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Importance of Mammary Deoxyribonucleic Acid and Precursor Incorporation on Milk Secretion Rate in Rats (38402)

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The amount of milk produced by a mammal is dependent upon the number of cells in the mammary glands and the rate of activity per cell. The number of cells is primarily dependent upon the growth of the gland during pregnancy and early lactation and the rate of involution after peak lactation. The rate of activity of each cell is dependent upon a number of factors, among which are (1) available precursors and energy for milk synthesis, (2) accumulation of milk in the glands in that milk must be secreted against a pressure gradient, (3) level of hormones in the animal body, and (4) rate-limiting biochemical reactions that may be controlled by the enzyme levels in the mammary gland.

Considerable research has been directed towards determining factors responsible for mammary gland growth, relationship of blood precursors to milk secretion, relationship of mammary gland pressure to milk secretion rate, enzyme levels, and rate-limiting biochemical reactions. Most of this work has been on a qualitative basis and only limited quantitative information is available. The objective of this study was to measure the quantitative importance of DNA content of the mammary gland and incorporation of radioactive precursors into milk components in determining the amount of milk produced by lactating rats.

Methods and Materials. Forty female Sprague–Dawley rats, weighing between 180 and 200 g, were bred and housed in individual cages in a room maintained at 22°. On the day after parturition, rats were placed under light ether anesthesia, the thoracic mammary glands were ligated leaving the six inguinal–abdominal mammary glands to be suckled, and litter sizes were reduced to either six or ten healthy pups to provide two nursing intensities. The dam and pups were weighed on the day after parturition, and on days 5, 10, 15, and 20 postpartum.

Five lactating rats, selected at random from each of the two groups (six and ten pups per litter) were sacrificed on days 5, 10, 15, and 20 postpartum. The young were separated from their dams for 2 hr and then reunited with their dams for 1 hr before sacrifice to produce a nearly complete evacuation of milk from the mammary gland. At sacrifice, the dam was removed from the young, stunned by a blow to the head, decapitated, and the trunk blood collected in small beakers containing 0.2 cc of a saturated solution of sodium citrate. The blood was centrifuged in a refrigerated centrifuge and the plasma was stored at −20° until time of assay for prolactin.

The skin was quickly removed from the abdominal surface and a small piece of the abdominal mammary glands was removed and placed in Media 199, a synthetic media for tissue culture (1) obtained from Grand Island Biological Company, Grand Island, NY. The tissue was blotted dry, weighed, and replaced in Media 199. After removal of surface connective tissue, the tissue was cut into explants weighing approximately 2 mg each. Eight explants, chosen at random, were removed, blotted dry, weighed, and placed in a 25 ml Erlenmeyer flask containing 2 ml of Media 199. Six flasks of explants were prepared from each rat. Each ml of Media 199 contained 20 units prolactin, 50 units penicillin, and 50 μg streptomycin. Media 199 also contained one of the following isotopes obtained from New England Nuclear Corporation: 1 μCi DL-leucine-1-^{14}C/ml, 1/2 μCi Na acetate-1-^{14}C/ml, or 1 μCi D-glucose-1-^{14}C/ml. Duplicate flasks were used for each isotope. The flasks were placed in a Dubnoff incubator and

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the hood was flushed for 30 sec with a mixture of 95% air and 5% CO_2 at the beginning and once every hour. The flasks were shaken at 60 cycles per min at a temperature of 37°. Incubation was continued for 4 hr, at which time the flasks were removed and placed in an ice bath to stop the metabolic processes. The tissue pieces were removed, placed between two pieces of parafilm, and frozen at −20° until processed. The remaining six abdominal–inguinal mammary glands were removed, weighed, and frozen at −20° until processed.

The nonincubated tissue was thawed, homogenized in ice cold water, and analyzed for DNA and RNA contents by the methods of Munro and Fleck (2) and Croft and Lubran (3) to determine mammary gland DNA and RNA contents.

Tissue explants incubated in ^{14}C-leucine were homogenized in 30 ml of the buffer described by Jergens *et al.* (4) which contained 1 mg carrier casein/ml. The casein was precipitated in a mixture of glacial acetate acid, NaOH, and ethanol according to the procedure of Ashworth (5). The precipitate from this procedure was dissolved in 4 ml of 5 *M* acetic acid of which 0.5 ml was placed with 10 ml Bray's liquid scintillation counting solution (6). This solution was counted in a Nuclear-Chicago Mark II Liquid Scintillation Spectrometer and the counts per minute (cpm) of ^{14}C leucine incorporated into casein were calculated.

Tissue explants incubated in the ^{14}C acetate media were homogenized in 3 ml saline and triglycerides extracted with 3 ml Dole's extraction mixture (7). Following addition of 3 ml heptane, 1 ml of the lipid phase was placed in a liquid scintillation vial, evaporated to dryness, and counted in 15 ml Bray's solution. Likewise tissue incubated with ^{14}C glucose was homogenized in 3 ml saline and the lactose was separated by the method of de Langen (8) using a charcoal-celite column. The eluate containing lactose was placed in a liquid scintillation counting vial, evaporated to dryness, and counted in 10 ml Bray's solution. The counts per minute of ^{14}C glucose, acetate, and leucine incorporated into milk components were expressed per milligram DNA in the tissue. These three measures were used to estimate the secretory activity of the cells. The total leucine, acetate, and glucose values shown in Table III were calculated by multiplying the cpm of the precursors/mg DNA by the total DNA content of the mammary glands.

The prolactin in the blood plasma was assayed by the radioimmunoassay procedure of Neill and Reichert (9). NIAMD rat prolactin-RP-1[2] was used as the standard for prolactin. An analysis of variance was conducted for each of the parameters shown in Table I. The effect of number of young and day of lactation and the interaction between the two variables were tested in each analysis. Thirty-two degrees of freedom were available for the error mean square. Treatment means were compared by Duncan's multiple range test. Simple correlations within days and within groups were calculated among all parameters. Multiple regression values were determined on the data using pup weight and pup gain as the dependent variables and mammary gland DNA and RNA values and secretory rate measurements as the independent variables.

Results. Means for pup growth rate, mammary gland measurements, and plasma prolactin levels are shown in Table I. The differences in pup weights at the various times after parturition were significantly different as expected. The average weight and average gain of each pup for 5 days before sacrifice were significantly higher ($P < 0.01$) when dams nursed six pups instead of ten. The weight gains during the 5 days before sacrifice ending at days 10, 15, and 20 were significantly higher ($P < 0.01$) than the average weight gain during the first 5 days postpartum.

Significant increases in mammary gland weights and DNA and RNA measurements occurred with advancing lactation; however, there were no significant differences between mammary gland weights and nucleic acid contents of dams nursing six and ten pups. The RNA/DNA ratio was 2.1, 3.2, 3.8, and 3.4, for days 5, 10, 15, and 20 days postpartum, respectively. The ratios for days 10, 15, and 20 were significantly higher than those for day 5 ($P < 0.05$).

There were no significant differences in leucine or glucose incorporation per mg DNA; however a marked increase in acetate incorporation per milligram of DNA occurred on day 15, which was significantly higher than that on day

[2] Rat radioimmunoassay kit obtained from the Rat Pituitary Hormone Program of the National Institutes of Arthritis and Metabolic Diseases.

TABLE I. Effect of Stage of Lactation and Number of Suckling Young on Mean Growth Rate of Young Rats, Mean Mammary Gland Measures of the 6 Abdominal-Inguinal Glands, and Mean Blood Prolactin Levels.

	Number of young								
	6	10	Av	6	10	Av	6	10	Av
Day of lactation	Average wt. gain of each pup (g)			Average pup gain 5 days before sacrifice (g)			Mammary gland wt. (g)		
5	9.8	8.4	9.1^{d}	3.0	1.8	2.4^{b}	9.2	7.4	8.3^{f}
10	16.2	13.7	14.9^{c}	6.0	4.7	5.4^{a}	11.4	11.8	11.6^{e}
15	25.7	18.2	22.0^{b}	8.1	4.9	6.5^{a}	14.6	11.7	13.2^{e}
20	33.3	27.5	30.4^{a}	8.9	6.1	7.5^{a}	11.8	12.0	11.9^{e}
Av.	21.3*	17.0+	—	6.5*	4.4+	—	11.7	10.7	—
	Mammary DNA (mg)			Mammary RNA (mg)			cpm Leucine/mg DNA/4 hr. ($X10^{4}$)		
5	18.9	11.4	15.2^{b}	39.8	25.9	32.9^{b}	217	236	226
10	19.8	18.0	18.9^{b}	59.2	58.1	58.7^{b}	286	254	270
15	28.3	27.3	27.8^{a}	93.8	102.8	98.3^{a}	330	306	318
20	30.0	31.9	30.9^{a}	98.2	112.9	105.6^{a}	299	270	284
Av.	24.3	22.1	—	72.8	74.9	—	283	266	—
	cpm Acetate/mg DNA/4 hr ($X10^{5}$)			cpm Glucose/mg DNA/4 hr ($X10^{3}$)			Plasma prolactin (ng/ml)		
5	548	360	454^{ab}	972	412	692	415	295	355^{a}
10	572	392	482^{ab}	632	504	568	382	416	399^{a}
15	724	600	662^{a}	760	680	720	235	316	275^{ab}
20	284	256	270^{b}	572	644	608	97	158	128^{b}
Av	532	402	—	734	560	—	282	296	—

$^{a, b, c, d}$ ($P < 0.01$), $^{e, f}$ ($P < 0.05$) for day of lactation.

$^{*,+}$ ($P < 0.01$) for number of young per litter.

20 of lactation ($P < 0.05$). The lack of significant differences in glucose incorporation may be due in part to the possibility that some of the secreted lactose moved from the tissue into the media.

Plasma prolactin levels for days 5 and 10 postpartum were significantly higher than those for day 20 ($P < 0.01$). There were no significant differences in prolactin levels due to the number of pups nursed per dam.

The simple correlations among the variables are shown in Table II. The variable most highly related to pup weight and pup gain was total mammary gland weight. Total DNA and total RNA were positively correlated with pup weight and pup gain; however the values were not statistically significant ($P < 0.05$). Negative correlations existed between mammary gland weight and DNA and RNA concentrations per milligram of tissue.

The measures of secretory activity were negatively correlated with pup weight and pup gain, however these correlations were not significantly different from zero ($P > 0.05$). The measurements of activity were negatively correlated to mammary gland weight and positively correlated with total DNA, with total RNA, and with DNA and RNA concentrations per mg of mammary gland tissue. The activity measurements were also correlated to each other.

The squared multiple correlation coefficients (R^2) for pup weight and pup gain are shown in Table III. Mammary gland DNA accounted for only 5.8% and 1% of the variation in pup weight and pup gain, respectively. The addition of the secretory activity variables increased the amount of variation accounted for in comparison to mammary gland DNA alone. The correlations

TABLE II. Simple Correlations Among Variables Related to Mammary Gland Parameters and Pup Growth.[a]

Variable	1	2	3	4	5	6	7	8	9	10	11
1. Pup wt.	1.00										
2. Pup gain–5 days before sacrifice	0.82	1.00									
3. Total mammary gland (MG) wt.	0.50	0.37	1.00								
4. Total DNA	0.24	0.10	0.42	1.00							
5. Total RNA	0.26	0.24	−0.10	0.47	1.00						
6. DNA/mg MG	−0.19	−0.21	−0.34	0.67	0.53	1.00					
7. RNA/mg MG	−0.13	−0.10	−0.60	0.22	0.79	0.73	1.00				
8. RNA/DNA ratio	−0.01	0.08	−0.43	−0.40	−0.57	0.07	1.58	1.00			
9. cpm Leu/DNA	−0.15	−0.17	−0.33	0.54	0.51	0.80	0.65	−0.02	1.00		
10. cpm Ac/DNA	−0.16	−0.11	−0.46	0.30	0.45	0.70	0.70	0.13	0.75	1.00	
11. cpm Glu/DNA	−0.18	−0.10	−0.45	0.16	0.33	0.53	0.52	0.14	0.58	0.57	1.00

[a] Values greater than 0.33 are significant at the 5% level of probability and those above 0.42 at the 1% level.

were always higher with pup weight than pup gain. No single measure of incorporation of radioactive precursors into milk components accounted for more variation than the others. The combination of mammary gland DNA, RNA, total leucine incorporation, total acetate incorporation, and total glucose incorporation accounted for 30.9% of the pup weight variation and 23.5% of the pup gain before sacrifice variation.

Discussion. The mammary gland weights, nucleic acid contents, and the secretory activity measures indicate that milk secretion rate increases until day 15 of lactation. Decreases in mammary gland weight and acetate incorporation into triglycerides from days 15 to 20 of lactation may point to a decrease in milk secretion rate and the beginning of the involution; however nucleic acid contents continued to increase to day 20. Tucker (10) also observed similar results in that the DNA content of six abdominal–inguinal mammary glands, suckled by six young, more than doubled between the day of parturition and day 16 of lactation; however, he found that the mammary gland DNA content decreased between days 16 and 20 of lactation. The increase in milk secretion rate to day 15 would be expected since the young are increasing in size with advancing age and exist on milk only through day 16 or 17 postpartum. By day 20, the young consume solid feed, thus diminishing the requirement for milk from their dams. The decreased suckling stimulus by day 20 due to more solid feed consumption by the young may also explain the significant drop in plasma prolactin levels.

Most DNA measurements on mammary gland tissue are done to determine cell numbers under the assumption that the DNA content per cell is constant. Research work in our laboratory on rats and rabbits (11, 12) questions this assumption and evidence has been presented to indicate that additional DNA per cell is present during lactation in comparison to epithelial cells during pregnancy and involution of the mammary glands. This extra DNA is hypothesized to be "metabolic" DNA.

Evidence for the existence of "metabolic" DNA is provided by the positive correlations between the activity measurements (cpm leucine, acetate and glucose/mg DNA), total DNA, and the DNA content per milligram of

TABLE III. Percentage of the Total Variation (R^2) in Pup Weight and Pup Gain (Dependent Variables) That Is Accounted for by the Various Combinations of Mammary Gland Parameters Calculated on a Within Days and Within Group Basis.

Independent variables	R^2 values (%)	
	Pup weight	Pup gain — 5 days
Mammary gland (MG) DNA	5.8	1.0
MG DNA, cpm Leu/DNA	17.4	9.6
MG DNA, total Leu	17.0	10.0
MG DNA, cpm Ac/DNA	14.5	5.9
MG DNA, total Ac	15.8	5.8
MG DNA, cpm Glu/DNA	13.8	4.2
MG DNA, total Glu	14.3	4.1
MG DNA, RNA/DNA ratio	13.1	3.8
MG DNA, MG RNA	16.8	7.8
MG DNA, MG RNA, total Leu, total Ac, total Glu	30.9	23.5

mammary gland tissue. The activities per unit DNA thus increase as the quantity of DNA increases, an indication that the DNA synthesized during lactation increases the rate of milk secretion.

Because the DNA content per cell may not be constant in lactating mammary gland tissue, the incorporation of labeled leucine, acetate, and glucose into tissue casein, triglycerides, and lactose per unit of DNA is not the best estimate of the synthetic activity per cell. Further research work will have to be conducted whereby the mammary gland cells are dispersed, incubated in a culture containing labeled precursors, and the activity expressed per enumerated cell and unit of DNA.

In addition to the problem of measuring the secretory activity of mammary tissue, another limiting factor is that pup weight and pup gain are indirect and relatively crude measures of the amount of milk produced by rats. Even with these major limitations, the combination of mammary gland DNA, RNA, and total leucine, acetate, and glucose incorporation accounted for approximately 31% of the variation in pup weight and 24% in pup gain. The results indicate that no single measure of activity or cell number influences pup growth independently, but show that quantitating the relationships of DNA contents and activity rates to milk secretion is possible.

Summary. Rats nursing either six or ten young on six abdominal–inguinal mammary glands, were sacrificed on days 5, 10, 15 and 20 postpartum. Nucleic acid measurements were made on the mammary glands and explants were prepared and incubated with radioactive precursors. Incorporation of ^{14}C acetate into tissue triglycerides, ^{14}C glucose into lactose, and ^{14}C leucine into tissue casein per milligram DNA for 4 hr was used as an index of secretory activity. Dams nursing ten young did not have higher mammary gland weights, mammary gland nucleic acid contents or secretory activity measurements than those nursing six young. Mammary gland RNA and DNA contents increased with advancing lactation. Acetate incorporation into triglycerides was higher on day 15 of lactation than at other times. Plasma prolactin was higher on days 5 and 10 of lactation than day 20. The variable most highly correlated with pup weight and pup gain was mammary gland weight.

Mammary gland DNA content accounted for 5.8% of the total variation in pup weight. Addition of one measure of leucine, acetate or glucose incorporation to DNA content increased the variation accounted for to 13.8–17.4%. The addition of RNA content, total leucine, total acetate, and total glucose incorporation to the mammary gland DNA values increased the variation for pup weight accounted for to 30.9%. The variation accounted for in pup gain by these variables was less than for pup weight. These results indicate that both DNA content and secretory activity of mammary glands play a significant role in accounting for variation in pup weight and pup gain.

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