Relationship of Pituitary Prolactin and LH to Mammary and Uterine Growth of Pubertal Rats During the Estrous Cycle*† (34007)

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On the basis of morphological appearance, the mammary gland proliferates during the estrogenic and regresses during the progestational phase of the estrous cycle (1). The uterus also undergoes marked changes with stages of the estrous cycle. For example, uterine weight, water (2), nitrogen, and noncollagen protein (3) increase dramatically during the estrogenic phase of the cycle. But changes in number of cells (deoxyribonucleic acid—DNA) and protein synthetic activity (ribonucleic acid—RNA) of these tissues during consecutive postpubertal estrous cycles have not been determined. Although some hormones are known to influence both mammary and uterine development in adult animals, these relationships, to our knowledge, have not been studied with respect to phase of the estrous cycle of pubertal animals. Thus, the present experiments were designed to compare the normal pattern of biochemical changes in the mammary gland and uterus of rats during various phases of the five estrous cycles after vaginal opening and to relate these changes with pituitary prolactin and luteinizing hormone (LH) content.

Materials and Methods. Female Sprague-Dawley rats (Spartan Research Animals, Haslett, Michigan) obtained at 25 days of age were housed in a temperature- and light-controlled room (24 ± 1°; 6 AM to 6 PM) and checked twice daily for vaginal opening. Thereafter, stage of the estrous cycle was determined by daily examination of vaginal smears. A group of 12 rats was decapitated between 1 and 3 PM on each of the following days: proestrus, estrus, metestrus, and the first day of diestrus of the first, second, third, fourth, and fifth estrous cycle after vaginal opening. No distinction was made between rats with 4 or 5 day estrous cycles.

The six abdominal-inguinal mammary glands were removed at autopsy and placed in 95% ethyl alcohol for nucleic acid determination (4). Uteri were blotted free of any luminal fluid and weighed. A sample of uterine horn was placed in Bouin's fluid and processed for histological examination. The remainder was stored in 0.25 *M* sucrose at −20° until a 40–60 mg sample was taken for DNA and RNA analysis (5).

Anterior pituitary glands were removed within 2 min after decapitation, weighed, and stored at −20°. Four, 6, or 12 pituitaries within a group were pooled, depending upon the amount of tissue available, homogenized, and assayed for prolactin (6) and LH (7). Low and high dose in prolactin assays consisted of 0.5 and 2.0 mg of anterior pituitary tissue which were compared with 1.0 and 4.0 μg of NIH-P-S_5. For LH, the doses were 0.2 and 0.8 mg of pituitary and 0.4 and 1.6 μg of NIH-LH-B_2.

Results. Mammary nucleic acids. When averaged across all five estrous cycles mammary DNA increased 8% ($p<.01$) between proestrus and estrus, whereas changes between estrus and diestrus were not significant ($p>.05$) (Table I). Changes in mammary DNA content among stages were not always consistent from cycle to cycle. For example, although not shown in Table I, in cycles 1 and 2, the increase in mammary DNA initiated during proestrus or estrus continued through metestrus, whereas in cycles 3, 4, and 5, the peak DNA content occurred at estrus. Except for the first two cycles, there was a decline in mammary DNA between diestrus and the subsequent proestrus. None-

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TABLE I. Mammary Nucleic Acid Content of Rats during the Five Estrous Cycles after Vaginal Opening.

Estrous cycle	Age (days)	BW (g)	mg/100 g BW DNA	mg/100 g BW RNA	RNA/DNA
Stage[a]					
Proestrus	47	153	1.41 ± 0.03	1.86 ± 0.06	1.30 ± 0.02
Estrus	48	154	1.52 ± 0.04	2.10 ± 0.07	1.40 ± 0.02
Metestrus	50	158	1.52 ± 0.03	2.10 ± 0.06	1.40 ± 0.02
Diestrus	52	164	1.50 ± 0.02	1.92 ± 0.04	1.28 ± 0.02
Number[b]					
1	37	123	1.30 ± 0.03	1.90 ± 0.05	1.46 ± 0.03
2	46	150	1.43 ± 0.03	1.92 ± 0.06	1.36 ± 0.02
3	50	161	1.54 ± 0.03	2.02 ± 0.05	1.32 ± 0.02
4	54	176	1.58 ± 0.02	2.00 ± 0.04	1.28 ± 0.02
5	60	176	1.59 ± 0.03	2.10 ± 0.05	1.32 ± 0.02

[a] Each value is an average for 60 rats ± standard error.
[b] Each value is an average for 48 rats ± standard error.

theless, cumulative increases in mammary DNA averaged 10% ($p<.01$) between the first and second, 8% ($p<.01$) between second and third, 3% between third and fourth, but less than 1% between the fourth and fifth estrous cycle.

Changes in mammary RNA content and RNA/DNA ratios during the estrous cycle (Table I) paralleled changes in DNA except that the declines in RNA and RNA/DNA were initiated 1 day earlier (between metestrus and diestrus). The increases between proestrus and estrus and decreases between metestrus and diestrus were significant for RNA ($p<.01$) and RNA/DNA ($p<.05$). In addition, the RNA/DNA was significantly greater during the first estrous cycle after vaginal opening than during later cycles.

Uterine nucleic acids. Within each cycle, uterine DNA was maximum at proestrus or estrus, but then decreased 18% ($p<.01$) to a minimal value at metestrus (Table II). This suggests that a significant loss of uterine cells occurred during the early luteal phase of the cycle. Histological appearance of the uterus supported this concept because the endome-

TABLE II. Uterine Nucleic Acid Content of Rats during the Five Estrous Cycles after Vaginal Opening.

Estrous cycle	mg/100 g BW Weight	mg/100 g BW DNA	mg/100 g BW RNA	RNA/DNA
Stage[a]				
Proestrus	211 ± 6	0.88 ± 0.03	1.50 ± 0.08	1.70 ± 0.03
Estrus	183 ± 4	0.85 ± 0.03	1.22 ± 0.06	1.42 ± 0.03
Metestrus	125 ± 3	0.70 ± 0.02	0.68 ± 0.03	1.00 ± 0.01
Diestrus	134 ± 3	0.70 ± 0.02	0.86 ± 0.03	1.22 ± 0.01
Number[b]				
1	136 ± 6	0.62 ± 0.02	0.82 ± 0.05	1.32 ± 0.05
2	162 ± 7	0.76 ± 0.03	1.06 ± 0.07	1.36 ± 0.05
3	171 ± 6	0.88 ± 0.04	1.16 ± 0.08	1.30 ± 0.04
4	168 ± 6	0.83 ± 0.03	1.16 ± 0.07	1.38 ± 0.05
5	179 ± 8	0.82 ± 0.03	1.10 ± 0.05	1.36 ± 0.04

[a] Each value is an average for 60 rats ± standard error.
[b] Each value is an average for 48 rats ± standard error.

TABLE III. Pituitary Prolactin Concentration of Rats during the Five Estrous Cycles after Vaginal Opening.

Estrous cycle	No. of assays[a]	IU/mg		
		Average potency ± SE	Combined potency[b]	95% Confidence limits[c]
Stage				
Proestrus	10	0.054 ± 0.015	0.032	0.015–0.070
Estrus	12	0.023 ± 0.008	0.015	0.008–0.028
Metestrus	12	0.068 ± 0.020	0.041	0.024–0.068
Diestrus	12	0.015 ± 0.003	0.015	0.011–0.020
Number				
1	8	0.046 ± 0.018	0.025	0.010–0.064
2	8	0.050 ± 0.027	0.027	0.012–0.063
3	8	0.032 ± 0.015	0.017	0.008–0.038
4	11	0.041 ± 0.011	0.030	0.017–0.052
5	11	0.031 ± 0.011	0.018	0.009–0.036

[a] The 46 pools of pituitaries were assayed at random.

[b] Values are weighted average of the individual assays combined by the procedure of Bliss (18).

[c] 95% combined confidence interval of the combined potency.

trium and uterine glands appeared considerably reduced during metestrus and diestrus compared with proestrus or estrus. These losses in uterine DNA, however, were regained consistently between diestrus and the next proestrus. Uterine DNA increased cumulatively 42% ($p<.01$) between the first and third estrous cycle but no significant cumulative increases occurred thereafter.

Uterine RNA content and RNA/DNA ratio decreased ($p<.01$) from maximal values at proestrus to minimal values at metestrus followed by 26 and 22% ($p<.01$) increases, respectively, between metestrus and diestrus. Cumulatively, uterine RNA content increased 41% between the first and third estrous cycle with no significant increases thereafter. Uterine RNA/DNA ratios, unlike other uterine measurements, did not change significantly ($p>.05$) between the first and fifth estrous cycle.

Pituitary prolactin. Two peaks of prolactin concentration, one during proestrus and the other during metestrus, were apparent in three of the five estrous cycles. A preliminary analysis of the data by the hierarchical method showed no significant ($p>.05$) change in average prolactin concentration from the first through fifth estrous cycle. Therefore, to test for differences among stages of the estrous cycle, data from all cycles were pooled and analyzed with a one-way analysis of variance. This analysis revealed that pituitary prolactin concentrations at proestrus and metestrus were significantly greater ($p<.025$) than at estrus and diestrus (Table III). Changes in prolactin content per gland paralleled changes in prolactin concentration.

Correlation coefficients of average pituitary prolactin content with average mammary DNA, RNA, and RNA/DNA ratio were 0.09, 0.01, and −0.05, respectively ($p>.05$).

Pituitary LH. Average LH concentration from the first to fifth estrous cycle did not change significantly ($p>.05$), but among stages of the estrous cycle, the differences were significant (Table IV). From a maximum of 0.72 μg/mg at proestrus, LH concentration declined 57% ($p<.01$) to 0.31 μg/mg at estrus, remained relatively constant at metestrus and increased 22% ($p>.05$) at diestrus. Changes in LH content per gland were similar to changes in LH concentration.

Correlation coefficients between average pituitary LH content and average uterine weight, RNA/DNA ratio and DNA were 0.59, 0.58 ($p<.01$), and 0.44 ($p<.05$), respectively.

TABLE IV. Pituitary Luteinizing Hormone Concentration of Rats during the Five Estrous Cycles after Vaginal Opening.

Estrous cycle	No. of assays[a]	μg/mg		
		Average potency ± SE	Combined potency[b]	95% Confidence limits[c]
Stage				
Proestrus	9	0.67 ± 0.11	0.72	0.43–1.29
Estrus	11	0.33 ± 0.05	0.31	0.22–0.43
Metestrus	9	0.28 ± 0.04	0.27	0.20–0.38
Diestrus	11	0.34 ± 0.04	0.33	0.26–0.42
Number				
1	5	0.41 ± 0.06	0.40	0.29–0.54
2	8	0.29 ± 0.12	0.37	0.25–0.56
3	8	0.47 ± 0.10	0.40	0.29–0.55
4	10	0.28 ± 0.06	0.23	0.14–0.38
5	9	0.43 ± 0.08	0.39	0.27–0.57

[a] The 40 pools of pituitaries were assayed at random.

[b] Values are weighted average of the individual assays combined by the procedure of Bliss (18).

[c] 95% combined confidence interval of the combined potency.

Discussion. Pubertal growth (DNA) and increases in metabolic activity (RNA and RNA/DNA) of the mammary gland and uterus were completed largely by the fourth estrous cycle after vaginal opening. Most of the increases in nucleic acids in the mammary gland occurred between proestrus and estrus whereas in the uterus they occurred between diestrus and proestrus. Except for the first two estrous cycles after vaginal opening there were losses of nucleic acid in the mammary gland between diestrus and proestrus whereas the uterus lost nucleic acid between estrus and metestrus during every cycle. The delay of 1 day between mammary and uterine tissue in achieving maximal nucleic acid contents and the start of involution may reflect differences in latent periods and/or optimal titers of hormones necessary for maximal stimulation. The cyclic changes observed in nucleic acid contents of the mammary gland and uterus are in agreement with histological observations (1, 8). The increase in prolactin content of the pituitary between diestrus and proestrus agrees with the reports of Reece and Turner (6) and Sar and Meites (9), but they did not observe a decrease at estrus or an increase at metestrus as found in the present study. However, presumptive evidence that prolactin may be secreted during proestrus as well as metestrus is provided by the recent findings of Hashimoto *et al.* (10) that progesterone in ovarian venous plasma increased during each of these two stages of the cycle.

Our finding that uterine RNA and RNA/DNA ratios were elevated at proestrus supports the assumption that estrogen secretion is elevated at this time. Assuming that elevated levels of prolactin in the pituitary reflected elevated secretion of the hormone, it seems reasonable that prolactin and estrogen probably contributed to the mammary growth between proestrus and estrus. There was a latent period between peak prolactin secretion at proestrus and peak mammary cell proliferation at estrus. This latent period may partially account for the low correlation between prolactin content of the pituitary and mammary DNA and mammary RNA/DNA ratio. The failure to maintain mammary cell numbers throughout the estrous cycle may be caused by cyclic imbalance between pituitary and ovarian mammogens. For example, it is known that it takes very large amounts of prolactin (probably more than is secreted during the estrous cycle) to stimulate mammary growth in the

absence of estrogen (11).

Although it is hazardous to infer definitively from the content of a hormone in the pituitary gland, the high concentration of LH in the pituitary observed on the day of proestrus was construed to signify high level of synthesis and release. The parallel changes in pituitary LH content and uterine RNA and RNA/DNA ratios support this concept because it is known that LH is required together with FSH to stimulate estrogen secretion from growing follicles (12), and estrogen promotes RNA synthesis in the uterus (13, 14). Hence we interpret these data as indicating that LH promoted estrogen secretion which resulted in increased uterine RNA/DNA ratio.

The marked decline in pituitary LH between proestrus and estrus in each estrous cycle after vaginal opening is in agreement with the findings of others (15) using mature animals and probably is associated with release of LH.

Metestrus was the period of lowest uterine protein synthetic activity (RNA/DNA ratio) as well as lowest LH concentration in the pituitary. But during diestrus the uterine RNA/DNA ratio increased significantly. This enhancement of uterine metabolic activity may indicate stimulation of the uterus by estrogen at this time. Since it is known that there is a 6–24 hr latent period between initial estrogen treatment and rise in RNA content of the uterus (13), it would appear that significant estrogen secretion is probably initiated early in diestrus. This hypothesis is supported by the 22% increase in the pituitary LH concentration between metestrus and diestrus. Although this increase was not significant in the present study, Schwartz and Bartosik (15) observed a similar increase in pituitary LH content at this time. The LRF content of the stalk median eminence (16, 17) is also elevated during diestrus and further supports the idea of increased LH and hence estrogen secretion being initiated during diestrus.

Summary. Mammary and uterine development and their relationships to the pituitary content of prolactin and LH were studied during the five estrous cycles after vaginal opening in rats. Cumulative mammary and uterine growth was completed largely by the fourth estrous cycle after puberty. Although there was a lag of 1 day between peak mammary and uterine development, virtually all of this growth occurred during the estrogenic phase of the estrous cycle. Involutionary changes occurred in both organs during the progestational phase of the estrous cycle. Pituitary prolactin concentration was greater at proestrus (0.032 IU/mg) and metestrus (0.041 IU/mg) than at estrus (0.015 IU/mg) or diestrus (0.015 IU/mg). Pituitary LH concentration, which averaged 0.72, 0.31, 0.27, and 0.33 μg/mg at proestrus, estrus, metestrus, and diestrus, respectively, was correlated ($r=0.58$, $p<.01$) with changes in uterine RNA/DNA ratio.

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Fatty Acid Oxidation, Citric Acid Cycle Activity, and Morphology of Mitochondria in Diabetic Rat Liver* (34008)

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Fatty acid oxidation and citric acid cycle activity in the liver are thought to be important factors in ketogenesis. Either increased breakdown of fatty acid (FA) or impairment of citric acid cycle activity may cause ketonemia, but the mechanism regulating these two systems has not been adequately clarified. Insulin has been found to spare the oxidation of intravenously injected palmitate-1-^{14}C when measured in diabetic rat liver slices. (1). In other studies, no significant increase was demonstrated in the oxidation of palmitate-1-^{14}C when added directly to slices and isolated mitochondria (MT) from diabetic rat liver (2, 3). These conflicting reports warrant further studies in isolated MT where FA oxidation primarily takes place.

In this paper we report that increases in palmitate and oleate oxidation in isolated MT are accompanied by changes in the ultrastructure of MT. Metabolic and structural changes in MT of diabetic rat liver were reversed by insulin treatment. The activities of citric acid cycle enzymes were not altered in MT of diabetic rat liver.

Materials and Methods. Diabetes was induced in male Wistar rats weighing 150-250 g by intravenous injection of alloxan monohydrate, 6 mg/100 g body wt. Rats with blood sugar exceeding 250 mg/100 ml were used. MT were isolated by a modification of Schneider's method (4). After initial centrifugation of the homogenate at 620*g* for 10 min to remove the nuclear fraction, the supernatant was centrifuged at 13,000*g* for 10 min. After washing once in 0.25 *M* sucrose containing 0.5 m*M* EDTA at pH 7.5, MT were suspended in twice as much sucrose solution as the original fresh liver weight. Protein determinations in MT and supernatant fractions were done by the biuret method (5).

FA oxidation was determined according to a modification of Bjorntorp's method (6). The rate of incorporation of radioactivity in $^{14}CO_2$ and ^{14}C acetoacetate from ^{14}C FA was found to be proportional to the amount of MT suspension added under the following test conditions. The assay system (3 ml) contained (in micromoles) sucrose, 100; $MgCl_2$, 10; phosphate buffer (pH 7.5), 40; ATP, 6; glucose, 50; FA, 0.3 (containing 0.04–0.06 μCi of palmitate-1(U)-^{14}C or oleate-U-^{14}C and bovine albumin in ratio of FA/albumin=7), hexokinase, 6 units; cytochrome *c*, 0.39 mg, and 0.4 ml of MT suspension.

Materials for ultrastructural study (pellets of isolated MT or biopsy specimens from the left lateral lobe of the liver) were fixed in ice cold veronal-buffered 2% osmium tetroxide (7) for 1 hr, progressively dehydrated through graded alcohols, and embedded in

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