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Nucleic Acid Estimates of Mammary Tissue and Nuclei.*† (27878)

H. ALLEN TUCKER AND RALPH P. REECE
Department of Dairy Science, New Jersey Agricultural Experiment Station, New Brunswick

The use of deoxyribonucleic acid (DNA) content as a measure of cell numbers was first suggested by Davidson and Leslie(1). DNA and ribonucleic acid (RNA) content of mammary tissue have been used, respectively(2,3), as indices of growth and of metabolic activity. DNA as an index of growth is valid only when DNA content per nucleus remains constant(1). DNA per nucleus was found to be constant in mammary and pooled kidney and spleen tissue during pregnancy but at parturition it declined sharply and it was not until late lactation that it approached normal(4). Folley(5) recently expressed the opinion that further work was necessary to establish the validity of DNA content as a measure of mammary development.

Experiments were designed to determine: (a) the applicability of a modified Schmidt and Thannhauser(6) procedure for nucleic acid extraction from mammary glands of rats, and (b) the DNA content per mammary cell nucleus during pregnancy and lactation.

Methods. Pregnant and lactating primiparous hooded Norway rats were used. Day 1 of pregnancy was the day sperm were noted in vaginal smears, and day 1 of lactation was the day of parturition. Litter size was adjusted to 8 pups per mother. To develop a nucleic acid procedure, rats pregnant 14, 18, or 20 days and rats lactating 12 or 14 days were used. In the DNA per nucleus experiment rats pregnant 12 or 20 days and rats lactating 1, 4, 8, 14, or 21 days were used.

Animals were sacrificed by cervical dislocation, and 6 abdominal-inguinal mammary glands were removed and washed quickly in ice-cold sucrose-bicarbonate buffer(7). After freezing on Dry Ice, glands were stored at –17°C until analyzed.

Tissue for nucleic acid determinations was defatted in a Bailey-Walker fat extraction apparatus, using 95% ethanol for 10 hours followed by anhydrous ether for 14 hours. After drying (6 hours at 37°C), the dried, ethanol-ether extracted tissue (DEEET) was weighed, frozen, and ground to a fine powder in a Wiley mill. Duplicate samples of 25 mg were extracted once with 5 ml of ethanol:chloroform (3:1, v:v). Samples were extracted twice with 5 ml of ice-cold 10% trichloroacetic acid (TCA), which was removed by washing the residue with ice-cold ethanol. The residue was digested in 3 ml of 1N KOH for 15 hours at 37°C. The digest was acidified with 0.4 ml ice-cold 6N HCl and 3.4 ml ice-cold 10% perchloric acid (PCA). The precipitate was washed twice with ice-cold 5% PCA. The combined supernatants were analyzed for ribose (RNA) by the colorimetric orcinol procedure(8). Mammary gland RNA content was calculated from a standard curve of highly purified yeast RNA.‡ DNA was extracted from

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the precipitate with 5 ml of 5% PCA at 70°C for 15 minutes. After washing the precipitate twice with 5% PCA, the combined supernatants were read directly in a Beckman DU spectrophotometer at 268 mμ. Highly polymerized DNA (Nutritional Biochemicals Corp.) was used as the standard.

Loss of DNA into the ice-cold TCA fraction and RNA fraction was determined by the colorimetric diphenylamine test of Dische (9) and the colorimetric indole test of Ceriotti(10). RNA contamination of the DNA fraction was estimated from its ribose content(8).

Optimum acid hydrolytic time and temperature required to completely extract DNA and simultaneously minimize interfering protein contamination were estimated from 4 duplicate samples of 6 different mammary glands, each duplicate being hydrolyzed with 5% PCA for 5, 10, or 15 minutes at 70°C or for 15 minutes at 90°C. DNA was determined from the purine-pyrimidine absorption maximum (268 mμ) and from the deoxypentose-diphenylamine absorption maximum (605 mμ). Protein in these DNA fractions was estimated(11), using a bovine serum albumen standard (Armour).

Nuclei were isolated in a cold room (0-3°C) from frozen mammary tissue which was homogenized for 1.5 minutes in a Waring Blendor containing 100 ml of 0.25M sucrose-1% citric acid. The homogenate was filtered through 2 and then 4 layers of #60 grade cheesecloth. Two 35-ml samples were removed and centrifuged at 1050 × *g* for 30 minutes. The sediment was washed 3 successive times with 25 ml of sucrose-citric acid; centrifuging times were 15, 10, and 10 minutes, and forces were 850, 670, and 600 × *g*, respectively. The final pellet was suspended in 1.5 ml of sucrose-citric acid. A 0.5-ml sample for counting in a hemocytometer was removed and placed in a 3-ml silicone-coated test tube, to which was added 1.5 ml of a 0.1% solution of polyoxyethylene sorbitan monooleate (Tween 80) and 0.2 ml of 0.1% crystal violet in 0.1M citric acid. To estimate DNA content of nuclei, the remaining 1 ml was defatted with 5 ml of ethanol for at least 24 hours at 5°C. The residue was reextracted at room temperature with ethanol and twice extracted with ether. This defatted residue was then extracted with ethanol-chloroform, and the DNA content was determined as previously described.

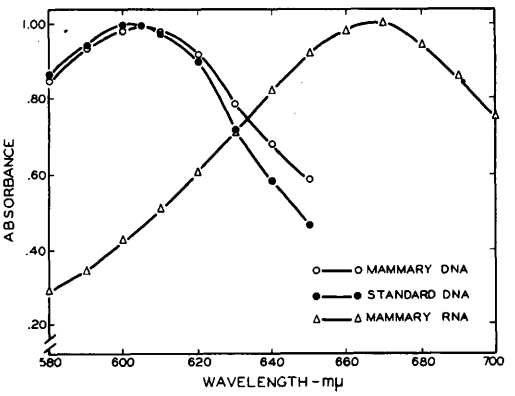

FIG. 1. Diphenylamine absorption curves of mammary DNA, standard DNA, and mammary RNA. Curves based on absorbance of 1.00 at the absorption maxima.

Results and discussion. Measurable lipid material was removed up to 16 hours; extraction times of 10 and 14 hours with alcohol and ether, respectively, were used as a safeguard against lipid contamination. Ethanol: chloroform treatment further increased the amount of fat extracted.

DNA tests with diphenylamine and indole were negative when applied to the cold TCA fraction.

Contamination of the RNA fraction with DNA, using the diphenylamine test, was not observed (Fig. 1). The material reacting with diphenylamine was not DNA because of a bathochromic shift from the normal spectra of standard DNA and mammary gland DNA. Since it is well known, however, that the diphenylamine test lacks sensitivity and several substances interfere with the test, the indole procedure was used to check further the RNA fraction for DNA. Various hydrolyzing times, alkali strengths, and types of alkali were employed. In every instance an absorbance reading was obtained at the absorption maximum (492 mμ); it was, however, either on the lower limit or below the known sensitivity of the test. The indole test also has disadvantages. Using

TABLE I. Influence of Acid Hydrolytic Time and Temperature on DNA and Protein Content of Mammary Glands of Pregnant and of Lactating Rats.

Hydrolytic condition	Reproductive state	DNA (μg/mg DEEET)* 268 mμ†	DNA (μg/mg DEEET)* diphenylamine†	% difference	Protein (μg/mg DEEET)†
5 min – 70°C	Pregnant	30.6	19.4	36.6	5.7
	Lactating	21.6	13.3	37.6	5.5
10 " – 70°C	Pregnant	37.7	29.9	20.7	6.1
	Lactating	25.0	19.4	22.4	6.1
15 " – 70°C	Pregnant	38.8	35.4	8.8	5.5
	Lactating	27.1	24.9	8.1	4.8
15 " – 90°C	Pregnant	42.5	31.0	27.1	8.4
	Lactating	29.8	21.0	29.5	8.9

* Dried, ethanol-ether extracted tissue.
† Mean.

standard DNA, very large day-to-day differences were observed in standard curves, and in addition, pure yeast RNA gave a slight response. It was concluded there was little, if any, DNA contamination of RNA.

RNA contamination of the DNA fraction was at all times below the known sensitivity of the orcinol test for ribose.

Results with the indole method were much too variable; the diphenylamine test lacked sensitivity and serious difficulties arose when the latter method was applied to mammary tissue(12). Therefore, DNA was usually determined by absorption at 268 mμ, but optimum acid hydrolysis of the mammary DNA fraction was determined using both 268 mμ absorption and the diphenylamine method. The 268 mμ-DNA increased with each increase in time or temperature, whereas diphenylamine-DNA increased up to the 15 minutes-70°C condition after which increased temperatures caused destruction of a portion of the deoxypentose component of DNA. Optimum temperature-time condition chosen was 15 minutes-70°C, since at this point DNA was not being destroyed, 268 mμ-DNA and diphenylamine-DNA contents agreed most closely, and protein contamination was least. Since protein contamination and percent difference between the two estimates of mammary DNA were strikingly constant among glands within treatment, they can be disregarded (Table I).

Average DNA content per nucleus is given in Table II. No significant differences ($P > 0.40$) existed in mean DNA per nucleus among glands from rats in various stages of pregnancy or lactation. These results are opposed to those of Griffith and Turner(4) and indicate that DNA content may be used directly as a measure of mammary cell numbers, using the nucleic acid extraction procedure described.

TABLE II. Mean DNA per Nucleus of Rat Mammary Gland During Pregnancy and Lactation.

Reproductive state	No. of duplicate determinations	DNA (picograms/nucleus) mean ± S.E.*
Pregnant 12 days	6†	9.19 ± .44
" 20 "	6	8.47 ± .35
Lactating 1 day	8	8.33 ± .24
" 4 "	6	8.57 ± .22
" 8 "	6	8.66 ± .24
" 14 "	6	8.49 ± .16
" 21 "	6	8.53 ± .25

* No signif. difference among means ($P > 0.40$).
† Determinations on 12-day pregnant group based on 2 animals per duplicate determination; all others on one animal.

Summary. Various biochemical determinations showed that a modified Schmidt and Thannhauser nucleic acid procedure was applicable for extraction of nucleic acids from rat mammary tissue. Since DNA per mammary cell nucleus was not found to be significantly different in glands from either pregnant or lactating rats, DNA content of mammary tissue may be used directly as an index of development.

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Differential Extraction of Lymphocytic and Reticulum Cell Fractions from Thymus.* (27879)

AILENE HERRANEN AND WILLA K. BRUNKHORST (Introduced by R. I. Dorfman)
Worcester Foundation for Experimental Biology, Shrewsbury, Mass.

Most fractionation schemes place little emphasis on quantitative aspects of tissue composition. A procedure for qualitative and quantitative extraction of thymus has been reported(1). The method does not, however, distinguish between lymphocytic and reticulum cells, the 2 predominant cell types in thymus. Since these 2 cells differ in their sensitivity to hormone treatment and irradiation(2,3) a fractionation procedure which differentiates between lymphocytic and reticular components is desirable. The following report describes methods which are developed for this purpose. One of the procedures permits separation of cytoplasmic and nuclear components from lymphocytes. A subsequent paper(4) concerns the effect of ionizing radiation and cortisol treatment on the fractions obtained by these methods.

Methods. Calf thymus or thymuses from male rabbits, 3 to 4 months old, were used. The tissue was chilled and immediately fractionated by one of the methods described below. All operations were carried out at 3-4°C. The discussion stresses the data obtained from rabbit thymus since the results can be expressed more reliably as yield per thymus. Some variation in the quantity of fractions was observed. These differences could be correlated with the condition of the thymus in the experimental animals and did not alter interpretation of the data.

A. Method I. The procedure is based on sequential extraction. Thymus was minced, put through an onion press and stirred in water for 10 minutes (10 ml/g wet weight). The supernatant after centrifugation at 8500 $\times$ *g* for 4 minutes was designated as the water soluble Fraction A. The sediment from the preceding step was stirred for 10 minutes in a volume of 0.3 M NaCl equal to the volume of the sediment. The supernatant after centrifugation at 8500 $\times$ *g* for 4 minutes was called Fraction B. Re-extraction of the sediment with 0.15 M NaCl yielded a small amount of saline soluble material with the same characteristics as those of Fraction B. Finally, the sediment from the third step was homogenized in 0.15 M NaCl in a Waring blendor at top speed for 1 minute. The resulting brei was centrifuged at 8500 $\times$ *g* for 4 minutes. Homogenization was repeated 4 times. The combined 8500 $\times$ *g* supernatants were designated as Fraction D. The 8500 $\times$ *g* supernatants from each fraction were centrifuged at 23,000 $\times$ *g* for 1 hour to obtain the corresponding supernatant and sediment fractions. The insoluble ma-

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