Alkaline Ribonuclease and Ribonuclease Inhibitor in Mammary Gland During the Lactation Cycle and in the R3230AC Mammary Tumour

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(Received 17 September 1974)

Alkaline RNAase (ribonuclease) and RNAase inhibitor were assayed to determine the potential role of the degradative process in regulating the amount of RNA in the mammary gland and mammary tumour. Very little free alkaline RNAase activity was found in the cytosol fraction of the mammary gland of virgin, pregnant, lactating or involuting Fischer rats. However, addition of *p*-chloromercuribenzoate to the assay medium revealed latent RNAase which, when expressed on a DNA basis, decreased during pregnancy and lactation. The cytosol latent RNAase is stable in 0.125M-H₂SO₄. The non-cytosol RNAase activity also decreased during pregnancy and lactation. Addition of Triton X-100 produced slightly higher activity at all stages tested. The inhibitor activity in rat mammary gland was very low before pregnancy, increased gradually during pregnancy and more dramatically at parturition, continued to increase throughout lactation and returned to resting-gland values by the sixth day of involution. The increase during pregnancy may be due to the increased cellularity of the gland, whereas the gain during lactation was more than could be accounted for by increases in cell number. The R3230AC transplantable mammary tumour resembles the normal gland in early lactation with respect to both its cytosol and non-cytosol alkaline RNAase activities and its moderately high content of RNAase inhibitor. The relatively high inhibitor and low RNAase activities in both the gland of the lactating rat and in the tumour are of potential significance in maintaining high amounts of RNA and increased rates of protein synthesis in these tissues.

Intracellular RNAase* activity often changes with the metabolic state of a tissue. A species of alkaline RNAase, distributed in cytosol and mitochondria and characterized by a pH optimum around 7.8 (Roth, 1958*b*, 1967; Beard & Razzell, 1964), is known to decrease in developing rat liver (Rahman *et al.*, 1969) and in some transplantable hepatomas (Roth, 1967). The activity of this enzyme increases in tissues of hormonally depleted (Brewer *et al.*, 1969) and irradiated (Kraft *et al.*, 1969) or starved (Sheppard *et al.*, 1970) rats. The liver cytosol enzyme is normally present in a latent form that is suppressed by the presence of a natural protein inhibitor (Pirotte & Desreux, 1952; Roth, 1958*a,b*). This cytosol inhibitor is widely distributed in various tissues (Roth, 1956; Murthy & McKenzie, 1974) and is capable of stabilizing newly synthesized nuclear RNA (Hymer & Kuff, 1964) and polyribosomes (Blobel & Potter, 1966), which in turn results in maintenance of amino acid incorporation into protein in a cell-free system (Gribnau *et al.*, 1969). The inhibitor activity tends to increase in tissues under growth-stimulating conditions such as in regenerating liver (Shortman, 1962; Moriyama *et al.*, 1969), and in phytohaemagglutinin-

transformed lymphocytes (Kraft & Shortman, 1970). Thus the interplay between the alkaline RNAase and the inhibitor within the cell has been regarded as an important regulatory factor in protein synthesis (Kraft & Shortman, 1970; Brewer *et al.*, 1969).

During the normal lactation cycle the mass of the mammary epithelium increases severalfold owing to proliferation (Munford, 1963) and increased cell size (Hollman, 1969). This enlargement is accompanied by cytodifferentiation characterized by the accumulation of rough endoplasmic reticulum (Hollman, 1969; Wellings, 1969) and increased RNA/DNA ratios (Tucker & Reece, 1963*a,b*), and culminates in the synthesis and secretion of milk proteins, carbohydrates and lipids. Thus the mammary gland provides a useful system for studying the relationship between the alkaline RNAase and its inhibitor and their role in regulating protein synthesis. We have used the well-differentiated transplantable Fischer rat mammary adenocarcinoma R3230AC (Hilf *et al.*, 1965) to compare the functional state of closely related normal and tumour tissues. We now report the presence of an alkaline RNAase and its inhibitor in rat mammary gland, at different stages of the lactation cycle, and in the R3230AC mammary tumour.

* Abbreviation: RNAase, ribonuclease.

Experimental

Animals

The female Fischer rats used in these experiments were maintained in a room at $24 \pm 1^\circ\text{C}$ with 12 h of artificial light provided from 07:00h to 19:00h. Purina Laboratory Chow (Ralston Purina Company, St. Louis, Mo., U.S.A.) and tap water were provided *ad libitum*. Normal mammary gland was obtained from animals weighing 160–210 g as virgins or during their first pregnancy, lactation or involution; only females with litters of eight or more pups were used. Lactating females were kept with their litters until the time of death and were usually nursing the pups when taken from the cage. The pregnant animals were obtained from timed matings and the foetuses were examined to verify the stage of pregnancy. The R3230AC mammary tumour was given to us originally by Dr. Russell Hilf of the University of Rochester, and has been maintained by serial subcutaneous transplantation of fragments measuring 1–2 mm, into mature virgin females. For the study of early tumour stages, up to six pieces were transplanted into a single host.

Preparation of tissue

The animals were killed by decapitation. Liver, mammary gland and tumour were excised while the carcass was chilled on ice. The tissues were placed in cold Medium A, consisting of 0.35 M-sucrose, 10 mM-Tris-HCl (pH 7.4) and 6 mM-EDTA. All the following steps were carried out at 4°C . Surface connective tissue, lymph nodes and major blood vessels were removed from the mammary gland under a dissecting microscope. The membranous connective tissue on the outside and any necrotic tissue in the interior of the tumour were removed. After being rinsed with Medium A, the tissues were blotted dry, weighed, minced finely with scissors and homogenized in 4 vol. of Medium A with a motor-driven Teflon pestle (approx. 4000 rev./min) in a Potter-Elvehjem homogenizer. Fifteen up-and-down strokes were used for the gland and tumour. The homogenate was then centrifuged at 105 000g for 1 h at 4°C in a Spinco L2-65B centrifuge. The supernatant was taken as the cytosol fraction. The pellet was resuspended in Medium A in a homogenizer with a loosely fitted Teflon pestle and re-centrifuged under the same conditions as before. The pellet was then resuspended a second time in the original volume of Medium A under the same conditions as the initial homogenization but with only five up-and-down strokes. This final suspension is subsequently referred to as the particulate fraction. Since we were primarily interested in changes in the amount of total alkaline RNAase and RNAase inhibitor of the cytosol fraction, we did not attempt to separate this fraction into its com-

ponent subcellular particles. Both cytosol and particulate fractions were kept at -20°C until assay of enzyme and inhibitor activities. The RNAase and its inhibitor were stable in both fractions during 10 days of storage. The assays were usually carried out within 2–3 days.

RNA and RNAase

Yeast RNA was obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). It was dialysed for 12 h (three changes) against each of 10 mM-EDTA, 0.15 M-NaCl and water. The final concentration of RNA was adjusted to 1% (w/v), assuming that extinction of a 1 mg/ml solution at 260 nm is 21 (Crestfield *et al.*, 1955). A 1 mg/ml stock solution of bovine pancreatic RNAase (Boehringer Mannheim Corp., New York, N.Y., U.S.A.) was made in 0.02% (w/v) bovine serum albumin (Sigma) and kept at -20°C . A 1% (w/v) bovine serum albumin solution was dialysed overnight against 0.1 mM-EDTA, pH adjusted to 7.0 by addition of 1 M-NaOH. This solution was diluted with water to 0.02% albumin and used to prepare a working solution of pancreatic RNAase immediately before each experiment (Thach & Boedtker, 1969).

Assay of RNAase

The RNAase activity was assayed in triplicate by the method of Shortman (1961) modified by the use of Tris-HCl buffer (Gribnau *et al.*, 1970). The reaction medium contained: 0.1 ml of 0.5 M-Tris-HCl, pH 7.8; 0.1 ml of 1% (w/v) yeast RNA; 0.05 ml of 0.02% bovine serum albumin; 0.05 ml of enzyme solution containing 0.001–0.01 unit of activity. The assay medium for latent RNAase activity was the same except that 0.05 ml of 4 mM-*p*-chloromercuribenzoate (Sigma), adjusted to pH 7.8 by addition of 1 M-NaOH, in 0.02% bovine serum albumin replaced the plain albumin solution. The reaction was started by adding enzyme solution and the mixture was incubated in a Dubnoff shaking bath at 37°C for 30 min. At the end of the incubation the reaction tubes were placed in an ice-water bath and after 1 min 0.3 ml of cold ethanol-HCl mixture [76% (v/v) ethanol in 1 M-HCl] was added to stop the reaction. The tubes were centrifuged at 3000g for 15 min at 4°C and 0.2 ml of supernatant was diluted six-fold with water. The acid-soluble nucleotides produced in the reaction were determined by measuring the 260 nm absorption in a Zeiss PMQII spectrophotometer. A blank assay in which the enzyme solution was added after adding the ethanol-HCl was carried out simultaneously under the same conditions. A unit of enzyme activity is the amount that produces 1 μmol of nucleotide residues ($\epsilon = 11\,000$ at 260 nm)/min (Beard & Razzell, 1964).

Assay of inhibitor

The inhibitory activity of the cytosol fraction was tested in the same system as the RNAase assay, by using 3ng of pancreatic RNAase (0.0278 unit) in 0.05 ml of 0.02% bovine serum albumin and 0.05 ml of an appropriate dilution of the cytosol fraction. The reaction was started by adding the RNAase and a portion of the cytosol fraction. The blanks contained the same components, but the RNAase and the portion of the cytosol fraction were added after the ethanol-HCl. After centrifuging at 3000g for 15 min the supernatant was diluted 11-fold and the absorption was measured at 260nm. A unit of inhibitory activity is defined as the inhibition of 50% of the activity of 3ng of RNAase (0.0278 unit) under the assay conditions (Roth, 1958a).

DNA measurements

The tissue homogenate was treated with 10 vol. of cold 10% (w/v) trichloroacetic acid. The 9000g (10min) pellet was washed with trichloroacetic acid, and once each with ethanol and ethanol-ether (1:1, v/v). DNA was extracted from the pellet with 5% (v/v) HClO₄ at 90°C for 20min and measured by the diphenylamine reaction (Schneider, 1957).

Results

We found that the amount of DNA in rat mammary gland increases approximately fourfold during pregnancy, with an additional twofold increase in early lactation (Fig. 1). Thus for the interpretation of our RNAase and inhibitor data to approximate more closely to cellular events we have expressed them per mg of DNA, as well as per g of tissue.

Fig. 1. Changes in DNA content in rat mammary gland during the lactation cycle

Mammary-gland homogenates were prepared and DNA was determined as described in the Experimental section. Each point represents the mean $\pm$ s.e.m. of determinations from three or four rats, except the virgin stage which represents seven animals.

Alkaline RNAase activity in developing mammary gland

The cytosol free alkaline RNAase activity of the mammary gland at all developmental stages tested was very low or undetectable (Fig. 2). This is similar to the situation in the liver, where alkaline RNAase activity is not detectable unless thiol-blocking agents are added to prevent the action of the RNAase-bound inhibitor (Roth, 1958b, 1967). Our samples were also assayed for RNAase activity in the presence of *p*-chloromercuribenzoate, to reveal any latent activity. As shown in Fig. 2, latent RNAase was found in the cytosol at all stages of gland development. When expressed on a tissue-weight basis latent RNAase activity rose briefly at parturition and again at weaning to about twice the values in the virgin animals. At days 15 and 22 of lactation the activity was lower than that found early in lactation. There is a drastic increase in cellularity (the number of cells per unit weight) of the gland during pregnancy and early lactation, owing to both the decrease in fat-cell size (Elias *et al.*, 1973) and the proliferation of epithelial cells (Munford, 1963; Grahame & Bertalanffy, 1972). These events result in an increase in the number of cells per g of tissue, as indicated in the increased DNA concentration shown in Fig. 1. Therefore, as the

Fig. 2. Free and latent alkaline RNAase activities in the cytosol fraction of rat mammary gland at different stages of the lactation cycle

The cytosol (105000g supernatant) was prepared and RNAase activity assayed as described in the Experimental section. Each point represents the mean $\pm$ s.e.m. of determinations from three or four rats, except the virgin stage, which represents seven animals. (a) Free (○), and latent (▲) RNAase activities expressed on a tissue-weight basis. (b) Free (○) and latent (●) RNAase activities expressed on a DNA basis.

gland develops, the RNAase activity is distributed in a larger volume of cytosol per g of tissue. Thus the higher RNAase activity per g of tissue in early lactation may only reflect this increased cellularity of the gland. When expressed on a DNA basis, the activity was highest in the resting gland and actually decreased during pregnancy and lactation (Fig. 2b). Expressed in this way the activity during the middle and late lactation period was less than 15% of that found in the resting gland. Thus, as lactation progressed, the latent RNAase activity decreased.

In the particulate fraction, the RNAase activity was in general less than half that of the latent cytosol RNAase at all stages of development tested (Fig. 3). Addition of Triton X-100 has been reported to increase RNAase activity in the mammary-gland homogenate (Slater, 1961). In our assay, blank values were elevated by the addition of Triton X-100 (Sigma) to a final concentration of 0.2%, and the variability in results was larger. No significant differences in activity were found after the addition of Triton, although the values observed were generally higher. Whether Triton X-100 was present or absent, the overall pattern of change in the particulate enzyme

activity during mammary-gland development was similar: the resting gland contained the highest RNAase activity and the lactating glands contained very low activity. When the concomitant changes in DNA content of the gland are taken into consideration (Fig. 3b), the particulate RNAase activity per cell is seen to decrease during the proliferative phases and to rise during the involuting phase of the lactation cycle.

Addition of *p*-chloromercuribenzoate to the RNAase assay of the particulate fraction resulted in little or no additional RNAase activity, which indicates that not only was there little or no latent RNAase activity, but also that there was a negligible amount of cytosol contamination in the particulate preparation. When 0.2% Triton X-100 was used in the assay of the cytosol fraction, no latent RNAase activity was observed, thus indicating that the slightly higher RNAase activity observed in our particulate RNAase assay in the presence of Triton X-100 most likely is not due to the presence of cytosol latent RNAase.

Effect of H₂SO₄ on latent cytosol RNAase

Because the liver cytosol latent RNAase but not the RNAase inhibitor is known to be stable in 0.125M-H₂SO₄ (Roth, 1956; Beard & Razzell, 1964), we were interested in whether the same is true for the mammary-gland enzyme and inhibitor. The effect of 0.125M-H₂SO₄ treatment on cytosol RNAase from glands at different stages of development is shown in Fig. 4. The acid-treated cytosol indeed showed RNAase activity in the absence of *p*-chloromercuribenzoate, although slightly lower than the activity of untreated cytosol of the same sample assayed in the presence of *p*-chloromercuribenzoate. Since the acid treatment presumably destroys most other RNAases, as well as the alkaline RNAase inhibitor, the slightly higher RNAase activity measured in the presence of *p*-chloromercuribenzoate without acid treatment is most likely attributable primarily to alkaline RNAase.

Alkaline RNAase activity in R3230AC mammary tumour

The free RNAase activity in the tumour cytosol fraction was low or not detectable under these assay conditions (Table 1). Activity in these extracts increased 15–20-fold when *p*-chloromercuribenzoate was included in the assay; the same relative increase was seen in normal gland. The activities were comparable with those of the normal gland during early lactation, whether expressed per g of tissue or per mg of DNA. The particulate fraction of the tumour extracts contained less alkaline RNAase activity than did the

Fig. 3. Alkaline RNAase activity in the particulate fraction of rat mammary gland at different stages of the lactation cycle

The particulate fraction of rat mammary gland was prepared and the RNAase activity was assayed in the presence (●) and absence (○) of Triton X-100 as described in the Experimental section. Each point represents the mean $\pm$ S.E.M. of determinations from three or four rats, except the virgin stage, which consists of seven animals. (a) RNAase activity expressed on a tissue-weight basis; (b) RNAase activity expressed on a DNA basis.

Fig. 4. Comparison of cytosol acid-stable alkaline RNAase activity with the latent RNAase in rat mammary gland at different stages of the lactation cycle

The cytosol fraction was prepared as described in the Experimental section. A chilled 1.8M-H₂SO₄ solution was added to the cytosol fraction to 0.125M final concentration (Beard & Razzell, 1964). The same volume of water was added to a control sample. The samples were kept at 4°C overnight, and the pH was adjusted to 7.4 with 1 M-NaOH before centrifugation at 3000g for 10min. RNAase activity was then assayed as described in the Experimental section. *p*-Chloromercuribenzoate was added to the assay of the control sample (▲) but not to that of the acid-treated sample (□). Each point represents the average of determinations from two animals, except at day 5 of lactation, where three animals were used.

cytosol fraction, as was the case in the mammary gland during the various stages of the lactation cycle. Also, treatment of the tumour particulate fraction with Triton X-100 usually resulted in higher RNAase activities. Expressed per g of tissue, the tumour particulate RNAase activity resembled that of the normal gland from virgin, pregnant or parturient animals. Particulate activity per mg of DNA, however, was intermediate between the values for days 1 and 5 of lactation.

Alkaline RNAase inhibitor activity in developing mammary gland

The results of some typical assays of RNAase inhibitor in mammary gland are shown in Fig. 5. There was no inhibition of pancreatic RNAase by extracts of the gland from virgin or early-pregnant animals when up to 20µl of the cytosol fraction was present in the assay; however, a 30% inhibition occurred when 50µl was used. In contrast, extracts of the gland from animals at both day 1 and day 15 of lactation produced marked inhibition of the pancreatic RNAase when as little as 5µl or less of the cytosol fraction was used for the assay. The gland from animals on day 15 of lactation appeared to contain higher inhibitor activity. The inhibitor activity declined during involution.

To explore the nature of the inhibitor, cytosol

Table 1. Alkaline RNAase activity in the R3230AC mammary tumour and in mammary gland of tumour-bearing and non-tumour-bearing virgin rats. Preparation of cytosol and particulate fractions and assay conditions were as described in the Experimental section. The numbers of animals are given in parentheses. Times given are weeks after implantation of tumour. n.d. indicates DNA content of the sample was not determined. n.a. indicates RNAase was not assayed. The values represent means ± s.e.m.

Tissue and stage	RNAase in cytosol fraction			RNAase in particulate fraction		
	Free		Free+latent	-Triton		+Triton
	(unit/g)	(unit/g)		(unit/g)	(unit/g)	(unit/mg of DNA)
	(unit/mg of DNA)	(unit/mg of DNA)		(unit/mg of DNA)	(unit/mg of DNA)	
Gland from non-tumour rat (7)	0.0	0.386 ± 0.014	1.062 ± 0.072	0.126 ± 0.026	0.206 ± 0.066	0.542 ± 0.280
Tumour host gland	0.040 ± 0.022	0.502 ± 0.054	1.408 ± 0.172	0.218 ± 0.040	0.208 ± 0.056	0.592 ± 0.186
5-7 weeks (3)	0.020 ± 0.022	0.342 ± 0.050	n.d.	0.154 ± 0.052	n.a.	n.d.
10 weeks (3)	0.046	0.936	n.d.	0.016	n.a.	n.d.
R3230AC tumour	0.048	0.922	n.d.	0.046	n.a.	n.d.
2 weeks (1)	n.a.	0.756 ± 0.090	0.122 ± 0.022	0.078 ± 0.026	0.156 ± 0.034	0.024 ± 0.004
3 weeks (1)	n.a.	0.562 ± 0.122	n.d.	0.038 ± 0.020	n.a.	n.d.
5-7 weeks (3)						
10 weeks (3)

Fig. 5. Cytosol alkaline RNAase inhibitor determinations in rat mammary gland at different stages of the lactation cycle

The cytosol fraction was prepared and inhibitor activity was assayed as described in the Experimental section. Serial dilutions of the cytosol fraction were made in Medium A and 50 μ l of the dilutions were used in the assay. The abbreviations are: V (virgin) (●), P₁₀ (day 10 of pregnancy) (○), L₁ (day 1 of lactation) (△), L₁₅ (day 15 of lactation) (▲), I₆ (day 6 of involution) (□) and I₂₄ (day 24 of involution) (■).

Table 2. Effects of dialysis and heat treatment on cytosol alkaline RNAase inhibitor activity

Preparation of cytosol fraction and assay of inhibitor activity were as described in the Experimental section. A portion of the cytosol fraction was dialysed against Medium A for a total of 20h, with three changes of Medium A at about 6h intervals. Another portion of the cytosol fraction was heated in a 100°C water bath for 15min, cooled on ice, centrifuged at 3000g for 10min and assayed for inhibitor activity as described in the Experimental section. n.a. indicates RNAase was not assayed.

Tissue and stage	Activity (units/10mg of tissue)		
	Original	Dialysed	Heated
Mammary gland			
Pregnancy, day 22	4.3	4.1	0
Lactation, day 15	35.5	33.8	0
Lactation, day 22	30.9	30.9	0
R3230AC tumour			
10 weeks	21.5	n.a.	0

preparations were tested after dialysis or heat treatment. No changes in inhibitory activity were detected in the dialysed cytosol of glands from pregnant or lactating animals. In contrast, the heat-treated fractions no longer showed inhibitory activity (Table 2). These results are consistent with a protein nature, which is known for the liver inhibitor (Gribnau *et al.*, 1969).

The inhibitory activity at different stages of mammary-gland development is summarized in Fig. 6. The activity increased rapidly during pregnancy, and near parturition was ten times greater on a tissue-weight basis, and 3.5 times greater on a DNA basis, than in the resting gland. Activity continued to rise throughout lactation, another 7-fold on a weight basis, another 3.5-fold on a DNA basis. After weaning, the inhibitor activity quickly fell to that of the resting gland.

Alkaline RNAase inhibitor activity in R3230AC mammary tumour

The tumour contained high RNAase inhibitor activity (Table 3), comparable with those in the normal gland early in lactation. Although there were small differences in activity between individual tumours, the differences were apparently not related to the time elapsed since implantation. The mammary gland of the tumour-bearing rat, like the resting gland of the non-tumour-bearing animal, showed very

Fig. 6. Alkaline RNAase inhibitor activity in rat mammary gland at different stages of the lactation cycle

Preparation of the cytosol fraction, assay of inhibitor activity and DNA determination were as described in the Experimental section. Each point represents the mean $\pm$ S.E.M. of inhibitor activity in the mammary gland of several animals (seven for virgin, three each for day 16 of pregnancy, day 1 and day 5 of lactation and four each for day 15 and day 22 of lactation and day 6 of involution), expressed both per g of tissue (○) and per mg of DNA (●).

Table 3. *Alkaline RNAase inhibitor activity in R3230AC mammary tumour and mammary gland of tumour-bearing and non-tumour-bearing virgin rats*

Preparation of cytosol fraction and assay of inhibitor activity were as described in the Experimental section. The values represent means $\pm$ s.e.m., for the numbers of animals indicated in parentheses. Time-periods indicated for tumour are weeks after tumour implantation. n.d. indicates DNA content of the sample was not determined.

Tissue and stage	DNA (mg/g of tissue)	Inhibitor activity	
		(units/g of tissue)	(units/mg of DNA)
Gland from non-tumour rat	0.38 $\pm$ 0.01 (7)	66.0 $\pm$ 13.7 (7)	179 $\pm$ 44 (7)
Tumour host gland	0.37 $\pm$ 0.02 (4)	59.8 $\pm$ 16.8 (4)	161 $\pm$ 49 (4)
R3230AC tumour			
2 weeks	n.d.	2270 (1)	n.d.
3 weeks	n.d.	2400 (1)	n.d.
5-7 weeks	6.32 $\pm$ 0.50 (3)	2892 $\pm$ 1497 (3)	429 $\pm$ 188 (3)
10-13 weeks	n.d.	3028 $\pm$ 1056 (4)	n.d.

Table 4. *Summary of alkaline RNAase and RNAase inhibitor activities in mammary gland at different stages and in the R3230AC tumour*

The values for alkaline RNAase are the sum of the mean values in Fig. 2(a) (latent) and 3(a) (Triton). The inhibitor data are mean values from Fig. 6. The tumour data are the sum of the values for cytosol (free+latent) and particulate (Triton) fractions in Table 1.

Tissue and stage	Total alkaline RNAase (units/g of tissue)	Inhibitor (units/g of tissue)	Inhibitor RNAase
Mammary gland			
Virgin	0.592	66	112
Pregnancy			
day 16	0.640	460	719
day 22	0.636	678	1066
Lactation			
day 1	0.876	1280	1461
day 5	0.524	2733	5216
day 15	0.470	3780	8043
day 22	0.496	4565	9204
Involution			
day 6	0.872	223	256
day 24	0.812	84	103
Tumour host gland			
5-7 weeks	0.720	60	83
R3230AC tumour			
2 weeks	0.952	2270	2384
3 weeks	0.968	2400	2479
5-7 weeks	0.912	2892	3171
10 weeks	0.496	3028	6105

low inhibitor activity. Inhibitor activity in the tumour is comparable with that of the normal gland at about day 5 of lactation, on either a tissue-weight or a DNA basis (Table 3, Fig. 6).

Discussion

Our results show that the subcellular distribution of both alkaline RNAase and RNAase inhibitor in the mammary gland is similar to their distributions in liver: free RNAase primarily in the particulate

fractions of the cell, acid-stable latent RNAase and inhibitor in the cytosol fraction. On a tissue-weight basis the cytosol and total tissue RNAase (Table 4) increased about twofold during early lactation but decreased in late lactation. However, on a DNA basis it decreased during both pregnancy and lactation (Figs. 2 and 3). This apparent disagreement is primarily due to the change in cellularity of the gland during the lactation cycle. Since there is no single satisfactory base to which to refer the data at all

stages of the cycle, we have expressed our results in both ways. Our data on RNAase agree for the most part with those of Slater (1961); in particular, when expressed on a DNA basis we both observed a decrease in RNAase during pregnancy and lactation. However, on a weight basis we did not observe the nearly 6–7-fold increase in alkaline RNAase activity during mammary-gland development reported by Slater (1961). Slater (1961) apparently did not use *p*-chloromercuribenzoate, but used Triton X-100 in his assay, hence release of latent cytosol activity would not be expected. The possibility that Triton X-100 might reveal latent RNAase is not supported by the report of Sirakov & Schulga (1971), who found inhibitor activity to be unaffected by Triton X-100 concentrations as high as 2%. We have observed that 0.2% Triton X-100 had no effect on inhibitor assays or on release of latent RNAase. Therefore we would expect our assays of the particulate fraction in the presence of Triton X-100 to correspond to those of Slater (1961), who used whole homogenates.

According to Rahman (1966), the inclusion of Triton X-100 in the assay causes maximal release of alkaline RNAase activity showing maximal activity at pH 9.5 from the membrane fraction. Thus the use of Triton X-100 in the assay for alkaline RNAase of total homogenate or particulate fractions could result in the release of alkaline RNAase having a pH optimum of 9.5 from cell membranes. Whether this enzyme made a significant contribution to the activity observed by Slater (1961), we cannot ascertain. Slater (1961) exposed the mammary-gland homogenates to Triton X-100 in the range 0.3–0.7% before the enzymic assay, whereas we added it during the assay. The contribution of the pH 9.5 enzyme would be minimized by the fact that both Slater (1961) and we used a substrate concentration (10 mg/ml) somewhat inhibitory (about 40%) to the pH 9.5 enzyme. Our assay medium also contains 1 mM-EDTA, which should preferentially inhibit the pH 9.5 enzyme (Rahman, 1966), but Slater (1961) reported that addition of EDTA over a range of 5–15 mM to his assays had little effect on activity.

Our determination of inhibitor by serial dilution showed differences of two orders of magnitude between tumour or lactating gland and the gland of the virgin, whereas the latent RNAase activity showed no more than a twofold difference between any two stages of gland tested. Therefore the use of *p*-chloromercuribenzoate may only demonstrate the presence of an inhibitor–RNAase complex and the amount of latent RNAase, but not the amount of inhibitor. These results also indicate that even in the gland of the virgin the amount of inhibitor is sufficient to suppress cytosol RNAase activity. However, in the lactating gland and in the tumour a great excess of inhibitor is present, as shown by the increase in the inhibitor/RNAase ratio (Table 4).

The higher amount of RNA in the glands of pregnant and lactating animals (Munford, 1964; Richards & Hilf, 1972) may be the result of an increase in synthesis and/or a decrease in degradation. There is evidence that RNA synthesis is increased during this time. Banerjee & Banerjee (1973) have shown that the mammary glands from pregnant and lactating mice incorporate [³H]uridine into rapidly labelled RNA whereas those of virgin mice do not. They also demonstrated by radioautography that the nucleoli of mammary gland on days 5 and 10 of lactation incorporate more RNA precursor than do the nucleoli from the glands of pregnant animals (Banerjee & Banerjee, 1971). [³H]Uridine incorporation by nucleoplasm, however, was similar in pregnancy and lactation. Wang & Greenbaum (1962) have shown that there is a marked increase in the rate of [2-¹⁴C]-glycine and [6-¹⁴C]orotic acid incorporation into total rat mammary-gland RNA during early lactation. This apparent upsurge in the rate of RNA synthesis during pregnancy and early lactation may contribute to the increased concentration of RNA during the same period. However, there is an apparent decrease in the rate of precursor incorporation as lactation advances (Wang & Greenbaum, 1962), whereas the RNA concentration continues to increase (Munford, 1964; Richards & Hilf, 1972); hence something other than alteration of the rate of synthesis must be responsible for the increased concentration of RNA. It may be that decreased degradation has more effect on the concentration of RNA during this late lactation period than at earlier stages. There is also evidence, however, that the apparent changes in synthetic rate may not prevail for all types of RNA (Banerjee & Banerjee, 1971; Mendelson & Anderson, 1972, 1973).

The amount of RNAase inhibitor found in the developing mammary gland increases with time on both tissue and DNA bases, that is, the amount per cell increases (Fig. 6). This increase is nearly parallel to the known increase in RNA. The RNA content on a DNA basis increases 2.5-fold during pregnancy and another twofold during lactation (Munford, 1964; Richards & Hilf, 1972), whereas the RNAase inhibitor content rises 3.5-fold during pregnancy and a further 3.5-fold during lactation (Fig. 6). Total protein and many enzymes in the gland also accumulate during pregnancy and lactation (Munford, 1964; Baldwin & Milligan, 1966; Richards & Hilf, 1972). Thus it may be that the increasing amounts of RNAase inhibitor stabilize the RNA molecules which enable these proteins to be synthesized. These biochemical events in the differentiation of the mammary gland are consistent with the suggestion that the inhibitor/RNAase ratio is high in tissues relatively active in protein synthesis (Kraft & Shortman, 1970).

One might predict that the high inhibitor content

of the cytosol fraction from lactating gland would maintain the protein-synthetic capacity of polyribosomes in a cell-free system, as the liver RNAase inhibitor does (Blobel & Potter, 1966; Gribnau *et al.*, 1969). Data consistent with this hypothesis have been published by Sirakov & Rychlik (1969). They found that amino acid incorporation by cell-free systems from mammary gland was eight times greater in lactation than in pregnancy, and that this difference was primarily due to the 105000g supernatant fraction which had been passed through Sephadex G-25. These authors did not ascertain the nature of the stimulatory factor.

Alkaline RNAase activity per mammary cell declines during pregnancy and lactation as the non-epithelial cells make up a progressively smaller proportion of the tissue. Similarly, non-epithelial cells comprise a quarter or less of the R3230AC tumour (Hilf *et al.*, 1965). Thus it is possible that the low RNAase activity observed in the lactating gland and R3230AC tumour may have been derived solely from connective tissues. Conversely, the RNAase inhibitor is most likely localized in the epithelial cell population. Daoust & Amano (1963) and Daoust (1972) utilized a histochemical technique to examine various experimental and human cancer tissues, including breast carcinoma, and demonstrated that RNAase activity is detectable only in the connective tissue and necrotic regions. He concluded that cancer cells are generally deficient in RNAase. However, since inhibitor is undoubtedly present in these tissues, the histochemical film method probably demonstrates primarily enzyme in the organelles which comprise the particulate fraction. The lower activity in sections of tumour could be due to a large increase in the amount of inhibitor, rather than a decrease in enzyme.

To the list of biochemical events during the cellular differentiation which accompanies the changes in functional capacity of the rat mammary gland we can now add the increasing amount of RNAase inhibitor in lactating tissue. RNAase inhibitor has also been found in the mammary gland of the cow (E. W. Bingham, personal communication), indicating that this parameter of mammary-gland differentiation is not restricted to rats. The presence of high concentrations of RNAase inhibitor and low RNAase activity in the R3230AC transplantable mammary tumour suggests that RNA metabolism in this tumour may be similar to that of the gland in early lactation. These observations are consistent with the previously noted biochemical resemblance of the tumour to partly differentiated mammary gland (Hilf *et al.*, 1965, 1967; McGuire, 1969; Turkington, 1970). It is noteworthy that in the tumour this level of differentiation occurs in the absence of the hormonal milieu which elicits lactogenesis in the normal gland.

This investigation was supported in part by a grant (CA 12808) from the United States Public Health Service.

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