Polyamine Biogenesis in the Rat Mammary Gland during Pregnancy and Lactation

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(Received 15 May 1972)

Polyamines and RNA accumulate in the rat mammary gland during pregnancy, but the major increases occur after parturition. Therefore the major increases occur after the gland has obtained its maximal complement of epithelial cells. During lactation, the spermidine concentration rises above 5 mM and RNA content in the lactating mammary gland reaches a value 16 times that of the unstimulated mammary gland. The ratio of spermidine/spermine, an increase of which initially signals an elevation in biosynthetic activity, is near 1 in the normal mammary gland and is greater than 10 in the lactating mammary gland. Putrescine concentration is very low during the entire course of mammary-gland development, with the exception of early pregnancy. The low putrescine concentration probably reflects the very rapid conversion of putrescine into spermidine. Both ornithine decarboxylase, the enzyme that synthesizes putrescine, and putrescine-stimulated *S*-adenosyl-L-methionine decarboxylase, the enzyme that synthesizes spermidine, increase in activity during middle and late pregnancy; during lactation, both enzyme activities are elevated until the 21st day of lactation, and then decline. These declines are concomitant with involution. Also, it was found that the amount of ribonuclease activity in the mammary gland was very high during lactation, almost double that in the gland during pregnancy.

Polyamine synthesis and accumulation as an early event in rapid-growth systems may well be correlated with the regulation of rRNA synthesis (Raina & Cohen, 1966; Russell, 1970; Russell & Lombardini, 1971; Gfeller & Russell, 1971). It has been shown, for example, that the synthesis of putrescine and spermidine in *Xenopus laevis* embryos corresponds with the onset of rRNA synthesis and the formation of a viable nucleolus in the embryonic cell (Russell, 1971). Even more suggestive is the observation that anucleolate-mutant embryos of *Xenopus*, which do not synthesize and accumulate rRNA, also do not synthesize and accumulate spermidine. This is the first evidence indicating that the DNA missing in the anucleolate mutants may contain more than just rRNA genes; i.e. it may contain genes for regulatory mechanisms that affect rRNA synthesis. Experiments on nuclear preparations and on nuclear fractions from rat liver indicate that the polyamines act to increase the effective activity of the RNA polymerase which specifies rRNA synthesis (Russell *et al.*, 1971, 1972). If this is true, those systems which synthesize new rRNA during growth should always show an earlier increment in polyamine synthesis.

The resting mammary gland contains almost no epithelial gland cells but is largely fat and connective tissue. On pregnancy, an enormous increase in the epithelial gland cells occurs. It appears that by

mid- to late-pregnancy, the mammary gland reaches its maximal concentration of these gland cells (F. Stockdale, personal communication). Therefore the changes in both RNA and polyamines that occur during pregnancy probably reflect the increase in the number of epithelial gland cells. However, the changes in polyamine concentrations and RNA content during lactation must reflect increases in these components within a fairly fixed number of gland cells, as very little DNA synthesis is occurring during lactation (F. Stockdale, personal communication).

No study has been undertaken to illustrate the pattern of polyamine biosynthesis and accumulation during pregnancy, lactation and involution of the rat mammary gland. Neish & Key (1968) have reported high amounts of spermidine in the lactating mammary gland of the rat compared with the unstimulated gland. However, in this study spermidine concentration was measured at one point during lactation and no attempt was made to measure the enzymes of the biosynthetic pathway.

In this paper we report on relationships between polyamines and RNA in the rat mammary gland. These relationships can be studied effectively since the mammary gland is easily cycled through growth and regression. We found that maximal concentrations of the polyamines, particularly spermidine,

slightly precede maximal concentrations of RNA and are concomitant with the most rapid rates of protein synthesis (Suetina, 1966). We also found that by measuring the enzymes in the polyamine biosynthetic pathway, we could predict increased endogenous pools by the increased enzymic activities. This is not always true of enzyme systems, but in all studies to date, increases in the polyamine biosynthetic pathway are reflected in increases in polyamine pools.

Materials and Methods

Materials

DL-[1- 14 C]Ornithine monohydrochloride (2.6 mCi/mmol), [1,4- 14 C]putrescine dihydrochloride (11.3 mCi/mmol), [14 C]spermidine trihydrochloride (10.7 mCi/mmol), and [14 C]spermine tetrahydrochloride (9.9 mCi/mmol) were obtained from New England Nuclear Corp., Boston, Mass., U.S.A. *S*-Adenosyl-L-[carboxyl- 14 C]methionine (47 mCi/mmol) was obtained from Amersham-Searle, Arlington Heights, Ill., U.S.A.

Methods

RNA was prepared from baker's yeast by the method of Crestfield *et al.* (1955).

Sperm-positive Sprague-Dawley rats (150–200 g) and matched controls were obtained from Zivic-Miller, Allison Park, Pa., U.S.A. All rats were maintained in a room with a 0700–1900 h photoperiod and were given food and water *ad libitum*. To avoid the possibility of cyclic enzymic changes, all animals were killed between 0900 and 1100 h. Litter sizes were kept uniform (between 9 and 11 rats).

Enzyme preparation. The mammary gland was excised, chilled on ice, weighed and immediately homogenized in 5 vol. of cold 0.05 M-sodium-potassium phosphate buffer, pH 7.2, containing 1 mM-dithiothreitol. After clarification at 10000g for 10 min, the crude homogenate was centrifuged at 100000g for 90 min. The pellet was discarded and the supernatant solution was used as the source of enzyme in all subsequent assays.

Assay for ornithine decarboxylase activity. Ornithine decarboxylase activity was determined by measuring the liberation of $^{14}\text{CO}_2$ from carboxyl-labelled ornithine as described in detail by Russell & Snyder (1968, 1969). The substrate concentration (0.1 mM as L-ornithine) used as a routine was non-saturating. However, in some experiments an excess of ornithine (2 mM) was used as substrate, and the same general changes were observed.

Assay for *S*-adenosyl-L-methionine decarboxylase activity. Enzyme activity was determined by measuring the liberation of $^{14}\text{CO}_2$ from *S*-adenosyl-L-[carboxyl- 14 C]methionine as described by Pegg & Williams-Ashman (1969), with minor changes.

Incubation mixtures consisted of 0.2 mM-*S*-adenosyl-L-[carboxyl- 14 C]methionine, 25–50 mM-sodium-potassium phosphate buffer, pH 7.2, 50 μM -pyridoxal phosphate, 2.5 mM-putrescine, and 0.1–0.15 ml of enzyme supernatant solution [3–6 mg of protein, measured by the method of Lowry *et al.* (1951)], prepared as described above, to make a total volume of 0.2 ml. Reaction was linear in this range of protein concentration and was linear for 30 min. The substrate concentration (0.2 mM as *S*-adenosyl-L-methionine) used as a routine was non-saturating. However, in some experiments excess of *S*-adenosyl-L-[carboxyl- 14 C]methionine (1 mM) was used as substrate, and the same general changes were observed. In certain cases, [14 C]putrescine was added, and unlabelled *S*-adenosyl-L-methionine [unlabelled *S*-adenosyl-L-methionine iodide obtained from Sigma was purified by application to and elution from a Dowex 50 (H^+ form) column, as described by Russell *et al.* (1970)]. The [14 C]spermidine formed was determined as previously described (Russell *et al.*, 1970). There was a stoichiometric relationship between the amount of [14 C]spermidine formed under these conditions and the evolution of $^{14}\text{CO}_2$ when *S*-adenosyl-L-[carboxyl- 14 C]methionine was added.

Determination of endogenous concentrations of putrescine, spermidine, and spermine. Tissues were excised, immediately chilled, and homogenized in 4 vol. of 0.1 M-HCl, then 4 vol. of cold 10% (w/v) trichloroacetic acid was added and the mixture was centrifuged. Trichloroacetic acid was removed by ether extraction. The concentrations of the polyamines in the supernatant solution were assayed by extraction into alkaline butan-1-ol, separation by high-voltage electrophoresis, and staining with acid ninhydrin as previously described (Russell *et al.*, 1970).

Ribonuclease assay. Ribonuclease activity was determined as described by Roth (1967). The enzyme source was the supernatant solution (20000g for 20 min) of mammary glands from normal, pregnant or lactating rats. The reaction mixture contained 100 μmol of tris-HCl buffer (pH 7.0), 300–600 μg of yeast RNA and 0.1–0.2 ml of the enzyme preparation in a total volume of 1 ml. After incubation at 37°C for 30 min, the reaction was stopped with 1 ml of 25% (v/v) HClO_4 containing 0.75% uranyl acetate. The mixture was kept on ice for 1 h and then centrifuged at 50000g for 10 min. The increase in the absorbance of the supernatant solution was measured at 260 nm in a Gilford model 2400 spectrophotometer. This assay gave linear results with time and enzyme concentration.

RNA content was determined by the method of Munro & Fleck (1966). In some experiments, RNA was isolated from mammary-gland homogenates by hot phenol-sodium dodecyl sulphate extraction and separated by sedimentation through discontinuous

sucrose gradients, as previously described (McConkey, 1967).

Results

Putrescine and spermidine synthesis in rat mammary gland during pregnancy, lactation and involution

Ornithine decarboxylase activity was measured in the mammary gland by collection of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]\text{ornithine}$ as described in the Materials and Methods section. Ornithine decarboxylase activity does not differ from control values until day 6 of pregnancy, when it starts to increase; at parturition it reaches a maximum 20-fold above controls (Fig. 1). After birth, it declines markedly but is elevated again around day 7 of lactation (Fig. 2).

S-Adenosyl-L-methionine decarboxylase activity was determined in the mammary gland by measuring the liberation of $^{14}\text{CO}_2$ from S-adenosyl-L-[carboxyl- $^{14}\text{C}]\text{methionine}$ as described in the Materials and Methods section. S-Adenosyl-L-methionine decarboxylase activity increases before birth (Fig. 1), but increases most dramatically after birth (Fig. 2), reaching a value 160-fold above that of controls at day 16 of lactation. It remains elevated until the initiation of involution and then rapidly declines to the baseline.

Fig. 1. Time-course of increases of ornithine decarboxylase and S-adenosyl-L-methionine decarboxylase activities in the mammary gland of the pregnant rat

Ornithine decarboxylase (●) was measured by assaying the release of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]\text{ornithine}$ and S-adenosylmethionine decarboxylase (○) by assaying the release of $^{14}\text{CO}_2$ from S-adenosyl-L-[carboxyl- $^{14}\text{C}]\text{methionine}$ as described in the Materials and Methods section. Each point represents the mean $\pm$ S.E.M. of five separate determinations on five separate mammary glands.

Endogenous concentrations of polyamines and of RNA in rat mammary gland during pregnancy, lactation and involution

Putrescine is present in measurable amounts in the unstimulated mammary gland and in the mammary gland up to day 6 of pregnancy. Only trace amounts of putrescine are present during the rest of pregnancy and lactation.

Spermidine concentrations increase gradually during pregnancy and reach a value 13-fold above those of controls just before birth (Table 1). After birth, spermidine concentrations continue to rise, reaching a value above 5 mM by day 13 of lactation. Both the initiation of involution and of decreased spermidine concentration begin simultaneously (Table 2).

The spermine pool in the mammary gland during pregnancy increases gradually and is double the control value within 16 days. Thereafter the spermine concentration declines to a value below that of the controls near birth (Table 1). The striking relationship is the dramatic variation in the spermidine/spermine ratio during pregnancy. This ratio is less than 1 in control mammary glands and is more than 10 in mammary glands of rats that are at days 18–22 of lactation.

The RNA content of the unstimulated mammary gland is low (0.7 mg/g wet wt.) (Table 1). During the course of pregnancy, the RNA content in the mammary gland increases to approx. 2.5 mg/g wet wt. near birth. This amount of RNA is still relatively low, since normal rat liver contains approx. 7 mg/g wet wt. The RNA content undergoes rapid changes after birth and reaches a value 16 times that of controls 13 days after parturition. This maximum corresponds to the

Fig. 2. Fluctuations of ornithine decarboxylase and S-adenosyl-L-methionine decarboxylase activities in the mammary gland of the lactating rat

The details are as given in Fig. 1. ●, Ornithine decarboxylase activity; ○, S-adenosylmethionine decarboxylase activity.

Table 1. *Endogenous polyamine concentrations and RNA content in rat mammary gland during pregnancy*

Polyamine concentrations were determined in mammary-gland homogenates by extraction of the amines into butan-1-ol and separation by high-voltage electrophoresis as described in the Materials and Methods section and by Roth (1967). RNA was determined by the method of Munro & Fleck (1966). Each value represents the mean $\pm$ S.E.M. of five separate determinations on five separate mammary glands.

	Concn. of polyamine (nmol/g wet wt.)			Concn. of RNA (mg/g wet wt.)
	Putrescine	Spermidine	Spermine	
Control (not pregnant)	160 $\pm$ 20	120 $\pm$ 10	160 $\pm$ 20	0.7 $\pm$ 0.1
Day 6	230 $\pm$ 30	220 $\pm$ 15	180 $\pm$ 20	1.7 $\pm$ 0.2
Day 10	<40	580 $\pm$ 80	240 $\pm$ 20	2.0 $\pm$ 0.2
Day 13	<40	520 $\pm$ 60	240 $\pm$ 20	2.3 $\pm$ 0.2
Day 15	<40	1040 $\pm$ 120	300 $\pm$ 30	2.2 $\pm$ 0.2
Day 16	<40	1370 $\pm$ 150	400 $\pm$ 40	2.6 $\pm$ 0.3
Day 18	<40	1470 $\pm$ 180	360 $\pm$ 30	2.4 $\pm$ 0.2
Day 21	<40	1470 $\pm$ 160	170 $\pm$ 20	2.6 $\pm$ 0.3
Day 22	<40	1550 $\pm$ 190	130 $\pm$ 10	2.5 $\pm$ 0.3

Table 2. *Endogenous polyamine concentrations and RNA content in rat mammary gland during lactation and involution*

Details are as given in Table 1.

Time (days)	Concn. of polyamine (nmol/g wet wt.)			Concn. of RNA (mg/g wet wt.)
	Putrescine	Spermidine	Spermine	
1	<40	2210 $\pm$ 210	320 $\pm$ 40	4.7 $\pm$ 0.6
2	<40	2730 $\pm$ 240	330 $\pm$ 30	5.7 $\pm$ 0.6
7	<40	3760 $\pm$ 410	240 $\pm$ 20	8.9 $\pm$ 0.9
9	<40	3190 $\pm$ 320	260 $\pm$ 30	7.8 $\pm$ 0.8
10	<40	2540 $\pm$ 270	330 $\pm$ 40	6.1 $\pm$ 0.7
12	<40	4540 $\pm$ 450	420 $\pm$ 40	10.4 $\pm$ 1.3
13	<40	5140 $\pm$ 540	540 $\pm$ 60	11.7 $\pm$ 1.6
14	<40	4390 $\pm$ 420	450 $\pm$ 50	9.4 $\pm$ 1.2
16	<40	4960 $\pm$ 500	590 $\pm$ 60	10.6 $\pm$ 1.2
20	<40	5160 $\pm$ 520	290 $\pm$ 30	6.9 $\pm$ 0.8
21	<40	3050 $\pm$ 310	420 $\pm$ 40	5.0 $\pm$ 0.5
26	<40	1870 $\pm$ 190	590 $\pm$ 60	3.8 $\pm$ 0.4

maximal spermidine concentration (over 5 mM) present at this time. RNA concentrations decline after 2 weeks of lactation, whereas that of spermidine remains elevated until day 20 and then declines.

Fluctuations in polyamine concentration and RNA content in response to premature involution

In this experiment the young were removed from certain mothers on day 18 of lactation and the

mammary glands of these rats were assayed on day 20 for the concentrations of polyamines and RNA. Control values were obtained with mammary glands from rats with young on day 20 of lactation. Both the spermidine concentration and the RNA content in the experimental animals were less than half that present in the control animals (Table 3). Again, when the young were removed on day 21 of lactation, and the mammary gland was assayed on day 26, similar results were obtained (Table 3).

Table 3. *Endogenous polyamine concentrations and RNA content in rat mammary gland during premature involution*

	Concn. of polyamine (nmol/g wet wt.)			Concn. of RNA (mg/g wet wt.)
	Putrescine	Spermidine	Spermine	
Day 20 (with young)	<40	5160 ± 520	290 ± 30	6.9 ± 0.8
Day 20 (young removed on day 18)	<40	2420 ± 230	360 ± 30	2.9 ± 0.3
Day 26 (with young)	<40	1870 ± 190	590 ± 60	3.8 ± 0.4
Day 26 (young removed on day 21)	<40	720 ± 70	390 ± 40	1.6 ± 0.2

Table 4. *Ribonuclease activity in lactating mammary gland*

Ribonuclease activity was determined as described in the Materials and Methods section. A 200 μ l portion of the incubation mixture was diluted to 3ml and the E_{260} recorded. Each value is a measurement on a separate mammary gland. These values were obtained when 600 μ g of yeast RNA were used and 100 μ l of the enzyme preparation (see the Materials and Methods section).

Group	E_{260}
Normal	0.278
	0.213
	0.250
Pregnancy (7 day)	0.317
	0.361
	0.355
Lactating (14 day)	0.416
	0.502
	0.519

low-molecular-weight RNA was obtained. This indicated that a very stable ribonuclease which was unaffected by the hot phenol-sodium dodecyl sulphate extraction might be present in the mammary gland.

By using a standard technique for ribonuclease detection (see the Materials and Methods section), it was found that supernatants from lactating mammary gland contained considerable ribonuclease activity (Table 4), as much as 200% that of controls. Moreover, ribonuclease activity in the mammary gland during pregnancy was significantly higher than controls.

Discussion

There are some basic trends of polyamine and RNA accumulation in the stimulated mammary gland that should be emphasized. Both polyamine and RNA accumulation increase during pregnancy starting from very low values. By the tenth day of pregnancy the RNA content is approx. 2.0mg/g and it remains near that value until parturition, after which it increases rapidly to a value as high as 12mg/g. Spermidine concentration never exceeds 1.5mM before parturition, but thereafter increases to a concentration above 5mM in the lactating mammary gland. Although the hormonal regulation of mammary-gland functions is tremendously complicated, it seems that parturition results in alterations in the hormonal patterns, which then allow for the rapid synthesis and accumulation of polyamines (particularly spermidine) and RNA.

Other investigators have reported a very high spermidine/spermine ratio in the lactating mammary gland of *Rattus rattus* (Neish & Key, 1968). The ratio reported was approx. 11 for rats that were lactating from 5 to 17 days. In normal mammary gland, the ratio was near 1. This agrees well with our results,

Determination of ribonuclease activity of mammary gland

We found in experiments in which RNA was extracted with hot phenol and sodium dodecyl sulphate from mammary glands that were non-stimulated (not pregnant) and from lactating glands, that the RNA sedimented on a 15-30% (w/v) sucrose gradient exhibited only a large E_{260} in the range of 4S RNA (for method see McConkey, 1967). RNA processed in the same manner from rat liver resulted in a characteristic profile of 28S, 18S and 4S RNA. However, when the rat liver RNA and mammary-gland RNA were extracted in the same tube, only a large peak of

which indicate a spermidine/spermine ratio of over 10 from days 18 to 22 of lactation. In normal animals, the ratio is less than 1. Generally, this enhancement of the spermidine/spermine ratio initially signals increased biosynthetic activity of a tissue and, more specifically, signals increased biosynthetic capacity for RNA and protein. It is also noteworthy that this high spermidine/spermine ratio is established after all the gland cells of the mammary gland are present.

It is important at this time at least to hypothesize a mechanism by which polyamine biogenesis might be a regulatory factor in rRNA synthesis. Other investigators found that the DNA content per nucleus is 20–37% higher in mammary-gland nuclei during late pregnancy and lactation as compared with the quantity present during involution (Simpson & Schmidt, 1969; Sod-Moriah & Schmidt, 1968). Pelc (1968) has postulated that this 'extra' DNA consists of extra copies of genes that regulate and code for RNA synthesis, presumably rRNA synthesis. These ideas fit well with results from our laboratory which indicate that the polyamine spermidine, incubated with isolated rat nuclei, increases nucleolar DNA-dependent RNA polymerase activity by first increasing the amount of DNA present at the nucleolus (Russell *et al.*, 1972).

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