Variation of 5'-Nucleotidase Activity of the Rat Mammary Gland during Pregnancy, Lactation and Involution

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The increase in synthetic activity at parturition is paralleled by an increase in RNA and DNA content of the rat mammary gland (Greenbaum & Slater, 1957*a*; McLean, 1958*a*) and therefore involves nucleotides both as coenzymes in the synthesis of milk components and as nucleic acid precursors. At the same time, there are increases in the concentrations and total amounts of acid-soluble ribonucleotides in mammary tissue (Wang & Greenbaum, 1960) and milk (Denamur, Fauconneau & Guntz, 1958), and there is an increased rate of synthesis (Wang & Greenbaum, 1962). Although the higher concentrations could be due to increased synthesis, these might also be partly due to a decrease in catabolic activity. Therefore the activities of the 5'-nucleotidases over the lactation cycle have been examined.

METHODS

Animals. Adult female black-and-white Norwegian hooded rats of the Medical Research Council strain were used and the number in the litter was restricted to six to eight in the lactating group. The length of pregnancy was ascertained from the mating date and averages 22 days for this colony. Animals were taken at three stages of pregnancy: early pregnant (5 days), mid pregnant (10 days) and late pregnant (17–19 days). For lactating animals, early, mid and late lactation represent animals taken at 3–5, 9–11 and 17–19 days after parturition respectively. In the involuting group, animals were weaned at the twentieth day of lactation. The animals were fed *ad lib.* on stock diet M.R.C. no. 41 B (Bruce & Parkes, 1946).

Animals were killed by cervical dislocation and the lower abdominal glands quickly excised and placed in ice-cold water. Tissue suspensions in water (1:10, w/v) were prepared by homogenizing in an Emanuel-Chaikoff homogenizer (Emanuel & Chaikoff, 1957) with clearances of 64 and 17 μ . The suspensions were strained through muslin after treatment with the 64 μ plunger and before treatment with the 17 μ plunger, to remove any intractable connective tissue. The enzymic and chemical determinations were done on such prepared homogenates.

Chemicals. All the nucleotides were used as the sodium salts. AMP and IMP were purchased from the Sigma Chemical Co. and UMP, CMP and GMP from Pabst Laboratories. All other reagents used were of analytical reagent grade.

Nucleic acid. DNA was determined by the method described by Greenbaum & Slater (1957*a*) but modified by

using a shorter boiling time of 5 min., as recommended by Slater (1961*c*). The DNA concentration has been expressed in terms of DNA phosphorus.

Lactose. Lactose was measured by the orcinol procedure (Slater, 1957) and corrections were made for retained fluid as described by Slater (1961*b*).

5'-Nucleotidase assay. 5'-Nucleotidase activity was assayed by the procedure of Heppel & Hilmoe (1958). The assay system consisted of 0.2 ml. of 0.1 M-MgCl₂, 0.2 ml. of 1 M-glycine buffer (pH 8.5), 0.5 ml. of tissue homogenate and 6 μ moles of the respective nucleotide; the volume was made up to 2.5 ml. with water. The mixture was incubated at 37° for 10 or 15 min. and the reaction stopped by the addition of 2.5 ml. of 10% trichloroacetic acid solution; the tube was placed in ice-water to facilitate the precipitation of protein and fat. After centrifuging, the inorganic phosphate of the supernatant was measured by the method of Allen (1940), except that the final volume was reduced to 10 ml. The calibration of this modified method showed a linear relationship between inorganic phosphate concentration and chromogen in the ranges employed.

The amount of inorganic phosphate liberated was proportional to the incubation time (up to 25 min.) and to the volume of homogenate (0.1–1.0 ml.) for all the substrates used. The amount of dephosphorylation was the same whether 4, 6 or 12 μ moles of substrate were present.

A difficulty encountered in 5'-nucleotidase estimations was the presence of interfering phosphatases. Alkaline-phosphatase activity has been reported for the rat mammary gland (Folley & Kay, 1935) and could therefore contribute appreciably to the amount of dephosphorylation of nucleotides measured. To investigate this difficulty the incubation of 6 μ moles of β -glycerophosphate and AMP under identical conditions was carried out and the dephosphorylations were compared. The results obtained indicated that the dephosphorylation of AMP is seven times as great as that for β -glycerophosphate. This comparison of the breakdown of β -glycerophosphate and the other nucleotides was made for every estimation and Table 2 shows that the level of dephosphorylation of β -glycerophosphate throughout the lactation cycle remains low in relation to the nucleotide dephosphorylation. Hence the error introduced by the dephosphorylation of nucleotides by alkaline phosphatase was not of major importance.

RESULTS

Table 1 gives the activities of the 5'-nucleotidases over the lactation cycle. From the results, expressed as μ moles of inorganic phosphate liberated/min./mg. of DNA phosphorus, the 5'-nucleotidase

Table 1. *Variation in activity of 5'-nucleotidase of rat mammary gland over the lactation cycle*

All values are means from five estimations ($\pm$ s.e.m.). Results are given in terms of inorganic phosphate liberated: (a) μ moles/min./mg. of DNA phosphorus; (b) μ moles/min./100 g. of wet tissue after correction for retained fluid.

Status	AMP		GMP		IMP		CMP		UMP	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Early pregnant*	62.35 ($\pm$ 9.25)	94.40 ($\pm$ 17.35)	34.30 ($\pm$ 6.25)	51.70 ($\pm$ 6.95)	37.95 ($\pm$ 7.30)	56.80 ($\pm$ 6.90)	59.50 ($\pm$ 10.55)	90.60 ($\pm$ 22.60)	81.95 ($\pm$ 13.20)	124.30 ($\pm$ 19.55)
Mid pregnant*	76.25 ($\pm$ 10.10)	131.00 ($\pm$ 47.15)	42.15 ($\pm$ 11.15)	72.50 ($\pm$ 24.20)	44.65 ($\pm$ 1.45)	77.10 ($\pm$ 18.55)	76.20 ($\pm$ 8.30)	131.00 ($\pm$ 44.40)	88.15 ($\pm$ 8.30)	151.30 ($\pm$ 48.40)
Late pregnant*	80.55 ($\pm$ 15.80)	214.00 ($\pm$ 19.55)	34.40 ($\pm$ 8.45)	91.70 ($\pm$ 7.40)	35.55 ($\pm$ 8.25)	94.20 ($\pm$ 6.10)	70.05 ($\pm$ 18.80)	186.00 ($\pm$ 13.70)	90.20 ($\pm$ 19.65)	239.50 ($\pm$ 15.05)
Early lactating†	33.00 ($\pm$ 10.90)	357.30 ($\pm$ 14.30)	15.40 ($\pm$ 2.65)	167.10 ($\pm$ 35.50)	14.30 ($\pm$ 2.15)	155.60 ($\pm$ 29.50)	31.55 ($\pm$ 4.10)	342.90 ($\pm$ 29.50)	34.75 ($\pm$ 4.70)	377.50 ($\pm$ 21.85)
Mid lactating†	25.75 ($\pm$ 8.25)	323.70 ($\pm$ 16.65)	11.90 ($\pm$ 0.95)	148.50 ($\pm$ 14.55)	10.65 ($\pm$ 1.30)	133.80 ($\pm$ 23.35)	25.70 ($\pm$ 1.50)	323.00 ($\pm$ 11.35)	27.95 ($\pm$ 2.30)	350.50 ($\pm$ 15.00)
Late lactating†	27.20 ($\pm$ 8.20)	340.20 ($\pm$ 12.80)	14.10 ($\pm$ 2.90)	176.50 ($\pm$ 9.15)	10.90 ($\pm$ 1.40)	136.40 ($\pm$ 15.95)	26.95 ($\pm$ 2.50)	337.30 ($\pm$ 7.70)	32.30 ($\pm$ 4.00)	404.70 ($\pm$ 15.35)
Involuting (3 days)*	37.15 ($\pm$ 6.00)	284.60 ($\pm$ 42.25)	23.15 ($\pm$ 4.65)	176.50 ($\pm$ 31.20)	20.90 ($\pm$ 4.20)	160.10 ($\pm$ 13.65)	42.15 ($\pm$ 7.20)	322.90 ($\pm$ 17.40)	50.30 ($\pm$ 7.70)	385.80 ($\pm$ 56.00)
Involuting (6 days)*	49.45 ($\pm$ 4.15)	248.50 ($\pm$ 13.05)	30.55 ($\pm$ 3.85)	153.50 ($\pm$ 21.60)	30.00 ($\pm$ 3.80)	151.00 ($\pm$ 21.10)	57.20 ($\pm$ 5.50)	288.70 ($\pm$ 71.00)	69.50 ($\pm$ 7.40)	349.60 ($\pm$ 16.25)

* Pooled tissue from two animals was used in each estimation.

† A single animal was used in each estimation.

Table 2. *Relative rates of dephosphorylation of 5'-nucleotides by homogenates of rat mammary gland*

All values are means from five estimations ($\pm$ s.e.m.), expressed as percentages and calculated as:

$$\frac{\text{amount of dephosphorylation of the substrate per unit time per unit of homogenate}}{\text{total dephosphorylation (including } \beta\text{-glycerophosphate) per unit time per unit of homogenate}} \times 100.$$

Status	AMP	GMP	IMP	CMP	UMP	β -Glycerophosphate
Early pregnant*	22.1 ($\pm$ 0.53)	12.1 ($\pm$ 0.61)	13.3 ($\pm$ 0.98)	21.2 ($\pm$ 1.60)	29.1 ($\pm$ 0.55)	2.5 ($\pm$ 0.60)
Mid pregnant*	22.6 ($\pm$ 1.09)	12.5 ($\pm$ 0.61)	13.3 ($\pm$ 0.95)	22.6 ($\pm$ 0.63)	26.1 ($\pm$ 0.20)	2.9 ($\pm$ 0.28)
Late pregnant*	25.2 ($\pm$ 0.67)	10.8 ($\pm$ 0.28)	11.1 ($\pm$ 0.27)	21.9 ($\pm$ 0.78)	28.2 ($\pm$ 0.47)	2.9 ($\pm$ 0.21)
Early lactating†	24.8 ($\pm$ 0.98)	11.6 ($\pm$ 0.45)	10.8 ($\pm$ 0.39)	23.8 ($\pm$ 0.47)	26.2 ($\pm$ 0.75)	2.9 ($\pm$ 0.48)
Mid lactating†	24.2 ($\pm$ 1.21)	11.1 ($\pm$ 0.92)	10.0 ($\pm$ 0.60)	24.1 ($\pm$ 0.68)	26.2 ($\pm$ 0.95)	4.4 ($\pm$ 0.60)
Late lactating†	23.7 ($\pm$ 0.87)	12.3 ($\pm$ 1.74)	9.5 ($\pm$ 0.40)	23.5 ($\pm$ 1.29)	28.2 ($\pm$ 0.88)	2.7 ($\pm$ 0.24)
Involuting (3 days)*	20.8 ($\pm$ 0.88)	12.9 ($\pm$ 1.02)	11.7 ($\pm$ 0.61)	23.6 ($\pm$ 0.32)	28.2 ($\pm$ 0.60)	3.0 ($\pm$ 1.56)
Involuting (6 days)*	20.4 ($\pm$ 0.42)	12.6 ($\pm$ 0.38)	12.4 ($\pm$ 0.42)	23.7 ($\pm$ 0.52)	28.7 ($\pm$ 0.30)	3.5 ($\pm$ 1.00)

* Pooled tissue from two animals was used in each estimation.

† A single animal was used in each estimation.

activity is seen to fall by about 65 % from pregnancy to lactation but at involution the activity rises above those at lactation although not to the values for pregnancy. Results in Table 1 are also expressed as μ moles of inorganic phosphate liberated/min./100 g. of tissue after correction for retained fluid (Slater, 1961b). In contrast with the above-mentioned, the activities expressed in this way seem to increase from pregnancy to lactation some two- to three-fold. This value is then maintained throughout lactation and involution with the possible exception of that for AMP, where there is a decrease in activity in involution, this pattern being similar to that reported by Folley & Greenbaum (1947) for alkaline phosphatase. Table 2 shows the proportions of the various 5'-nucleotidases expressed as percentages of the total activity of all the 5'-nucleotidases and β -glycerophosphatase. Although there is an indication of a slight change in the AMP-nucleotidase activity over the lactation cycle, the other 5'-nucleotidases remain surprisingly constant. The dephosphorylation of β -glycerophosphate accounts for only some 3 % of the total.

This constancy in the relative rates of dephosphorylation suggests that the 5'-nucleotidase activities may be due to only one enzyme. In an attempt to elucidate this point two experiments were performed, one employing mixed substrates and the other investigating the effect of inactivation by heat of the various 5'-nucleotidase activities (see Dixon, 1938).

Table 3 shows the amounts of dephosphorylation of all the substrates employed, compared with the

amounts of dephosphorylation when 6 μ moles of AMP were added to each of these nucleotides. The degree of dephosphorylation for the mixed substrates is far less than that for the added individual amounts of dephosphorylation.

Table 4 gives the relative amounts of dephosphorylation of all the nucleotides and β -glycerophosphate after various periods of heating the tissue extract at 60°. The relative rates of dephosphorylation remain remarkably constant even when only 35 % of the original activity remains. The inactivation of the β -glycerophosphatase is slower than that of the 5'-nucleotidase.

The 5'-nucleotidase activity in milk was also measured. The milk was obtained after overnight weaning by manual milking of the glands, with the injection of 5 i.u. of pitocin (Parke, Davis and Co. Ltd.) intraperitoneally. The milk was diluted with 9 vol. of water and assayed for 5'-nucleotidase activity in the usual manner. The inorganic phosphate liberated (μ moles/min./100 g. of milk) was 12.8, 13.6, 9.2, 12.4, 8.4 and 36.0 for AMP, GMP, IMP, UMP, CMP and β -glycerophosphate respectively. By comparing these results with those in Table 1 (columns headed b) it can be seen that essentially all the nucleotide dephosphorylation is caused by the mammary tissue, even if the gland contained as much as 40 % of retained fluid.

Preliminary experiments to determine the sub-cellular localization of 5'-nucleotidase in the rat mammary gland, with 0.25 M-sucrose medium, homogenizing in an Emanuel-Chaikoff homogenizer (Emanuel & Chaikoff, 1957) and differential centrifuging, suggested that 60 % of the activity

Table 3. *Dephosphorylation by mammary-gland homogenates of mixed nucleotide substrates*

Results are expressed in arbitrary units of dephosphorylation per unit time per unit amount of homogenate.

Additions	AMP	GMP	IMP	UMP	CMP
Nil	190	140	195	305	230
AMP (6 μ moles)	190	205	175	255	190
Calc. value if dephosphorylation additive	—	330	385	495	420

Table 4. *Relative dephosphorylation of 5'-nucleotides by mammary-gland homogenates after varying degrees of heat-inactivation*

Results are expressed as percentages and calculated as:

$$\frac{\text{amount of dephosphorylation of the substrate per unit time per unit of homogenate}}{\text{total dephosphorylation (including } \beta\text{-glycerophosphate) per unit time per unit of homogenate}} \times 100.$$

Time of heating at 60° (min.)	Percentage of original enzyme activity	AMP	GMP	IMP	CMP	UMP	β -Glycerophosphate
0	100	22.2	11.9	12.3	22.1	28.4	3.1
5	95	22.2	11.2	10.5	22.0	30.6	3.6
10	87	21.9	12.7	11.2	21.8	27.6	4.9
15	69	22.0	12.1	11.0	21.0	28.7	5.1
20	49	20.2	10.8	12.9	20.9	29.3	6.0
30	35	20.5	11.2	10.4	21.6	30.0	6.2

resided in the cytoplasmic fraction (supernatant fraction after centrifuging for $100\,000 \times 30\text{g}\cdot\text{min.}$) and 25 % in the microsomal fraction.

DISCUSSION

The mammary gland in its transition from the metabolically quiescent state to the actively synthesizing lactating state exhibits many enzymic changes that have been correlated with this corresponding change in metabolic status (Folley & Greenbaum, 1947; Greenbaum & Greenwood, 1954; Greenbaum & Slater, 1957*b*; McLean, 1958*a*). The content of nucleotide coenzymes also increases in this period (Wang & Greenbaum, 1960; Denamur, Fauconneau & Guntz, 1959; McLean, 1958*b*), and may reflect the increasing demand by the tissue in its synthetic role for coenzymes.

The results in this paper indicate differences in the activity of 5'-nucleotidases over the lactation cycle. The cellular content of enzymes decreases to about one-third from the pregnant to the lactating state (Table 1, results in columns headed *a*). At involution the enzymic content per cell appears to increase, although it does not reach the pregnancy level. The cellular content of 5'-nucleotidases in lactation remains relatively constant and in this respect resembles acid and alkaline ribonuclease (Slater, 1961*a*) and diphosphopyridine nucleotidase (McLean, 1958*b*). 5'-Nucleotidase also resembles acid ribonuclease (Slater, 1961*a*) and β -glucuronidase (Greenbaum & Greenwood, 1954) in its increase at involution. Therefore this pattern for cellular enzyme content suggests that at lactation, when the requirement for acid-soluble ribonucleotides would be thought to be maximal, the 5'-nucleotidase activity is minimal. When expressed per unit weight of tissue (Table 1, columns headed *b*), the changes of enzyme activity resemble that for alkaline phosphatase, especially in that the involution level of both enzymes is about three times as great as that at early pregnancy (Folley & Greenbaum, 1947). However, these variations are quite different from the others shown in Table 1.

Thus the total acid-soluble ribonucleotides of the rat mammary gland increase some threefold from pregnancy to late lactation on a weight basis (Wang & Greenbaum, 1960), and this increase is similar in magnitude to that for 5'-nucleotidase. If, however, the total acid-soluble-ribonucleotide content of the gland had been expressed on a DNA basis, then, from the data of Slater (1961*a*), a decrease would have been seen in the period from pregnancy to lactation. This implies that the 5'-nucleotidase activity parallels the changes in the concentration of acid-soluble ribonucleotides, if both are expressed in the same tissue units. Thus the increase observed by Wang & Greenbaum (1960) for the

total acid-soluble ribonucleotides from pregnancy to lactation is not accounted for by a lowered catabolic activity of the 5'-nucleotidases; further, if this enzyme is rate-limiting in the breakdown of nucleotides, the increases must be due wholly to synthesis.

As stated above, the dephosphorylation of β -glycerophosphate is approximately 14 % of that found for AMP under identical conditions and this indicates that the alkaline phosphatase in the homogenate contributes only a small percentage to the breakdown of nucleotides. With milk the breakdown of β -glycerophosphate was greater than that for 5'-nucleotidases (two- to three-fold) and allows an approximate measure of nucleotide dephosphorylation that is due to the presence of alkaline phosphatase. If it is assumed that the alkaline phosphatase in milk is similar to the enzyme in rat mammary tissue, the 3 % breakdown of β -glycerophosphate shown in Table 2 represents an error of less than 1 % on each of the other substrates.

Tables 3 and 4 indicate that the enzymic dephosphorylation of all the nucleotides investigated is due to only one enzyme, although this is not conclusive. Table 4 also reveals that the alkaline phosphatase as measured by the dephosphorylation of β -glycerophosphate is inactivated at a lower rate than the 5'-nucleotidase and hence it is a different enzyme.

If the activities are indeed due to one enzyme, it is not surprising that the relative activities shown in Table 2 remain so constant over the lactation cycle. In Table 2 UMP is shown to be dephosphorylated at the greatest rate, followed by CMP and AMP; in this respect it is similar to the 5'-nucleotidase isolated by Klein (1957) from potato, but unlike the liver microsomal enzyme studied by Segal & Brenner (1960), for which the greatest rate of dephosphorylation occurred for AMP.

SUMMARY

1. 5'-Nucleotidase activities in the rat mammary gland have been measured for the monophosphates of adenosine, guanosine, inosine, cytidine and uridine over the lactation cycle.

2. All the activities, when expressed per unit amount of glandular deoxyribonucleic acid, showed a decrease from pregnancy to early lactation and then an increase from late lactation to involution. The activities remained constant over lactation.

3. The ratios of 5'-nucleotidase activities for all the substrates investigated remained constant throughout the lactation cycle.

4. Evidence is presented which indicates that the 5'-nucleotidase activities found in the rat mammary gland are due to only one enzyme.

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Anthocyanins in Petals of Tulip var. President Eisenhower

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The anthocyanins of cultivated tulip flowers were first investigated by Willstätter & Bolton (1916), who identified cyanidin diglucoside in some scarlet-red varieties. Robinson & Robinson (1931) examined 34 varieties of tulips and found that they contained a mixture either of cyanidin and pelargonidin biosides or of cyanidin bioside and delphinidin diglucoside. In these studies most pigments were not fully identified. Shibata (1956) and Shibata & Sakai (1958) isolated crystalline delphinidin 3-rhamnoglucoside and cyanidin 3-rhamnoglucoside. Halevy & Asen (1959) used paper chromatography and spectrographic methods to identify cyanidin 3-glucoside, pelargonidin 3-glucoside and pelargonidin 3-rhamnoglucoside in two tulip varieties in addition to the two pigments isolated by Shibata. Shibata & Ishikura (1960), in a chromatographic examination of 105 tulip varieties, identified also delphinidin 3-glucoside and delphinidin 3,5-diglucoside.

Five anthocyanins have now been separated from the petals of tulip var. President Eisenhower. One of them, pelargonidin 3-glucoside, was not found by Shibata & Ishikura (1960).

EXPERIMENTAL

Separation of crude anthocyanins. Petals (300 g.) were dried at 50° in a forced-draught oven; they were ground and extracted in 500 ml. of methanol containing 1% of HCl for 24 hr. at room temperature. The extract was filtered and the anthocyanins were precipitated with basic lead acetate. The lead salt of the anthocyanins was dried at room temperature and blended with methanol containing 2% (v/v) of concentrated HCl. The precipitated lead chloride was removed by filtration; any residual lead was precipitated by treatment with H₂S. The methanolic solution was evaporated to dryness *in vacuo* at 40° and the residue was dissolved in a minimum volume of methanol. The anthocyanins were precipitated with ether and kept at 3° for 3 days before filtration. The anthocyanins were again dissolved in methanol (fraction A).

The supernatant remaining after precipitation with lead acetate was red-brown, indicating that not all the anthocyanins were precipitated. Chromatography in butan-1-ol-acetic acid-water (6:1:2, by vol.) and 15% (v/v) acetic acid showed that it contained at least two anthocyanins in addition to other flavonoid pigments. The supernatant was shaken several times with ethyl acetate and then extracted in a liquid-liquid extractor for 24 hr. with ethyl acetate, to remove yellow flavonoid pigments. Finally it was