with dehydroepiandrosterone (DHA) to localize the site of DHA- 3β -HSD, as an indicator of the biosynthesis of estrogens. Figures 78-91 have a final magnification of 175x.

PLATE XII

- Fig. 78. An ovary removed during estrus (E) depicting weakly positive to maximal DHA- 3β -HSD activity. Maximal (+4) intensity for DHA- 3β -HSD in I. T., and C. L.; strong intensity in T. I. of T. F.; and weakly positive intensity in G. of T. F.
- Fig. 79. Another section of ovary described in fig. 78, removed during E., and showing most intense and maximal (+4) DHA- 3β -HSD activity in theca interna of A. F., in I. T. and in C. L.; weakly positive (+1) in granulosa of A. F.
- Fig. 80. An ovary of a metestrous (ME) rat displaying moderate to strong DHA- 3β -HSD activity in G. and T. I. of T. F. and C. L. respectively. Note antrum (A) is typically negative.
- Fig. 81. Another section of ovary described in fig. 80, removed during ME, and showing strongly intense activity for DHA- 3β -HSD in T. I. of A. F., in C. L., and in I. T.; moderate intensity in T. I. of S. F.; and trace to weakly positive intensities in granulosa of S. F. and A. F. respectively.

PLATE XII - Cont'd.

Fig. 82. An ovary removed during early diestrus (D_1), and depicting variable reaction sites for DHA- 3β -HSD. Moderate intensity of diformazan located in T.I. of T.F. and in I.T.; negative to trace intensities located in G. of P.E. and T.F. Antra (A.) and ovum (O.) of T.F. are negative for DHA- 3β -HSD activity.

Fig. 83. A corpus luteum (C.L.) appearing in another area of the ovary described in fig. 82, showing moderate DHA- 3β -HSD activity in luteal cells.

Fig. 84. Another section of the D_1 ovary, described in fig. 82, depicting variable sites of DHA- 3β -HSD activity. Moderate intensities of diformazan deposits in T.I. of A.F. and T.F. as well as in I.T.; and trace to weakly positive intensities in G. of T.F., A.F., and S.F. The antrum (A.) is typically negative.

PLATE XII

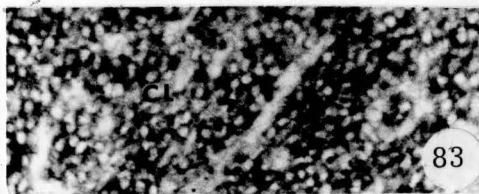
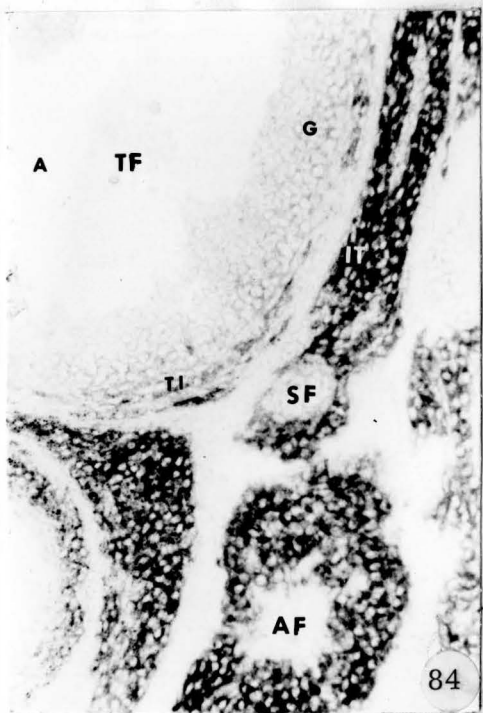
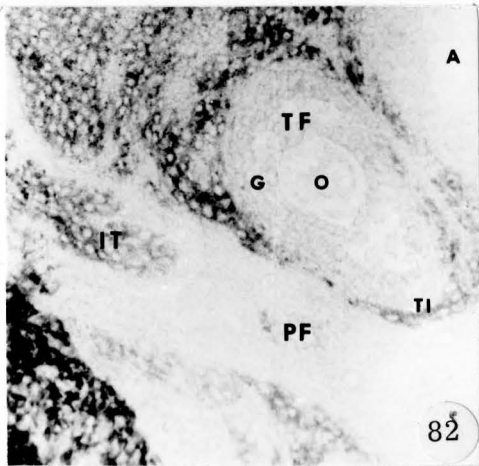
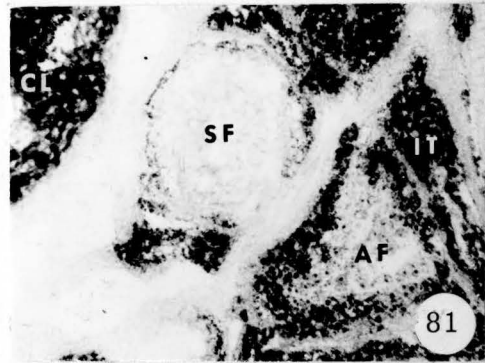
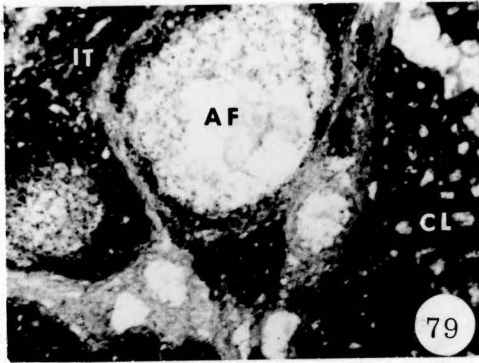
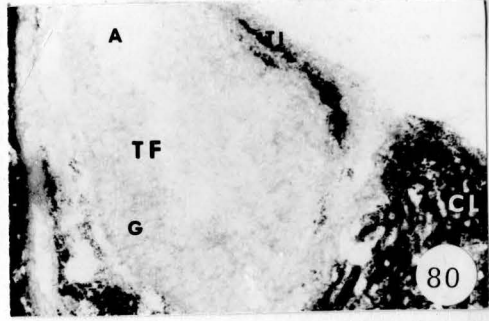
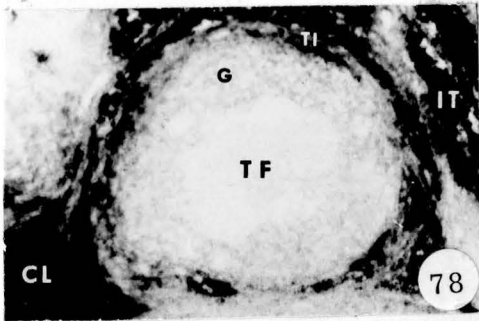


PLATE XIII

Fig. 85. An ovary removed during late diestrus, showing strong intensities of DHA-3 β -HSD activity in T. I. of A. F. and weakly positive sites in G. of A. F.

Fig. 86. Another section of the ovary described in fig. 85, depicting strong intensities of DHA-3 β -HSD activity in C. L., in I. T. and in T. I. of T. F.; moderate intensities in G. of T. F. Both ovum (O.) and antrum (A.) in T. F. lack DHA-3 β -HSD activity.

Fig. 87. An ovary removed during preproestrus (PPE) and showing strong DHA-3 β -HSD activity localized in the cells of the C. L.; S. F. displays only trace to weakly positive activity in the G. and T. I. respectively.

Fig. 88. Another section of the ovary described in fig. 87 which was removed from a rat during PPE. Intensities of diformazan localized in I. T. are quite variable, but usually strong in intensity; moderate intensities in T. I. of T. F.; and weakly positive intensities in G. of T. F. Note antrum (A) is typically negative.

Fig. 89. An ovary removed during the proestrous stage depicting maximal intensities of DHA-3 β -HSD in I. T.; strong intensities in T. I. of T. F.; and moderate intensities in G. of T. F. The antrum (A.) is negative.

Fig. 89. Another region of the ovary displayed in fig. 89, showing maximal DHA-3 β -HSD activity localized in the theca interna cells of A. F.; strong activity in theca interna cells of S. F.; and moderate activity in granulosa cells of S. F.

Fig. 91. A corpus luteum (C. L.) appearing in another area of the ovary described in fig. 89, depicting maximal DHA-3 β -HSD activity in the luteal cells.

PLATE XIII

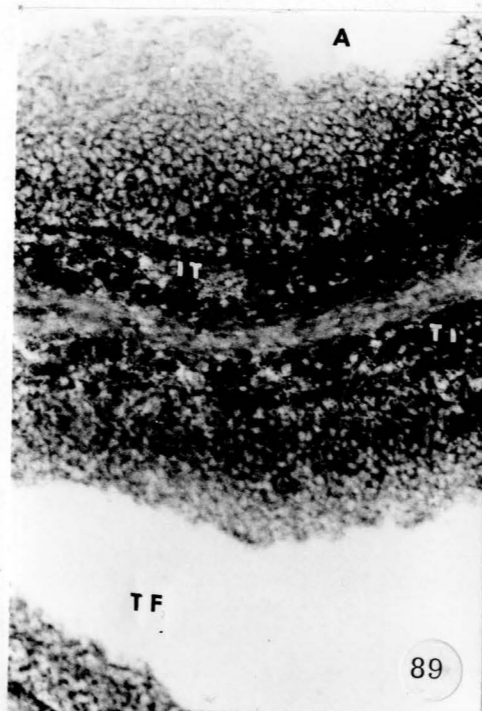
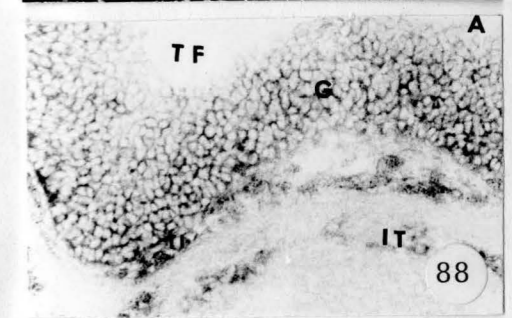
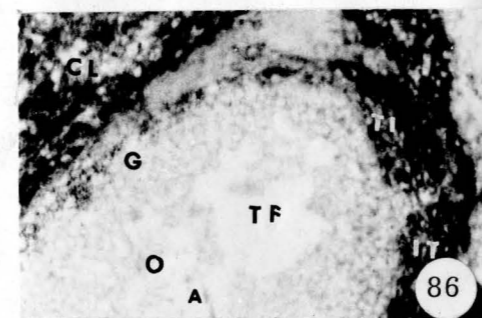
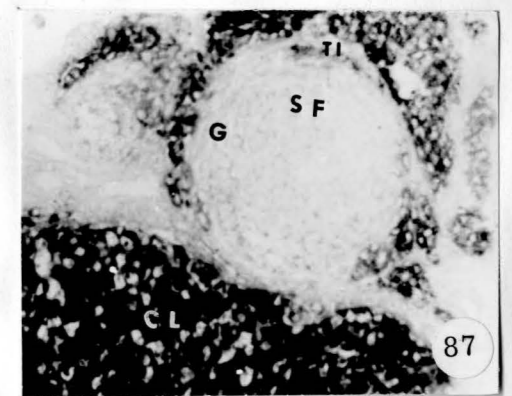
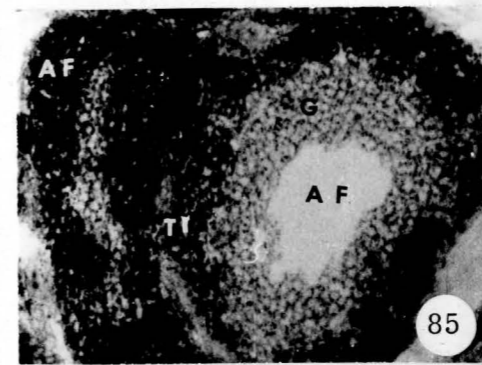


PLATE XIV

Plate XIV is comprised of high-powered photomicrographs of DHA-3 β -HSD indicated by different intensities of diformazan deposits in the ovaries of the R- group of animals. The left column consists of examples of photomicrographs depicting lowest observable intensities, while the right column consists of examples of highest intensities in similar components, e. g. lowest P.F. (0, i. e. zero) fig. 92, highest P.F. in fig. 98. All figures which comprise this plate have a final magnification of 875x.

- Fig. 92. Primary follicle (P.F.) - lowest DHA-3 β -HSD activity in ovary of rat in metestrus; cf. fig. 98, highest activity observed during PE, increasing from negative (0) to weakly positive (+1).
- Fig. 93. Secondary follicle (S.F.) - lowest DHA-3 β -HSD activity in ovary of rat in PPE; cf. fig. 99, highest activity observed during E, increasing from trace to moderate in G. and weakly positive to maximal in T.I.
- Fig. 94. Tertiary follicle (T.F.) - lowest DHA-3 β -HSD activity in ovary of rat in early diestrus; cf. fig. 100, highest activity observed in metestrus, increasing from moderate to strong in T.I. and weakly positive to moderate in G.
- Fig. 95. Atretic follicle (A.F.) - lowest DHA-3 β -HSD activity in ovary of rat during early diestrus (D₁); cf. fig. 101, highest activity observed during estrus, increasing from moderate to maximal in T.I. with comparable weakly positive intensities of G.

PLATE XIV - Cont'd.

- Fig. 96. Interstitial tissue (I. T.) - lowest DHA-3 β -HSD activity in ovary of rat during D₁; cf. fig. 102, highest and maximal activity during proestrus (exhibited) and also during E, increasing from moderate to maximal.
- Fig. 97. Corpus luteum (C.L.) - lowest DHA-3 β -HSD activity in ovary of rat during D₁; cf. fig. 103, highest and maximal activity observed during proestrus and estrus (the latter exhibited), increasing from moderate to maximal.
- Fig. 98. Primary follicle (P.F.) - highest DHA-3 β -HSD (+1) activity during PE; cf. fig. 92, lowest during ME.
- Fig. 99. Secondary follicle (S.F.) - highest DHA-3 β -HSD (G. - +2 and T.I. - +4) activity during E.; cf. fig. 93, lowest during PPE.
- Fig. 100. Tertiary follicle (T.F.) - highest DHA-3 β -HSD (G. - +2 and T.I. - +3) activity during ME; cf. fig. 94, lowest during D₁).
- Fig. 101. Atretic follicle (A.F.) - highest DHA-3 β -HSD (G. - +1 and T.I. - +4) activity during E; cf. fig. 95, lowest during D₁.
- Fig. 102. Interstitial tissue (I. T.) - highest DHA-3 β -HSD (+4) activity during PE (exhibited) and E.; cf. fig. 96, lowest during D₁.
- Fig. 103. Corpus luteum (C.L.) - highest DHA-3 β -HSD (+4) activity during PE and E (the latter exhibited); cf. fig. 97, lowest during D₁.

PLATE XIV

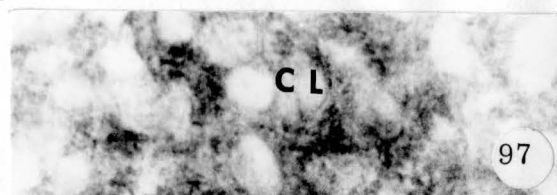
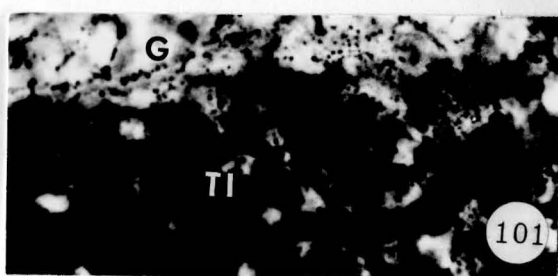
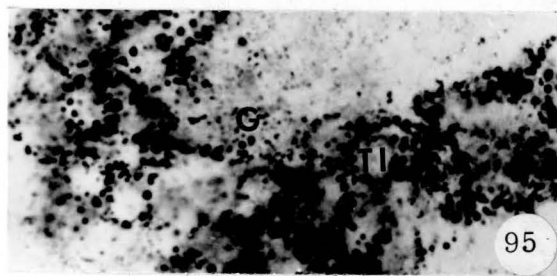
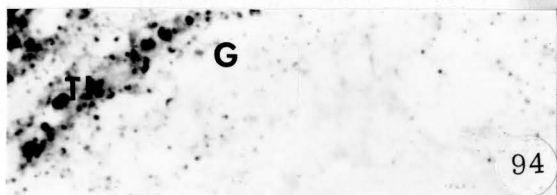
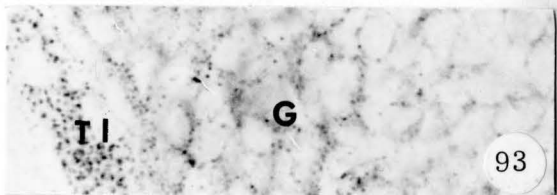
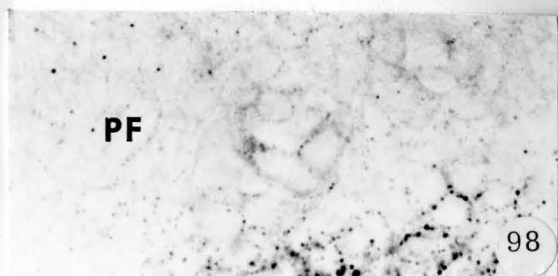
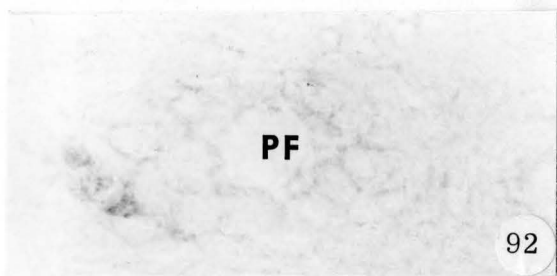


Plate XV is comprised of photomicrographs of ovarian sections of a rat on day 10 of normal pseudopregnancy. Figures 104 and 105 have a final magnification of .175x, whereas figures 104a, and 106 through 111 have a final magnification of 875x.

Fig. 104. The histochemical control section of a corpus luteum in which the substrates are omitted. Neither mono- nor diformazan material is present in any of the histochemical control sections.

Fig. 104a. Control ovarian section. NADH_2 substrate omitted. The section is devoid of any noticeable diformazan; thus, the section does not show any detectable NADH_2 diaphorase activity.

Fig. 105. A section of an ovary showing the presence of NADH_2 diaphorase localized in P. F., S. F., T. F., A. F., I. T., and C. L. Note the dense diformazan deposits in the ovarian sections which are indicative of the intensity of intrinsic enzymatic activity. Note antrum (A.) in T. F. lacks diformazan material.

Fig. 106. Another section taken from ovary depicted in fig. 105, which illustrates the intensity of diformazan deposition in P. F. and S. F. The ovum (O.) contains a relatively moderate intensity of reaction product.

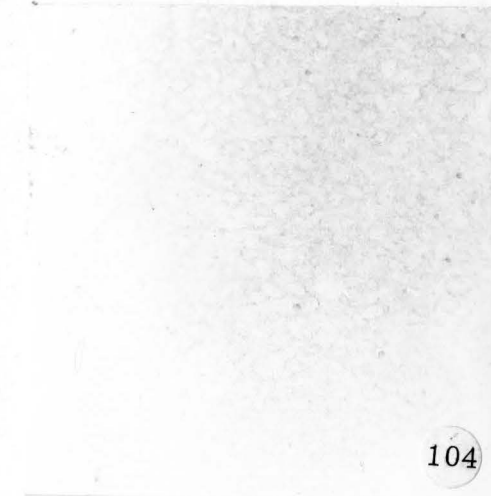
Fig. 107. Tertiary follicle taken from another area of the same ovary depicted in fig. 105 and showing highest and maximal NADH_2 diaphorase activity (+4) in T. I. and moderate activity (+2) in G.

Fig. 108. Follicle in the early stages of atresia taken from another region of the same ovary depicted in fig. 105. Note the T. I. appears maximally intense, and has increased in size, cf. fig. 107, whereas the G. appear moderately intense and are not as compactly arranged as is T. F. above.

Fig. 109. Atretic follicle (A. F.) which illustrates a later stage in follicular degeneration in which the G. cells are greatly reduced and the T. I. are hypertrophied when compared with the T. F. (fig. 107).

Fig. 110. Interstitial tissue - maximally reactive for NADH_2 .

Fig. 111. Corpus luteum - maximal (+4) deposition of diformazan; clear regions are the sites of the nuclei in the luteal cells.



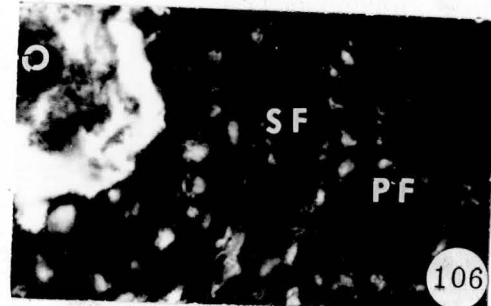
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104a



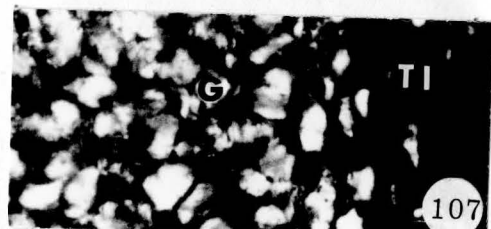
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106



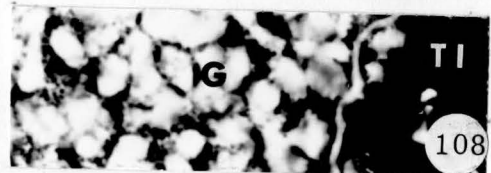
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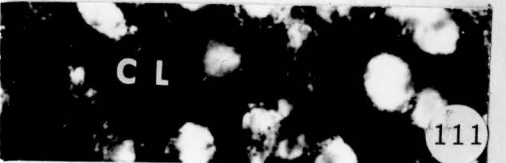
107



110



108



111

PLATE XVI

Plates XVI and XVII are comprised of photomicrographs of sections taken from ovaries removed from animals during normal pseudopregnancy which were incubated with pregnenolone (P-one) to localize the sites of biosynthesis of progesterone. Figures 112-121 have a final magnification of 175x.

PLATE XVI

Fig. 112. An ovary removed on day 6 showing maximal (+4) P-one- 3β -HSD activity localized in theca interna of A.F. and T.F. as well as I.T.; G. of T.F. appear moderately responsive while G. of A.F. shows trace (\pm) intensities for P-one- 3β -HSD. The antrum (A) in T.F. lacks positive P-one- 3β -HSD material.

Fig. 113. Another section taken from ovary depicted in fig. 112, showing maximal to strong activity in P-one- 3β -HSD localized in the theca interna of T.F. and S.F., respectively and moderate in G. in both T.F. and S.F. The antrum (A.) is negative.

Fig. 114. A corpus luteum (C.L.) appearing in another area of the ovary shown in fig. 112. Note maximal P-one- 3β -HSD activity in the C.L. cells.

Fig. 115. An ovary removed on day 8 depicting variable reaction sites for P-one- 3β -HSD. Strong (+3) diformazan located in T.I. of T.F., I.T. and C.L.; moderate (+2) intensities in thecal cells of S.F.; weak diformazan present in G. of S.F. and T.F.

Fig. 116. Another section taken from ovary in fig. 115, showing strong activity in P-one- 3β -HSD localized in thecal cells of A.F., I.T. and C.L. G. of T.F. appears weakly positive, whereas granulosa cells of A.F. and P.F. appear devoid of any reaction product. The ovum (O.) and antrum (A.) are typically negative.

PLATE XVI

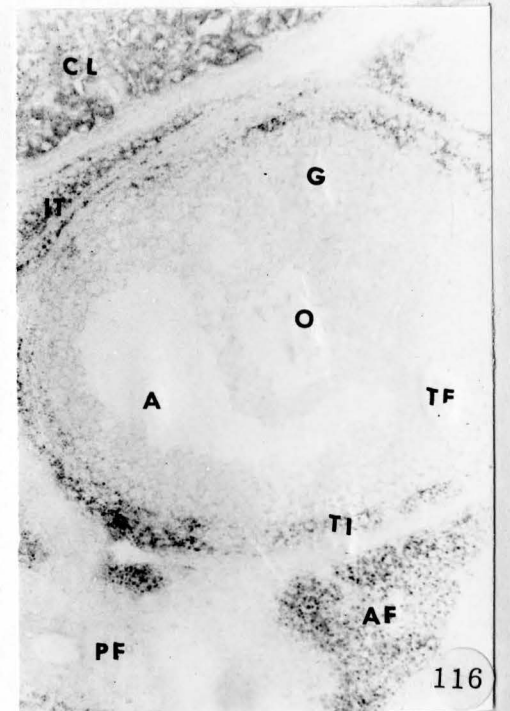
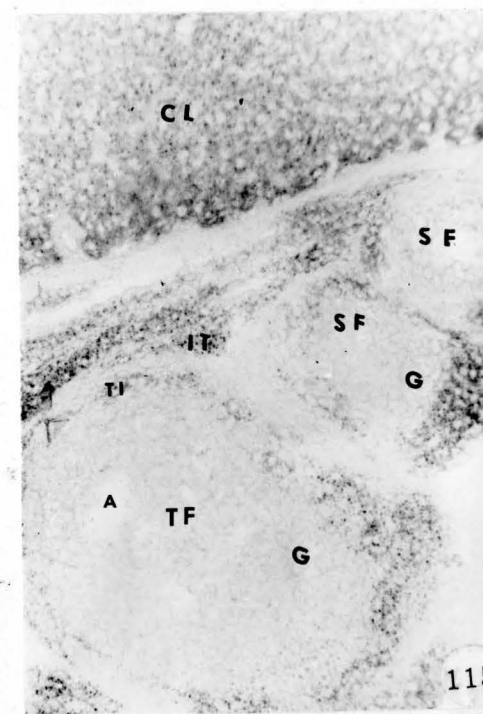
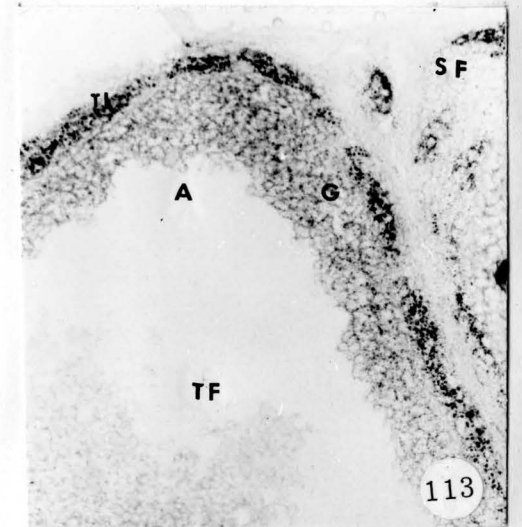
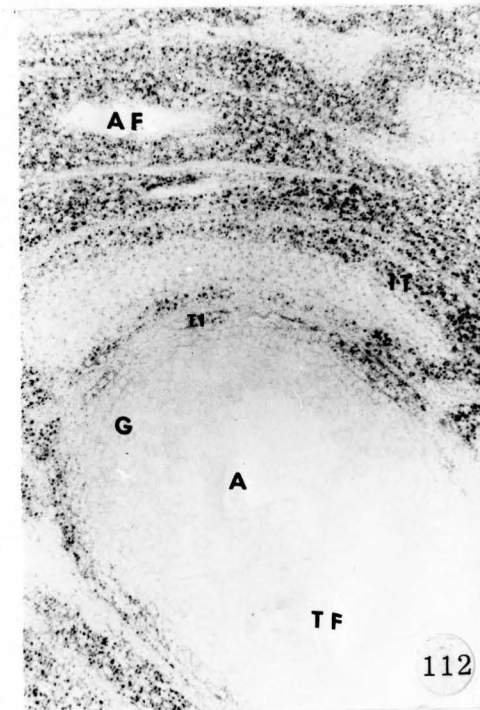


PLATE XVII

Fig. 117. An ovary removed on day 10 depicting strong deposition of diformazan granules in T.I. of T.F. as well as in I.T. Moderate P-one- 3β -HSD intensity in G. of T.F. The antrum (A.) is again negative.

Fig. 118. Another section of ovary described in fig. 117, showing strong P-one- 3β -HSD localized in the thecal cells of A.F. and in I.T. Moderate reactive enzymatic activity in T.I. of S.F., and only weakly appearing activity in G. of S.F.

Fig. 119. A moderately intense P-one- 3β -HSD deposition in a C.L. of the ovary described in fig. 117.

Fig. 120. An ovary removed on day 13. The T.I. of T.F. and T.I. of A.F. are moderately intense for P-one- 3β -HSD; the I.T. is strongly intense, whereas the G. of T.F. is weakly positive in activity. Monoformazan, trace intensities located in G. of A.F.

Fig. 121. Weakly positive activity in a C.L. of the ovary shown in fig. 120.

PLATE XVII

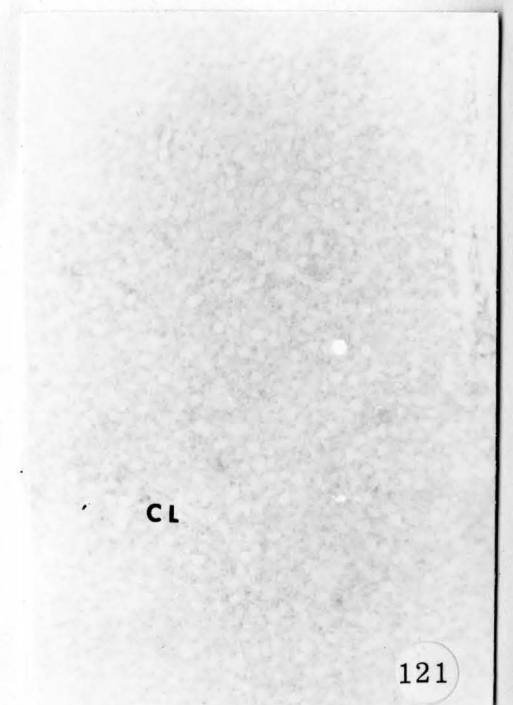
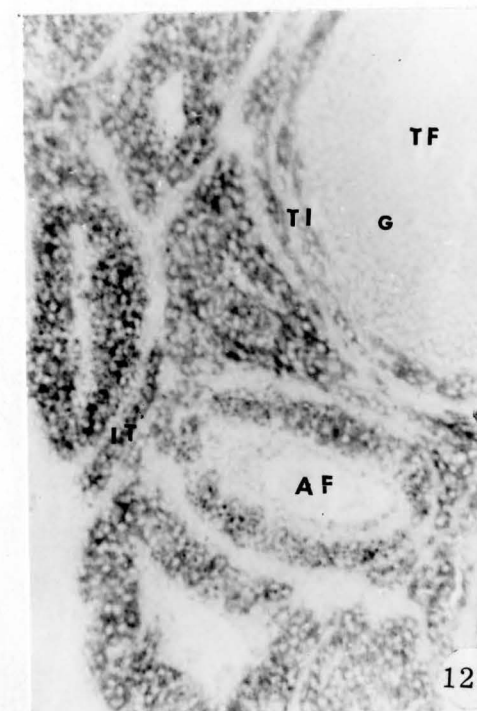
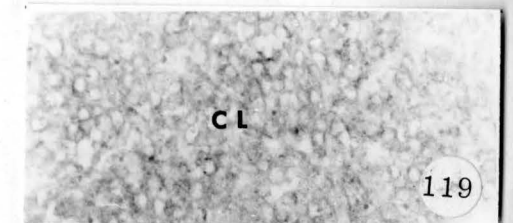
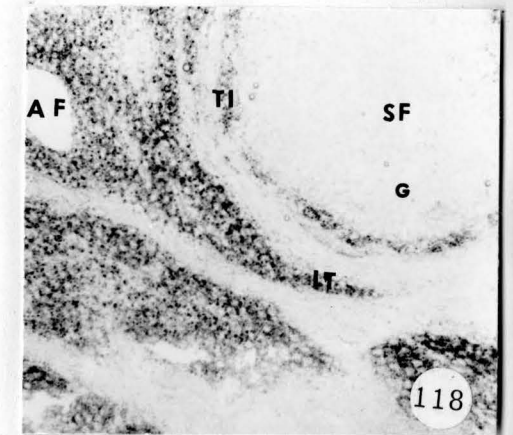
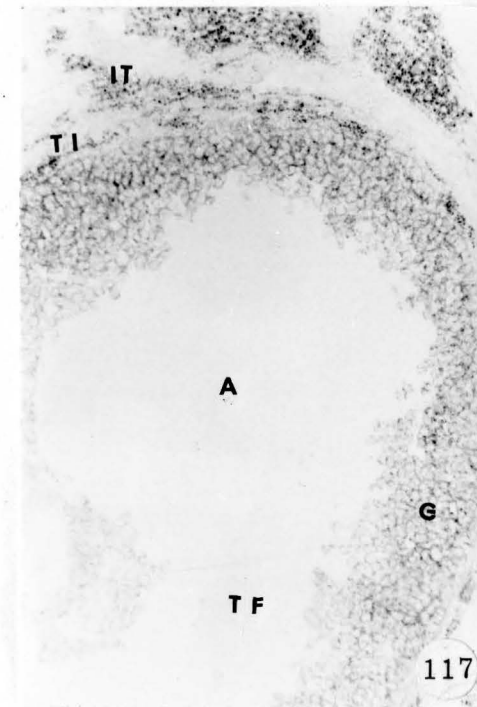


PLATE XVIII

Plate XVIII is comprised of high-powered photomicrographs of diformazan deposits observed in the P-one- 3β -HSD series of normal pseudopregnant rats. The left column consists of examples of photomicrographs depicting lowest observable intensities while the right column consists of exhibits of highest intensities in similar ovarian components; e. g. lowest P.F. (0, i. e. zero) fig. 122, highest P.F. in fig. 128. All figures which comprise this plate have a final magnification of 875x.

- Fig. 122. Primary follicle (P.F.) - lowest P-one- 3β -HSD activity in ovary of rat on day 13 (exhibited), also on days 6 and 8; cf. fig. 128, highest activity observed on day 10, increasing only slightly from negative (0) to trace (\pm).
- Fig. 123. Secondary follicle (S.F.) - lowest and minimal P-one- 3β -HSD activity in ovary of rat on day 13; cf. fig. 129, highest activity on day 6, increasing from trace to moderate (+2) in G. and trace to strong (+3) in T. I.
- Fig. 124. Tertiary follicle (T.F.) - lowest observable P-one- 3β -HSD activity in ovary of rat on day 13; cf. fig. 130, highest activity on day 6, increasing from weakly positive (+1) to moderate in G., and moderate to maximal (+4) in T. I.
- Fig. 125. Atretic follicle (A.F.) - lowest observable P-one- 3β -HSD activity in ovary of rat on day 13; cf. fig. 131, highest activity on day 6, increasing from trace to weakly positive in G. and moderate to maximal in T. I.
- Fig. 126. Interstitial tissue (I.T.) - lowest observable P-one- 3β -HSD activity in ovary of rat on day 10 (exhibited) and also on days 8 and 13; cf. fig. 132, highest activity on day 6, increasing from strong to maximal (+4).

PLATE XVIII - Cont'd.

- Fig. 127. Corpus luteum (C.L.) - lowest observable P-one- 3β -HSD activity in ovary of rat on day 13; cf. fig. 133, highest activity on day 6, increasing from weakly positive to maximal (+4).
- Fig. 128. Primary follicle (P.F.) - highest P-one- 3β -HSD (\pm) activity on day 10; cf. fig. 122, lowest on days 6, 8 and 10 (the latter exhibited).
- Fig. 129. Secondary follicle (S.F.) - highest P-one- 3β -HSD (G. - +2 and T.I. - +3) activity on day 6; cf. fig. 123, lowest on day 13.
- Fig. 130. Tertiary follicle (T.F.) - highest P-one- 3β -HSD (G. - +2 and T.I. - +4) activity on day 6; cf. fig. 124, lowest on day 13.
- Fig. 131. Atretic follicle (A.F.) - highest P-one- 3β -HSD (G. - +1 and T.I. - +4) activity on day 6; cf. fig. 125, lowest on day 13.
- Fig. 132. Interstitial tissue (I.T.) - highest P-one- 3β -HSD (+4) activity on day 6; cf. fig. 126, lowest on days 8, 13 and 10 (the latter exhibited).
- Fig. 133. Corpus luteum (C.L.) - highest P-one- 3β -HSD (+4) activity on day 6; cf. fig. 127, lowest on day 13.

PLATE XVIII

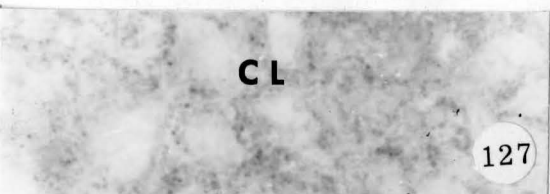
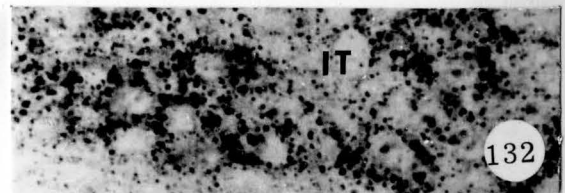
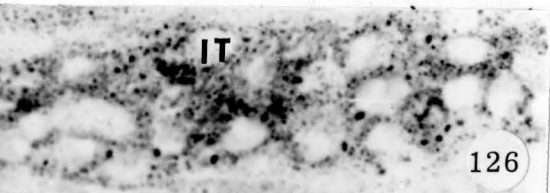
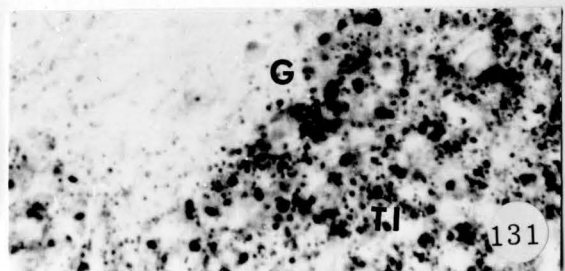
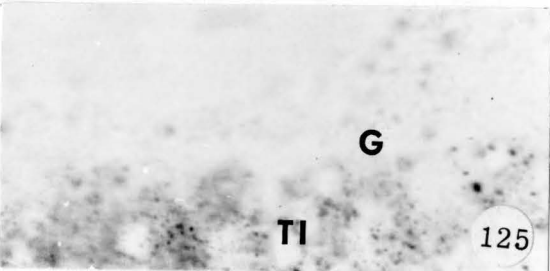
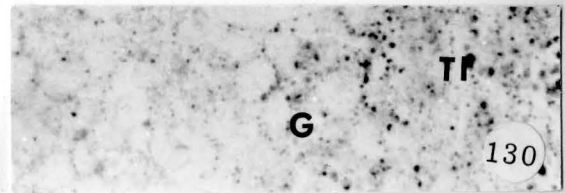
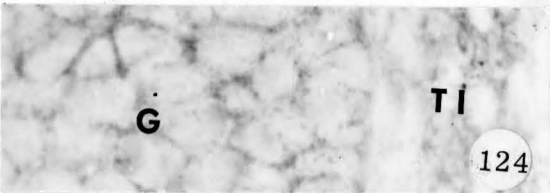
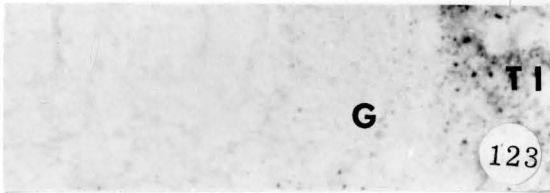
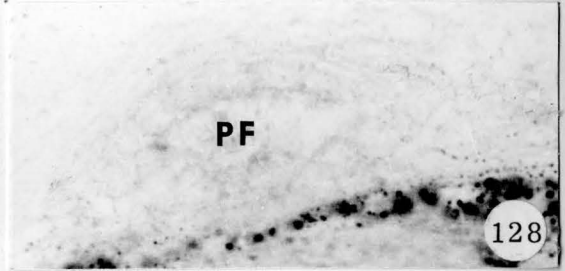
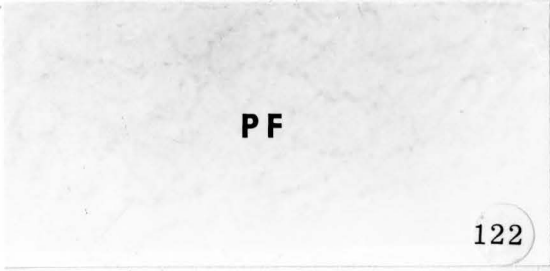


PLATE XIX

Plates XIX and XX are comprised of photomicrographs of sections taken from the ovaries removed from animals during normal pseudo-pregnancy. The ovarian sections were incubated with dehydroepiandrosterone (DHA) to localize the sites of DHA-3 β -HSD as an indicator of the biosynthesis of estrogens. Figures 134-143 have a final magnification of 175x.

PLATE XIX

Fig. 134. An ovary removed on day 6 depicting weakly positive to strong DHA-3 β -HSD activity. Strong (+3) intensity for DHA-3 β -HSD in C. L.; moderate (+2) intensity in T. I. of A. F. and in I. T.; and weakly positive (+1) in T. I. of S. F. and G. of S. F., T. F. and A. F. The ovum (O.) of T. F. is typically negative.

Fig. 135. Another section of ovary described in fig. 134, removed on day 6, and showing moderate DHA-3 β -HSD activity in thecal cells of A. F. and T. F.; weakly positive in granulosa cells of T. F. and A. F. The antrum (A.) is typically negative.

Fig. 136. An ovary removed on day 8 depicting strong intensities of diformazan deposition in thecal cells of A. F. and T. F. as well as in interstitial tissue (I. T.); moderate DHA-3 β -HSD localization in G. of T. F.; trace intensities in granulosa cells of A. F. Note antra (A.) are negative.

Fig. 137. Another section of ovary described in fig. 136, and showing strong intensities of diformazan granulation in thecal cells of A. F.; weakly positive intensities in T. I. of S. F.; and trace intensities in G. of S. F. and A. F.

Fig. 138. A corpus luteum (C. L.) appearing in another area of the ovary described in fig. 136, and showing strong DHA-3 β -HSD activity in luteal cells.

PLATE XIX

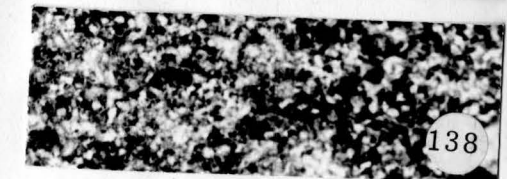
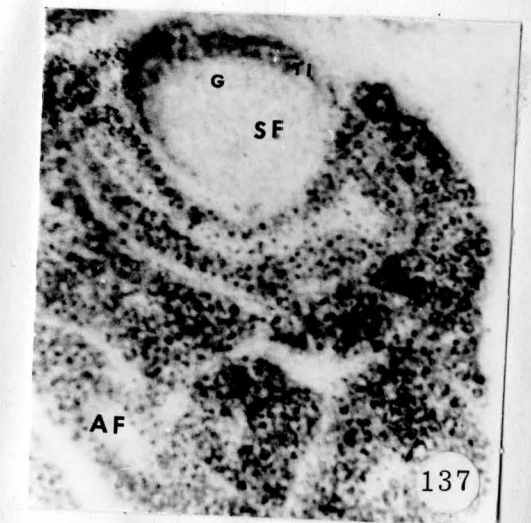
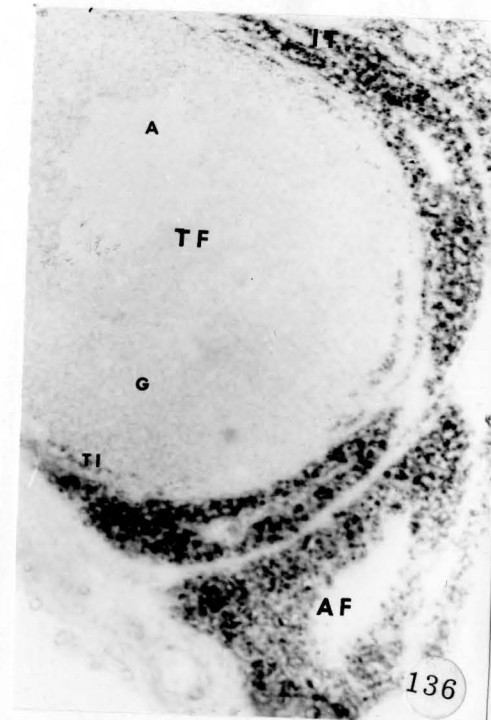
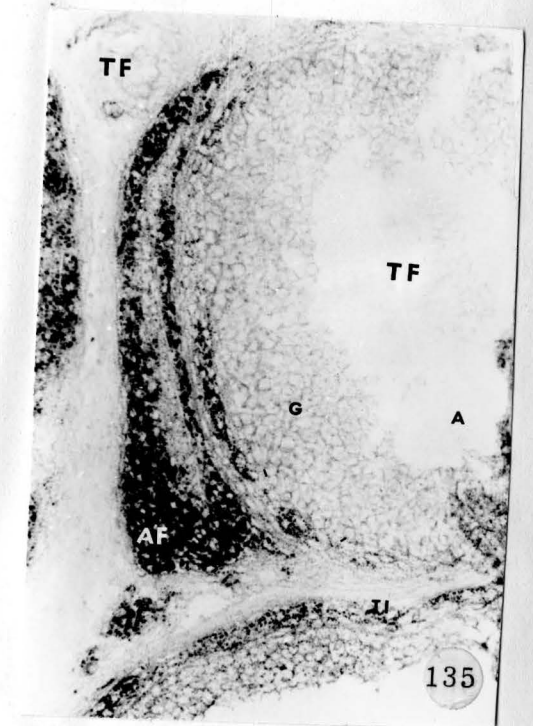
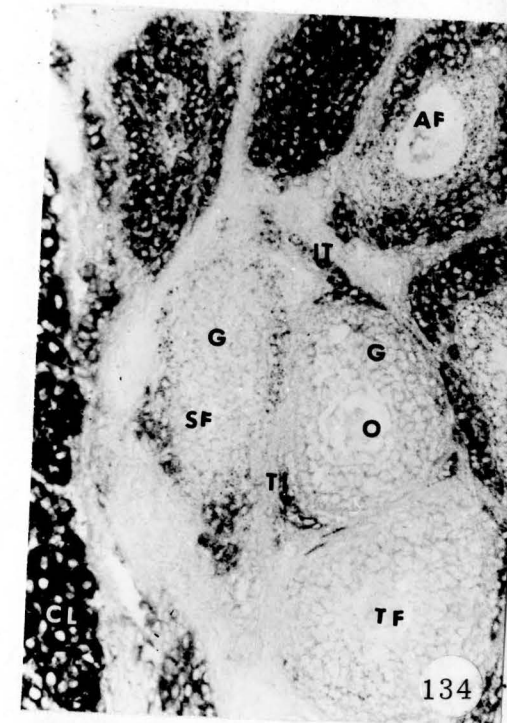


PLATE XX

Fig. 139. An ovary removed on day 10 of normal pseudopregnancy, showing maximal (+4) intensities of DHA-3 β -HSD activity localized in C. L.; strong (+3) intensities in T. I. of T. F.; moderate (+2) intensities in I. T.; and weakly positive (+1) intensities in G. of T. F. The antrum (A.) is negative.

Fig. 140. Another region of the ovary described in fig. 139, and showing maximal DHA-3 β -HSD activity in C. L.; moderate activity in thecal cells of S. F. and weakly positive activity in granulosa cells of S. F.

Fig. 141. Another area of the ovary described in fig. 139; thecal cells of A. F. appear strongly intense for DHA while granulosa cells of A. F. are minimally intense.

Fig. 142. An ovary removed on day 13 of normal pseudopregnancy showing maximal (+4) intensities of diformazan granules localized in I. T.; strong (+3) intensities in thecal cells of T. F. and A. F.; moderate (+2) intensities in G. of T. F. and A. F.; trace (monoformazan material) in granulosa cells of S. F. Note the ovum (O.) and antrum (A.) are typically negative.

Fig. 143. Another region of the ovary described in fig. 141 and showing maximal DHA-3 β -HSD activity localized in C. L. Intensities of diformazan localized in I. T. are quite variable, but usually appear maximal in intensity.

PLATE XX

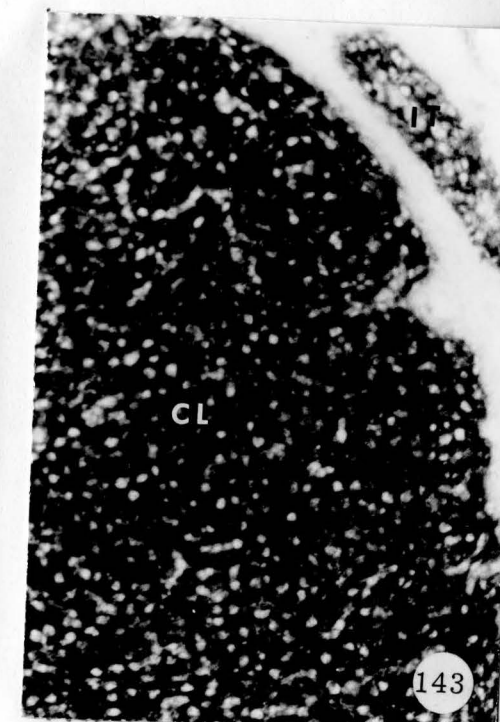
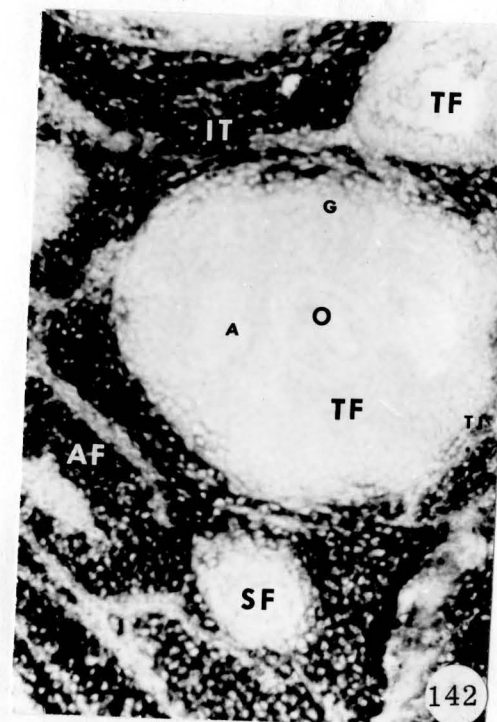
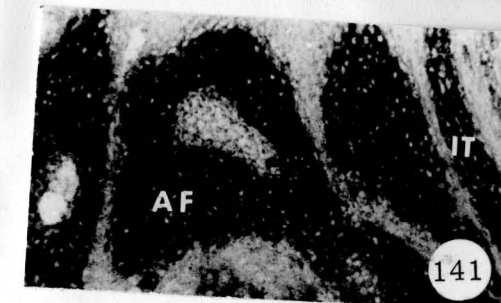
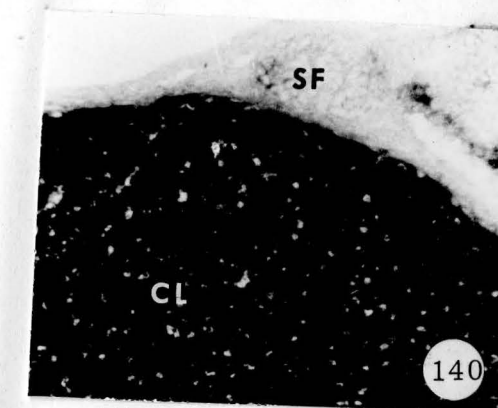
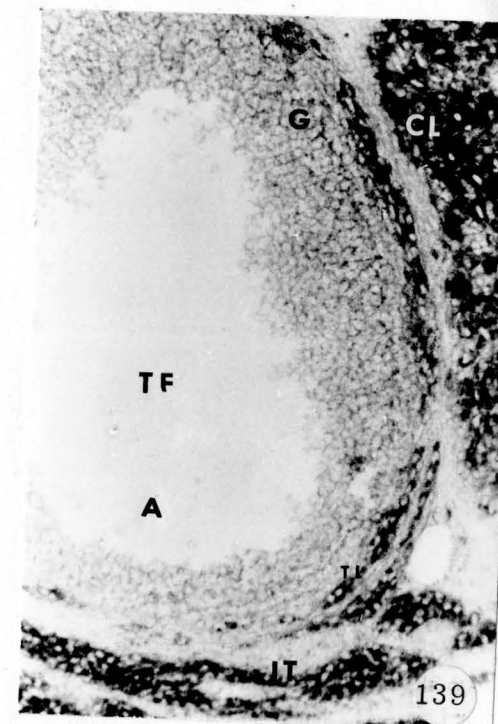


PLATE XXI

Plate XXI is comprised of high-powered photomicrographs of DHA-3 β -HSD indicated by different intensities of diformazan deposits in the ovaries, of the normal pseudopregnant series of animals. The left column consists of examples of photomicrographs depicting lowest observable intensities, while the right column consists of examples of highest intensities in similar components, e. g. lowest P.F. (0, i. e. zero) fig. 144, highest P.F. in fig. 150. All figures which comprise this plate have a final magnification of 875x.

Fig. 144. Primary follicle (P.F.) - lowest DHA-3 β -HSD activity in ovary of rat on day 10 (exhibited) also on days 8 and 13; cf. fig. 150, highest activity observed on day 6, increasing from negative (0) to trace (\pm).

Fig. 145. Secondary follicle (S.F.) - lowest DHA-3 β -HSD activity in ovary of rat on day 6; cf. fig. 151, highest activity observed on day 13, T.I. increasing from weakly positive to moderate, while G. decline from weakly positive to trace.

Fig. 146. Tertiary follicle (T.F.) - lowest DHA-3 β -HSD activity in ovary of rat on day 6; cf. fig. 152, highest activity observed on day 8, increasing from weakly positive to moderate in G. and moderate to strong in T.I.

Fig. 147. Atretic follicle (A.F.) - lowest DHA-3 β -HSD activity in ovary of rat on day 6; cf. fig. 153, highest activity on day 13, increasing from moderate to strong in T.I. with comparable weakly positive intensities in G.

Fig. 148. Interstitial tissue (I.T.) - lowest DHA-3 β -HSD activity in ovary of rat on day 6 (exhibited, also on day 10); cf. fig. 154, highest and maximal activity on day 13, increasing from moderate to maximal.

PLATE XXI - Cont'd.

- Fig. 149. Corpus luteum (C.L.) - lowest DHA-3 β -HSD activity in ovary of rat on day 6 (exhibited also on day 8); cf. fig. 155, highest and maximal activity observed on days 10 and 13 (the latter exhibited), increasing from strong to maximal.
- Fig. 150. Primary follicle (P.F.) - highest DHA-3 β -HSD (\pm) activity on day 6; cf. fig. 144, lowest on days 8, 10 and 13.
- Fig. 151. Secondary follicle (S.F.) - highest DHA-3 β -HSD (G. - \pm and T.I.-+2) activity on day 13; cf. fig. 145, lowest on day 6.
- Fig. 152. Tertiary follicle (T.F.) - highest DHA-3 β -HSD (G. - +2 and T.I.-+3) activity on day 8; cf. fig. 146, lowest on day 6.
- Fig. 153. Atretic follicle (A.F.) - highest DHA-3 β -HSD (G. - +1 and T.I.-+3) activity on day 13; c.f. fig. 147, lowest on day 6.
- Fig. 154. Interstitial tissue (I.T.) - highest DHA-3 β -HSD (+4) activity on day 13; cf. fig. 148, lowest on days 6 and 10.
- Fig. 155. Corpus luteum (C.L.) - highest DHA-3 β -HSD (+4) activity on days 10 and 13; cf. lowest on days 6 and 8

PLATE XXI

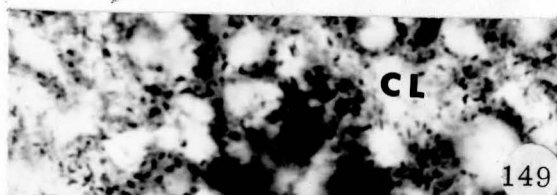
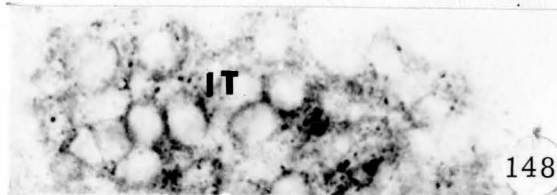
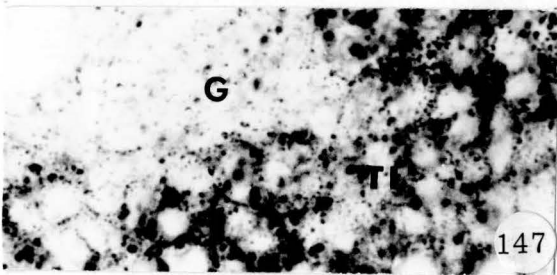
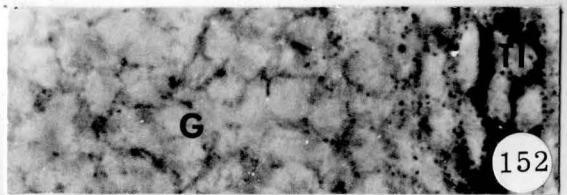
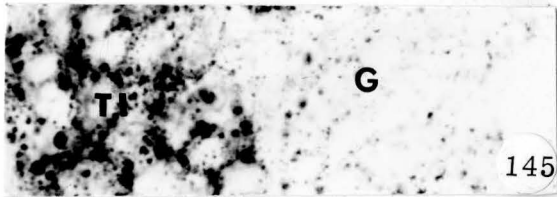
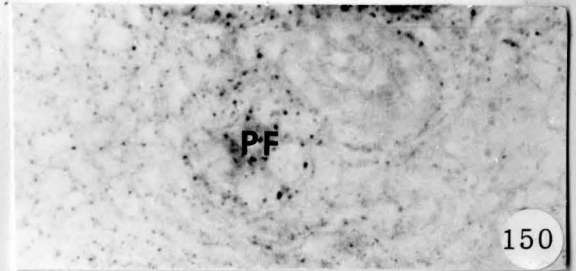
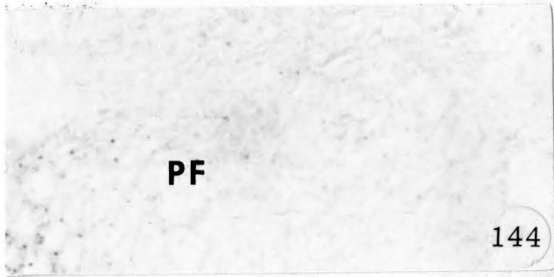


PLATE XXII

Plate XXII is the ovarian hilus of animals necropsied on day 10 of normal pseudopregnancy. These ovarian tissue sections were incubated with each of the substrates utilized in this study in order to localize the sites of enzymatic activity. Figures 156-158 have a final magnification of 175x.

Fig. 156. An ovarian section showing the presence of NADH_2 diaphorase localized in the walls of the blood vessels and interstitial tissue. Note the dense (maximal, +4) diformazan deposits in the I. T. and blood vessels. The remaining cells in the hilar region of the ovary appear weakly positive for NADH_2 diaphorase activity.

Fig. 157. Section of an ovary in the hilar area for the demonstration, localization and appearance of P-one- 3β -HSD in this region. Intensities of P-one- 3β -HSD activity appeared between weakly positive and moderate in the interstitial tissue cells. In the ovarian hilus, these interstitial cells appeared to be the sole source of potential progesterone production.

Fig. 158. Section of an ovary in the hilar region for the demonstration, localization and appearance of DHA- 3β -HSD in the cellular components in this region. Moderate intensities of DHA- 3β -HSD activity are localized in the interstitial tissue cells. Due to the refractile index of the tunics of the blood vessels, the vascular organs seem to contain reaction material; upon careful examination of these regions, no discernable mono- nor diformazan deposits were localized in any of the cellular components of the blood vessels.

PLATE XXII

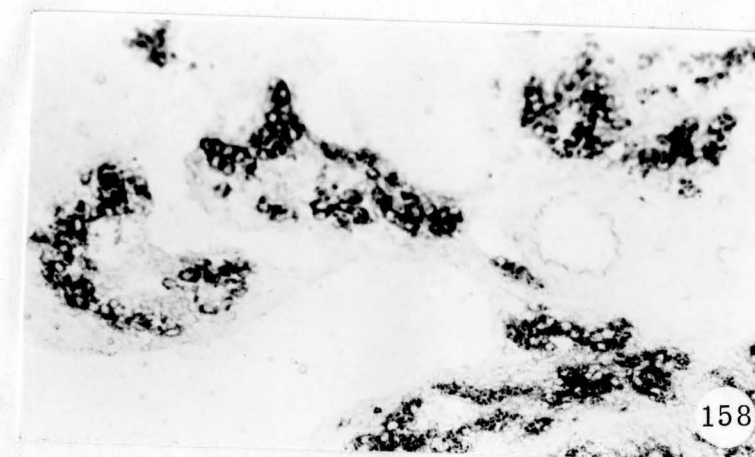
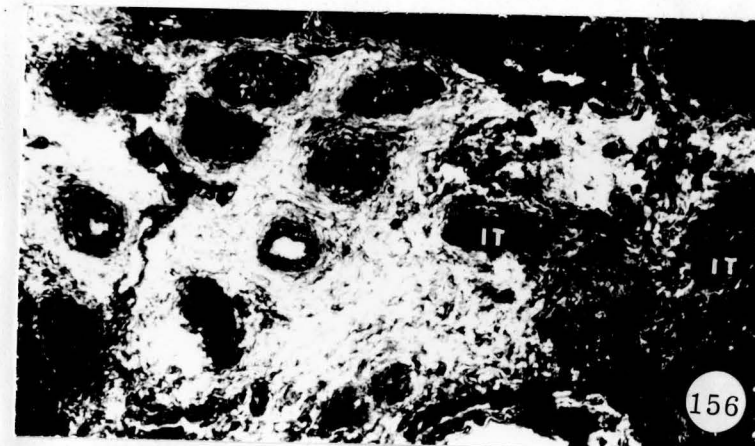


PLATE XXIII

PLATE XXIII

Plates XXIII through XXVI are comprised of photomicrographs of sections taken from ovaries removed during prolonged pseudopregnancy which were incubated with pregnenolone (P-one) to localize the sites of biosynthesis of progesterone. Figures 159-179 have a final magnification of 175x.

PLATE XXIII

- Fig. 159. An ovary removed on day 6 showing maximal (+4) P-one- 3β -HSD activity localized in theca interna of A.F., I.T. and C.L.; strong (+3) intensities of T.I. of S.F. and T.F.; and weakly positive (+1) intensities in G. of S.F., T.F. and A.F. The antrum (A.) of T.F. is negative.
- Fig. 160. Another section of ovary described in fig. 159 showing maximal (+4) diformazan intensities in theca interna cells of A.F., and I.T.; strong (+3) intensities of P-one- 3β -HSD in T.I. of T.F.; weakly positive (+1) intensities in G. of T.F. and A.F.
- Fig. 161. An ovary removed on day 8 and showing strong (+3) P-one- 3β -HSD activity in T.I. of T.F. and A.F. as well as in I.T.; moderate (+2) activity in theca interna cells (patches) of S.F.; and weakly positive (+1) in G. of S.F., T.F. and A.F. Ovum (O.) and antrum (A.) are typically negative.
- Fig. 162. A corpus luteum appearing in another area of the ovary shown in fig. 161. Note maximal (+4) P-one- 3β -HSD activity in C.L. cells.

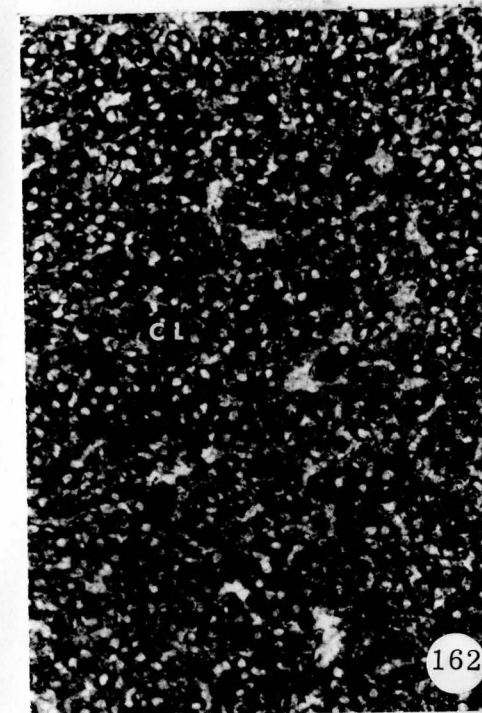
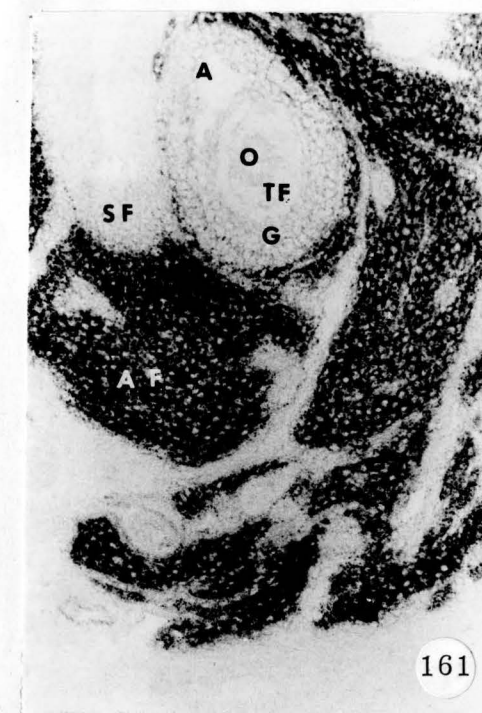
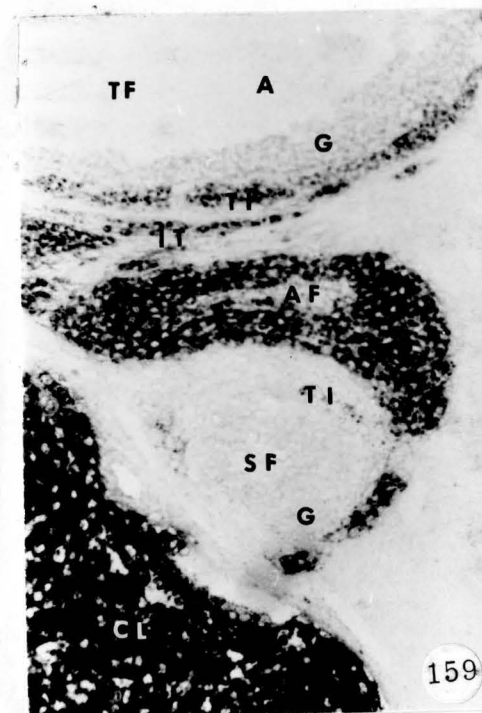


Fig. 163. An ovary removed on day 10 depicting maximal (+4) P-one- 3β -HSD activity in theca interna of A.F. and I.T.; strong activity in T.I. of T.F.; moderate (+2) activity in G. of T.F.; and trace (\pm) activity in granulosa cells of A.F. The antrum (A.) lacks P-one- 3β -HSD activity.

Fig. 164. A maximally active P-one- 3β -HSD deposition in a C.L. of the ovary described in fig. 163.

Fig. 165. Another region of the ovary described in fig. 163 showing maximal intensities of diformazan in theca interna cells of A.F. as well as in I.T.; strong (+3) but patchy intensities in T.I. of S.F.; and trace (\pm) intensities in G. of S.F. and A.F.

Fig. 166. An ovary removed on day 13 depicting strong P-one- 3β -HSD deposition in T.I. of T.F. and A.F. and in I.T.; moderate (+2) in G. of T.F.; and weakly positive in granulosa cells of A.F. Both antra (A.) are negative.

Fig. 167. Another region of the ovary described in fig. 166 and showing strong P-one- 3β -HSD activity localized in theca interna of A.F. as well as in I.T.; moderate in T.I. of S.F.; and weakly positive in granulosa cells of S.F. and A.F.

Fig. 168. Another area of the ovary depicted in fig. 166 and showing maximal (+4) P-one- 3β -HSD activity in C.L.

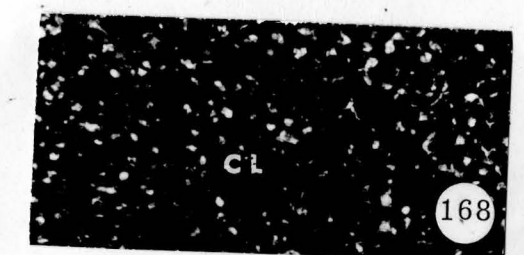
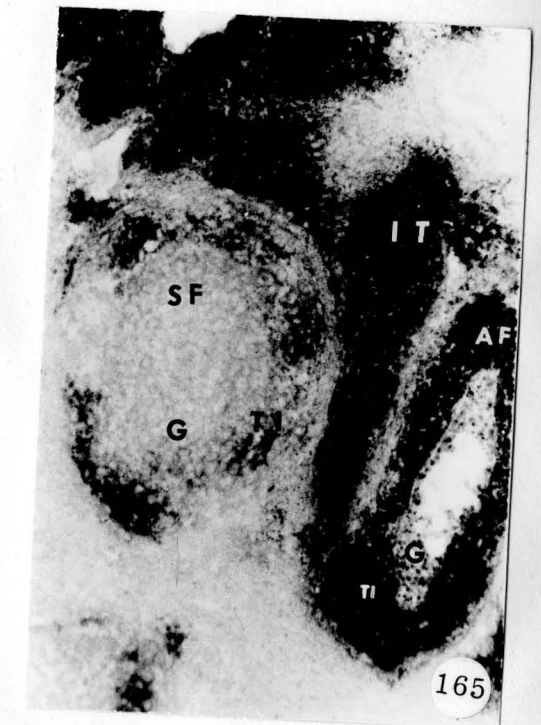
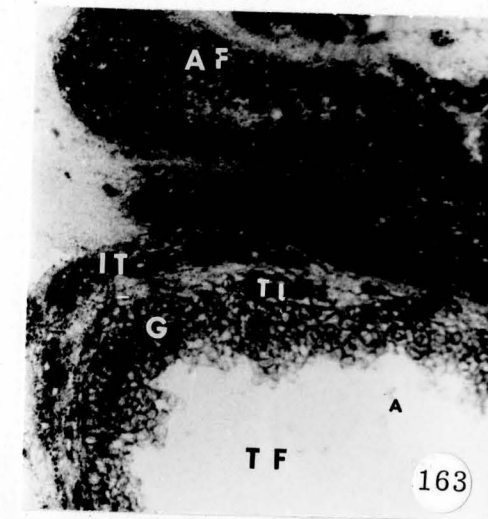


PLATE XXV

Fig. 169. An ovary removed on day 15 depicting moderate (+2) activity of P-one- 3β -HSD in I. T. as well as in T. I. of T. F.; and weakly positive (+1) activity in G. of T. F. The antrum (A.) of T. F. is negative.

Fig. 170. Another region of the ovary described in fig. 169 showing moderate P-one- 3β -HSD activity localized in theca interna cells of A. F. as well as in I. T.; granulosa cells of A. F. are weakly positive.

Fig. 171. A corpus luteum (C. L.) appearing in another area of the ovary shown in fig. 169 and depicting variable P-one- 3β -HSD activity ranging from weakly positive to moderate.

Fig. 172. An ovary removed on day 18 displaying moderate intensities of diformazan deposition in I. T. and T. I. of S. F. and T. F.; and trace (monoformazan material) in granulosa cells of S. F. and T. F.

Fig. 173. Another area of the ovary described in fig. 172 showing moderate (+2) P-one- 3β -HSD activity in theca interna cells of A. F. as well as in I. T. and weakly positive (+1) activity in granulosa cells of A. F.

Fig. 174. A corpus luteum (C. L.) appearing in another region of the ovary shown in fig. 172 and exhibiting strong (+3) P-one- 3β -HSD activity in the luteal cells.

PLATE XXV

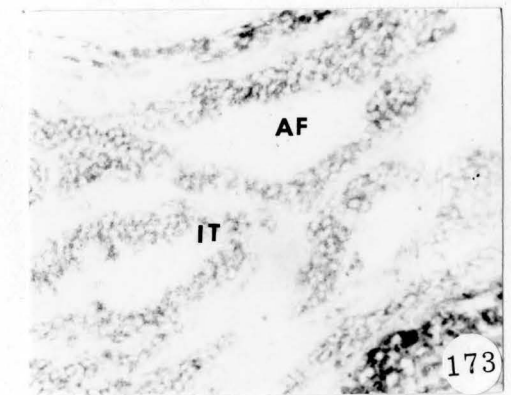
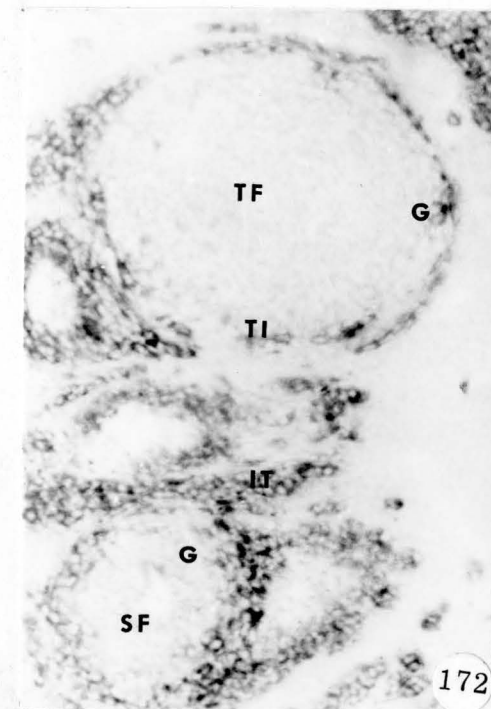
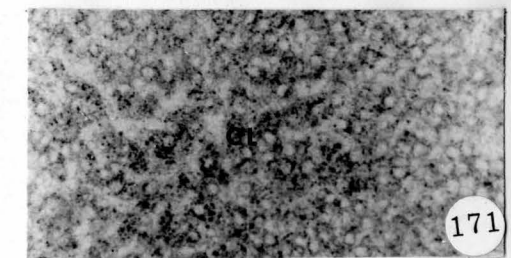
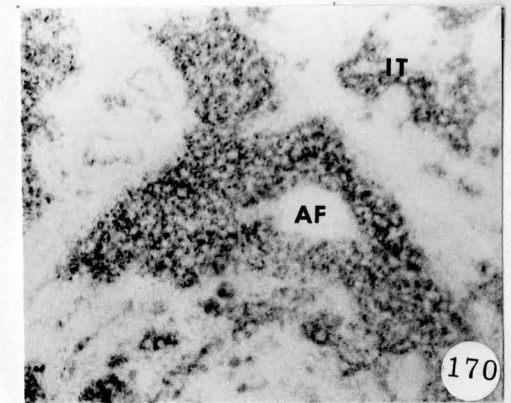
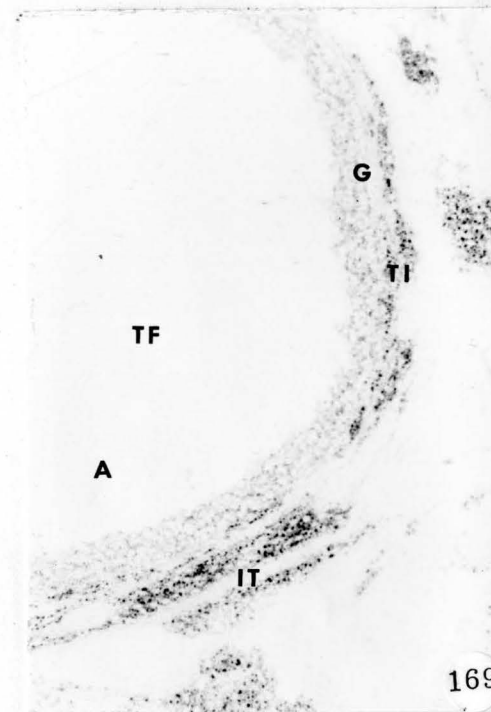


PLATE XXVI

Fig. 175. An ovary removed on day 20 of prolonged pseudopregnancy and showing strong P-one- 3β -HSD activity localized in I. T. ; moderate (+2) activity in T. I. of T. F. and C. L. ; and weakly positive (+1) activity in G. of T. F. The antrum (A.) of T. F. is negative.

Fig. 176. Another region of the ovary described in fig. 175, illustrating P-one- 3β -HSD reaction intensities of strong in theca interna cells of A. F. and in I. T. ; moderate intensities in theca interna cellular patches of S. F. ; weakly positive intensities in granulosa cells of A. F. ; and trace intensities in granulosa cells of S. F.

Fig. 177. An ovary removed on day 21 displaying moderate (+2) diformazan intensities in theca interna of S. F. and A. F. as well as I. T. ; and trace (monoformazan material) in G. of S. F. and A. F.

Fig. 178. Another region of the ovary described in fig. 177 showing moderate (+2) P-one- 3β -HSD activity in T. I. of T. F. and weakly positive (+1) activity in G. of T. F. The antrum (A.) is typically negative.

Fig. 179. A corpus luteum appearing in another region of the ovary shown in fig. 177. Note weakly positive (+1) intensities of diformazan in luteal cells.

PLATE XXVI

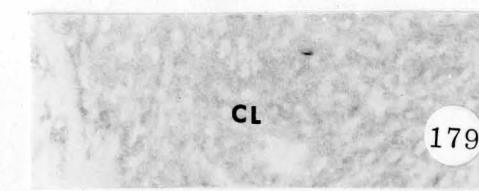
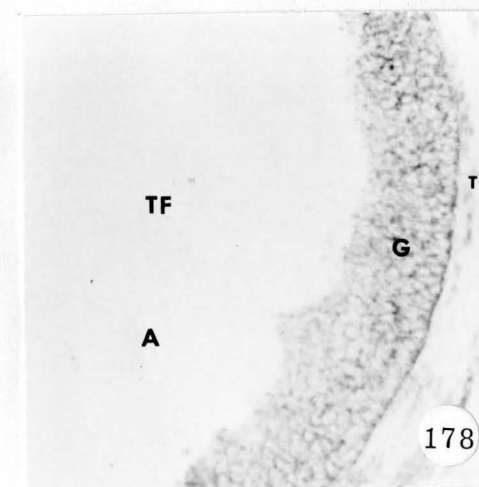
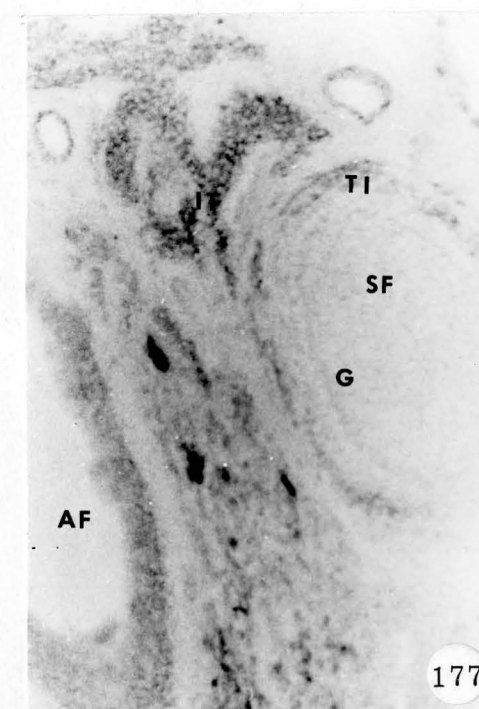
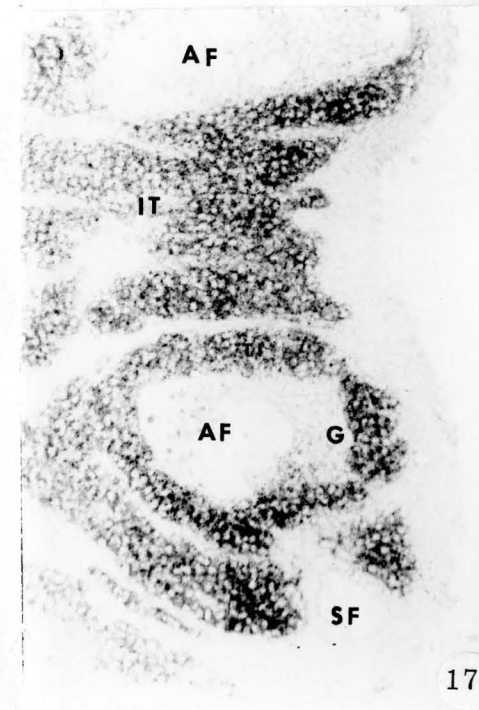
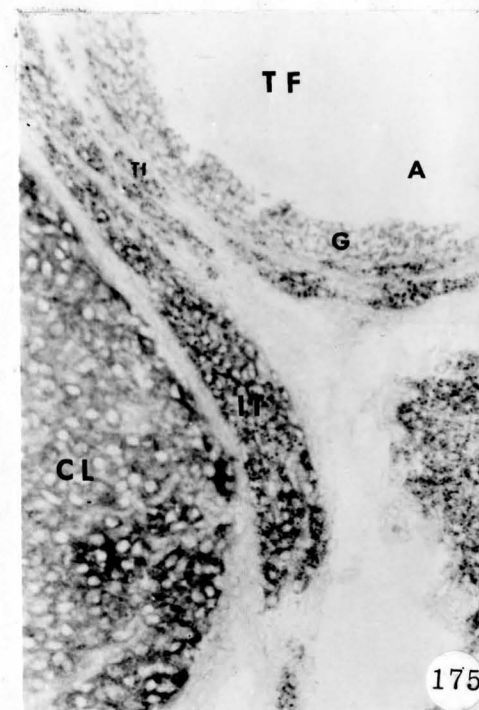


PLATE XXVII

Plate XXVII is comprised of high-powered photomicrographs of P-one- 3β -HSD appearing as diformazan deposits in the ovaries of prolonged pseudopregnant rats. The left column consists of examples of photomicrographs depicting lowest observable intensities while the right column consists of exhibits of highest intensities in similar ovarian components, e. g. lowest P.F. (0, i. e. zero) fig. 180, highest P.F. (+1) in fig. 186. All figures which comprise this plate have a final magnification of 875x.

Fig. 180. Primary follicle (P.F.) - lowest P-one- 3β -HSD activity in ovary of rat on day 21 (exhibited) and also on day 20; cf. fig. 186, highest activity observed on days 8, 10, 13, and 15 (exhibited), increasing from negative (0) to weakly positive (+1).

Fig. 181. Secondary follicle (S.F.) - lowest P-one- 3β -HSD activity in ovary of rat on days 18 and 21 (exhibited); cf. fig. 187, highest on day 6, increasing from trace to weakly positive (+1) in G. and moderate (+2) to strong (+3) in T. I.

Fig. 182. Tertiary follicle (T.F.) - lowest observable P-one- 3β -HSD activity in ovary of rat on day 18; cf. fig. 188, highest activity on days 10 (exhibited) and 13, increasing from trace to moderate in G., and moderate to strong (+3) in T. I.

Fig. 183. Atretic follicle (A.F.) - lowest observable P-one- 3β -HSD activity in ovary of rat on day 21, cf. fig. 189, highest activity on day 6, increasing from trace to weakly positive in G. and moderate to maximal (+4) in T. I.

Fig. 184. Interstitial tissue (I. T.) - lowest observable P-one- 3β -HSD activity in ovary of rat on days 15, 18 and 21 (exhibited); cf. fig. 190, highest and maximal activity on days 6 (exhibited) and 10, increasing from moderate (+2) to maximal (+4).

PLATE XXVII - Cont'd.

- Fig. 185. Corpus luteum (C.L.) - lowest observable P-one- 3β -HSD activity in ovary of rat on day 21; cf. fig. 191, highest activity on days 6 (exhibited), 8, 10, 13, increasing from weakly positive to maximal (+4).
- Fig. 186. Primary follicle (P.F.) - highest P-one- 3β -HSD activity (+1) on days 8, 10, 13, and 15 (exhibited); cf. fig. 180, lowest on days 20 and 21 (exhibited).
- Fig. 187. Secondary follicle (S.F.) - highest P-one- 3β -HSD activity (G. - +1; T.I. - +3) on day 6, cf. fig. 181, lowest on days 18 and 21 (exhibited).
- Fig. 188. Tertiary follicle (T.F.) - highest P-one- 3β -HSD activity (G. - +2, T.I. - +3) on days 10 (exhibited) and 13; cf. fig. 182, lowest on day 18.
- Fig. 189. Atretic follicle (A.F.) - highest P-one- 3β -HSD activity (G. - +1, T.I. - +4) on day 6; cf. fig. 183, lowest on day 21.
- Fig. 190. Interstitial tissue (I.T.) - highest P-one- 3β -HSD activity (+4) on days 6 (exhibited) and 10; cf. fig. 184, lowest on days 15, 18 and 21 (exhibited).
- Fig. 191. Corpus luteum (C.L.) - highest P-one- 3β -HSD activity (+4) on days 6 (shown), 8, 10 and 13; cf. fig. 185, lowest on day 21.

PLATE XXVII

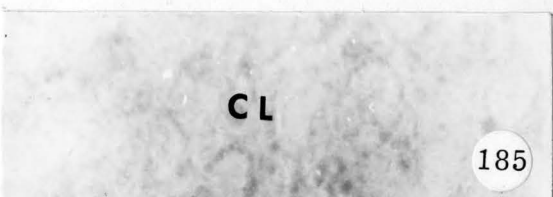
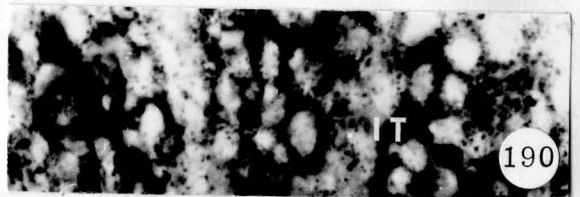
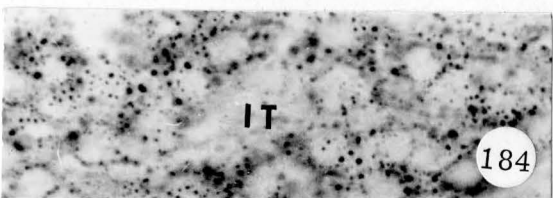
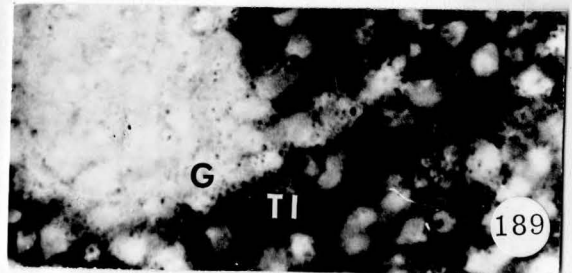
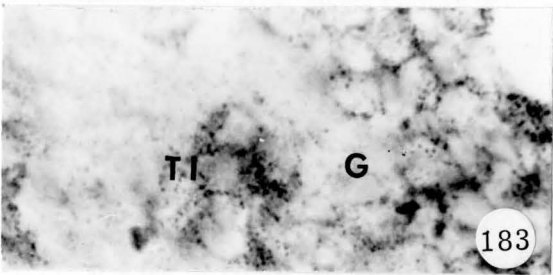
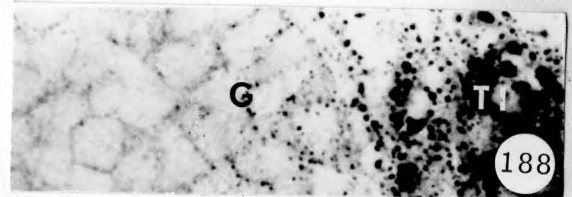
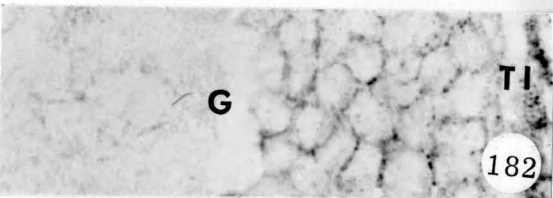
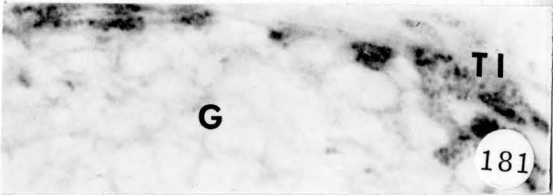
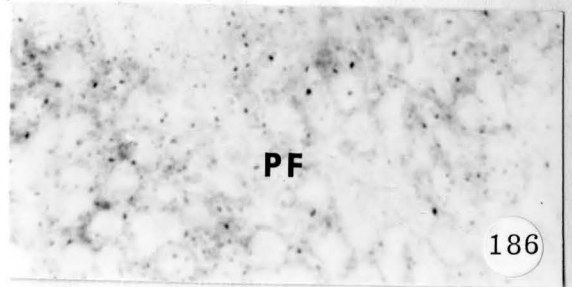
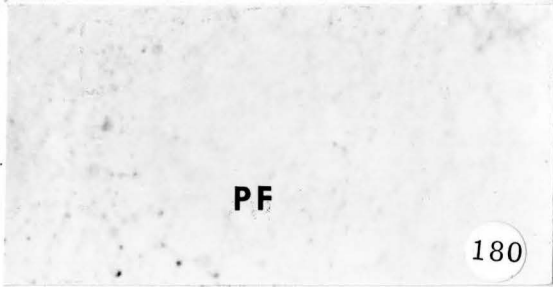


PLATE XXVIII

Plates XXVIII through XXX are comprised of photomicrographs of sections taken from ovaries removed from animals during prolonged pseudopregnancy which were incubated with dehydroepiandrosterone (DHA) to localize the sites of the biosynthesis of estrogens. Figures 192-209 have a final magnification of 175x.

PLATE XXVIII

Fig. 192. An ovary removed on day 6 showing maximal (+4) DHA-3 β -HSD activity localized in the theca interna of A. F.; moderate (+2) activity in T. I. of S. F. and T. F.; weakly positive (+1) activity in G. of T. F. and A. F.; and trace (\dagger) activity in G. of S. F.

Fig. 193. Another region of the ovary described in fig. 192 showing maximal (+4) diformazan intensities in C. L. and I. T.; moderate (+2) in T. I. of T. F.; and weakly positive (+1) intensities in G. of T. F. The antrum (A.) is negative.

Fig. 194. An ovary removed on day 8, depicting variable DHA-3 β -HSD activity ranging from strong to maximal (+4) in I. T.; strong (+3) activity in theca interna of A. F. and T. F.; moderate (+2) activity in thecal cells of S. F.; weakly positive (+1) activity in G. of T. F.; and variable activity ranging between (\dagger) in granulosa cells of S. F. and A. F.

Fig. 195. A corpus luteum (C. L.) appearing in another area of the ovary shown in fig. 194. Note strong (+3) DHA-3 β -HSD activity in the C. L. cells.

Fig. 196. An ovary removed on day 10 depicting maximal (+4) DHA-3 β -HSD activity in theca interna cells of A. F. and in I. T.; strong (+3) to maximal activity in C. L.; and weakly positive (+1) activity in granulosa cells of A. F.

Fig. 197. Another region of the ovary described in fig. 196 showing strong DHA-3 β -HSD activity in T. I. of T. F. and weakly positive activity in G. of T. F. The antrum (A.) is negative.

PLATE XXVIII

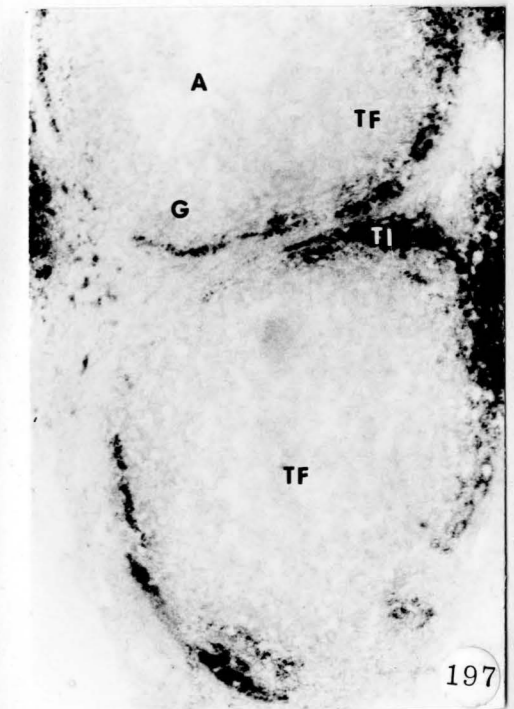
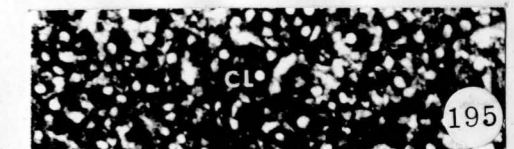
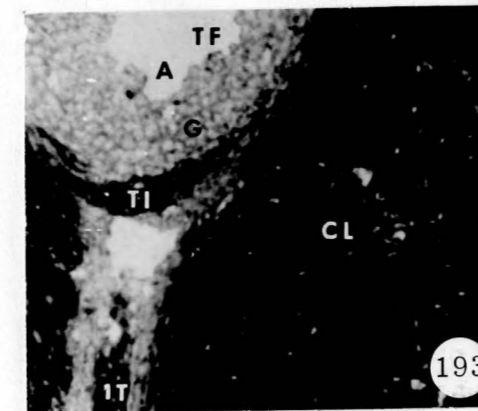
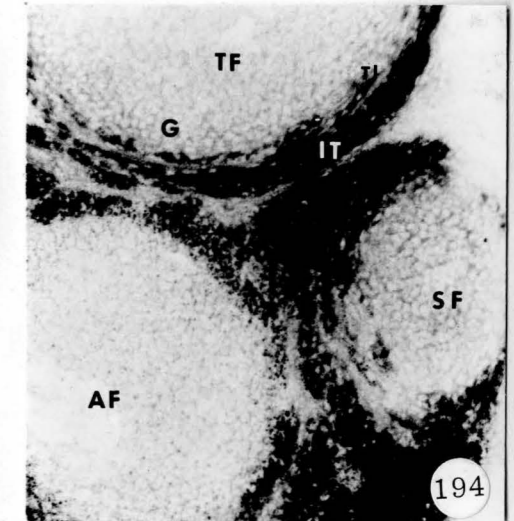
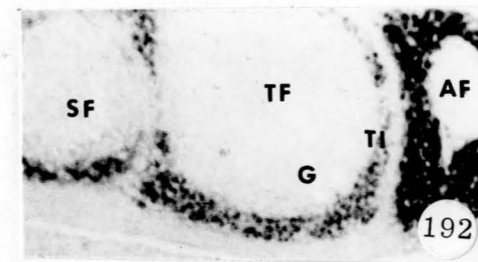


PLATE XXIX

Fig. 198. An ovary removed on day 13 of prolonged pseudopregnancy showing maximal (+4) DHA-3 β -HSD activity in theca interna of A. F., and in I. T.; moderate (+2) activity in T. I. of S. F.; and weakly positive (+1) to moderate (+2) activity in G. of S. F. and A. F.

Fig. 199. Another region of the ovary described in fig. 198, depicting maximal intensities of diformazan in theca interna cells of A. F. and T. I. of T. F.; and weakly positive intensities in G. of T. F. and A. F. The antrum (A.) is negative.

Fig. 200. Another area of the ovary shown in fig. 198 displaying maximal (+4) DHA-3 β -HSD activity in theca interna cells of A. F. and in I. T.; strong (+3) to maximal activity in C. L., and weakly positive (+1) activity in granulosa cells of A. F.

Fig. 201. An ovary removed on day 15 depicting strong DHA-3 β -HSD activity localized in I. T. and C. L.; moderate activity in patches of T. I. of T. F.; and weakly positive activity in G. of T. F. The antrum (A.) is typically negative.

Fig. 202. Another portion of the ovary described in fig. 201 showing strong (+3) diformazan deposits in theca interna cells of A. F. and in I. T.; monoformazan material (+) in granulosa cells of A. F.

Fig. 203. An ovary removed on day 18 depicting maximal (+4) DHA-3 β -HSD activity in I. T.; strong (+3) activity in T. I. of T. F. as well as in C. L.; weakly positive (+1) activity in G. of T. F.

Fig. 204. Another area of the ovary described in fig. 203 showing maximal (+4) diformazan intensities in thecal cells of A. F. as well as in I. T.; strong (+3) intensities in T. I. of T. F.; weakly positive (+1) to moderate (+2) intensities in G. of T. F.; and trace (+) intensities in G. of A. F.

PLATE XXIX

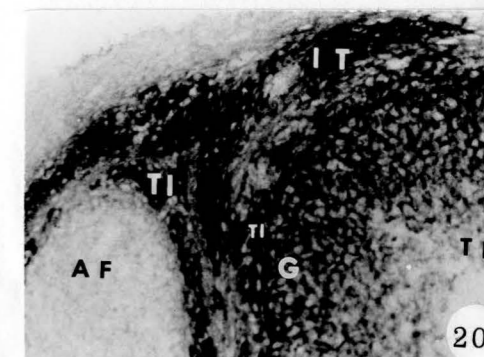
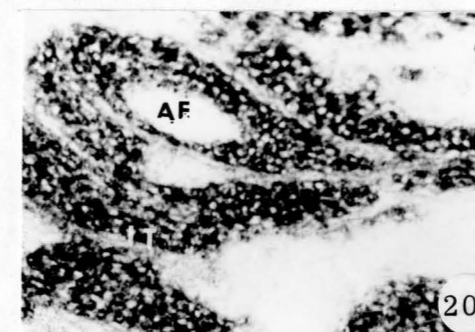
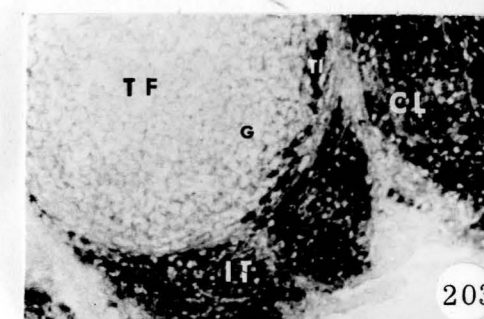
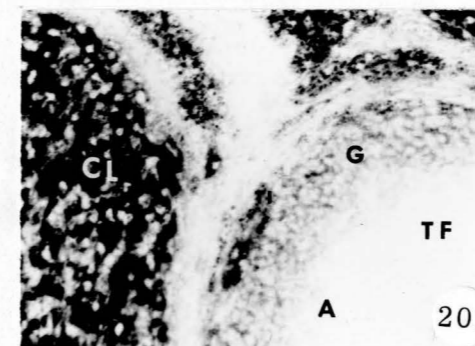
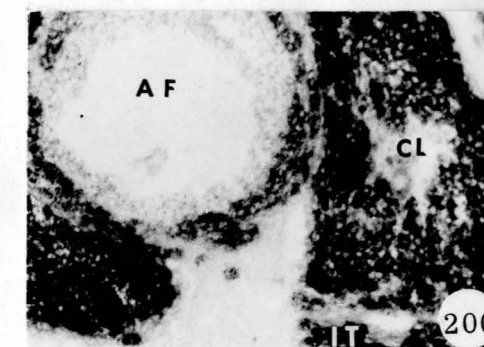
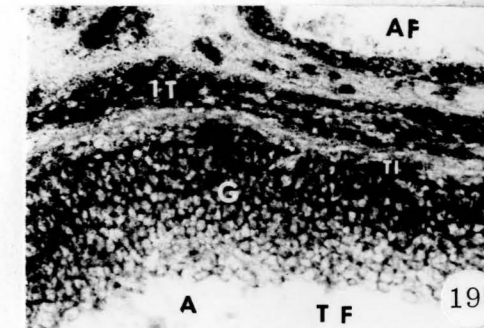


PLATE XXX

Fig. 205. An ovary removed on day 20 showing strong (+3) DHA-3 β -HSD activity in the theca interna of A. F. as well as in I. T.; moderate (+2) activity in thecal cells of S. F.; and weakly positive (+1) activity in granulosa cells of S. F. and A. F.

Fig. 206. Another region of the ovary described in fig. 205 depicting variable intensities of diformazan deposition in C. L. ranging from moderate to strong; strong intensity in T. I. of T. F. as well as in I. T.; and moderate intensity in G. of T. F. The antrum (A.) is negative.

Fig. 207. An ovary removed on day 21 of prolonged pseudopregnancy showing moderate to strong DHA-3 β -HSD activity in T. I. of T. F.; moderate (+2) activity in thecal cells of A. F. as well as in I. T. and G. of T. F.; weakly positive (+1) in granulosa cells of A. F. The antrum (A.) is typically negative.

Fig. 208. Another region of the ovary described in fig. 207, depicting moderate intensities of diformazan material in theca interna cells of A. F., in I. T. and in C. L., and weakly positive intensities in granulosa cells of A. F.

Fig. 209. Another area of the ovary shown in fig. 207 displaying moderate DHA-3 β -HSD activity in C. L., weakly positive activity in theca interna of S. F.; and trace activity in granulosa cells of S. F.

PLATE XXX

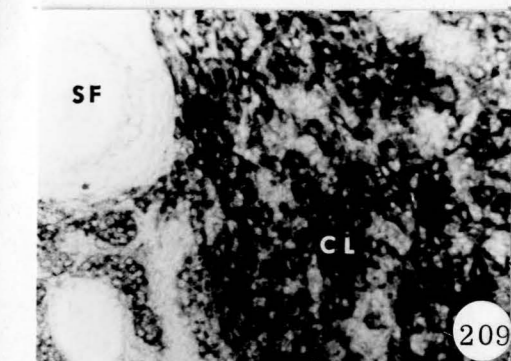
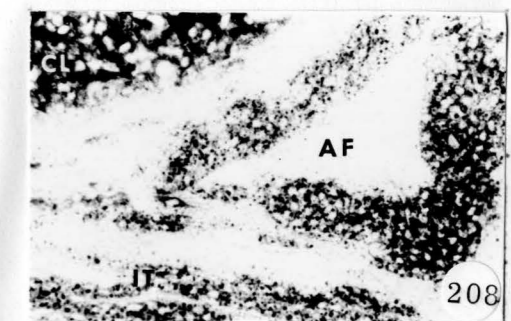
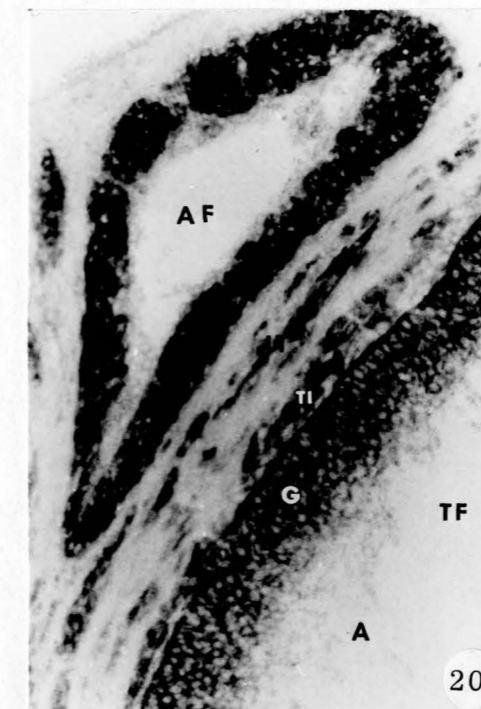
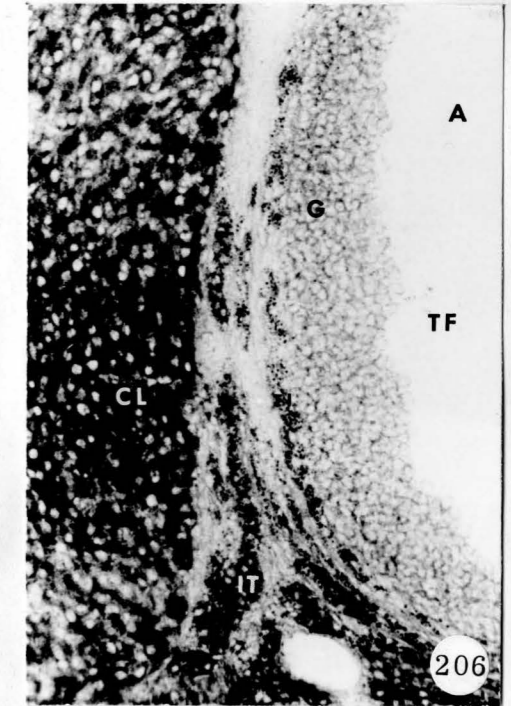
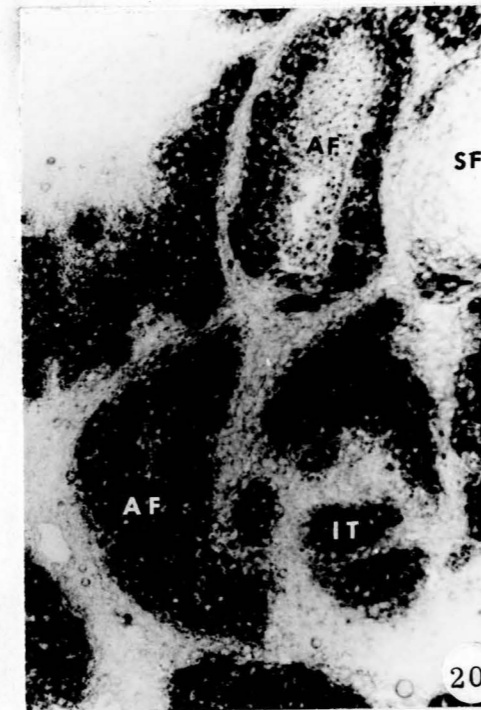


PLATE XXXI

Plate XXXI is comprised of high-powered photomicrographs of DHA- 3β -HSD appearing as diformazan deposits in the ovaries of the prolonged pseudopregnant series of animals. The left column consists of examples of photomicrographs depicting lowest observable intensities while the right column consists of exhibits of highest intensities in similar ovarian components; e. g. lowest P.F. (\pm) fig. 210, highest P.F. (+1) in fig. 216. All figures which comprise this plate have a final magnification of 875x.

Fig. 210. Primary follicle (P.F.) - lowest DHA- 3β -HSD activity in ovary of rat on days 6, 18 (exhibited) and 21; cf. fig. 216, highest activity observed on days 8 (exhibited), 10, 13, 15, and 20, increasing from trace (\pm) to weakly positive (+1).

Fig. 211. Secondary follicle (S.F.) - lowest DHA- 3β -HSD activity in ovary of rat on days 18 (exhibited) and 21; cf. fig. 217, highest activity observed on days 10 (exhibited), 13, 15, and 20, increasing from trace (\pm) to weakly positive (+1) in G. and weakly positive to moderate (+2) in T. I.

Fig. 212. Tertiary follicle (T.F.) - lowest DHA- 3β -HSD activity in ovary of rat on days 6, 13 and 15 (shown); cf. fig. 218, highest activity observed on days 20 and 21 (exhibited), increasing from weakly positive (+1) to moderate (+2) in G. and moderate to strong (+3) in T. I.

Fig. 213. Atretic follicle (A.F.) - lowest DHA- 3β -HSD activity in ovary of rat on day 21; cf. fig. 219, highest activity observed on days 6 (exhibited), 10 and 13; both G. are weakly positive (+1) whereas T. I. increases from moderate (+2) to maximal (+4).

PLATE XXXI - Cont'd.

- Fig. 214. Interstitial tissue (I. T.) - lowest DHA-3 β -HSD activity in ovary of rat on day 21; cf. fig. 220, highest activity observed on days 6 (exhibited), 10, 13, and 18, increasing from moderate to maximal (+4).
- Fig. 215. Corpus luteum (C. L.) - lowest DHA-3 β -HSD activity in ovary of rat on days 20 and 21 (exhibited); cf. fig. 221, highest activity observed on day 6, increasing from moderate to maximal.
- Fig. 216. Primary follicle (P. F.) - highest DHA-3 β -HSD activity (+1) on days 8 (shown), 10, 13, 15, and 20; cf. fig. 210, lowest on days 6, 18 (shown) and 21.
- Fig. 217. Secondary follicle (S. F.) - highest DHA-3 β -HSD activity (G. - +1, T. I. - +2) on days 10 (shown), 13, 15 and 20; cf. fig. 211, lowest on days 18 (shown) and 21.
- Fig. 218. Tertiary follicle (T. F.) - highest DHA-3 β -HSD activity (G. - +2, T. I. - +3) on days 20 and 21 (exhibited); cf. fig. 212, lowest on days 6, 13 and 15 (exhibited).
- Fig. 219. Atretic follicle (A. F.) - highest DHA-3 β -HSD activity (G. - +1, T. I. - +4) on days 6 (shown), 10 and 13; cf. fig. 213, lowest on day 21.
- Fig. 220. Interstitial tissue (I. T.) - highest DHA-3 β -HSD activity (+4) on days 6 (shown), 10, 13, and 18; cf. fig. 214, lowest on day 21.
- Fig. 221. Corpus luteum (C. L.) - highest DHA-3 β -HSD activity (+4) on day 6; cf. fig. 215, lowest on days 20 and 21 (shown).

PLATE XXXI

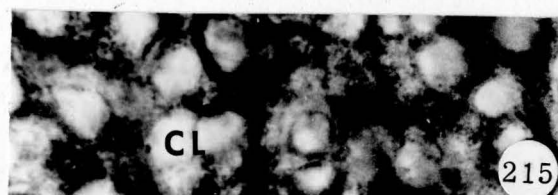
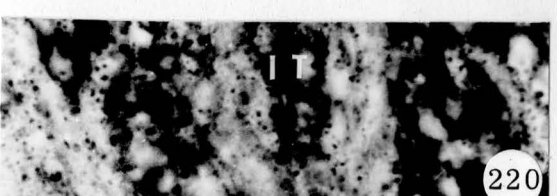
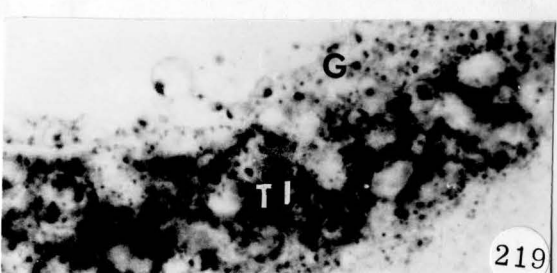
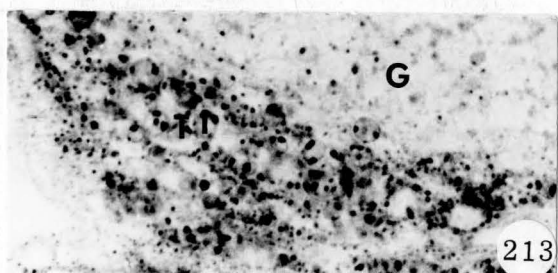
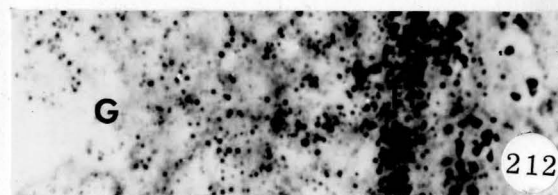
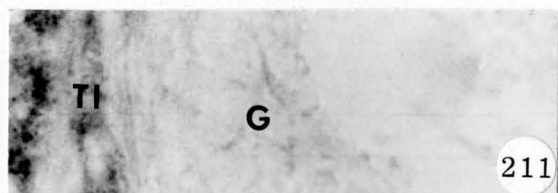
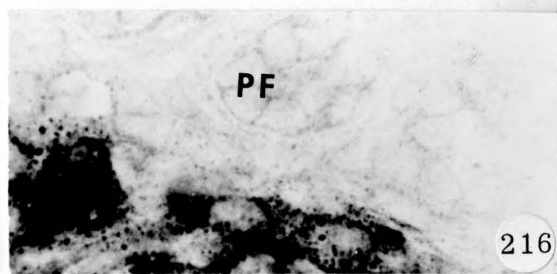
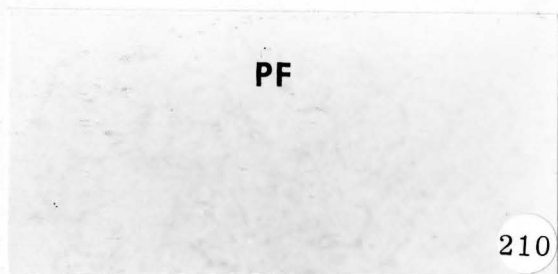


PLATE XXXII

Plates XXXII through XXXVI are comprised of photomicrographs of sections taken from ovaries removed during pregnancy which were incubated with pregnenolone (P-one) to localize the sites of biosynthesis of progesterone. Figures 222 - 242 have a final magnification of 175x.

PLATE XXXII

Fig. 222. An ovary removed on day 6 showing maximal (+4) P-one- 3β -HSD activity localized in the theca interna of A.F. and in I.T.; moderate (+2) intensities in theca interna of S.F.; and weakly positive (+1) intensities in granulosa cells of S.F. and A.F.

Fig. 223. Another section of ovary described in fig. 222 showing maximal (+4) intensities of diformazan in I.T.; strong (+3) activity in C.L. and T.I. of T.F.; moderate (+2) activity in T.I. of S.F.; weakly positive to moderate activity in G. of T.F.; weakly positive (+1) activity in P.F. and G. of S.F. The antrum (A.) of T.F. is typically negative.

Fig. 224. An ovary removed on day 8 and showing strong (+3) P-one- 3β -HSD activity in theca interna of T.F. and A.F., as well as in I.T.; C.L. appears to vary between moderate and strong activity; moderate (+2) activity in G. of T.F.; weakly positive (+1) activity in G. of S.F. and A.F. as well as in T.I. of S.F. The ovum (O.) and antrum (A.) are negative.

Fig. 225. A corpus luteum observed in another area of the ovary described in fig. 224. Strong (+3) P-one- 3β -HSD activity is localized in the luteal cells.

PLATE XXXII

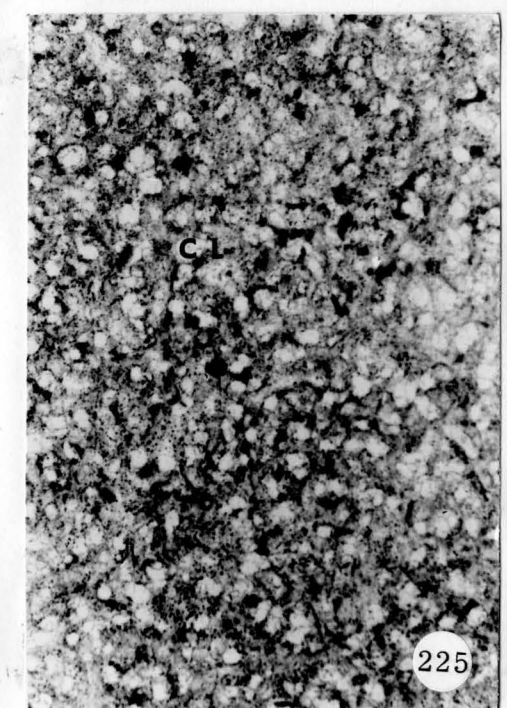
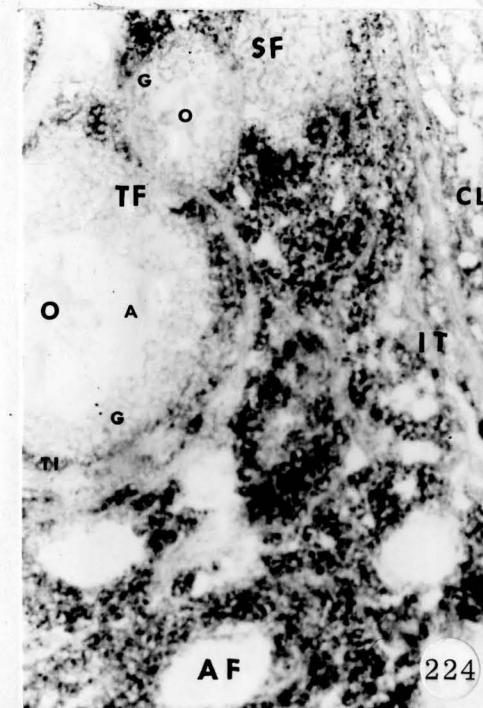
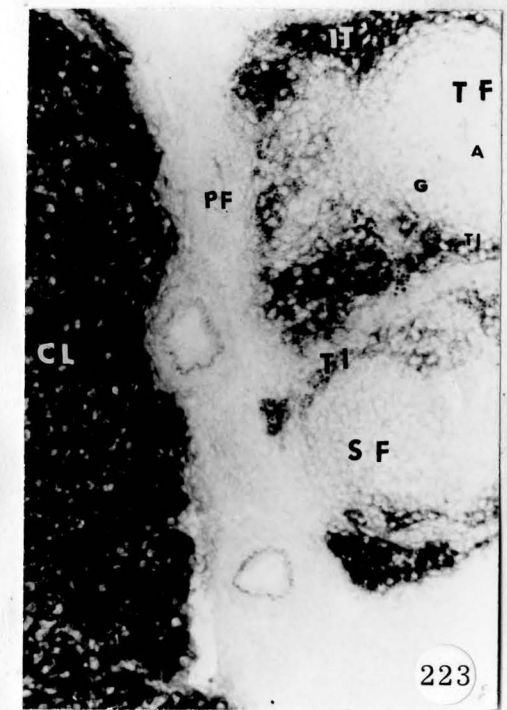
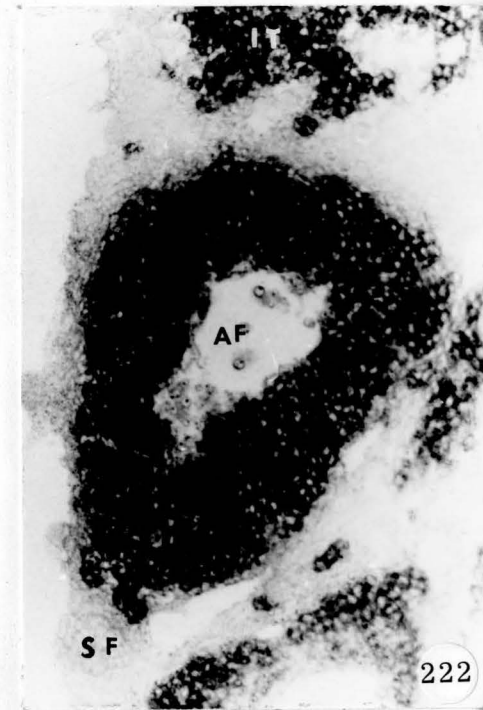


PLATE XXXIII

Fig. 226. A section of an ovary removed on day 10 and incubated with P-one showing strong (+3) intensities of diformazan in C.L. and I.T.; T.I. of T.F. varies between moderate to strong intensities; weakly positive to moderate intensities in G. of T.F.: The antrum (A.) is negative.

Fig. 227. Another region of the ovary described in fig. 226 and incubated with P-one depicting strong P-one- 3β -HSD activity localized in the theca interna of A.F.; T.I. of S.F. and granulosa cells of A.F. weakly positive activity; G. of S.F. as well as P.F. show trace (\pm) activity.

Fig. 228. A corpus luteum appearing in another area of the ovary described in fig. 226. Note strong (+3) P-one- 3β -HSD activity in C.L. cells.

Fig. 229. A section of an ovary removed on day 13 of pregnancy depicting strong (+3) P-one- 3β -HSD deposition in T.I. of A.F. as well as in I.T.; moderate (+2) in T.I. of S.F. and T.F.; weakly positive (+1) in G. of A.F. and T.F.; and G. of S.F. appears to vary between trace (\pm) to weakly positive.

Fig. 230. Another area of the ovary described in fig. 229 and showing strong (+3) P-one- 3β -HSD activity in C.L.

PLATE XXXIII

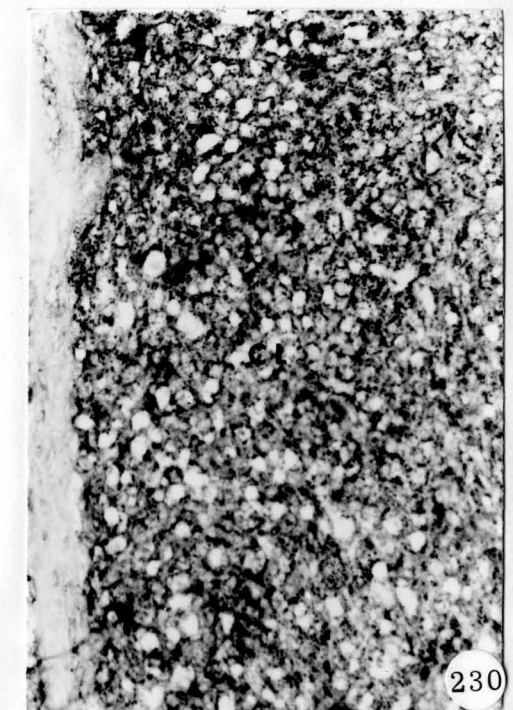
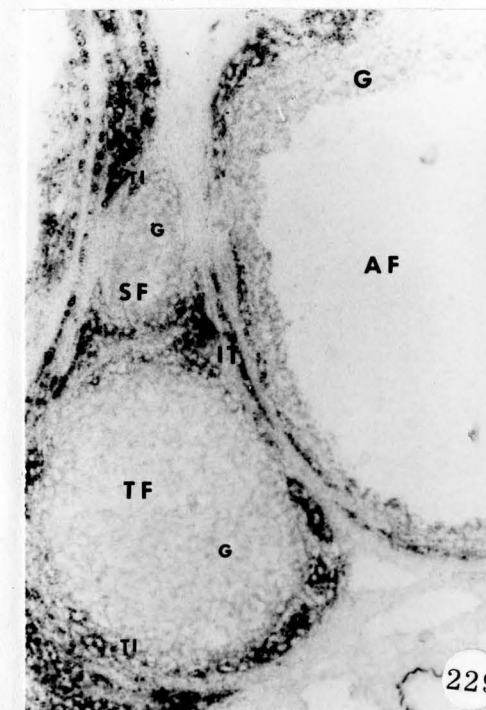
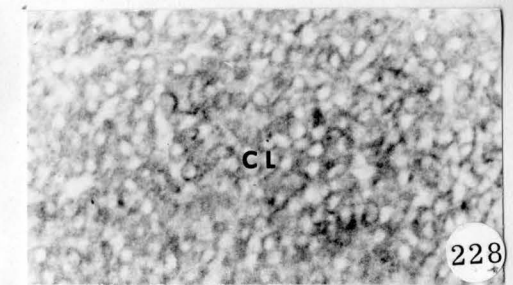
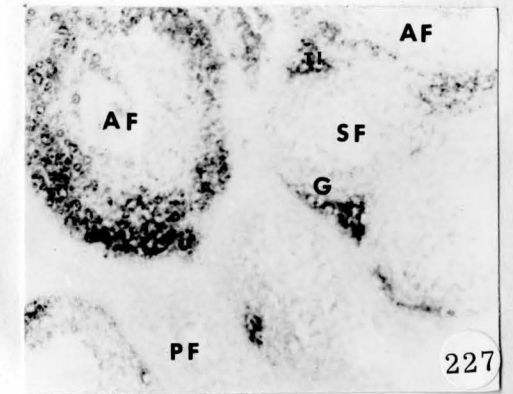


PLATE XXXIV

Fig. 231. A section of an ovary removed on day 14 of pregnancy and incubated with P-one depicting variable maximal (+4) to strong (+3) P-one- 3β -HSD activity in C.L. ; strong (+3) activity in theca interna of A.F. as well as in I. T. ; moderate (+2) activity in T.I. of T.F. ; weakly positive in G. of T.F. and A.F. The antrum (A.) is typically negative.

Fig. 232. Maximal (+4) P-one- 3β -HSD activity in a C.L. of the ovary described in fig. 231.

Fig. 233. A section of an ovary removed on day 15 depicting strong to moderate P-one- 3β -HSD activity in I. T. ; moderate activity in T.I. of S.F. and T.F. ; varying activity, trace (+) to moderate activity in G. of S.F. and T.F. The antrum (A.) is typically negative.

Fig. 234. Another region of the ovary described in fig. 233 and showing maximal (+4) intensity of diformazan in theca interna of A.F. ; strong (+3) intensity in C.L. and I. T. ; weakly positive (+1) intensity in granulosa cells of A.F.

PLATE XXXIV

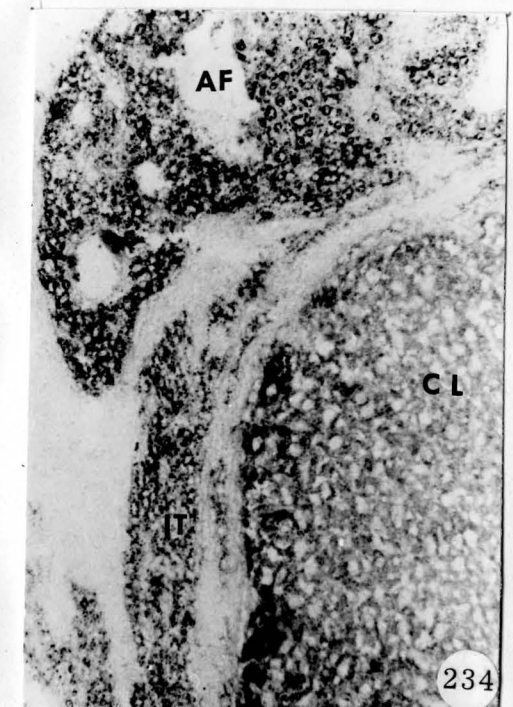
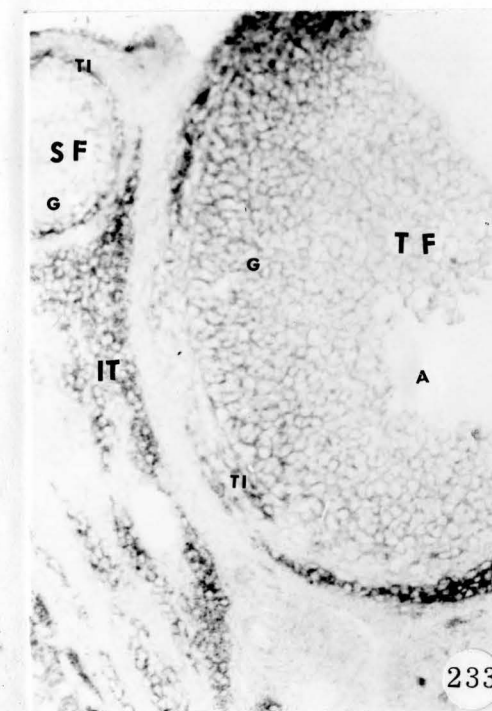
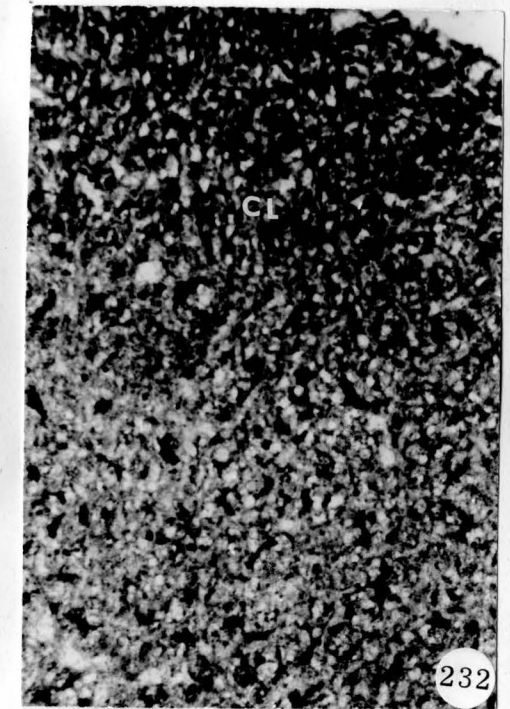
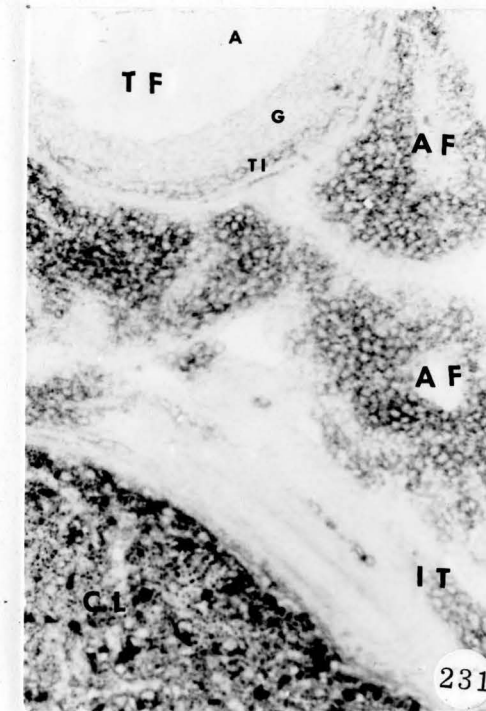


Fig. 235. A section of an ovary removed on day 18 of pregnancy and incubated with P-one depicting strong (+3) intensities of diformazan in theca interna of A.F. as well as in I.T. and C.L.; moderate (+2) intensities in T.I. of S.F.; weakly positive (+1) intensities in G. of S.F.; trace (monoformazan) intensities in granulosa cells of A.F. The ovum (O.) of S.F. is typically negative.

Fig. 236. Another region of the ovary described in fig. 235 and showing strong P-one- 3β -HSD activity in I.T.; moderate activity in T.I. of T.F.; trace (\pm) activity in G. of T.F. The antrum (A.) is negative.

Fig. 237. Strong P-one- 3β -HSD activity in a C.L. of the ovary described in fig. 235.

Fig. 238. A section of an ovary removed on day 20, showing moderate (+2) P-one- 3β -HSD activity in T.I. of A.F. and T.F. as well as in I.T.; weakly positive activity in T.I. of S.F.; trace (\pm) activity in G. of S.F., T.F. and A.F. The antra (A.) are negative.

Fig. 239. Another region of the ovary described in fig. 238 depicting moderate P-one- 3β -HSD activity in patches of T.F.; trace to weakly positive activity in G. of T.F. The ovum (O.) and antrum (A.) are typically negative.

Fig. 240. A corpus luteum in another area of the ovary shown in fig. 238 incubated with P-one depicting moderate intensity of diformazan deposition.

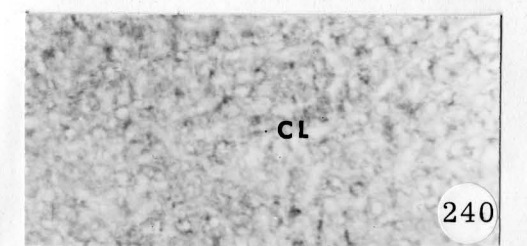
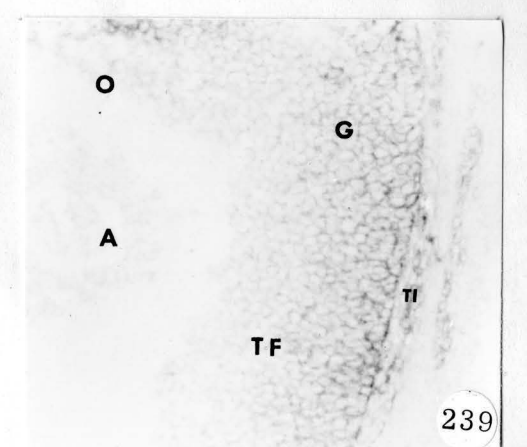
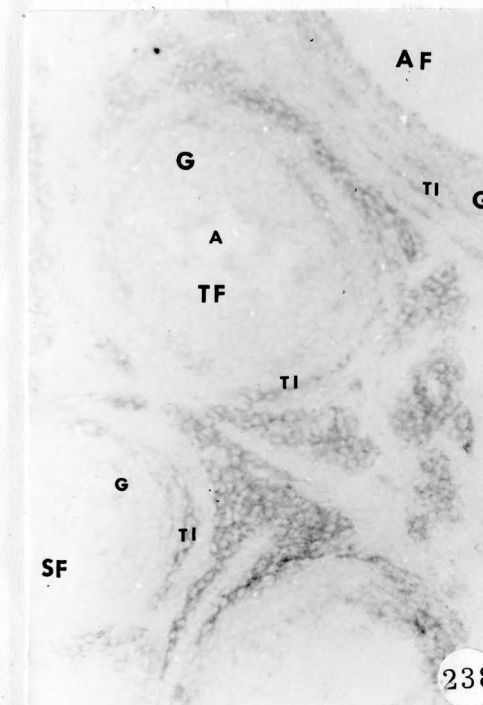
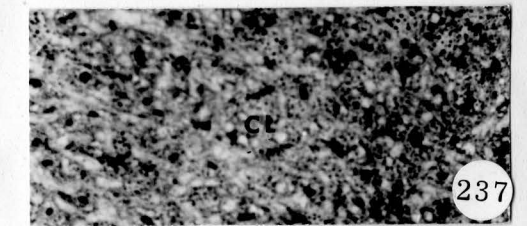
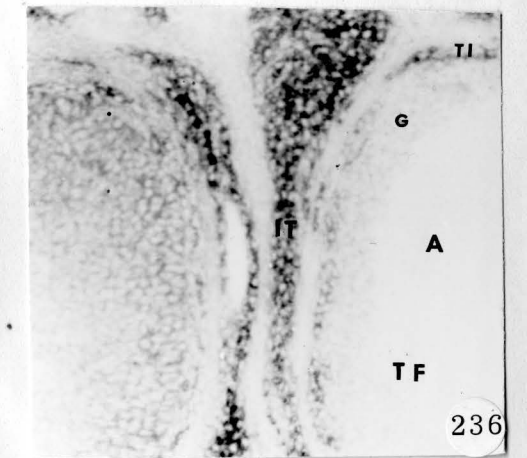
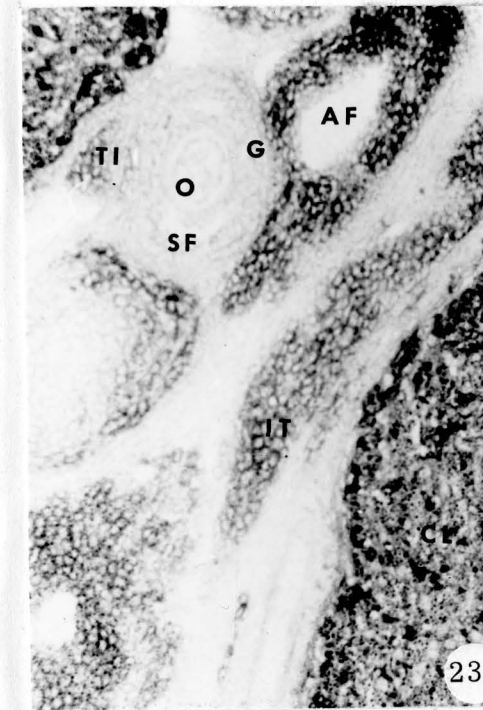


PLATE XXXVI

Fig. 241. A section of an ovary removed on day 21 of pregnancy showing moderate to strong P-one- 3β -HSD activity in T.I. of T.F.; moderate activity in T.I. of A.F. and S.F. as well as in I.T.; weakly positive activity in G. of S.F. and T.F.; trace (monoformazan) activity in granulosa cells of A.F. The antrum (A.) is typically negative.

Fig. 242. Weakly positive intensities of diformazan deposits in a corpus luteum incubated with P-one and located in another region of the ovary described in fig. 241.

PLATE XXXVI

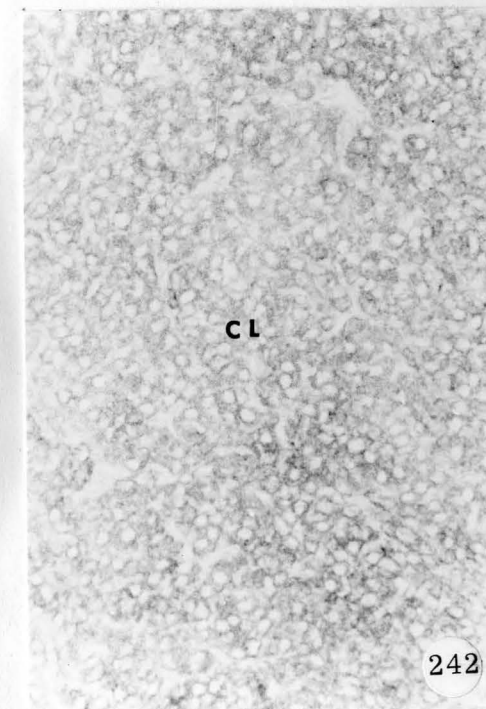
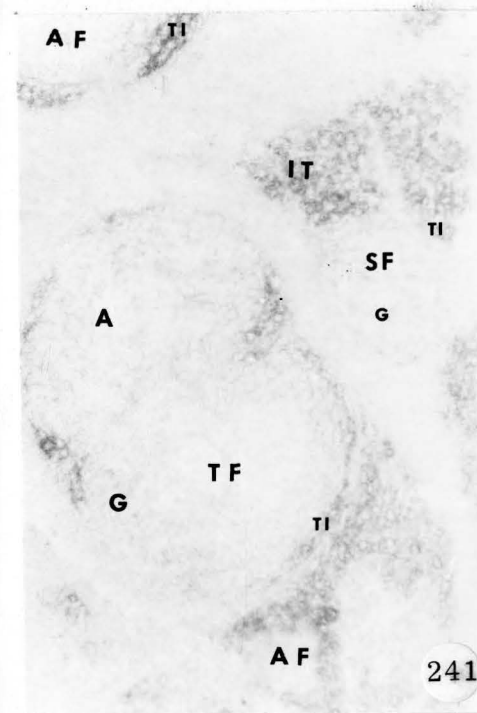


PLATE XXXVII

Plate XXXVII is comprised of high-powered photomicrographs of P-one- 3β -HSD appearing as diformazan deposits in the ovaries of pregnant rats. The left column consists of examples of photomicrographs depicting lowest observable intensities while the right column consists of exhibits of highest intensities in similar ovarian components, e. g. lowest P.F. (\pm) in fig. 243, highest P.F. (+1) in fig. 249. All figures which comprise this plate have a final magnification of 875x.

- Fig. 243. Primary follicle (P.F.) - lowest P-one- 3β -HSD activity in ovary of rat on days 8, 10, 13, 14, 18, and 21 (exhibited); cf. fig. 249, highest activity observed on days 6 (exhibited), 15 and 20, increasing from trace (\pm) to weakly positive (+1).
- Fig. 244. Secondary follicle (S.F.) - lowest P-one- 3β -HSD activity in ovary of rat on days 10 (exhibited) and 18; cf. fig. 250, highest activity observed on days 6, 13, 14 (exhibited), 15, 20, and 21 increasing from trace (\pm) to weakly positive (+1) in G. and weakly positive (+1) to moderate (+2) in T. I.
- Fig. 245. Tertiary follicle (T.F.) - lowest P-one- 3β -HSD activity in ovary of rat on days 18 (exhibited) and 20; cf. fig. 251, highest activity observed on days 6 (exhibited) and 8, increasing from trace (\pm) to moderate (+2) in G. and moderate (+2) to strong (+3) in T. I.
- Fig. 246. Atretic follicle (A.F.) - lowest P-one- 3β -HSD activity in ovary of rat on days 20 and 21 (exhibited) cf. fig. 252, highest activity observed on days 6 (exhibited) and 15, increasing from trace (\pm) to weakly positive (+1) in G. and moderate (+2) to maximal (+4) in T. I.

PLATE XXXVII - Cont'd.

- Fig. 247. Interstitial tissue (I. T.) - lowest observable P-one- 3β -HSD activity in ovary of rat on days 20 and 21 (exhibited); cf. fig. 253; highest activity observed on day 6, increasing from moderate (+2) to maximal (+4).
- Fig. 248. Corpus luteum (C.L.) - lowest observable P-one- 3β -HSD activity in ovary of rat on day 21; cf. fig. 254, highest activity observed on day 14, increasing from weakly positive (+1) to maximal (+4).
- Fig. 249. Primary follicle (P.F.) - highest P-one- 3β -HSD activity (+1) on days 6 (exhibited), 15 and 20; cf. fig. 243, lowest on days 8, 10, 13, 14, 18, and 21 (exhibited).
- Fig. 250. Secondary follicle (S.F.) - highest P-one- 3β -HSD activity (G. - +1; T.I. - +2) on days 6, 13, 14 (exhibited), 15, 20, and 21; cf. fig. 244, lowest on days 10 (exhibited) and 18.
- Fig. 251. Tertiary follicle (T.F.) - highest P-one- 3β -HSD activity (G. - +2; T.I. - +3) on days 6 (exhibited) and 8; cf. fig. 245, lowest on days 18 (exhibited) and 20.
- Fig. 252. Atretic follicle (A.F.) - highest P-one- 3β -HSD activity (G. - +1; T.I. - +4) on days 6 (exhibited) and 15; cf. fig. 246, lowest on days 20 and 21 (exhibited).
- Fig. 253. Interstitial tissue (I. T.) - highest P-one- 3β -HSD activity (+4) on day 6; cf. fig. 247, lowest on days 20 and 21 (exhibited).
- Fig. 254. Corpus luteum (C.L.) - highest P-one- 3β -HSD activity (+4) on day 14; cf. fig. 248, lowest on day 21.

PLATE XXXVII

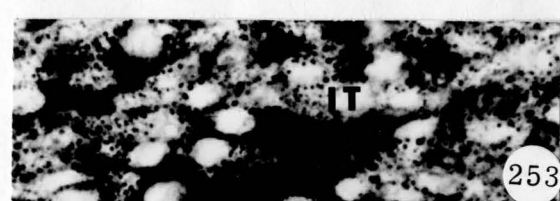
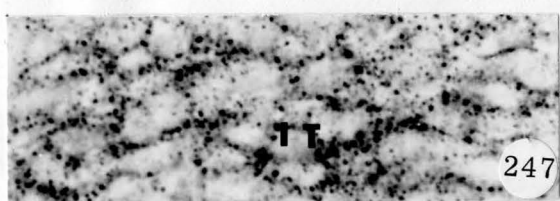
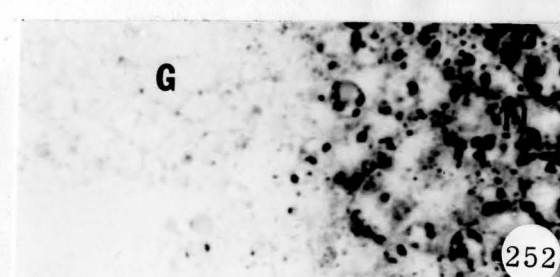
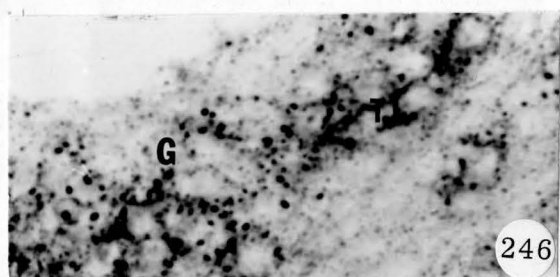
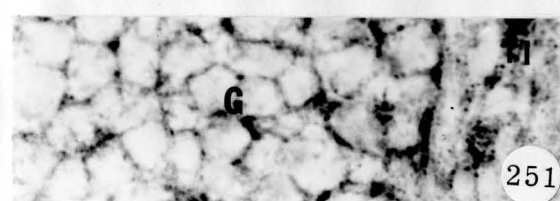
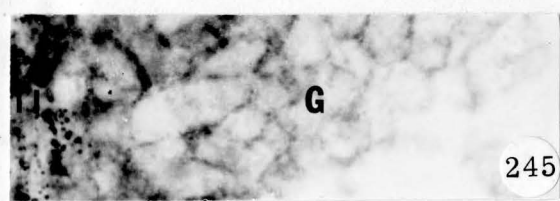
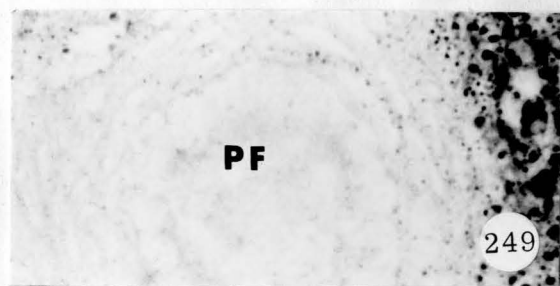
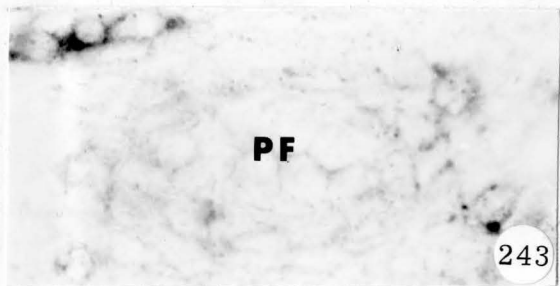


PLATE XXXVIII

Plates XXXVIII through XXXXII are comprised of photomicrographs of sections taken from ovaries removed during pregnancy and incubated with dehydroepiandrosterone (DHA) to localize the sites of biosynthesis of estrogens. Figures 255-277 have a final magnification of 175x.

PLATE XXXVIII

Fig. 255. Section of an ovary removed on day 6 of pregnancy and incubated with DHA depicting maximal (+4) intensities of diformazan deposits in I. T. and in theca interna of A. F.; strong (+3) intensities in patches of T. I. in S. F.; moderate (+2) intensities in patches of T. I. of T. F.; weakly positive (+1) intensities in G. of S. F., T. F. and A. F. The antrum (A.) is negative.

Fig. 256. Another region of the ovary described in fig. 255 showing maximal (+4) DHA- β -HSD activity localized in I. T. as well as in C. L.

Fig. 257. A section of an ovary removed on day 8 showing maximal DHA- β -HSD activity in C. L. and strong activity in I. T.

Fig. 258. Another section of the ovary described in fig. 257 and incubated with DHA depicting strong (+3) intensities of diformazan deposits in T. I. of T. F. as well as I. T.; moderate to strong intensities in G. of T. F. The antrum (A.) is typically negative.

Fig. 259. Another area of the ovary in fig. 257 showing strong DHA- β -HSD activity in G. of A. F. and S. F.; trace (\pm) intensities in P. F.

PLATE XXXVIII

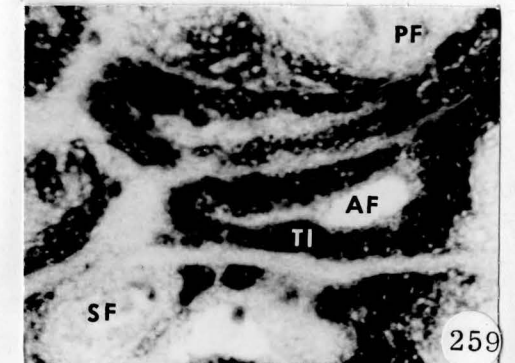
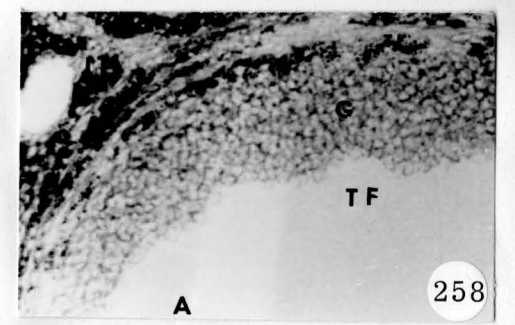
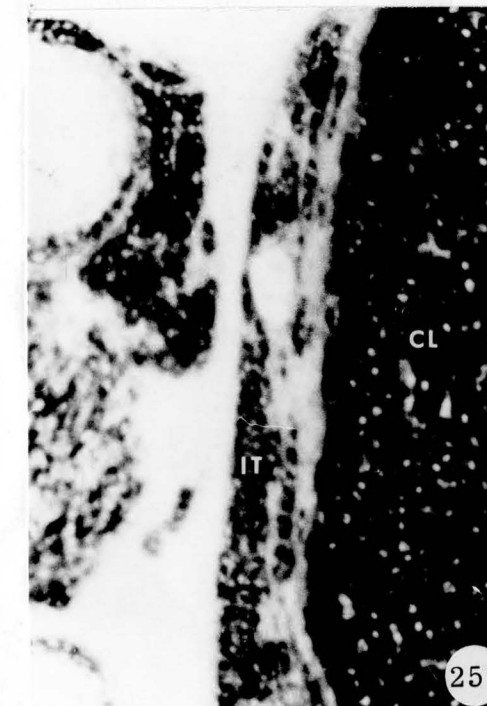
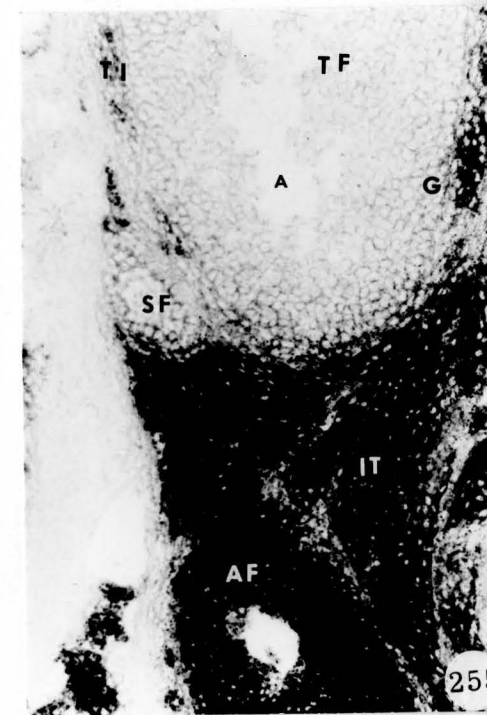


Fig. 260. A section of an ovary removed on day 10 showing strong (+3) DHA-3 β -HSD activity in T.I. of T.F. and A.F. as well as in I.T.; moderate (+2) activity in G. of T.F.; weakly positive (+1) activity in granulosa cells of A.F. The antrum (A.) is negative.

Fig. 261. Another region of the ovary described in fig. 260 depicting strong-maximal intensities of DHA-3 β -HSD activity in C.L., I.T. and T.I. of S.F. and A.F.; weakly positive intensities in G. of S.F. and A.F.

Fig. 262. Section of an ovary removed on day 13 and incubated with DHA showing strong (+3) to maximal (+4) intensities of diformazan in C.L.; strong intensities in theca interna of A.F. as well as in I.T.; moderate (+2) intensities in T.I. of T.F.; weakly positive (+1) intensities in granulosa cells (G.) of A.F. and T.F. The antrum (A.) is typically negative.

Fig. 263. Another region of the ovary described in fig. 262 depicting strong (+3) DHA-3 β -HSD activity in I.T. as well as in theca interna of A.F.; moderate (+2) activity in T.I. of T.F.; trace (\pm) activity in granulosa cells (G.) of A.F. and T.F. The ovum (O.) and antrum (A.) are negative.

Fig. 264. Another area of the ovary shown in fig. 262 depicting strong DHA-3 β -HSD activity in theca interna of A.F. and weakly positive activity in granulosa cells of A.F.

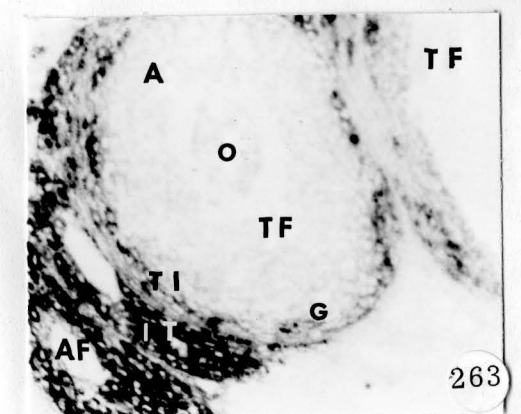
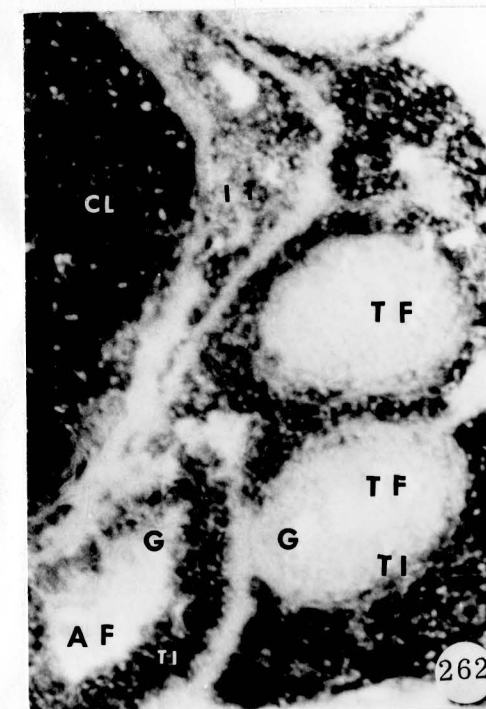
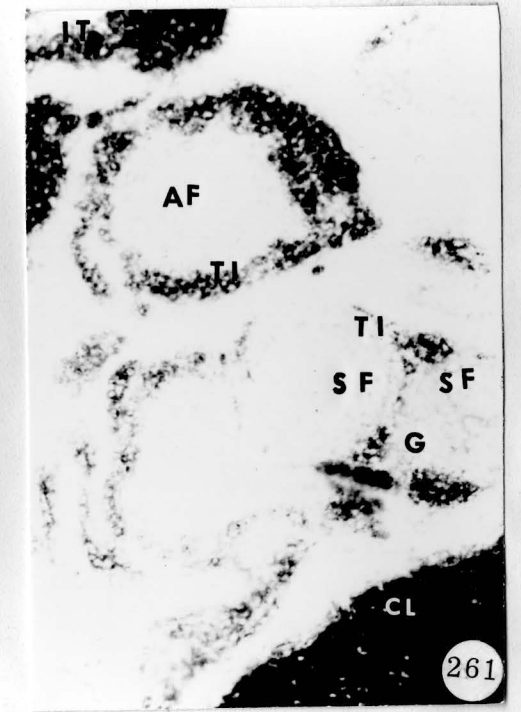
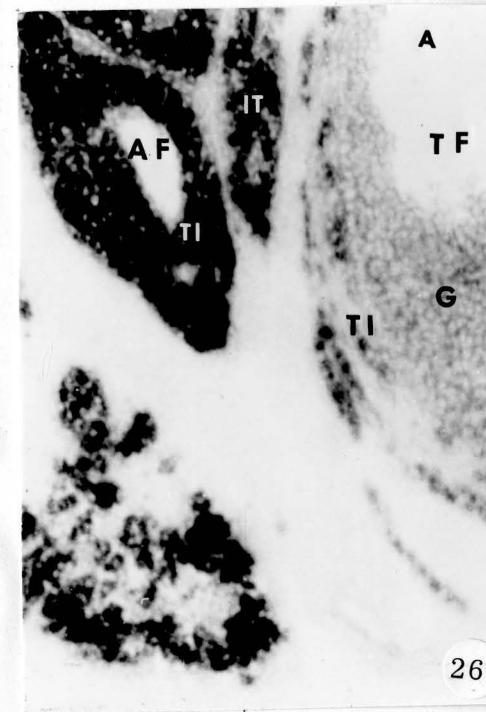


PLATE XXXX

- Fig. 265. Section of an ovary removed on day 14 of pregnancy and incubated with DHA showing maximal (+4) intensities of diformazan deposits in C.L.; strong (+3) intensities in theca interna of A.F. as well as I.T.; weakly positive intensities in granulosa cells of A.F.
- Fig. 266. Another region of the ovary described in fig. 265 depicting strong DHA-3 β -HSD activity in T.I. of S.F. and T.F. as well as in I.T.; moderate activity in G. of T.F.; weakly positive activity in granulosa cells of S.F. The antrum (A.) is typically negative.
- Fig. 267. A corpus luteum showing maximal (+4) DHA-3 β -HSD activity in another area of the ovary described in fig. 265.
- Fig. 268. A section of a tertiary follicle (T.F.) observed in an ovary removed on day 15 of pregnancy and incubated with DHA. Strong (+3) intensities of diformazan in T.I. and moderate (+2) intensities in G. The ovum (O.) and antrum (A.) are both negative.
- Fig. 269. Another region of the ovary described in fig. 268 showing strong DHA-3 β -HSD activity in T.I. of T.F. and A.F.; moderate (+2) activity in G. of T.F. as well as in theca interna of S.F.; weakly positive (+1) activity in granulosa cells of S.F.; trace (\pm) activity in granulosa cells of A.F. The antrum (A.) is negative.
- Fig. 270. Another area of the ovary depicted in fig. 268 and incubated with DHA. Maximal (+4) intensities of diformazan deposits in C.L.; strong (+3) intensities in I.T.

PLATE XXXX

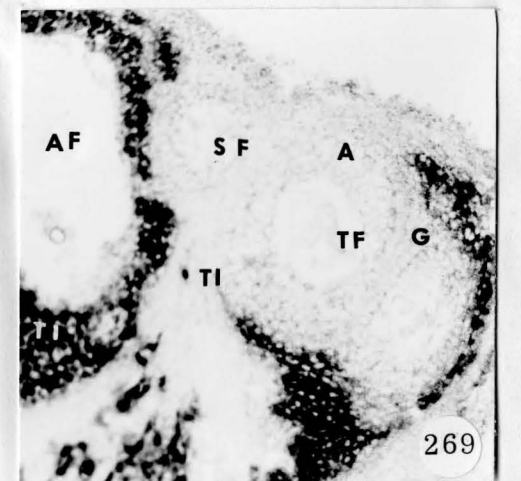
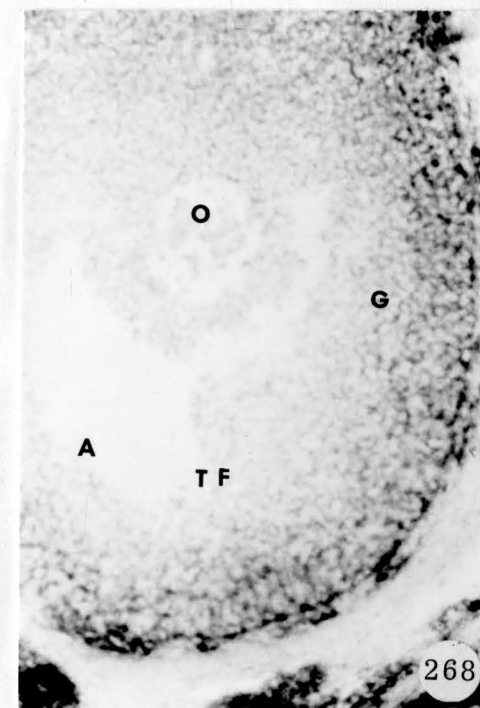
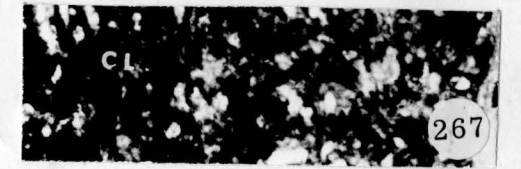
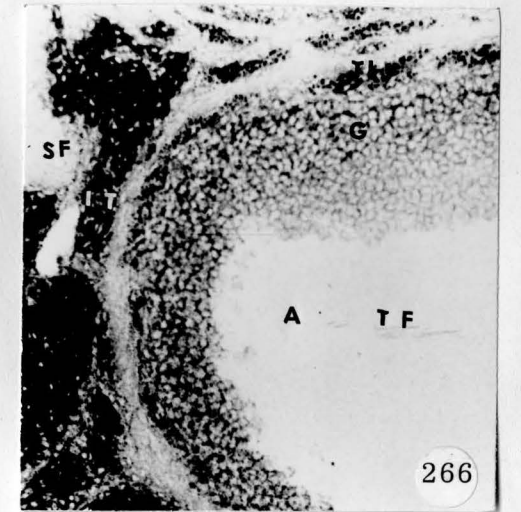
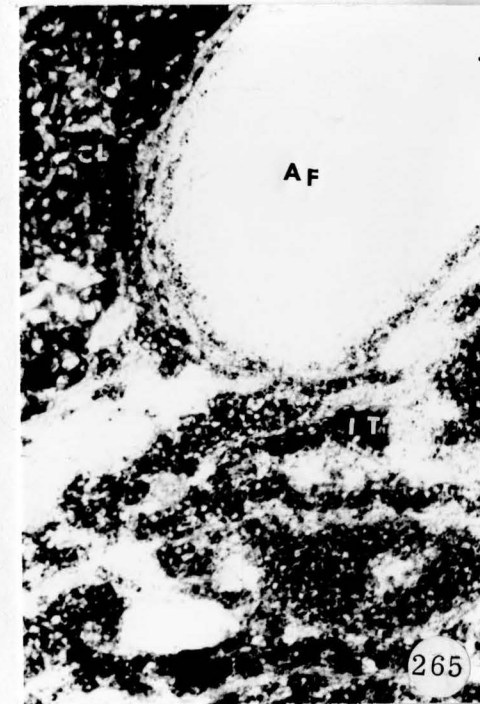


PLATE XXXXI

Fig. 271. Section of an ovary removed on day 18 and incubated with DHA showing maximal (+4) intensity of diformazan in C.L.; strong (+3) intensity in theca interna (T.I.) of A.F. and T.F. as well as in I.T.; moderate (+2) intensity in G. of T.F. as well as in theca interna of S.F.; weakly positive (+1) intensity in granulosa cells of A.F. and S.F. as well as in P.F. The antrum (A.) is typically negative.

Fig. 272. Another region of the ovary described in fig. 271 depicting maximal DHA-3 β -HSD activity in C.L.; strong activity in T.I. of T.F. and A.F. as well as in I.T.; moderate activity in G. of T.F.; weakly positive in granulosa cells of A.F.

Fig. 273. Section of an ovary removed on day 20 of pregnancy incubated with DHA showing strong (+3) intensity of diformazan in C.L.; moderate (+2) intensities in T.I. of T.F. as well as in I.T.; weakly positive intensities in granulosa cells and theca interna of S.F. and in G. of T.F. The antrum (A.) is negative.

Fig. 274. Another region of the ovary described in fig. 273 depicting strong (+3) DHA-3 β -HSD activity in C.L.; moderate to strong activity in theca interna of A.F.; trace (\pm) activity in P.F. and granulosa cells of A.F.

PLATE XXXXI

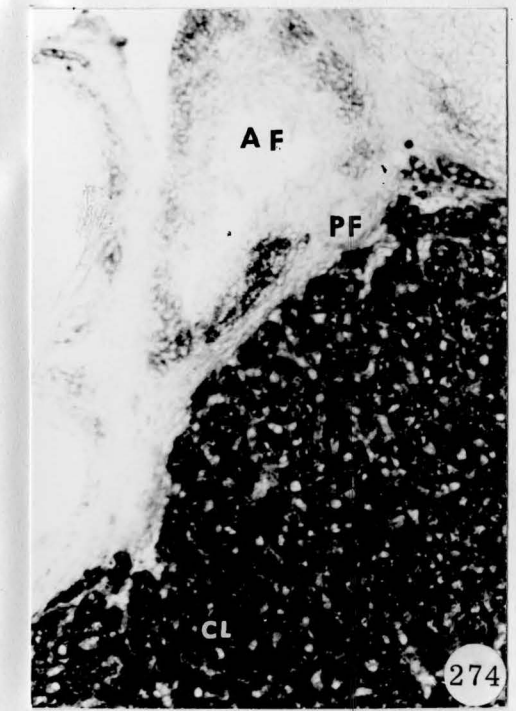
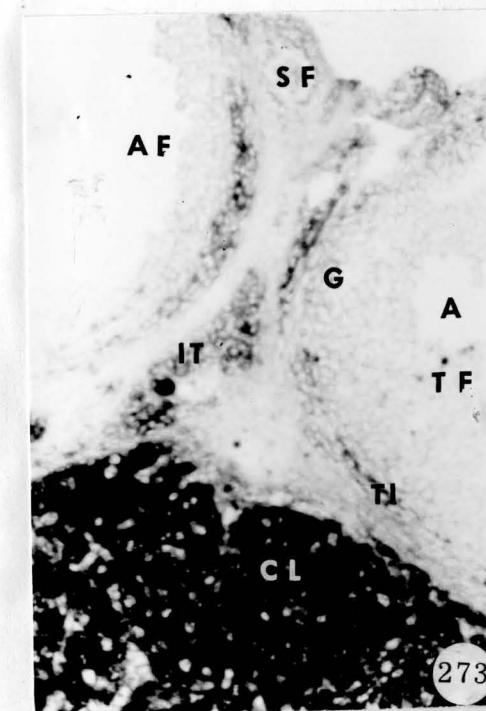
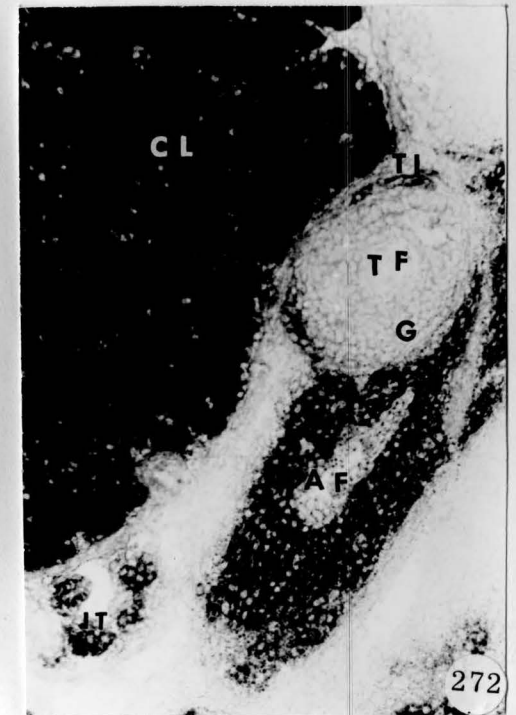


PLATE XXXXII

Fig. 275. Section of an ovary removed on day 21 of pregnancy, incubated with DHA showing strong to maximal intensities of diformazan in theca interna of A. F. ; moderate intensities in T. I. of T. F. ; weakly positive intensities in G. of A. F. and T. F. The antrum (A.) is typically negative.

Fig. 276. Another region of the ovary described in fig. 275 depicting strong to maximal DHA-3 β -HSD activity in theca interna of A. F. ; strong activity in I. T. ; moderate patches of activity in theca interna of S. F. ; weakly positive activity in granulosa cells of A. F. and S. F.

Fig. 277. Moderate intensities of diformazan deposited in C. L. cells in another area of the ovary described in fig. 275 and incubated with DHA.

PLATE XXXXII

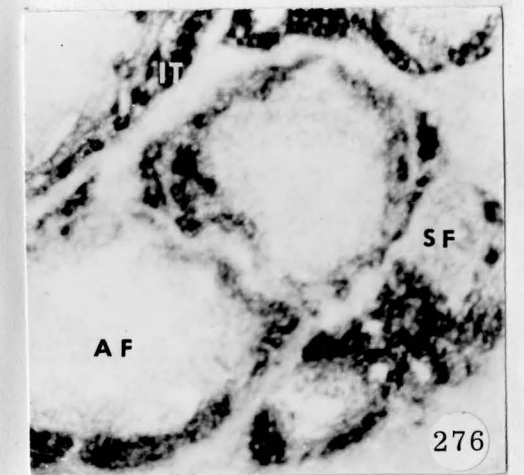
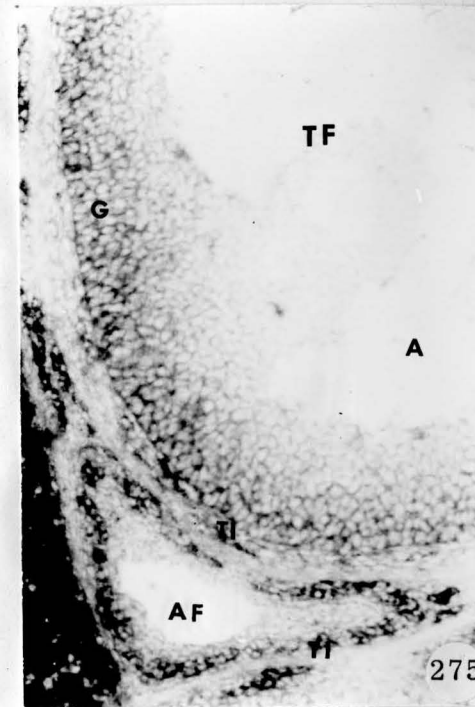


PLATE XXXXIII

Plate XXXXIII is comprised of high-powered photomicrographs of DHA-3 β -HSD appearing as diformazan deposits in the ovaries of pregnant rats. The left column consists of examples of photomicrographs depicting lowest observable intensities while the right column consists of exhibits of highest intensities in similar ovarian components, *e. g.* lowest P.F. trace (\pm) fig. 278, highest P.F. (+1) in fig. 284. All figures which comprise this plate have a final magnification of 875x.

Fig. 278. Primary follicle (P.F.) - lowest DHA-3 β -HSD activity in ovary of rat on days 8, 10, 13, and 20 (exhibited); cf. fig. 284, highest on days 6 (exhibited), 14, 15, 18, and 21, increasing from trace (\pm) to weakly positive (+1).

Fig. 279. Secondary follicles (S.F.) - lowest DHA-3 β -HSD activity in ovary of rat on day 20; cf. fig. 285, highest activity on days 6 (exhibited), 8, 10, and 14, increasing from weakly positive (+1) to strong (+3) in T.I., while G. remains weakly positive (+1).

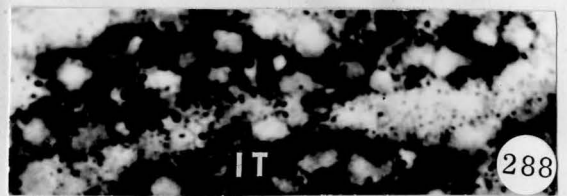
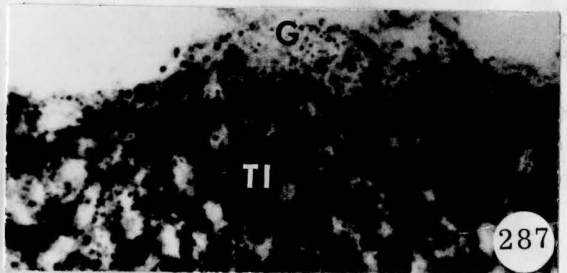
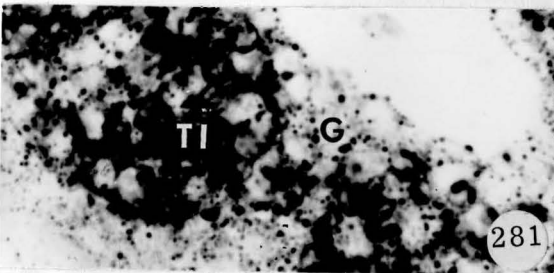
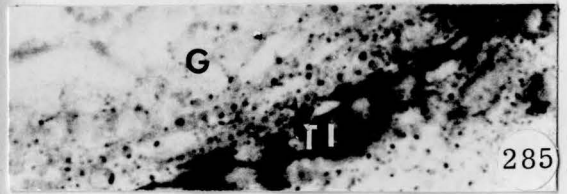
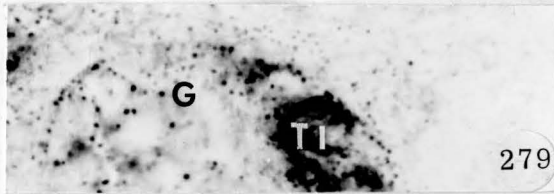
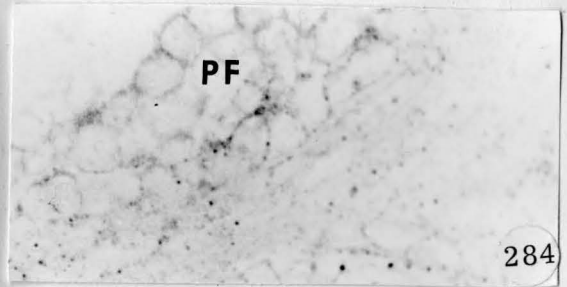
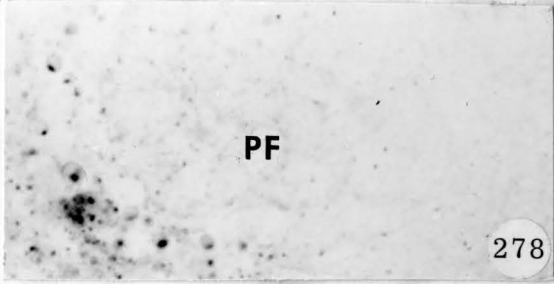
Fig. 280. Tertiary follicle (T.F.) - lowest DHA-3 β -HSD activity in ovary of rat on days 6 (exhibited), 13, 20, and 21; cf. fig. 286, highest activity on days 8, 10, 14, 15, and 18 (exhibited), increasing from weakly positive (+1) to moderate (+2) in G. and moderate to strong (+3) in T.I.

Fig. 281. Atretic follicle (A.F.) - lowest DHA-3 β -HSD activity in ovary of rat on day 20; cf. fig. 287, highest activity on days 6 (exhibited) and 21, increasing from trace (\pm) to weakly positive (+1) in G. and moderate (+2) to maximal (+4) in T.I.

PLATE XXXXIII - Cont'd.

- Fig. 282. Interstitial tissue (I. T.) - lowest DHA-3 β -HSD activity in ovary of rat on day 20; cf. fig. 288, highest activity on day 6, increasing from moderate (+2) to maximal (+4).
- Fig. 283. Corpus luteum (C. L.) - lowest DHA-3 β -HSD activity in ovary of rat on day 21; cf. fig. 289, highest activity on days 6 (exhibited), 8, 13, 14, 15, and 18, increasing from moderate (+2) to maximal (+4).
- Fig. 284. Primary follicle (P. F.) - highest DHA-3 β -HSD activity (+1) on days 6 (exhibited), 14, 15, 18, and 21; cf. fig. 278, lowest on days 8, 10, 13, and 20 (exhibited).
- Fig. 285. Secondary follicle (S. F.) - highest DHA-3 β -HSD activity (G. -+1; T.I. - +3) on days 6 (exhibited), 8, 10, and 14; cf. fig. 279, lowest on day 20.
- Fig. 286. Tertiary follicle (T. F.) - highest DHA-3 β -HSD activity (G. - +2; T.I. - +3) on days 8, 10, 14, 15, and 18 (exhibited); cf. fig. 280, lowest on days 6 (exhibited), 13, 20, and 21.
- Fig. 287. Atretic follicle (A. F.) - highest DHA-3 β -HSD activity (G. - +1; T.I. - +4) on days 6 (exhibited) and 21; cf. fig. 281, lowest on day 20.
- Fig. 288. Interstitial tissue (I. T.) - highest DHA-3 β -HSD activity (+4) on day 6; cf. fig. 282, lowest on day 20.
- Fig. 289. Corpus luteum (C. L.) - highest DHA-3 β -HSD activity (+4) on days 6 (exhibited), 8, 13, 14, 15, and 18; cf. fig. 283, lowest on day 21.

PLATE XXXXIII



APPROVAL SHEET

The dissertation submitted by Frances A. Kovarik has been read and approved by five members of the faculty of the Graduate School.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the dissertation is now given final approval with reference to content, form, and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

DATE May 22, 1972.

A handwritten signature in cursive script, appearing to read "Prof. James H. ...", written over a horizontal line.
Signature of Advisor