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Influence of Number of Suckling Young on Nucleic Acid Content of Lactating Rat Mammary Gland.* (29206)

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Single litters of 8 rat pups per 12 mammary glands provided sufficient suckling stimulus to maintain deoxyribonucleic acid (DNA) content, a measure of cell numbers, to 24 but not 28 days of lactation(1). Suckling by foster litters, however, maintained mammary DNA to 61 days(2) and maintained lobule-alveolar system to 70 days(3). Ribonucleic acid (RNA) content and RNA/DNA ratio, measures of protein synthetic activity, were maintained to 21 days of lactation(1) but markedly declined thereafter despite continued suckling(2). On the other hand, teat ligation of 3 abdominal-inguinal mammary glands of rats decreased the DNA and RNA content and RNA/DNA ratio when compared with contralateral, nonligated glands or glands of normal rats lactating similar periods of time(4). Thus, suckling stimulus, without milk removal, did not prevent cellular loss nor maintain the protein synthetic activity of the mammary gland. The nucleic acid content, however, of contralateral, nonligated glands was greater than those of glands of normal rats(4) and mice(5) lactating the same length of time.

The present research was undertaken to determine whether or not varying numbers of suckling pups would influence the number of cells (DNA) and protein synthetic activity (RNA and RNA/DNA) of intact abdominal-inguinal mammary glands of rats.

Methods. The litter size of lactating primiparous Sprague-Dawley rats was adjusted to 4, 6, 8, or 10 pups per 12 glands (groups 4/12, 6/12, 8/12, 10/12, respectively) on day 3 of lactation. In a second experiment, teats of the 6 thoracic glands were ligated on day 3 of lactation and 2, 4, or 6 pups (groups 2/6, 4/6, 6/6, respectively) were allowed to suckle the 6 intact abdominal-inguinal glands. Litter weight gain from the 7th to 15th day after parturition was used as an index of lactational performance.

Mother rats were sacrificed by cervical dislocation on the 21st day of lactation, and the 6 abdominal-inguinal mammary glands of rats of both experiments were removed, washed in ice-cold 0.25 M sucrose, blotted on paper towels, weighed, and then stored in 0.25 M sucrose at —20°C until analyzed.

The 6 abdominal-inguinal mammary glands were suspended in ice-cold distilled H_2O (1 g to 20 ml) and homogenized 2 min in a Waring blendor at top speed in a cold room. Duplicate 2-ml samples of homogenate were suspended in 8 ml of 95% ethanol for 24 hr at room temperature. The samples were extracted for 24 hr with 9 ml of methanol: chloroform (2:1) and for 24 hr with 9 ml of anhydrous ether(6) with constant agitation. The samples were extracted twice with 5 ml of ice-cold 10% trichloracetic acid which was removed by washing with ice-cold ethanol saturated with sodium acetate. The samples were digested in 2 ml of 1 N KOH for 15 hr at 37°C, and the digest was acidified with 0.3 ml ice-cold 6 N HCl and 5 ml ice-cold 10% perchloric acid (PCA). The residue was washed twice with 5 ml of 5% PCA and combined supernatants were analyzed for RNA-ribose(7). The remaining residue was extracted with 5 ml of 5% PCA at 70°C for 15 min and washed twice with 5 ml of

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TABLE I. Nucleic Acid Content of Mammary Glands of Lactating Rats with Varying Numbers of Suckling Young.

Pups/ glands	No. lactating rats	Avg body wt (g)	Avg fresh gland wt (g)	Total DNA (mg)*	Total RNA (mg)*	RNA/ DNA*
4/12	12	252	7.7766	18.07†± 1.14	31.06‡± 2.37	1.72‡± .08
6/12	12	260	9.4011	21.68 ± .51	53.32§± 2.66	2.46‖± .11
8/12	12	256	9.3633	21.87 ± 1.39	55.23‖± 3.39	2.53 ± .06
10/12	12	265	10.2810	23.93 ± .68	66.49 ± 3.71	2.78 ± .11
2/6	12	246	8.3368	18.14¶± .80	34.60¶± 3.77	1.91¶± .14
4/6	12	256	10.7436	23.90 ± 1.37	59.27 ± 4.57	2.48 ± .12
6/6	12	245	11.6131	27.18 ± 1.74	72.37 ± 5.70	2.66 ± .11

* Mean and standard error of mean.
† Significantly less than 6/12 and 8/12 ($P < 0.05$) and 10/12 ($P < 0.01$).
‡ Significantly less than 6/12, 8/12, and 10/12 ($P < 0.01$).
§ Significantly less than 10/12 ($P < 0.01$).
‖ Significantly less than 10/12 ($P < 0.05$).
¶ Significantly less than 4/6 and 6/6 ($P < 0.01$).

5% PCA. Combined supernatants were analyzed for DNA in a Beckman DB spectrophotometer at 268 mμ. Yeast RNA and highly polymerized DNA (Worthington Biochemical Corp.) were used as standards for mammary RNA and DNA, respectively.

Results. Means of mammary gland total DNA (mg), total RNA (mg) and RNA/DNA ratio, listed in Table I, ranged from 18.07, 31.06, and 1.72, respectively, for group 4/12, to 23.93, 66.49, and 2.78, respectively, for group 10/12 ($P<0.01$). Similar measurements of glands of rats whose thoracic glands were ligated ranged from 18.14, 34.60, and 1.91, respectively, for group 2/6 to 27.18, 72.37, and 2.66, respectively, for group 6/6 ($P<0.01$).

Litter weight gains were 66.5, 90.1, 110.7, and 133.5 g for groups 4/12, 6/12, 8/12, and 10/12, respectively, and 30.6, 70.3, and 77.8 g for groups 2/6, 4/6, and 6/6, respectively. The values represented an average gain per pup, within each of the respective groups, of 16.6, 15.0, 13.8, and 13.4 and 15.3, 17.6, and 13.0 g. The correlation of total RNA and RNA/DNA ratio with litter weight gain was 0.70 ($P<0.01$) and 0.53 ($P<0.01$), respectively, for all groups of pups per 12 glands and 0.69 ($P<0.01$) and 0.54 ($P<0.01$), respectively, for all groups of pups per 6 glands.

Gross observation of the mammary glands revealed that some individual glands of some rats of groups 4/12 and 2/6 apparently were not being suckled and may have been undergoing involution. Eight inguinal and 2 abdominal glands of this type, of a possible 48 inguinal and 24 abdominal glands, were found in group 4/12. Similar findings occurred in 16 inguinal glands and 1 abdominal gland in group 2/6.

Discussion. Mammary gland DNA and RNA content and RNA/DNA ratio were increased as the number of suckling pups per teat increased. It was not determined, however, whether the mammary gland was stimulated to grow and metabolize increased amounts of protein by the increased number of pups or whether this increased suckling stimulus merely prevented involution. The glands of the two groups with the fewest pups per teat contained significantly less nucleic acid than all other groups. These were the groups where certain glands were not being suckled or at least were not secreting milk. These observations support the previous finding that suckling will not prevent involution if milk is not removed(4).

Total RNA and RNA/DNA ratio were significantly correlated with average litter weight gain. It is suggested, however, that the correlation of these parameters would be even higher if rate of gain on the 15th day were correlated with the RNA content and RNA/DNA ratios of the 15th day of lactation.

Summary. The influence of varying numbers of suckling young on the number of cells (DNA) and protein synthetic activity (RNA and RNA/DNA) was determined in abdominal-inguinal mammary glands of rats lactat-

ing 21 days. The data indicated that mammary gland development and secretory activity increased as the number of suckling young per mother rat increased.

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A Method for Extraction of Evans Blue from Plasma and Tissues.* (29207)

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Dilution of Evans Blue (T-1824) following intravenous injection is the simplest method for estimation of plasma volume(1). The difficulties encountered with the method are principally associated with determination of the dye in plasma, and include turbidity due to lipaemia, occasional haemolysis, and the inherent color of the plasma, which may be exaggerated as in ruminants. The spectral characteristics of the dye, moreover, change on conjugation with plasma, and the changes are not the same for the plasmas of all species(2). This necessitates comparison with standards prepared by adding dye to the plasma of the same species. It is obvious therefore, that determination of the dye following its extraction from the plasma would have distinct advantages. Extraction is in fact obligatory if Evans Blue is used as a marker for plasma proteins for quantitative determination of the changes in the retentive properties of capillaries. One method of extraction from plasma was developed by Allen (3,4) which involves adsorption of the dye on cellulose, followed by prolonged elution (4-16 hr) with aqueous acetone. However, though consistently good recoveries (97%) were obtained with plasma, extraction of the dye from albumin in tissues gave only 60-80% recoveries(5). For this reason, a simpler and more versatile method was developed.

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The conjugation of Evans Blue with albumin involves the ionic interaction of the sulphonate anions of the dye with quaternary nitrogens of this protein, most probably the ϵ-amino group of lysine (pK 9.4-10.6) and possibly the guanidinium group of arginine (pK 11.6-12.6)(6). Suppression of the ionization of these amino groups by a sufficient rise in the pH should lead to disconjugation of the dye. Concentrated ammonia solution (S.G. 0.880) proved satisfactory for this purpose.

Methods. Human plasma was mixed with Evans Blue, and precipitated by addition of an equal volume of 10% (w/v) trichloracetic acid. The precipitate was washed twice with absolute ethyl alcohol (5 min at 70°C) followed by extraction with 1 ml of concentrated ammonia solution (25%, d^{20}_4 0.91) for 10 min. Four ml of alcohol was then added with thorough mixing and the supernatant decanted. This extraction with ammonia was repeated twice more, and the combined supernatants mixed with 3 ml of glacial acetic acid and made up to 25 ml with ethyl alcohol. Addition of sufficient alcohol to the ammoniacal extract is essential if a clear supernatant is to be obtained. The time and speed of centrifugation at every stage must be similarly adjusted to give clear extracts. A bench-type centrifuge (International Equipment Co., Boston, Mass., model HN) was used. Five minutes at 1000 times gravity sufficed for separation of the precipitate from the