15147

Growth of Corpus Luteum in Rats During Pregnancy and Following Injections of Testosterone.

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The corpus luteum increases in size during pregnancy in many species including man. The period of pregnancy during which the maximum size is reached varies among species[1] and corresponds, in part at least, to the length of time during which a functioning corpus luteum is the main source for progesterone. Experimental work during the past 20 years has shown that certain of the sex hormones when injected into suitable animals may produce an increase in the size of the corpus luteum comparable to that occurring spontaneously during pregnancy.[2,3,4] The experimental observations suggested that these hormones, particularly the estrogens, might contribute to the enlargement of the corpus luteum of pregnancy.[5]

During the past years, changes were noted in our rats following the injection of testosterone propionate during late estrus.[6] These changes included enlargement of the corpora lutea, extensive mucification of the vaginal epithelium, proliferation of ducts and alveoli in the mammary glands, and transformation of the endometrial stromal cells to decidua-like cells following proper traumatization of the uterus. Various phases of these experiments have been reported previously. The observations emphasized not only the importance of the corpus luteum with respect to certain effects of testosterone upon the rat, but also the similarity of the experimentally induced changes to the pregnant state. Hence, it became important to know to what extent the corpora lutea differed from or resembled those of pregnancy following injections of testosterone.

Data covering the growth and composition of the pregnancy corpus luteum were necessary first of all. In the literature such information concerns measurements of various diameters after fixation,[3,5,7,8,9] histochemical studies and extraction methods for the lipids,[7,10-14] and ascorbic acid.[15] The results may be briefly summarized by stating that neither lipid nor ascorbic acid content can quantitatively account for the increase in the size of the corpus luteum. Furthermore, differences in the species and methods make it difficult to gain an understanding of the changes within the corpus luteum during its growth and regression.

Therefore we decided to analyze the corpus

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TABLE I.
Groups and Periods of Experiments.

Pregnancy			Test. prop. 0.5 mg/day			Test. prop. 2.5 mg/day		
Day exp.	No. rats	No. of corp. lut.	No. rats	No. of corp. lut.	Total dose, mg	No. rats	No. of corp. lut.	Total dose, mg
Controls	65	646						
6			6	66	2.5			
11	19	184	25	238	5.0	16*	149	25.0
14	5	53						
16	15	171	17	162	7.5	10*	97	37.5
18	5	53						
21	16	171	42*	406	10.0	15*	159	50.0
24	4	42						
26	6	65	19*	198	12.5			
31	6	61	9*	87	15.0			
36	6	55						
Total	82	855	118	1157		41	405	

* The groups include rats with imperfect response.

luteum at various periods of pregnancy and to compare the results with those which might be obtained at corresponding periods of testosterone treatment. The present report describes the findings of the changes in growth and composition of the corpus luteum of the rat in these two conditions.

Material. A total of 306 female rats was used. They belonged to the Stanford strain of albino rats started from the University of Chicago stock in 1903. The strain had been observed particularly with respect to the reproductive phenomena during the past 3½ years. Because correct timing of the first injections with respect to the stage of the estrous cycle was of importance, many observations of the frequency and duration of the cycles were analyzed. A high degree of uniformity was found; 2966, or about 83%, of 3576 cycles lasted 4 to 5 days.

Methods. The vaginal estrous cycle of all animals was followed for 28 days after which all rats with cycles shorter than 4 and longer than 6 days were discarded. The age of the animals ranged from 90 to 150 days at the start of the experiment.

The control values were obtained from 65 rats which were killed within 24 hours after vaginal estrus had disappeared.

Eighty-two rats were used for the analysis of corpora lutea during pregnancy. They were placed with males during proestrus and the presence of spermatozoa in the vagina was assumed to indicate the onset of pregnancy. The animals were then housed individually and killed as indicated in Table I. Those rats which were reserved for the post partum period were permitted to nurse and the litter was uniformly adjusted to 6 sucklings.

The rats in which the corpora lutea were analyzed after hormone injections received the initial treatment during late estrus after cornified cells had been present in the smear for at least 15 hours. Two dosage levels were used. To 118 rats testosterone propionate† was given subcutaneously in daily doses of 0.5 mg in 0.1 cc of sesame oil over the periods given in Table I. This dosage was previously found to be the smallest amount which in the majority of our animals would result in persistent functioning corpora lutea and positive decidual reactions after uterine traumatization.[16] This experimental procedure was used in the 42 rats injected for 11 and 16 days respectively. Daily doses of 2.5 mg in 0.1 cc of sesame oil were given to 41 rats and they were killed at intervals which are indicated in Table I.

The animals were killed with ether 24 hours after the last injections and the corpora lutea individually dissected out under the microscope using the moist peritoneal surface as the operating field. As fast as possible, the

† Testosterone propionate (Perandren) was generously supplied by the Ciba Pharmaceutical Products, Inc., Summit, N.J.

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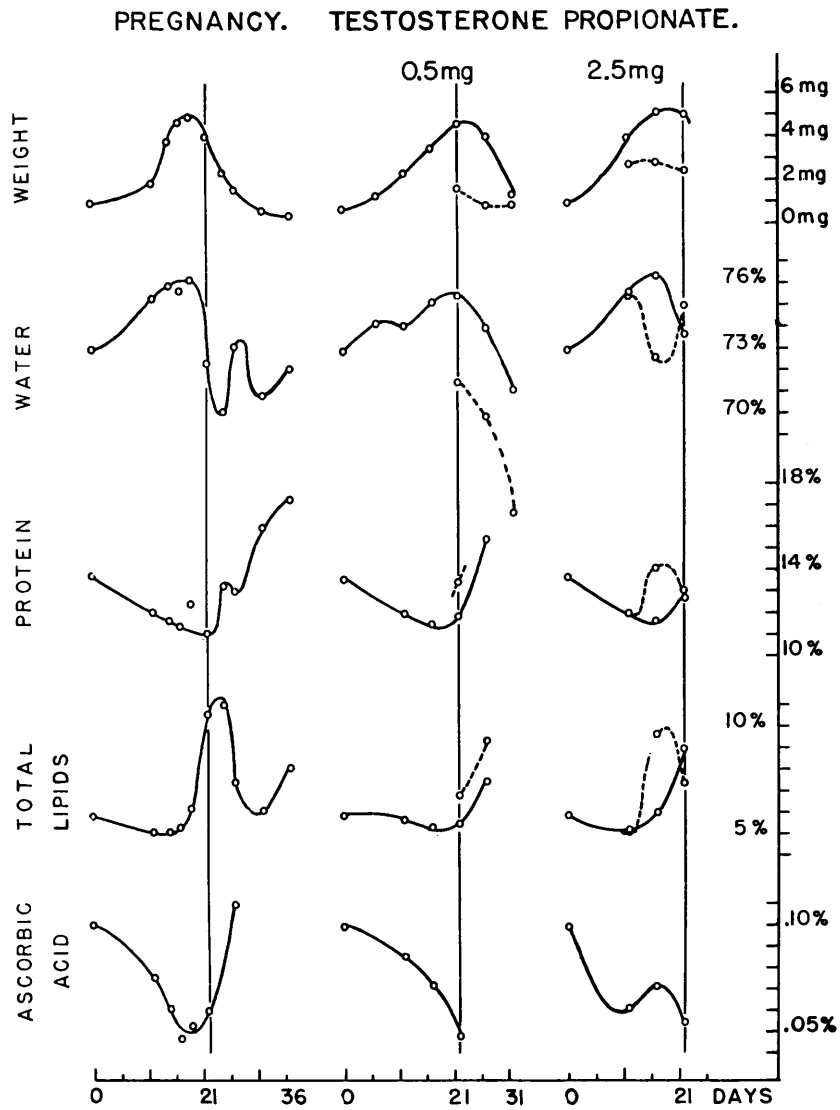

FIG. 1.

Weight curves and percentage values of water, protein, total lipids, and ascorbic acid of the corpus luteum in pregnancy and in experimentally induced hypertrophy.

corpora were transferred into weighing bottles to avoid drying of their surfaces. In the case of the controls, care was taken to dissect only the corpora of the last ovulation. These were easily distinguished from those of preceding ovulations by their greyish-white color and by a slight hyperemic surface protrusion marking the site of the stigma. Corpora lutea of previous ovulations were darker red and rounded. In the group of nursing rats, both sets of corpora lutea, those of pregnancy and those of lactation, were dissected out, weighed and analyzed separately. The data on corpora lutea of lactation are not reported here.

The moist corpora lutea were weighed in closed weighing bottles. The water content was determined by drying *in vacuo* over phosphoric anhydride to a constant weight which was usually reached within 18 hours.

The dried corpora were then extracted with ether in a micro-Soxhlet apparatus for 8 hours. The ether solution was transferred to a weigh-

ing bottle and the total lipids were weighed after evaporation of the ether. The lipids were then redissolved and aliquot parts used for colorimetric determinations by the Liebermann and the Zimmermann methods. The results were expressed as cholesterol and progesterone respectively by comparison with standard solutions of these substances. The non-steroid fraction was computed from the difference.

The total nitrogen content of the extracted corpora was then found by a semi-micro Kjeldahl method and this expressed as protein by the use of the factor 6.25.

Ascorbic acid was determined in the metaphosphoric acid extract of whole corpora lutea in separate specimens by titration with sodium 2.6 dichlorobenzenone-indophenol. Crystalline ascorbic acid was used as the standard.

The sum of the water, protein, and lipid expressed as percentages of the wet corpus luteum varied between 92.4 and 94.8, leaving between 5.2 and 7.6% of analyzed components such as carbohydrates and mineral salts.

Results and Discussion. The changes in the weight of the corpus luteum in mg and in the percentage of water, protein, lipids, and ascorbic acid calculated for wet tissue at various periods of the experiments are summarized in Fig. 1. The heavy vertical lines indicate the day of littering in the group of pregnant rats and the 21st day in testosterone treated rats respectively. The values obtained from animals which failed to respond in the usual way to the injected hormone are given in the curves with broken lines.

Before describing the findings, the values for day 0 require a brief consideration. They were obtained from our control animals killed during early diestrus, when the corpus luteum had become a rather solid anatomical structure. However, it should be pointed out that these corpora may not represent either the period of greatest corpus luteum function nor the earliest time of progesterone production in the normal animal. Recent experimental studies rather suggest that progesterone formation may occur even before ovulation.[14,17,18,19] It is for these reasons that we have not expressed the values in our experimental groups as percentages of the control values.

The 3 weight curves are essentially the same, indicating that the corpus luteum grows in the same fashion during periods of testosterone injections as it does during the course of pregnancy. There are but minor differences, in that the corpus luteum of pregnancy reaches the largest size at about the 18th day while the peak of the growth curve in rats injected with the small dosage is found at about the 21st day and that of rats receiving the larger dosage at about the 16th day. It seems reasonable to assume that there may be a dosage level between the two used here which may result in a growth curve fitting better yet that obtained during pregnancy. The dosage influences apparently the speed with which hypertrophy occurs. Whereas the results of diameter measurements suggested a plateaued weight between the 14th and 16th day of pregnancy and the day of littering, our curve indicates a maximum weight lasting only a short time and followed by a marked diminution in the size of the corpus luteum beginning several days before littering.

It is generally assumed that the enlargement of the corpus luteum is due to the increase in the size of the individual cells. No evidence has as yet been forthcoming which would indicate an increase in the number of specialized corpus luteum cells in pregnancy or after administration of steroid hormones. While an increase in cell size may be due to the deposition of unusual amounts of one or more substances, the analysis of the corpus luteum during its growth phase indicates that water, protein, lipids, and ascorbic acid increase simultaneously. This is readily seen when the actual weights of these various constituents are considered. If such weight curves are drawn they resemble that of the total weight of the corpus luteum. We have used the per cent values here in order to indicate the quan-

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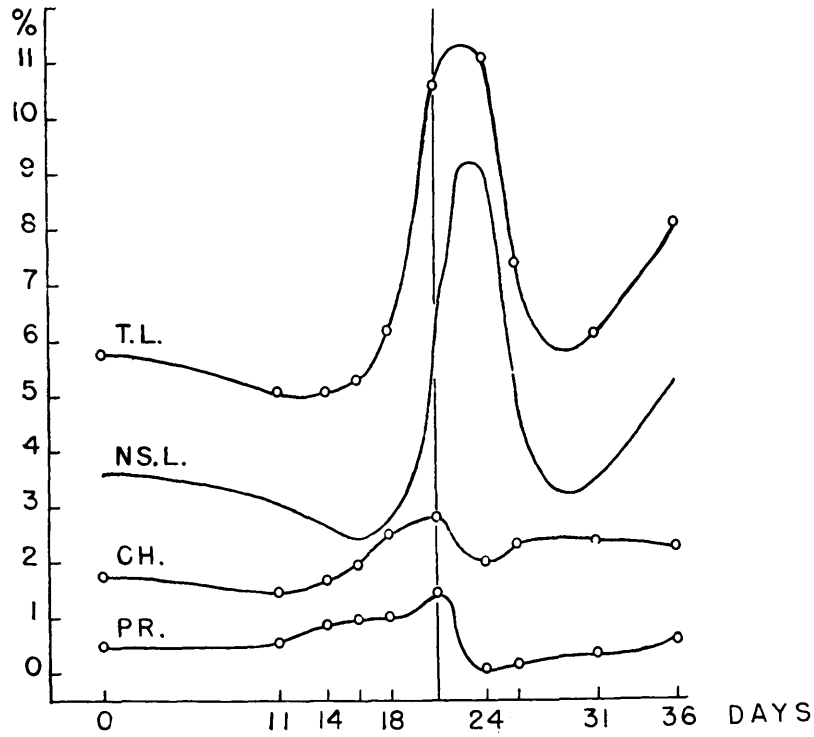

FIG. 2.
Percentage values for total lipids (T.L.), non-steroid lipids (NS.L), cholesterol (CH), and ketosteroids calculated as progesterone (PR) during the growth and regression of the corpus luteum of the pregnant rat.

titative relationship of the various cellular components to one another. It is seen then that during the period when the corpus luteum grows, water is present in larger proportions while protein, lipids, and ascorbic acid are found in relatively smaller amounts. Conversely, when the corpus luteum becomes smaller, the water content decreases both absolutely and relatively while the protein decreases absolutely although its concentration rises. These findings agree well with the general observation that growing tissues are richer in water than aged tissues.

The changes in the lipid fractions in relation to total lipids during the course of pregnancy and subsequent to parturition are given separately in Fig. 2. The total lipids show a slight decrease during the first 16 days which is followed by a marked rise evident at the 18th day of pregnancy. About 2 days following littering there is a drop in the total lipids which lasts but a few days, after which a second rise is observed. The decrease following parturition, though as yet unexplained, is of interest because it coincides with fluctuations in water and protein content. The possibility that the onset of lactation may have influenced the composition of the corpora lutea of pregnancy can only be suggested. Further analysis shows that the changes in total lipids are mainly due to those in the non-steroid fraction (glycerides and fatty acids), a finding which is in general agreement with the reports in the literature.[7,11,13]

The two lower curves in Fig. 2 give the values for cholesterol and ketosteroids, the latter calculated as progesterone. It is noteworthy that the ketosteroid fraction rises only slightly during the first half of pregnancy when progesterone action on the tissues is marked, while it increases sharply during the second half, reaching its highest concentration at about the day of littering. Taking into account the simultaneous growth of the cor-

pora lutea, the absolute amount of ketosteroids per corpus luteum can be calculated to have increased about 12-fold at that day. If this fraction represents principally progesterone, it may be possible that there is an increased release of this substance from the corpora lutea during the early periods of pregnancy. Such a mechanism is supported by the observations of Dempsey *et al.*, who found, using phenylhydrazine for histochemical demonstrations of ketosteroids, the smallest amounts during periods of greatest corpus luteum function. After littering a rapid lowering in ketosteroid concentration occurs, followed by a slow rise during the period of lactation.

During the first half of pregnancy the concentration of cholesterol actually decreases, but it rises during the second half of pregnancy, as does the ketosteroid fraction. It reaches its highest level at the day of littering, when it is increased sevenfold. Although the significance of cholesterol in the corpus luteum is unknown, the decrease in concentration may perhaps be connected with an increased permeability of the corpus luteum cell membrane or with the utilization of cholesterol in the production of progesterone.

The presence of ascorbic acid in the ovary was first emphasized in 1933[20] and attention was called to the corpus luteum as the tissue particularly rich in Vitamin C. It was further noted that the total amount of ascorbic acid varied with the functional state of the corpus luteum.[21-26] Although the importance of ascorbic acid and of its fluctuations with the physiologic states is unknown, it has been suggested that it is associated with the production of hormones in a non-specific way[27] and that an elevated amount suggests[24] or corresponds[15] to increased progestin secretion. When our data are expressed in absolute values, there is a rise and decline of the ascorbic acid values which corresponds to the general growth behavior of the corpus luteum. However, the ascorbic acid content, when expressed in relation to the weight of the corpus luteum, uniformly and significantly drops as the weight increases, and markedly rises as the weight falls. This indicates a dilution of extractable ascorbic acid rather than an increase in concentration. Because of the uncertainty as to the significance of ascorbic acid in corpus luteum function, the possibility still exists that there may be an increase in production of ascorbic acid which does not lead to an increased concentration in the corpus luteum because of increased utilization of ascorbic acid during the functional process. It should be remembered that the rat does not require exogenous ascorbic acid. This is in contrast to the species studied in the above mentioned articles and may possibly contribute to the differences noted.

As has been reported previously[16] deciduomata develop following traumatization of the uterus in rats injected with testosterone, provided functioning corpora lutea are induced. This test was used in the present experiment in order to supplement the analytical data with anatomical evidence of corpus luteum function. Of 42 rats injected with 0.5 mg daily for 11 and 16 days 37 animals, or 88%, developed deciduomata. This percentage was somewhat better than that obtained from all rats thus far subjected to the test, in which 24% of 137 rats failed to develop deciduomata. In the animals killed after 16 days of treatment, necrosis of the decidual cells was common, indicating that continued injections failed to maintain deciduomata beyond the usual 12 to 13 days.

Finally, the group of animals should be considered which responded differently to the injections than did the majority. These animals are represented in the curves with broken

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lines, and make up 39% of all the animals injected with testosterone. This reaction was observed more frequently during February and March than at any other time, the entire period of the experiments lasting from May, 1944, to April, 1945. The hormone preparation was the same before and after that period, so that variations in the testosterone can be excluded as the cause. Perhaps of still greater interest are the observations that these animals did actually show some degree of hypertrophy suggesting some form of response, and that they showed earlier the changes in composition which are typical for regression, such as loss in water and rise in protein and lipids. They formed a distinct group. No explanation can be offered as to the mechanism responsible for the lack of maximal response.

Summary. The changes in weight and in concentrations of water, protein, lipids, and ascorbic acid of the corpus luteum of the rat during pregnancy and during periods of injections with testosterone propionate at two dosage levels are reported. Comparison of the results permits the conclusion that the changes in weight and in chemical composition of the corpus luteum in experimentally induced hypertrophy are essentially the same as those occurring in untreated rats during the course of pregnancy.

15148

Comparison of the Cephalin-Cholesterol Flocculation with the Thymol Turbidity Test.

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The thymol turbidity test has been described recently by Maclagan[1] as an index of parenchymal liver dysfunction. Because of its simplicity the test gives promise of being of considerable aid in detecting disorders of the liver in which changes in the serum proteins are present. In clinical practice there is a marked parallelism between the thymol test and the cephalin flocculation test which also depends upon alterations of the serum pattern in certain liver diseases. Our studies indicate, despite this agreement, that the mechanisms of the two tests differ in important respects. In this paper some of these differences are described.

The thymol test is performed by adding 0.05 cc of the serum to be tested to 3 cc of saturated thymol in barbiturate buffered distilled water (pH 7.8). The intensity of the turbidity which develops within 10 minutes indicates the degree of active liver damage. Buffered distilled water without the addition of thymol does not give a positive test.

We have confirmed the findings of Maclagan that the test is usually positive in cirrhosis, hepatitis, etc., in which the cephalin flocculation is also positive. Notable exceptions have been observed, *e.g.*, the thymol test was positive and the flocculation negative in (1) certain lipemic sera such as in diabetes and in nephrosis, (2) biliary cirrhosis, (3) certain cases of metastatic neoplasm of the liver, (4) convalescent hepatitis, and (5) certain normal sera. The thymol test was negative and the flocculation positive in normal laboratory animals such as dogs and rabbits, and in certain rare cases of presumably normal individuals with no demonstrable liver disease.

Fundamental differences in the mechanism of the two tests can be demonstrated by studies on (1) effect of NaCl, (2) removal of lipids by

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