Discussion. It appears that complete methylation of FFA and bile acids (free or conjugated) can be achieved with diazomethane under conditions in which cholesterol, triglycerides and cholesterol esters appear to remain unaltered, as suggested by their mobility on thin-layer chromatography. Other components of plasma or bile lipid extracts may react with diazomethane under the conditions used, including bilirubin* and certain phospholipids(6). However, our results for plasma FFA suggest that there is no interference from such reaction products, or from the substances derived from diazomethane itself, for example, polymethylenes(7). Possible interference by phospholipids in the determination of bile acids in plasma or bile may be avoided by saponification, which removes phospholipids and gives a simplified mixture of non-conjugated bile acids (Fig. 2).

A recent report(8) describing the measurement of standard bile acids by methylation with C^{14}-diazomethane followed by autoradiography of paper chromatograms lends support to the procedure described above.

Summary. As an approach to the problem of microdetermination of acidic substances of biological interest, the procedure of introducing a C^{14}-methyl group and measuring activity associated with the methyl esters appears to offer promise. Treatment with C^{14}-diazomethane under mild conditions results in complete methylation of non-esterified fatty acids in plasma and of bile acids in bile. C^{14}-methyl esters of specific bile acids may be isolated by thin-layer chromatography free from other active substances. Preliminary studies suggest that absolute amounts of free acids present in plasma may be determined by the use of C^{14}-labeled standards.

* Unpublished observations.

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Nucleic Acid Content of Rat Mammary Gland After Teat Ligation.* (28471)

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Selye(1), using histological methods, reported that the suckling stimulus during lactation prevented involution of galactophore-ligated glands of rats up to 14 days. The effects of accumulated milk, however, eventually resulted in mammary involution despite continued suckling(2). Deoxyribonucleic acid (DNA) content of teat-ligated glands of lactating mice was similar to that of intact glands of normal mice whereas contralateral, non-ligated glands contained more DNA than intact glands(3). In guinea pigs suckling did not prevent involution of teat-ligated glands(4,5), and in mice ribonucleic acid (RNA) content and RNA/DNA ratio were very low in teat-ligated glands in comparison with contralateral, non-ligated glands.

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TABLE I. Deoxyribonucleic Acid Content of Mammary Glands with Ligated (L) and Non-Ligated (NL) Teats Within Lactating Rats.

Lactation day of				DEEET* (mg, mean)		Total DNA/100 g body wt (mg)†		Total DNA (mg)†	
Teat ligation	Sacrifice	No. animals	Body wt (g, mean)	L‡	NL‡	L‡	NL‡	L‡	NL‡
4	12	12	231	327.6	576.8	3.54 ±.10	6.13 ±.13	8.17 ±.34	14.16 ±.43
4	21	12	230	246.9	655.6	2.20 ±.08	6.03 ±.11	5.07 ±.18	13.86 ±.36
12	21	12	220	327.5	619.1	3.26 ±.11	5.91 ±.11	7.15 ±.25	12.98 ±.24

* Dried, ethanol-ether extracted tissue. † Mean and standard error of mean.
‡ One abdominal and 2 inguinal glands.

The present study was undertaken to determine whether or not the suckling stimulus would prevent mammary involution, as indicated by DNA content, and prevent reduction in protein synthesis, as measured by RNA and RNA/DNA ratio, in teat-ligated mammary glands of the rat.

Methods. Teats of one abdominal and 2 inguinal mammary glands of 2 groups of female rats (hooded Norway) were ligated (L) on day 4 of lactation to prevent milk removal. One group of rats was sacrificed on day 12 of lactation (4-12) and one group on day 21 of lactation (4-21). Comparable teats of a third group were ligated on day 12 of lactation and sacrificed on day 21 of lactation (12-21). Suckling of all glands was permitted, and the nucleic acid content, determined as previously reported(6), of teat-ligated glands (L 4-12, L 4-21, L 12-21) was compared with that of contralateral, non-ligated glands (NL 4-12, NL 4-21, NL 12-21). In addition, nucleic acid content of teat-ligated glands and of contralateral, non-ligated glands was compared with that of glands of normal rats sacrificed at comparable stages of lactation(7).

Results. Total mg of DNA per 100 g of body weight of teat-ligated and contralateral, non-ligated glands were 3.54 and 6.13 (4-12), 2.20 and 6.03 (4-21), and 3.26 and 5.91 (12-21). Total mg of DNA were 8.17 and 14.16 (4-12), 5.07 and 13.86 (4-21), and 7.15 and 12.98 (12-21) for teat-ligated and contralateral non-ligated glands, respectively. Teat ligation of groups 4-12, 4-21, and 12-21 decreased DNA per 100 g body weight 42.3, 63.5, and 44.8%, respectively, when compared with contralateral, non-ligated glands; total DNA was decreased similarly (42.3, 63.4, and 44.9%). Total DNA per 100 g of body weight and total DNA of the 3 teat-ligated glands were each multiplied by 2 and each compared with that of 6 corresponding glands of normal rats(7) sacrificed at comparable stages of lactation. Teat-ligated glands declined 36.0, 60.1, and 40.9% in DNA per 100 g of body weight and 38.0, 60.2, and 43.9% in total DNA. Both measures of DNA of teat-ligated glands were similar to those of glands of rats 3 to 12 days after weaning(8). Stage of lactation when teats were ligated had little effect on DNA content, but the longer the period of time after teat ligation the greater was the decline in both measures of DNA.

In groups NL 4-12, NL 4-21, and NL 12-21, total mg of DNA per 100 g of body weight, multiplied by 2, were 10.7, 9.3, and 7.2% higher, respectively, and total mg of DNA, multiplied by 2, were 7.5, 8.8, and 1.9% higher, respectively, than corresponding figures for 6 abdominal-inguinal glands of rats at corresponding stages of lactation (7). Analysis of variance of means of DNA content per 100 g of body weight showed that group NL 4-12 was highly significantly greater ($P<0.01$) than normal glands of rats lactating 12 days, and that groups NL 4-21 and NL 12-21 were significantly greater ($P<0.05$) than normal glands of rats lactating 21 days. Means of total DNA were not significantly different ($P>0.05$) in groups NL 4-12 and NL 12-21 from those of respective glands of normal lactating rats, but the mean of group NL 4-21 was significantly greater ($P<0.05$) than the mean of glands of normal rats lactating 21 days (Table I).

Total mg of RNA were 6.45 and 37.86 (4-12), 3.61 and 45.84 (4-21), and 5.91 and 40.59 (12-21) for teat-ligated and contralateral, non-ligated glands, respectively. Total

TABLE II. Ribonucleic Acid Content of Mammary Glands with Ligated (L) and Non-Ligated (NL) Teats Within Lactating Rats.

Lactation day of				DEEET* (mg, mean)		Total RNA (mg)†		RNA/DNA†	
Teat ligation	Sacrifice	No. animals	Body wt (g, mean)	L‡	NL‡	L‡	NL‡	L‡	NL‡
4	12	12	231	327.6	576.8	6.45 ±.22	37.86 ± 1.29	.79 ±.01	2.67 ±.04
4	21	12	230	246.9	655.6	3.61 ±.16	45.84 ± 1.39	.71 ±.02	3.31 ±.05
12	21	12	220	327.5	619.1	5.91 ±.31	40.59 ± 1.41	.83 ±.02	3.13 ±.09

* Dried, ethanol-ether extracted tissue. † Mean and standard error of mean.
‡ One abdominal and 2 inguinal glands.

RNA of the 3 teat-ligated glands, multiplied by 2, was 80.1 (L 4-12), 90.4 (L 4-21), and 84.3% (L 12-21) less than that of 6 abdominal-inguinal glands of normal rats sacrificed at comparable stages of lactation(7). RNA content of teat-ligated glands corresponded to that of glands involuted 3 to 12 days(8).

Total mg of RNA of the 3 contralateral, non-ligated glands, multiplied by 2, were 16.6 (NL 4-12), 22.1 (NL 4-21), and 8.2% (NL 12-21) higher than corresponding figures for 6 abdominal-inguinal glands of normal rats lactating the same length of time. Means of total mammary RNA of groups NL 4-12 and NL 4-21 were highly significantly greater than ($P<0.01$), but the mean of group NL 12-21 was not significantly different from ($P>0.05$), means of similar glands of normal rats sacrificed at comparable stages of lactation (Table II).

Average RNA/DNA ratios were 0.79 and 2.67 (4-12), 0.71 and 3.31 (4-21), and 0.83 and 3.13 (12-21) for teat-ligated and contralateral, non-ligated glands, respectively. RNA/DNA ratios of teat-ligated glands were 68.0 (L 4-12), 75.9 (L 4-21), and 71.9% (L 12-21) less than those of glands from normal rats lactating the same number of days. The protein synthetic activity per cell in glands with ligated teats corresponded to that found in glands involuted 3 to 12 days (8). RNA/DNA ratios of glands of groups NL 4-12 and NL 4-21 were highly significantly greater than ($P<0.01$), but the ratio of group NL 12-21 was not significantly different from ($P>0.05$), ratios of similar glands of normal rats sacrificed at comparable stages of lactation (Table II).

Discussion. The DNA content of teat-ligated glands of lactating rats was similar to that of glands of lactating rats 3 to 12 days after weaning. Hence, the suckling stimulus without milk removal did not prevent loss of mammary cells. The observations agree with those made in guinea pigs(4,5) but not in mice(3) in which DNA content of teat-ligated glands was similar to that of non-ligated glands. It should be noted that a significant decrease in mammary DNA occurred in the lactating mouse after day 14 of lactation(3), whereas a significant decrease did not occur in the lactating rat until after day 24 of lactation(7). Perhaps if a greater number of young per mother had been used it might have been possible to maintain cellular development in glands of normal lactating mice. Moreover, in mice and rats the DNA content of 3 contralateral, non-ligated glands was higher than that of corresponding glands of normal animals at comparable stages of lactation, which suggests that when the number of young per functional teat increased the number of mammary cells increased.

Total RNA and RNA/DNA ratios were lower in teat-ligated glands than in contralateral, non-ligated glands, which confirms results obtained in mice(3). It is suggested that suckling will not maintain protein synthesis unless secretory products are frequently removed. RNA content, multiplied by 2, and RNA/DNA ratio of 3 glands contralateral to teat-ligated glands were greater than those of 6 intact glands of normal lactating rats. These results are similar to those reported for mice(3). The probable reasons for increased protein-synthesizing activity of contralateral, non-ligated glands are that milk precursors are available to these glands in greater amounts and that there are more young per gland to remove increased amounts of milk.

Summary. Teat ligation of 3 abdominal-inguinal mammary glands of lactating rats markedly decreased DNA and RNA content and RNA/DNA ratio when compared with similar estimates of either contralateral, non-ligated glands or glands from normal rats lactating the same length of time. Suckling stimulus, without milk removal, did not prevent cellular loss and did not maintain level of protein synthesis. DNA and RNA content and RNA/DNA ratio of contralateral, non-ligated glands were greater than those of glands of normal rats lactating comparable periods of time.

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Relationship Between Fluoride Deposition and Metastatic Calcification in Soft Tissues of Rat and Guinea Pig. (28472)

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The relationship, if any, between fluoride metabolism and the deposition of fluoride in soft tissues of the rat is not known. In fact, only recently has it been recognized that fluoride may be deposited in soft tissue, it being thought previously that ingested fluoride was either retained in the skeleton (and teeth to a much lesser degree) or excreted. Several factors are now recognized which may influence fluoride deposited in the soft tissues, among which are degree of skeletal fluoride saturation(1,2), dietary fat level(3,4), age of the animal(2), dietary ascorbic acid content(5), and presence or absence of dietary molybdenum(6). However, little information is available concerning the mechanism by which this deposition of fluoride occurs. It seemed reasonable that soft tissue-retained fluoride may be deposited as calcium fluoride or a calcium fluoride-complex of some nature. These studies concern the relationship between the deposition of calcium, phosphorus, and fluoride in the soft tissues of albino rats and guinea pigs.

Methods. In Series I, 40 Sprague-Dawley strain albino rats from our breeding colony and ranging in age from 47 to 55 days were divided equally into 4 groups according to sex and body weight. The animals were housed in pairs in raised wire screen cages in an air-conditioned room and received redistilled fluoride-low water (F = 0.01 μg/ml) *ad libitum*. The animals in Groups A and B received a low fluoride stock corn diet* (F = 0.08 μg/g) while those animals in Groups C and D received the same diet plus 2% $CaCO_3$, 1% NaH_2PO_4, and 0.1% granular calciferol (850,000 units/gram) added at the expense of yellow corn meal. In addition, one-half of the animals in Groups B and D received 1.0 mg of fluoride (as aqueous NaF in 1.0 ml of solution) daily by stomach tube. After 30 days, all animals were sacrificed by ether inhalation and the hearts, kidneys, and livers removed and prepared for fluoride(6,7), calcium(8), and phosphorus(9) analyses. Each tissue was divided into approximate halves, one of which was placed in a 30 ml porcelain evaporating dish for calcium and phosphorus analysis and the other in a platinum crucible for fluoride analysis. Both portions were dried to a constant weight by means of a

* Composition of the diet (in %) was as follows: yellow corn meal, 64.0; powdered whole milk, 30.0; alfalfa meal, 4.8; iodized salt, 1.0; and irradiated yeast, 0.2.