6. The significance of the changes in DNA content of the mammary gland are discussed. It is suggested that the changes in DNA content of the gland at parturition indicate that a wave of cell division occurs in the gland around that time.

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Studies on the Particulate Components of Rat Mammary Gland

3. THE RELATIONSHIP BETWEEN ENZYME ACTIVITY AND PARTICLE COUNTS IN MAMMARY GLAND SUSPENSIONS

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The two preceding parts of this series (Greenbaum & Slater, 1957*a*, *b*) have reported the rapid changes in chemical content which occur in the rat mammary gland over the period of parturition. This paper describes changes in the enzymic activity of the gland throughout the lactation cycle.

Part 1 of this series showed that the increase in tissue nitrogen at parturition was both rapid and considerable, and Part 2 described the rapid increases in milk synthesis and nucleic acid content which occur over this same period. Thus the gland in late pregnancy contrasts vividly, both in secretory activity and in composition, with the gland a few days later when lactation is well established.

It is to be expected that the transition of the gland into the 'active' state would be accompanied by an increase in the activity of the oxidative-enzyme systems. That such an increase occurs is evidenced by the rapid rise in Q_{O_2} and R.Q. (Folley & French, 1949), the increases in succinic oxidase

and cytochrome oxidase activity (Tuba, Orr & Wiberg, 1955) and in glucose 6-phosphate and 6-phosphogluconate dehydrogenases (Glock & McLean, 1954).

Recent investigations have localized the majority of the oxidative enzymes in the particles of mitochondrial size (see Schneider, 1956). It seemed of interest, therefore, to follow in mammary gland suspensions the alterations in the activity of an enzyme which, in other tissues, is known to be associated with the mitochondrial fraction. In particular, it was of interest to see whether the rapid increase in synthetic activity found in the gland *post partum* was associated with changes in the number of mitochondrial particles, an increase in size of the existing particles or an increasing efficiency of the enzyme systems.

It has repeatedly been shown, in diverse tissues, that succinic oxidase is almost completely localized in the mitochondrial fraction. Although this enzyme has been studied in mammary tissue (guinea pig,

Moore & Nelson, 1952; rat, Tuba *et al.* 1955; mouse, Dmochowski & Strickland, 1953) it was thought that a re-investigation into the variations in activity in the full homogenate and in the mitochondrial fraction over the lactation cycle would be of interest and would provide information on the following points: (i) the intracellular localization of the enzyme at various stages of the lactation cycle; (ii) the variations in the total activity of the enzyme in the whole-tissue suspension and in the mitochondria at various stages of the lactation cycle; (iii) variation in the recovery of the enzyme in the mitochondrial fraction, under standard conditions of centrifuging, during the lactation cycle; (iv) the correlation between these variations and the changes in ribonucleic acid (RNA), deoxyribonucleic acid (DNA) and nitrogen which occur during the lactation cycle.

In order to carry the correlation of enzymic activity with mitochondrial fractions a stage further, counts were made of particle numbers in the original tissue suspension and in the mitochondrial suspension derived from it. These counts have been used to correlate recoveries of mitochondrial nitrogen, RNA and enzymic activity with the number of particles recovered.

METHODS

Animals. The animals used and the method of distribution of the rats into the various groups has already been described, as has the method of preparing the tissue suspension and the chemical procedures for lactose content, deoxyribonucleic acid phosphorus (DNA-P), ribonucleic acid phosphorus (RNA-P) and total nitrogen content of the gland (Greenbaum & Slater, 1957b).

Measurement of succinic oxidase activity. A method for the determination of succinic oxidase activity has been described by Schneider & Potter (1943). In most tissues, such as liver and kidney, this assay presents no difficulty, but in mammary gland the activity of the enzyme system is inhibited by the presence of a high endogenous level of trace metals in the milk and tissue. The Cu^{2+} ion concentration in rat milk may reach $7 \mu\text{g./ml.}$ ($1.1 \times 10^{-4} \text{M}$; Cox & Mueller, 1937), and considerable quantities of iron, calcium and zinc are also present. Succinic oxidase is strongly inhibited by trace concentrations of heavy metals (Hirade & Hayashi, 1953), although such inhibition can be removed by the addition of ethylenediaminetetra-acetic acid (EDTA) (Altmann & Crook, 1953). The addition of chelating agents is therefore of prime importance in enzyme assays on mammary material. Experiments on the activity of succinic oxidase in mammary tissue suspensions from both pregnant and lactating rats in the presence of variable concentrations of EDTA showed that maximum enzymic activity occurred with a final concentration of 0.003M . The conditions for the assay procedure used in this communication were as follows: 1 ml. of 0.1M -phosphate buffer, pH 7.4; 0.3 ml. of 0.5M -succinate, pH 7.4; 0.2 ml. of $2 \times 10^{-4} \text{M}$ -cytochrome *c*; 0.1 ml. of 0.1M -EDTA, pH 7.4; 0.4, 0.7 or 1 ml. of 1:10 tissue or mitochondrial suspension; 0.2 ml. of 20% KOH

in centre well and water to 3 ml. Temperature, 37° . Readings were taken at 10 min. intervals for 30 min. The succinic oxidase activity of mitochondrial and tissue suspensions remained constant for several hours when stored on ice.

Mitochondrial counting procedure. Counting, as a means of quantitative assay, has only recently been developed for use with mitochondrial suspensions (Allard, Mathieu, Lamirande & Cantero, 1952; Shelton, Schneider & Striebig, 1953; Agrell, 1955). In the present experiments particulate counts on suspensions of mammary tissue and on large-particle suspensions obtained by the differential centrifuging of these suspensions have been carried out by the following modification of the procedure described by Allard *et al.* (1952), with dark-ground illumination. The tissue or mitochondrial suspension was diluted by the addition of a calculated volume of ice-cold saturated (1.8M) sucrose. The volume added was so adjusted that each small square of the counting chamber contained about ten particles. The diluted suspension was then shaken vigorously and mounted in a Helber-type bacterial counting chamber with a grid-ruling of Thoma construction. The count was made with a dark-ground zonal condenser at a final magnification of $\times 1000$. All visible particles up to and including the mitochondrial size range were counted. In general, sixteen of the sixty-four large squares of the grid were counted, the sixteen squares being symmetrically distributed. To avoid bacterial contamination the 1.8M -sucrose was always prepared immediately before use and, as a check, counts on the suspending medium were always performed and used as blanks to correct the particle counts.

The hypertonicity of the saturated sucrose solution caused all particles to assume a spherical shape and this made it exceedingly difficult to differentiate the particles of different size ranges. Further, the use of dark-ground illumination permitted the detection and counting of much smaller particles than would have been possible with transmitted light. As a result of these two factors the counting procedure included particles much smaller than those usually counted as mitochondria. Thus the 'large-particle fraction' counted here includes both large and small mitochondria, and perhaps also a proportion of the larger type of microsomal particle, but excludes small microsomes. The wide range of particles counted is not, perhaps, wholly disadvantageous since it includes small mitochondria and large microsomes, fractions which have been shown to change under conditions of physiological or abnormal stress (Gavosto & Moyson, 1953; Thomson & Mikuta, 1954; Thomson & Moss, 1955; Greenbaum, Slater & Reid, 1955). The counting procedure appears to be accurate to $\pm 10\%$. In one typical count the sum of the counts of the particles in the separate nuclear, mitochondrial and supernatant fractions of a unit volume of suspension was 1009, while the count of the same volume of original suspension, from which these were prepared, was 905.

RESULTS

Mitochondrial counts

The results of particle counts obtained in mammary tissue and mitochondrial suspensions are given in Table 1, together with the percentage of the total suspension count sedimented in the large-particle fraction.

Table 1. Large-particle counts in suspensions of rat mammary tissue

Status	10 ⁻¹² × Particle count/100 g. wet tissue			Recovery of large particles in the mitochondrial fraction (%)	10 ⁻¹² × Homogenate count*		10 ⁻¹² × Homogenate count*		10 ⁻¹² × Homogenate count**	
	No. of animals	Whole suspension	Mitochondrial suspension		DNA-P	Mitochondrial N	DNA-P	Mitochondrial N	DNA-P	Mitochondrial N
Virgin	3†	103	—	—	21.4	12.6	—	—	—	—
Pregnant (20 days)	8	1240 ± 150†	890 ± 110	64 ± 9	97.3 ± 19.2	18.9	—	—	—	—
Lactating (3 days)	7	151 ± 38	73 ± 14	62 ± 8	7.6 ± 2.2	1.08	—	—	—	—
Lactating (18 days)	6	100 ± 22	64 ± 12	69 ± 5	4.9 ± 1.5	0.37	—	—	—	—
Involuting (2 days)	3	172 ± 10	105 ± 12	61 ± 6	14.1 ± 5.4	0.94	—	—	—	—

* Units: Count/100 g. wet wt. of gland
mg./100 g. wet wt. of gland

† Pooled sample obtained from three animals.
‡ In all tables the values given are group means ± standard error of the mean.

It can be seen that the number of particles/g. of milk-free mammary tissue rises to a maximum at the end of pregnancy. The number falls rapidly over the period of parturition and then remains approximately constant over the remainder of lactation. The mean percentage recovery of the particles, about 60 %, is approximately constant at all stages of the lactation cycle examined. If it is assumed that the number of cells in the tissue is directly proportional to the DNA content (see Greenbaum & Slater, 1957*a*) then the number of particles counted per cell is indicated by the ratio count:DNA-P content of the suspension (Table 1, column 6).

The table shows that the number of particles per cell rises to a maximum at the end of pregnancy and then falls precipitously over parturition.

Variations in succinic oxidase activity

The results of the estimations of succinic oxidase activity in mammary tissue suspensions are shown in Table 2. The table also shows the percentage of the activity sedimented in the large-particle fraction.

There is a similar pattern of change of succinic oxidase activity in both tissue and mitochondrial suspensions. In both there is a sharp rise in activity at parturition and maximum activity is reached in the second half of lactation. Table 2 also includes the activity of succinic oxidase calculated in terms of the total-nitrogen content of the mitochondrial fraction. If a fixed amount of enzyme activity were associated with a unit quantity of mitochondrial nitrogen then the ratio of activity to nitrogen would be constant throughout the lactation cycle. The ratio, however, varies considerably, which indicates that succinic oxidase activity and mitochondrial nitrogen can vary independently. Although cell numbers are practically doubled over parturition (Greenbaum & Slater, 1957*a, b*) the ratio of succinic oxidase activity:DNA-P rises at parturition (Table 2, column 9) which suggests a build up in the oxidative system per cell.

The percentage of the total activity sedimented in the large-particle fraction rises from 55 % in late pregnancy to 85 % at the end of lactation. The activity per particle counted in the large-particle fraction varies over the lactation cycle as shown by the ratio of succinic oxidase activity:particle count (Table 2, column 11).

DISCUSSION

Parturition is so obviously a transition period leading to a higher level of metabolic and synthetic activity in the mammary gland that the rapid decrease in particles counted per gram of tissue over this period is most striking and unexpected.

Table 2. *Changes in succinic oxidase activity in rat mammary gland*

Status	Activity in whole homogenate			Activity in mitochondrial suspension		
	No. of animals	$10^{-3} \times \mu\text{l. of O}_2$ uptake/hr./ 100 g. wet wt. of gland	$\mu\text{l. of O}_2$ uptake/hr./ mg. of N	No. of animals	$10^{-3} \times \mu\text{l. of O}_2$ hr./100 g. wet wt. of gland	$\mu\text{l. of O}_2$ /hr./ mg. of N
Pregnant (20 days)	9	34 $\pm$ 4	65.5 $\pm$ 5.0	8	17 $\pm$ 3.9	216 $\pm$ 51
Lactating (3 days)	10	101 $\pm$ 5.8	98.0 $\pm$ 9.0	9	74 $\pm$ 6.3	580 $\pm$ 97
Lactating (10 days)	6	144 $\pm$ 19	160 $\pm$ 19	6	113 $\pm$ 16.4	534 $\pm$ 75
Lactating (18 days)	8	189 $\pm$ 20	132 $\pm$ 17	6	144 $\pm$ 10.5	411 $\pm$ 52
Involuting (2 days)	3	46 $\pm$ 10.7	33 $\pm$ 4	2	37 $\pm$ 7.5	167
Involuting (4 days)	2	28	44	—	—	—

Status	Percentage of total activity in mitochondrial fraction	Homogenate activity* DNA-P†	Homogenate activity* Tissue N†	Activity of homogenate* Count†
Pregnant (20 days)	55 $\pm$ 12	1499 $\pm$ 660	184	22.7 $\pm$ 8.8
Lactating (3 days)	71 $\pm$ 5	4950 $\pm$ 586	150	1044 $\pm$ 260
Lactating (10 days)	76 $\pm$ 6	7200 $\pm$ 970	236	—
Lactating (18 days)	85 $\pm$ 8	8930 $\pm$ 1260	266	2618 $\pm$ 605
Involuting (2 days)	84	3251 $\pm$ 1030	39.7	266 $\pm$ 59
Involuting (4 days)	—	180 $\pm$ 39	—	—

* Units: $\mu\text{l. of O}_2$ uptake/hr./100 g. wet wt. of gland.

† Units: DNA-P in mg./100 g. wet wt. of gland. Tissue N in mg./100 g. wet wt. of gland. Count as $10^{-12} \times$ no. of particles/100 g. wet wt. of gland.

‡ Pooled sample obtained from three animals.

The fall in particle numbers at parturition is associated in time with increases in tissue nitrogen, RNA-P, DNA-P and of enzymic activity. Metabolic processes are so intimately associated with the particulate material that it seems unlikely that the particle content of the cell should be built up at a time when metabolism is relatively low and then dissipated into the very small submitochondrial range when the need is greater. It is not possible at this stage to decide whether the particles dissociate, whether they coalesce to form larger particles or whether the decrease results from a decrease in the large vesicular system of the cell. But whatever the explanation it seems fairly certain from the data presented here and in the previous paper (Greenbaum & Slater, 1957b) that at parturition there is a redistribution of the material between the intracellular fractions of the gland.

Succinic oxidase has been shown to be linked with mitochondria in a great variety of tissues other than rat mammary gland. The percentage sedimentation of the succinic oxidase activity is closely correlated with the percentage of particles recovered in the large-particle fraction. The mean percentage of succinic oxidase activity sedimented in late lactation is 85% and from this, and from the fact that changes in enzyme activity of the whole tissue suspension are exactly duplicated in the mitochondrial enzyme activity, it appears that succinic oxidase is wholly linked to the large-particle fraction sedimenting at 230 000 g min. in isotonic

sucrose. However, the fact that 230 000 g min. is required to sediment the greater part of the total succinic oxidase activity (smaller centrifugal fields give smaller recoveries) indicates that the average size of the particles containing succinic oxidase is smaller in the mammary gland than in the liver, where 50 000 g min. is sufficient to sediment 60% of the total activity (Schneider & Hogeboom, 1950). A similar conclusion may be inferred from the results previously reported by Dmochowski & Strickland (1953) on mouse mammary tissue.

The low level of succinic oxidase activity in the virgin tissue is an indication of the small metabolic requirement of a tissue which is synthetically non-functional. The onset of secretion, with the consequent demand for a tissue with highly developed and active enzyme systems witnesses a sharp rise in succinic oxidase activity which is carried on throughout lactation. In involution the activity falls precipitously. The pattern of change of succinic oxidase activity described here is different from that presented by Moore & Nelson (1952) who, however, worked on guinea pigs. In the guinea pig lactation is fully established almost immediately after parturition and then decreases throughout the remainder of lactation. In the rat lactation reaches its maximum near the end of the period of lactation (Brody & Nisbet, 1938). Thus the increasing succinic oxidase activity in lactation is in agreement with a view linking the activity of oxidative enzyme systems with milk synthesis.

The increase in succinic oxidase activity from late pregnancy to mid-lactation could be due to any of the following effects: (i) an increase in mitochondrial numbers with no increase in size or enzyme activity per particle; (ii) an increase in mitochondrial size, which, assuming the preservation of typical mitochondrial enzyme patterns, would lead to an increase in the amount of activity associated with each mitochondrion; (iii) an increase in the activity of the succinic oxidase system itself; (iv) any combination of the foregoing three possibilities.

There is little doubt that there is an increase in the number of mitochondria in the gland from late pregnancy to late lactation. Weatherford (1929) observed, cytologically, an increased number of mitochondria per cell over lactation and, when this is coupled with the doubling of cell numbers shortly after parturition (Greenbaum & Slater, 1957*a*), it is obvious that the total number of mitochondria in the gland must increase substantially over pregnancy and lactation. This is further indicated by the rapid rise in mitochondrial nitrogen (Greenbaum & Slater, 1957*b*), and quantitative counts by Howe, Richardson & Birbeck (1956) on guinea-pig tissue also lead to the view that mitochondrial numbers increase after parturition. This would imply that simultaneously with the increase in mitochondrial numbers there must be a decline in the number of particles other than mitochondria counted under the dark-ground conditions to account for the over-all fall in numbers observed.

Observations of an increased size of mitochondria in early lactation compared to late pregnancy have also been reported (Weatherford, 1929; Jeffers, 1935), with a change in shape from spheres predominantly in pregnancy to filaments and rods in lactation. The increasing sedimentation of the succinic oxidase in the large-particle fraction can thus be considered to indicate an increase in size of the particles containing succinic oxidase. Changes in size of mitochondria after stress have also been reported by Thomson & Mikuta (1954) and Thomson & Moss (1955).

No activators for the succinic oxidase system have, as yet, been reported (see Bonner, 1955), whereas many substances act as potent inhibitors. Metal ions, oestrogens, and mercaptide-forming agents have been reported to inhibit the succinic oxidase system. Of these, metal ions may be potent inhibitors in the mammary gland as they probably accumulate in the mammary gland during pregnancy; colostrum has, in general, a much higher content of these metal ions than milk secreted in active lactation (see Underwood, 1956). The increase in succinic oxidase activity which occurs over parturition may be due in part to the removal of some of these metal ions from the gland by the

secretion of milk. The copper content of rat milk is high when compared with other tissues [$2-3 \mu\text{g./g.}$ of rat liver (Cunningham, 1931) and $7 \mu\text{g./ml.}$ of milk (Cox & Mueller, 1937)] and, if the rat shows a similar pattern of change to all other species examined, colostrum should have an even greater content of copper than $7 \mu\text{g./ml.}$ Thus the initiation of lactation may result in the removal of metal-ion inhibitors and hence in an increased enzyme activity.

The influence of inhibitory agents may be evaluated approximately from the following considerations. The ratio of succinic oxidase activity to mitochondrial nitrogen rises at parturition (Table 2, column 7). This cannot be interpreted in terms of an increase either of number or size if it is assumed that the nitrogen content per unit of mitochondrial volume does not alter significantly at this period. The rise in the ratio must be interpreted in terms of the activity increasing more rapidly than the mass, which suggests that the effect is due to the removal of inhibitors. This effect accounts for some 50 % of the total rise in succinic oxidase activity at parturition.

Reasons can, therefore, be advanced for the operation of each of the three mechanisms proposed to account for the rise in succinic oxidase activity.

The results given here and in the previous paper (Greenbaum & Slater, 1957*b*) give a more precise picture of the mechanism by which the mammary gland responds to the increasing demands imposed on it by lactation. After the initial preparatory burst of mitosis there is a more protracted change in the nature of the cellular inclusions. It seems that advancing lactation causes a doubling in the amount of mitochondrial substance and probably a change in size towards larger particles. It is likely that a study of the changes in enzyme content of the different components of the mitochondrial population as lactation advances would throw more light on this adaptation.

SUMMARY

1. Succinic oxidase activity in suspensions of rat mammary tissue increases sharply at parturition and reaches a maximum value midway through lactation. The identical patterns of change of activity in the whole-tissue suspension and in the large-particle fraction, together with the high recoveries of activity in the large-particle fraction, indicate that the succinic oxidase activity of rat mammary tissue is very largely localized in this fraction.

2. The increase in succinic oxidase activity at parturition and in early lactation is probably the result of three effects: (i) an increase in mitochondrial numbers; (ii) an increase in mitochondrial size; (iii) the removal of inhibitory factors at parturition.

3. The large-particle count procedure indicated a rapid decrease in the number of particles present over parturition. It is suggested that this was the result of a rapid decrease in the number of particles other than mitochondria occurring simultaneously with a smaller increase in mitochondrial numbers.

4. Interrelationships between ribonucleic acid, deoxyribonucleic acid, tissue nitrogen, succinic oxidase and the large-particle counts are discussed.

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Studies on Pituitary Adrenocorticotropin

2. OXIDATION-REDUCTION PROPERTIES OF THE HORMONE*

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It is generally accepted that the biological activity of the pituitary adrenocorticotrophic hormone (corticotropin, ACTH) is unstable under oxidizing conditions. For example, Dixon, Moore, Stack-Dunne & Young (1951) have advocated the use of anti-oxidants to diminish oxidative inactivation during extraction and purification of the hormone, and the practice has often been adopted by later workers. No quantitative data on the subject are, however, available.

The first evidence that the oxidation process may be reversible, at least in part, was provided by Kuehl, Meisinger, Brink & Folkers (1953). These workers showed that corticotropin B, a highly active preparation made by partial peptic hydrolysis of purified pituitary extracts, could be oxidized by

the action of oxygen in the presence of ferric iron, to a product which was inactive in the ascorbic acid-depletion bioassay. The biological activity of the product could be restored by several days' contact with hydrogen sulphide. Attempts in several laboratories to repeat the reductive re-activation of oxidized hormone under the conditions advocated by Kuehl *et al.* (1953) have, however, been unsuccessful (Dixon & Stack-Dunne, 1955).

The observations of Kuehl *et al.* (1953) are of interest from both the chemical and biological standpoints. First, it is generally agreed that the best corticotropin preparations now available contain no detectable sulphhydryl groups, so that there is no obvious chemical explanation of the oxidation-reduction property. Secondly, the presence of an oxidation-reduction centre may also be of importance for the biological function of a

* Part 1: Cortis-Jones, Crooke, Henly, Morris & Morris (1950).