Ribonuclease Activity in the Rat Mammary Gland during Pregnancy, Lactation and Mammary Involution

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The rat mammary gland has a high content of ribonucleic acid during late pregnancy when the gland is functionally inactive (Greenbaum & Slater, 1957*b*). Slater (1956) suggested that this high level of ribonucleic acid might result from a very low activity of nucleic acid-degrading enzymes (ribonucleases) during late pregnancy. Rat mammary gland contains high concentrations of heavy-metal ions, e.g. copper and zinc, which inhibit crystalline pancreatic ribonuclease in low concentration (cf. McDonald, 1955). The activity of ribonucleases in the rat mammary gland over the lactation cycle was therefore investigated. Further, since a correlation has been reported for the concentration of ribonucleic acid and the activity of ribonucleases in normal and malignant tissues (Ledoux, Piler, Vanderhaeghe & Brändli, 1957), it was decided to investigate the changes in ribonuclease activity in the rat mammary gland in relation to the very abrupt increase in ribonucleic acid concentration which occurs during lactation (Greenbaum & Slater, 1957*b*; Slater & Planterose, 1958).

METHODS

Animals. Adult, female, black and white rats of the Medical Research Council strain were used in this investigation. The animals weighed approximately 200 g. For the lactating groups only animals with litters of 6–10 pups were used. 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 after they were killed. These examinations indicated that of the six animals used in this group one was at the eighteenth day of pregnancy, four were on the nineteenth day and one on the twentieth day of pregnancy. For the involuting group, pups were removed from the mothers at the 22nd day of lactation. For determinations on mammary gland from virgin animals, the glands from groups of three rats were pooled for each sample.

Animals were killed by cervical dislocation and the abdominal and upper inguinal mammary glands were quickly dissected and placed into ice-cold water. Tissue suspensions (1:10 in water) were prepared by using a high-speed top-drive Waring Blendor (as described by Folley & Watson, 1948); two periods of 1 min. were used for blending at top speed, after which the suspension was strained

through muslin to remove intractible connective tissue. All chemical estimations and enzyme assays were carried out on such strained suspensions.

Protein. Protein was determined by the colorimetric method of Lowry, Rosebrough, Farr & Randall (1951); standards of ox-plasma albumin were used on each occasion.

Nucleic acid. Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) were determined as reported by Greenbaum & Slater (1957*b*); results are expressed in terms of nucleic acid phosphorus (DNA-P and RNA-P respectively).

Lactose. Lactose was determined by the orcinol procedure (Slater, 1957).

Ribonucleases. Acid and alkaline ribonuclease were assayed at pH 5.8 and 8.0 respectively. RNA substrate was purified from a commercial sample of yeast nucleic acid by dissolving in dilute alkali followed by precipitation with acetic acid; the precipitate was dried with ethanol and ether essentially as described by Woodward (1944). The assay system was as follows: 0.1–0.5 ml. of enzyme was incubated at 37° for 1 min. with 0.2 ml. of 1% (v/v) Triton X-100, after which 1 ml. of RNA in buffered solution was added. The reaction was stopped 10 min. later by adding 2 ml. of 0.2% uranyl acetate in 10% perchloric acid. The tubes were then kept overnight at –20°, thawed, diluted to 20 or 40 ml. (according to activity) with 0.001% Triton X-100 and filtered through Whatman no. 42 paper. $E_{260}^{1\text{ml}}$ of the filtrate was then measured and compared with that of a blank obtained by adding tissue after the addition of the uranyl acetate-perchloric acid mixture. All values are based on measurements with two different amounts of tissue extract. The assay conditions reported below refer to mammary suspensions obtained from animals in late lactation; no differences in behaviour were noted when the influences of incubation time, concentration of tissue, addition of Triton X-100 and addition of ethylenediamine-tetra-acetate (EDTA) were investigated with mammary suspensions obtained from pregnant animals.

The effect of pH on ribonuclease activity was investigated by using veronal-acetate buffers at the same molarity. Two pH optima were found for the ribonuclease activity in mammary-gland suspensions, one at pH 5.8 and the other at pH 8.0. This result is similar to the results of Roth (1954) and of de Lamirande, Allard, da Costa & Cantero (1954) for rat-liver ribonuclease. During the preliminary investigation of enzyme activity and the effect on it of various additives such as metal ions and heparin, veronal buffer was used for assaying alkaline ribonuclease at pH 8.0 and acetate buffer was used for the acid ribonuclease at pH 5.8. For the major experiments (i.e. the variation in enzyme activity over the lactation cycle, Tables 2 and 3),

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2-amino-2-hydroxymethylpropane-1:3-diol (tris, recrystallized from water, 0.1M, pH 8.0) and phosphate (NaH_2PO_4 - Na_2HPO_4 , 0.1M, pH 5.8) buffers were used in place of veronal buffer and acetate buffer respectively. As reported by Roth (1957) phosphate buffer gave very similar activities to those obtained with acetate buffer and tris buffer gave similar results to veronal buffer.

The influence of the concentration of RNA in the incubation mixture was investigated with a constant amount of tissue; at pH 5.8 the extinction at 260 μ increased with the amount of RNA present up to a plateau level of 2 mg./ml. in the incubation mixture. At pH 8.0 the plateau level was reached only when approximately 10 mg. of RNA/ml. was present. These results are similar to the findings of de Lamirande *et al.* (1954) for rat-liver ribonucleases. For routine estimations 4 mg. of RNA was dissolved per ml. of phosphate buffer, pH 5.8, and 10 mg. of RNA was dissolved per ml. of tris buffer, pH 8.0. With optimum levels of ribonucleic acid substrate, $E_{260\text{ m}\mu}$ increased linearly with time of incubation up to 30 min. in the presence of mammary-gland suspensions at both pH 5.8 and 8.0. With a 10 min. incubation at 37°, $E_{260\text{ m}\mu}$ increased proportionately with the amount of tissue added (0–50 mg. wet wt. of mammary tissue).

The addition of Mg^{2+} or Cu^{2+} ions to the incubation medium inhibited the enzyme reaction at both pH 5.8 and 8.0 (Fig. 1). Heparin inhibited as described by de Lamirande *et al.* (1954) for rat-liver ribonucleases. EDTA had little effect on mammary-homogenate activity over a range of concentrations (0.005–0.015M). This would indicate that the total enzyme activities, as measured in this investigation, are not substantially inhibited by metal ions contributed either by the tissue or the incubating medium. The activity of mammary suspensions prepared in 0.25M-sucrose with a homogenizer of the type described by Emanuel & Chaikoff (1957) was greatly increased by freezing and thawing the homogenate, by high-speed blending, or by the addition of Triton X-100 (cf. Wattiaux & de Duve, 1956). These increases were approximately threefold at pH 5.8, and approximately sixfold at pH 8.0.

Unit of ribonuclease activity. The activity of acid (and alkaline) ribonuclease in the reaction mixture is defined as the increase in $E_{260\text{ m}\mu}$ per min. of incubation time multiplied by the total volume before filtering.

RESULTS

Table 1 gives the gland weights, lactose and retained fluid contents of the mammary suspensions

used in this investigation. Table 1 also includes the DNA-P, RNA-P and protein contents, which were similar to those of previous investigations (Greenbaum & Slater, 1957*b*; Slater & Planterose, 1958). The values for the percentage of retained fluid during late pregnancy (Table 1, column 6) are much less than previous figures (Folley & Greenbaum, 1947; Greenbaum & Slater, 1957*a*) because

Fig. 1. Inhibitory effect of Cu^{2+} and Mg^{2+} ions on acid and alkaline ribonuclease in rat mammary tissue. The ordinates express the activity of the enzyme in terms of arbitrary units; the abscissae represent the final concentration of metal ion added. O, Results for Cu^{2+} ions; ●, results for Mg^{2+} ions. Enzymes were assayed at pH 8.0 in veronal buffer (A) and at pH 5.8 in acetate buffer (B). Incubations were for 20 min. at 37° with 30 mg. (wet wt.) of mammary tissue taken from an animal in late lactation; the incubation mixture contained 0.2 ml. of 1% Triton X-100. Other details are in the Methods section.

Table 1. Concentrations of protein, lactose and nucleic acids in rat mammary-gland tissue at six stages during pregnancy, lactation and mammary involution

Mean values are given $\pm$ s.e.m. The percentages of retained fluid were calculated by using lactose contents for retained fluid of 1.6 g./100 g. of retained fluid in late pregnancy and 2.55 g./100 g. of retained fluid in lactation and early involution.								
Status	No. of animals	Gland wt. (g.)	Protein (g./100 g. of gland)	Lactose (g./100 g. of gland)	Retained fluid (%)	DNA-P (mg./100 g. of gland)	RNA-P (mg./100 g. of gland)	
Virgin	6	1.5	2.6	0.12	—	2.5	1.33	
Late pregnancy	6	3.1 $\pm$ 0.2	8.3 $\pm$ 0.6	0.16 $\pm$ 0.01	10 $\pm$ 1.0	10.3 $\pm$ 0.8	14.7 $\pm$ 0.9	
Early lactation	6	3.9 $\pm$ 0.3	10.2 $\pm$ 0.6	0.93 $\pm$ 0.08	36 $\pm$ 3.3	14.2 $\pm$ 1.2	24.7 $\pm$ 1.4	
Late lactation	6	5.7 $\pm$ 0.4	14.9 $\pm$ 1.1	1.16 $\pm$ 0.06	45 $\pm$ 2.5	18.3 $\pm$ 1.8	65.2 $\pm$ 4.2	
2-Day involution	6	5.7 $\pm$ 0.7	9.8 $\pm$ 1.7	0.91 $\pm$ 0.04	36 $\pm$ 5.6	11.7 $\pm$ 1.4	10.6 $\pm$ 1.0	
4-Day involution	2	3.0	9.6	0.33	13	11.8	9.4	

the chloramine-T procedure which was used previously for lactose estimations is now known to give values for the lactose content of mammary suspensions which are too high; the orcinol procedure used here is more accurate (Slater, 1961).

Table 2 shows the activity of acid ribonuclease at the various stages of the lactation cycle studied. Table 2 also shows the variation in the ratio of the activity of acid ribonuclease to DNA-P, to RNA-P and to total protein.

Table 3 shows similar results for alkaline ribonuclease, arbitrary activity for this enzyme being defined in the same manner as for acid ribonuclease.

It can be seen that both enzymes increase approximately threefold during pregnancy; there is a sharp rise in activity during the 5 days over parturition, followed by a small rise during lactation. Acid ribonuclease decreases slowly in mammary involution; the alkaline enzyme decreases much more rapidly.

DISCUSSION

The assays for the acid and alkaline ribonucleases were designed to measure total ribonuclease activities in mammary-gland suspensions. The activities of suspensions prepared in 0.25 M-sucrose are greatly stimulated by either freezing and thawing or by adding Triton X-100 (Greenbaum, Slater & Wang, 1960). Similar activation studies with enzymes like β -glucuronidase and cathepsin

(Greenbaum *et al.* 1960) have suggested that these enzymes are localized within particles similar to the lysosomes of liver (cf. Appelmans, Wattiaux & de Duve, 1955), and that breakage of the particles is necessary before the full activity of the enzyme can be realized (Wattiaux & de Duve, 1956). This investigation has therefore been concerned solely with the total activity of ribonucleases at two values of pH, 5.8 and 8.0; full activation was obtained by preparing the suspensions by high-speed blending in water and by adding Triton X-100 to the incubation medium.

The ribonuclease enzymes from rat mammary tissue show similarities to those of previous reports for many other tissues. Variation of the pH of the incubation mixture indicated the presence of an acid and an alkaline enzyme; pH optima were at pH 5.8 and 8.0. The activity was greatest at pH 8.0 so that in this respect the mammary tissue resembles kidney rather than liver (Roth, 1954). The addition of low concentrations of both copper and magnesium ions produced severe inhibition of both enzymes (Fig. 1); the inhibition is greatest for the alkaline enzyme. Although Roth (1954) reported inhibition of liver ribonuclease by magnesium, de Lamirande *et al.* (1954) reported an activation of acid ribonuclease and a slight inhibition of alkaline ribonuclease by a final concentration of 3 mM-Mg²⁺.

Tables 2 and 3 show that both acid and alkaline ribonucleases have a similar pattern of change

Table 2. *Variation in the activity of acid ribonuclease in suspensions of rat mammary-gland tissue at various stages of the lactation cycle*

The units of activity are defined in the Methods section. Values given are means $\pm$ S.E.M.

Status	No. of animals	Units/100 mg. wet wt. of tissue	10 ³ $\times$ Units/mg. of DNA-P	10 ³ $\times$ Units/mg. of RNA-P	10 ³ $\times$ Units/g. of protein
Virgin	6*	0.075	28.8	54.2	27.3
Late pregnancy	6	0.237 $\pm$ 0.021	23.6 $\pm$ 2.2	16.5 $\pm$ 1.8	29.7 $\pm$ 4.1
Early lactation	6	0.489 $\pm$ 0.051	31.2 $\pm$ 2.7	19.6 $\pm$ 1.6	49.2 $\pm$ 6.0
Late lactation	6	0.551 $\pm$ 0.018	31.9 $\pm$ 3.2	8.6 $\pm$ 0.6	40.0 $\pm$ 2.8
2-Day involution	6	0.418 $\pm$ 0.053	35.3 $\pm$ 2.3	39.7 $\pm$ 4.1	52.6 $\pm$ 4.1
4-Day involution	2	0.472	39.7	51.8	49.2

* Analyses of two pooled samples; three animals were used per sample.

Table 3. *Variation in the activity of alkaline ribonuclease in suspensions of rat mammary-gland tissue at various stages of the lactation cycle*

The units of activity are defined in the Methods section. Values given are means $\pm$ S.E.M.

Status	No. of animals	Units/100 mg. wet wt. of tissue	10 ³ $\times$ Units/mg. of DNA-P	10 ³ $\times$ Units/mg. of RNA-P	10 ³ $\times$ Units/g. of protein
Virgin	6*	0.22	101.4	190.1	98.0
Late pregnancy	6	0.60 $\pm$ 0.06	60.3 $\pm$ 8.6	41.5 $\pm$ 4.4	76.6 $\pm$ 11.7
Early lactation	6	1.31 $\pm$ 0.20	76.5 $\pm$ 9.2	52.5 $\pm$ 6.5	133.0 $\pm$ 25.4
Late lactation	6	1.43 $\pm$ 0.09	84.7 $\pm$ 13.1	22.7 $\pm$ 2.4	102.6 $\pm$ 13.7
2-Day involution	6	0.41 $\pm$ 0.02	37.5 $\pm$ 5.1	40.2 $\pm$ 3.2	58.9 $\pm$ 16.1
4-Day involution	2	0.47	39.8	49.7	48.6

* Analyses of two pooled samples; three animals were used per sample.

during pregnancy and lactation. The changes occurring in involution are particularly interesting. Acid ribonuclease shows only a small decrease during the first 4 days of mammary involution, whereas alkaline ribonuclease has decreased approximately threefold during this period. Even this threefold decrease is less than the precipitous drop in enzyme activity found to occur in early mammary involution for mitochondrial enzymes (Greenbaum & Greenwood, 1956; Greenbaum & Slater, 1957c) and for soluble enzymes (Glock & McLean, 1958). On the other hand, two enzymes which appear to be associated with lysosome-like particles in mammary tissue, β -glucuronidase and cathepsin, both increase during early mammary involution (Greenbaum & Greenwood, 1956). Thus if acid ribonuclease is localized in similar particles to β -glucuronidase and cathepsin (cf. Greenbaum *et al.* 1960) either independent variations in the individual enzymic activities can occur or there are several classes of lysosome-like particles with various enzymic constitutions (Conchie & Levvy, 1960; Beaufay & de Duve, 1959). Alkaline ribonuclease would seem by its behaviour to resemble more closely the mitochondrial enzymes which show a rapid decrease in activity over the period of early involution.

The variation in the ratio of enzyme activity to RNA-P is also of interest. Tables 3 and 4, column 5, show that the ratio is high in virgin animals, falls to a low level in late lactation and rises again in mammary involution; there is a slight rise in the ratio over parturition which indicates that ribonuclease increases rather more rapidly than RNA-P at this time. However, despite these variations in the ratios, there is an approximate relationship between enzyme activity and RNA-P content: as the enzyme activities increase so does the RNA-P content. The relationship is similar to several previous reports concerning a correlation between acid ribonuclease and RNA content in various rat and mouse tissues (Ledoux *et al.* 1957; Ledoux, Brändli & De Paepe, 1958) and in placental development (Brody, 1957).

The ratio of enzyme activity to DNA-P (Tables 2 and 3, column 4) shows further differences between the behaviour of the two ribonucleases over the lactation cycle. There is appreciable variation between the ratio of alkaline ribonuclease to DNA-P in the virgin state and in the late lactation period, whereas the same ratio for acid ribonuclease shows a smaller variation. Indeed for acid ribonuclease there appears to be an approximately constant amount of enzyme per cell at the various stages of the lactation cycle studied; this pattern is very different from that given by many other enzymes so far studied over the lactation cycle where the activity per cell usually shows a dramatic

increase from late pregnancy to late lactation (Greenbaum & Slater, 1957c; Glock & McLean, 1958; Slater & Planterose, 1960). It is difficult to understand the significance of this finding, but perhaps the solution lies in the fact that only total enzyme activities have been considered in this paper. It may be that the ratio of free to bound ribonuclease (cf. de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955) varies greatly over the lactation cycle so that the amount of available ribonuclease (i.e. free enzyme) is not directly related to the total activity as measured in this paper.

SUMMARY

1. The enzymic breakdown of ribonucleic acid by suspensions of rat mammary gland has been investigated. The variation in enzyme activity with pH indicated the presence of an acid (pH optimum 5.8) and an alkaline (pH optimum 8.0) enzyme.

2. The activities of both acid and alkaline ribonucleases have been studied at six stages in the lactation cycle: in the virgin animal, in late pregnancy, in early and in late lactation, and at 2 and 4 days of involution.

3. Both acid and alkaline ribonucleases increased during pregnancy, over parturition and during lactation. The acid ribonuclease decreased only slightly in early involution; alkaline ribonuclease decreased approximately threefold by the fourth day of mammary involution.

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Some Observations on the Form and Location of Invertase in the Yeast Cell

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Because it is so difficult to extract the invertase from yeast cells it has been generally supposed that the enzyme occurs in an insoluble form, requiring autolysis or the action of added enzymes for its release (cf. Gottschalk, 1958). At the same time various kinds of evidence (cf. Myrbäck & Willstaedt, 1955; Preiss, 1958) have indicated strongly that the enzyme is situated close to the cell surface and is associated with polysaccharide resembling the mannan of the cell wall.

Recently Myrbäck (1957) drew attention to the effect of ethyl acetate on baker's yeast. Cells treated overnight and washed thoroughly with water on the centrifuge lose much of their contents but retain the greater part of their invertase, even when stored for several months. Treatment with papain liberates the enzyme. Myrbäck explained this behaviour as follows: invertase is retained because it is firmly bound to insoluble structures of the cell; on plasmolysis by ethyl acetate only insignificant amounts of the enzyme are solubilized. On standing, no further enzyme is liberated because the proteinases of the yeast have been removed or destroyed during plasmolysis and washing. Only papain is known to take the place of the yeast proteinases and release the invertase.

We have described some preliminary experiments (Burger, Bacon & Bacon, 1958) which led us to a quite different conclusion, namely that the invertase is enclosed by membranes (one of which may be

the cell wall) and although it is freely soluble it cannot escape until these membranes are broken by mechanical or enzymic action. In this paper we describe further experiments supporting this conclusion.

MATERIALS AND METHODS

Yeasts. Baker's yeast (*Saccharomyces cerevisiae*) was obtained from a factory at Libáň and brewer's yeast (*S. carlsbergensis*) from the Smichov Brewery, Prague. A strain of *S. cerevisiae*, RXII, was grown in stationary culture on 8° wort (cf. Burger, Hejmová & Kleinzeller, 1959). *S. fragilis* was grown on the same medium, usually for 24 hr. with aeration.

Snail-crop juice. Edible snails, *Helix pomatia* (obtainable from A. Gaudin, 46 Greek Street, Soho, London, W. 1), were starved for at least 24 hr. before the crop juice was collected by the method of Bawden & Pirie (1946). It was centrifuged at 1500g for 15 min., frozen solid or freeze-dried, and kept at -15° .

Papain. A commercial sample (Merck) and a crystalline preparation made from it, and kindly given to us by Dr V. Liebl of this Institute, were used.

Estimation of invertase activity. Convenient dilutions of the solution or suspension to be tested were added to a solution at room temperature (20°) made by dissolving 2.5 g. of sucrose in sufficient water plus 1 ml. of m-sodium acetate buffer (pH 5.0) to make a final volume of 50 ml. A sample (10 ml.) was at once pipetted into a flask containing 10 ml. of 5% (w/v) Na_2CO_3 , filtered with the aid of a little Hyflo Supercel (Johns-Manville) and its optical rotation read in a 2 dm. tube in a polarimeter within 2 hr. Further samples (10 ml.) were taken and similarly treated at intervals (10 min. to 3 hr.) depending upon the activity of the solution being tested. From the fall in optical

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