Studies on the Particulate Components of Rat Mammary Gland

4. THE DISTRIBUTION OF RIBONUCLEIC ACID*

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Numerous investigations in the past few years have emphasized the relationship between the ribonucleic acid (RNA) content of a tissue and the rate of protein synthesis in that tissue (see Brachet, 1955). Unlike deoxyribonucleic acid (DNA), RNA is not localized in any one specific intracellular site, but is found to a greater or less extent in the nuclear, the mitochondrial, the microsomal and the soluble fractions of the cell.

Earlier studies on the mammary gland (Greenbaum & Slater, 1957*a, b*) have shown that the rapid increase in protein synthesis which occurs at parturition is accompanied by a similar change in the RNA content of the tissue. It was therefore of interest to determine the distribution of RNA in the mammary gland at various stages in the lactation cycle, to see if the increased content of RNA represents a generalized increase in the RNA content of all the separate intracellular fractions, or is the result of an outstanding increase in one particular intracellular fraction. This paper describes the intracellular distribution of RNA in the rat mammary gland at three stages in the lactation cycle: at the end of pregnancy, on the third day of lactation, and on the eighteenth day of lactation.

METHODS

Adult, female albino rats undergoing their first pregnancy or lactation were used in this investigation. For the lactating groups only animals with a litter of six to nine were used. The animals weighed approx. 200 g. For the late-pregnancy group, animals were selected whose appearance indicated a proximity to parturition. The length of the pregnancy was assessed more accurately by examination of the fetuses. These examinations indicated that of the four animals used in this group, one was on the eighteenth day, two were on the nineteenth day and one was on the twentieth day of pregnancy.

Animals were killed by dislocation of the cervical vertebrae and the lower abdominal glands were quickly dissected and placed in ice-cold isotonic sucrose (0.25M). Tissue suspensions (1:10) of mammary gland were prepared as described previously (Greenbaum & Slater, 1957*a*); the time of homogenization was kept constant for each.

The tissue suspensions in 0.25M-sucrose (1:10) were separated into five fractions by differential centrifuging. The procedure was as follows.

(a) 'Nuclear' fraction. This fraction (N) was obtained by centrifuging the homogenate at 2000 rev./min. for 10 min. at 0° in a MSE Angle 13 refrigerated centrifuge. The sediment was resuspended in 10 ml. of cold sucrose and respun under the same conditions. A pipette with a bent tip was used for separating the supernatant from the sediment in this and all other cases. The two supernatant suspensions were mixed and used for the isolation of the 'large-particle' fraction.

(b) 'Large-particle' fraction (M). The supernatant suspension from (a) was spun at 12 000 rev./min. for 15 min. in a MSE Angle 13 centrifuge. The sediment was resuspended in 10 ml. of cold sucrose and resedimented at the same speed. The centrifuge constants for this stage were (de Duve & Berthet, 1953):

$$r_{(\min.)} = 7.5 \text{ cm.}; \quad r_{(\max.)} = 12.5 \text{ cm.}; \\ \text{temp., } 0^\circ; \quad g_{(\max.)} = 14\,500.$$

(c) 'Intermediate-particle' fraction (I). The supernatant from (b) was spun at 13 000 rev./min. for 45 min. in a MSE Angle 13 centrifuge. The constants for this stage were:

$$r_{(\min.)} = 6.5 \text{ cm.}; \quad r_{(\max.)} = 12.5 \text{ cm.}; \\ \text{temp., } 0^\circ; \quad g_{(\max.)} = 17\,000.$$

(d) 'Small-particle' fraction (U). A volume (26 ml.) of the supernatant suspension from (c) was spun in a Spinco preparative centrifuge model L at 40 000 rev./min. for 30 min. The centrifuge constants for this stage were:

$$r_{(\min.)} = 3.8 \text{ cm.}; \quad r_{(\max.)} = 8.1 \text{ cm.}; \\ \text{temp., } 0^\circ; \quad g_{(\max.)} = 144\,700.$$

(e) 'Soluble' fraction. The supernatant solution from (d) is referred to as the 'soluble' fraction (S).

Fractions (N), (M) and (I) were finally suspended in 10 ml. of water; fraction (U) was suspended in 5 ml. of water.

Each fraction was analysed for total protein by the method of Lowry, Rosebrough, Farr & Randall (1951), ox-serum albumin being used as standard. The total protein recovered in the separate intracellular fractions was approx. 90% of the whole homogenate value. This low recovery probably results from the high fat content of the homogenate, which produces elevated extinction values owing to opalescence. This fat is to a large extent removed by solidification during the first centrifuging at 0°. The percentage of protein in the various fractions is therefore expressed in Table 1 as the percentage of the total protein recovered in the five fractions, and not as a percentage of the whole homogenate value. Lactose was estimated in the

* Part 3: Greenbaum & Slater (1957*c*).

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homogenates as described by Greenbaum & Slater (1957*a*). RNA was determined by a modification (Greenbaum & Slater, 1957*b*) of the procedure of Schneider (1945). The sample was treated with trichloroacetic acid (final concentration, 10%, w/v) in the cold to precipitate proteins and nucleic acids. The sediment was then carefully washed once with 5% (w/v) cold trichloroacetic acid and three times with cold 1% trichloroacetic acid. During each wash the sediment was well dispersed with a stirring rod. The nucleic acids were then extracted with two successive treatments of 5 ml. of 5% trichloroacetic acid at 90° for 15 min. and RNA was estimated on the combined extracts. In this investigation, measurements were made both at 520 and 670 m μ . The few extracts giving a low E_{670}/E_{520} ratio were corrected for the presence of sucrose as described by Slater (1958). That the method of washing used in the RNA estimations successfully eliminated sucrose was indicated by high ratios of E_{670}/E_{520} (see Slater, 1958). The recoveries of RNA in the five fractions totalled $96 \pm 4\%$ of the homogenate values.

As with the total protein results, the percentage of RNA sedimented in the various fractions is expressed with respect to the total 'recovered' in the five fractions, and not with respect to the whole-homogenate values. Results have been calculated in terms of ribonucleic acid phosphorus (RNA-P).

RESULTS

Results of the investigation are shown in Table 1. It can be seen that, as found earlier (Greenbaum & Slater, 1957*b*), the RNA-P content of the mammary gland increases sharply at parturition and increases further as lactation proceeds. Although this increase is in part a reflexion of a generalized increase in all the intracellular fractions, the increase in the total amount of the 'intermediate-particle' fraction RNA-P is much greater than the rise in any other fractions. Similarly, with total protein the most extensive change occurs in the 'intermediate-particle' fraction.

The 'specific concentration' of RNA-P (i.e. the RNA-P content of a fraction divided by the total protein content of that fraction) is also shown in Table 1. It is greatest in the 'small-particle' fraction, although the 'nuclear' fraction shows the greatest increase from late pregnancy to late lactation.

DISCUSSION

Before discussing the significance and interpretation of the results obtained in this investigation, the nature of the fractions isolated and the efficiencies of the procedures used for estimating protein and RNA-P will be considered.

Composition of the fractions isolated

The fraction spun down at approximately 6000 g min. in isotonic sucrose is conventionally termed the 'nuclear' fraction. In this investigation, however, owing to the difficulties of homogenizing mammary gland tissue, cell nuclei are to a

Table 1. *Distribution of total protein and of ribonucleic acid in the rat mammary gland*

All values quoted in this table are means obtained from four animals ($\pm$ s.e.m.). For preparation of fractions N, M, I, U and S see the Methods section. H represents the whole homogenate.

State of gland	Total protein in H (g./100 g. of wet tissue)	Total protein in fraction (%)					Total RNA-P in H (mg./100 g. of wet tissue)	RNA-P in fraction (%)					RNA-P/total protein ($\frac{\text{mg. of RNA-P/100 g. of wet tissue}}{\text{g. of total protein/100 g. of wet tissue}}$)					
		N	M	I	U	S		N	M	I	U	S	H	N	M	I	U	S
Late pregnancy	6.4 $\pm$ 1.1	6.5 $\pm$ 1.1	14.9 $\pm$ 2.1	4.8 $\pm$ 0.4	7.0 $\pm$ 0.9	66.7 $\pm$ 1.4	6.4 $\pm$ 1.5	4.5 $\pm$ 0.7	11.0 $\pm$ 0.5	11.0 $\pm$ 2.1	21.5 $\pm$ 3.9	51.7 $\pm$ 6.9	1.02 $\pm$ 0.17	0.75 $\pm$ 0.25	1.06 $\pm$ 0.33	1.42 $\pm$ 0.49	4.53 $\pm$ 1.54	0.91 $\pm$ 0.14
Lactating (3 days)	12.3 $\pm$ 1.2	4.9 $\pm$ 0.5	12.4 $\pm$ 0.6	7.2 $\pm$ 0.6	7.4 $\pm$ 0.4	68.5 $\pm$ 1.8	20.7 $\pm$ 3.4	4.5 $\pm$ 0.7	14.5 $\pm$ 2.1	21.5 $\pm$ 1.7	27.0 $\pm$ 3.0	31.7 $\pm$ 2.4	1.44 $\pm$ 0.24	1.11 $\pm$ 0.01	1.47 $\pm$ 0.26	3.33 $\pm$ 1.00	4.20 $\pm$ 1.20	0.58 $\pm$ 0.13
Lactating (18 days)	14.1 $\pm$ 0.5	5.0 $\pm$ 0.6	10.6 $\pm$ 0.7	9.0 $\pm$ 0.1	9.0 $\pm$ 0.6	66.2 $\pm$ 1.2	47.2 $\pm$ 3.9	5.5 $\pm$ 0.7	11.7 $\pm$ 0.8	24.5 $\pm$ 1.7	30.0 $\pm$ 1.8	27.7 $\pm$ 2.0	33.3 $\pm$ 1.20	4.33 $\pm$ 1.01	4.10 $\pm$ 0.52	4.10 $\pm$ 0.44	12.08 $\pm$ 0.75	1.29 $\pm$ 0.17

certain extent ruptured during homogenization, with a consequent loss of contents. Further, there is much greater content of cell debris in a mammary-tissue suspension than is usual for liver and kidney suspensions. The 'nuclear' fraction in this investigation cannot therefore be considered to represent the nuclei as a class of intracellular particle, but rather is composed of a mixture of unbroken nuclei and a large amount of connective tissue and other cell debris. It is likely, by consideration of the centrifuging technique used, that some unbroken nuclei remain in the supernatant fraction after removal of the 'nuclear' fraction and are subsequently sedimented in the 'large-particle' fraction at 200 000 g min. This would be expected to increase the percentage of total protein found associated with the 'large-particle' fraction. The 'large-particle' fraction was in fact found to possess some DNA-P, but the extent to which this is the result of adsorption of DNA-P liberated from ruptured nuclei or of the sedimentation of whole unbroken nuclei is not known. As the total protein content of the 'nuclear' fraction is only some 5% of the total homogenate value (see Table 1), the error introduced into the total protein content of the 'large-particle' fraction by the presence of some 10% of the 'nuclear' fraction will be very small.

The 'large-particle' fraction contains the greater part of the mitochondrial fraction in the mammary gland. This fraction in rat mammary tissue covers a wider range of size than in liver: sedimentation of cytochrome oxidase activity in mammary gland increases almost linearly with increasing g min. such that at 200 000 g min. only some 60% of the total homogenate activity is sedimented.

The 'intermediate-particle' fraction contains the remnants of the particles containing cytochrome oxidase and succinic oxidase plus, presumably, the larger members of the microsomal class, if one defines a microsome as a particle rich in RNA relative to protein. It is not possible to classify microsomal material on a glucose 6-phosphatase basis as is done in the liver (Hers, Berthet, Berthet & de Duve, 1951); rat mammary gland tissue possesses no detectable glucose 6-phosphatase activity.

In contrast with the liver, the mammary gland 'soluble' fraction is not largely composed of cell sap, and largely represents the milk retained in the ducts and alveoli after death. This retained fluid is rich in protein (Greenbaum & Slater, 1957*a*) so that direct usage of total soluble-protein values (and also homogenate-protein values) is pointless in calculating RNA-P:protein ratios. Such ratios can, however, be calculated approximately by estimating the percentage of milk retention and by using the percentage of protein in retained fluid estimated earlier (Greenbaum & Slater, 1957*a*).

Such corrections are only very approximate as the percentage of protein in retained fluid is not strictly constant for each stage of the lactation cycle, but varies rather widely according to such factors as the season, the diet and the size of the litter.

Distribution of ribonucleic acid phosphorus

The changes in homogenate RNA-P are similar to those previously reported by Greenbaum & Slater (1957*b*). The RNA-P values are lower than theirs, but this is probably the result of seasonal variations in gland composition. All the observations reported in this investigation were made in a period of 1 month, whereas those of Greenbaum & Slater (1957*b*) were spread throughout the whole year. The late-pregnancy group values are much lower than previously found by Greenbaum & Slater but, as mentioned in the Methods section, animals used varied between the eighteenth and twentieth day of pregnancy; it is known that the composition of the gland changes rapidly at the end of pregnancy.

The general pattern for the distribution of RNA-P is closely similar to that described for liver, kidney and other tissues (see Hogeboom & Schneider, 1955). The bulk of the cell RNA-P is in the 'small-particle' and 'soluble' fractions. There are, however, very significant alterations in this generalized pattern between the various stages of lactation studied.

In late pregnancy, for instance, 50% of the total RNA-P is in the 'soluble' fraction. This is much higher than the 16% reported for the soluble fraction of mouse liver (Schneider, Hogeboom & Ross, 1950). This high value (51.7% on the twentieth day of pregnancy) falls rapidly and significantly ($P < 0.05$) over parturition and throughout lactation to some 28% on the eighteenth day of lactation. At the same time the percentage of RNA-P in the 'small' and 'intermediate-particle' fractions is rising, although the percentage of RNA-P in the 'nuclear' and 'large-particle' fractions is remarkably uniform. The percentage of RNA-P in the 'intermediate-particle' fraction is more than doubled from late pregnancy to late lactation ($P < 0.01$). The results therefore show that as lactation advances and synthesis of milk rises towards its maximum, the distribution of RNA-P in the mammary gland cells changes, the emphasis moving from the 'soluble' to the 'small' and 'intermediate' particulate fractions. (These last two fractions may be termed the smaller-particle fractions of the cell.)

Distribution of protein

Table 1 shows that the percentages of total protein sedimented in the various fractions show little change over the lactation cycle, with the

exception of the 'intermediate-particle' fraction which shows an increase of almost 100% from late pregnancy to late lactation ($P < 0.02$); the other values show little change in spite of a large increase in total protein.

These values are, however, based on the total protein of the homogenate, which, as previously mentioned, includes the protein of the retained fluid. Considerable changes in the intracellular fractions may therefore be hidden by the very high protein contribution of the retained fluid. The protein sedimented in the 'large', 'intermediate' and 'small-particle' fractions has therefore been combined as 'particulate protein', and the percentage of this total value sedimented in each of the three subfractions contributing to it has been calculated. The values are shown in Table 2. It can be seen that considerable alterations occur in the distribution of protein among the intracellular particulate fractions over the lactation cycle. As protein synthesis commences on a large scale at parturition and rises to a peak towards the end of lactation, there is an increase in the relative concentration of protein material in the smaller particulate fractions of the cell. Since the total amount of 'large-particle' material increases over parturition and throughout lactation (Greenbaum & Slater, 1957*b*), results in Table 2 imply that the smaller-particle fractions increase even more rapidly over the lactation cycle.

Ratio of ribonucleic acid phosphorus:protein

The ratio RNA-P:total protein for the various fractions is shown in Table 1. The threefold increase in this ratio for the whole homogenates is again distorted by the inclusion of retained fluid protein. This objection does not, however, apply to the particulate fractions, where it can be seen that RNA-P is highly concentrated relative to protein in the 'intermediate-particle' and 'small-particle' fractions. In the 'small-particle' fraction the ratio in late lactation is similar to that obtained by Keller, Zamecnik & Loftfield (1954) for rat-liver microsomes.

By the use of values for milk composition previously published (Greenbaum & Slater, 1957*a*), the distribution of protein in the various fractions and the ratio of RNA-P:total protein for the various fractions have been recalculated on a milk-free-tissue basis. The results are shown in Table 3. As mean values for milk composition and percentage milk retention have been used no standard errors can be calculated. It can be seen that the distribution of total protein is not substantially altered by the correction for milk. The percentage of protein in the 'soluble' fraction is naturally decreased with a consequent increase in the percentage of total protein associated with the particulate fractions. The general pattern of distribution is, however, unchanged.

Table 2. *Distribution of protein in the 'large', 'intermediate', and 'small-particle' fractions of rat mammary gland*

All values quoted are means obtained from four animals ($\pm$ S.E.M.).

State of gland	Protein sedimented in each fraction (% of total protein in M+I+U)		
	'Large-particle' fraction (M)	'Intermediate- particle' fraction (I)	'Small-particle' fraction (U)
Late pregnancy	55.4 $\pm$ 3.1	18.5 $\pm$ 2.6	25.9 $\pm$ 2.6
Lactation (3 days)	45.9 $\pm$ 0.9	26.8 $\pm$ 1.7	28.1 $\pm$ 0.8
Lactation (18 days)	38.8 $\pm$ 2.0	30.9 $\pm$ 1.0	30.6 $\pm$ 1.2

Table 3. *Distribution of total protein and the value of ribonucleic acid phosphorus:total protein in milk-free fractions of rat mammary gland*

For preparation of fractions N, M, I, U and S see Methods section.

State of gland	Distribution of protein (% of total protein)					RNA-P:total protein (mg. of RNA-P/100 g. of milk-free tissue) (g. of total protein/100 g. of milk-free tissue)	
	N	M	I	U	S	H	S
Late pregnancy	9	22	7	9	52	1.5	1.6
Lactating (3 days)	7	15	9	9	60	2.0	1.5
Lactating (18 days)	7	16	13	13	50	5.2	3.1

The ratio of RNA-P:total protein, on the other hand, shows an increase in both the homogenate and 'soluble' fraction; this results from the lowering in the total protein value used in this calculation as a consequence of the removal of milk protein.

SUMMARY

1. The distribution of total protein and ribonucleic acid phosphorus in five fractions of the rat mammary gland have been determined at three stages of the lactation cycle: on the twentieth day of pregnancy and on the third and eighteenth days of lactation.

2. At the end of pregnancy, ribonucleic acid phosphorus is largely localized in the 'soluble' fraction of the mammary gland. Lactation produces a redistribution of total ribonucleic acid phosphorus such that the majority of it is found in the smaller particulate fractions of the tissue suspension.

3. Lactation produces a relative increase in the amount of protein associated with the smaller particulate fractions at the expense of the 'large-particle' fraction. All these fractions, however, show an increase in total amount from late pregnancy to late lactation.

4. The ratio of ribonucleic acid phosphorus to total protein is low in all fractions in late pregnancy

but has risen to a high value in the 'small-particle' fraction by the end of lactation.

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The Excretion of Urea, Ammonia and Purine End-products by the Newborn Animal

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It is usually taught that mammals other than primates, and insects except the Diptera, excrete 80–100 % of the uric acid they form only after oxidizing it to allantoin. In dogs in particular, apart from the Dalmatian coach-hound, it would seem that the amount of uric acid in the urine is normally minute, and that, even after injecting the acid, 98 % of it enters the urine as allantoin (Best & Taylor, 1955; Evans, 1956). The uric acid excreted by the Dalmatian coach-hound, once thought to be due to a purine metabolism resembling that of primates, is now attributed to there being no mechanism in the renal tubules for reabsorbing uric acid (Friedman & Byers, 1948; Miller, Danzig & Talbott, 1951). Yet Madsen, Earle, Heemstra & Miller (1944) reported finding uric acid infarcts in the kidneys of newborn piglets suffering from a

disease which they described as 'acute uremia'. Morrill (1952a, b) found accumulations of uric acid crystals in the kidneys of piglets which appear to have had a similar if not identical disease.

In another connexion it was noticed in this Department that in newborn puppies urea might form less than 30 % of the total N excreted. The turnover of protein in the body has not been so well explored in the newborn period as in adult life, and the information which can be obtained about cellular metabolism from a study of the excretion of the end-products of nitrogenous metabolism in the urine made it seem desirable to examine the excretion of the important end-products, uric acid and allantoin, and also that of urea and ammonia by newborn mammals. Human infants, piglets and puppies were chosen for the investigation.