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# Variations in the Activity of Several Enzymes in the Mammary Glands of Non-Pregnant, Pregnant and Lactating Rabbits

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1. The enzymes phosphofructokinase (EC 2.7.1.11), 6-phosphogluconate dehydrogenase (EC 1.1.1.44), phosphoglucomutase (EC 2.7.5.1), ATP–citrate lyase (EC 4.1.3.8), acetyl-CoA carboxylase (EC 6.4.1.2) and acetyl-CoA synthetase (EC 6.2.1.1) were assayed in rabbit mammary glands at various stages of the pregnancy–lactation cycle. 2. The activities of all enzymes were low during pregnancy and, with the exception of phosphofructokinase, in non-pregnant animals. Two- to ten-fold increases in enzyme activities occurred over the first 20 days of lactation. Although milk yield was considerably decreased, the enzyme activities remained elevated in late lactation (45 days after parturition). 3. These findings are discussed in relation to mammary-gland metabolism and compared with similar observations previously made on ruminants and other small mammals.

Studies on the control of metabolism in the lactating mammary gland are complicated by the varying hormonal requirements for the maintenance of lactation found in different species (see reviews by Folley, 1961; Cowie, 1966). In the hypophysectomized rat injections of prolactin and adrenocorticotrophin maintain milk secretion at about half its normal rate, and the hypophysectomized goat requires a combination of prolactin, growth hormone, corticoids, thyroxine and insulin to reinitiate milk secretion. Cowie, Hartmann & Turvey (1969) confirmed that prolactin alone was capable of restoring not only the yield but also the composition of milk to virtually normal values in rabbits hypophysectomized during lactation. This finding suggested that the rabbit might be a useful experimental animal for studies on the hormonal control of the metabolic activity of the mammary gland. However, few enzyme studies had been reported for the rabbit (McLean, 1958; Baldwin, 1966) and we decided, before embarking on work with hypophysectomized animals, to study the changes in the activities of several metabolically important enzymes throughout the pregnancy–lactation cycle in this species. Subsequently two reports on the time-course of changes in enzyme activity in the rabbit mammary gland have appeared (Heitzman, 1968; Gul & Dils, 1969), but only one of the five enzymes studied by us was also examined by these workers. In addition, the composition of rabbit milk was studied at different stages of lactation with the aim of correlating this with the enzyme changes.

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## MATERIALS AND METHODS

*Animals.* Dutch rabbits in their first or second lactations were used. They were allowed free access to water and provided *ad libitum* with a diet of equal parts of R.A.G. pellets (C. Hill Ltd., Poole, Dorset, U.K.) and Super Creep pellets (Spillers Ltd., Barking, Essex, U.K.). Three rabbits were killed at each of nine times (non-pregnant non-lactating; 10 days and 1 day before parturition, and 1, 5, 10, 15, 20 and 45 days after parturition) throughout the lactation cycle. The time of parturition was predicted by assuming a mean gestation period of 31 days (Asdell, 1946).

Six to eight young were allowed to suckle each lactating doe. The milk yield of the group of rabbits killed 1 day after parturition was measured over a period of 12h, whereas the yield of milk of the other lactating rabbits was measured over a 2-day period, as described by Cowie *et al.* (1969). Oxytocin (0.5–1.0i.u.) was injected intravenously at each nursing to ensure that there was an efficient milk ejection. Young, 8 days old, were used to suckle the groups 1 and 5 days after parturition. The lactating rabbits were killed immediately after the young suckled.

*Homogenates and particle-free supernatants.* Rabbits were killed by a blow on the head. The mammary tissue (except the clavical glands) was dissected free from muscle and adipose tissue, minced and homogenized in ice-cold medium (0.25M-mannitol–27mM-sucrose–1mM-EDTA, pH7.4) in a Folley & Watson (1948) top-drive blender. This suspension was then filtered through a double thickness of cheese-cloth to free the homogenate of intractable connective tissue and the volume noted.

A portion of the homogenate was retained for determinations of DNA by the method of Burton (1956) as modified by Munford (1963) and of total N (micro-Kjeldahl), and the remainder was centrifuged in a Spinco model L Ultracentrifuge at 105000*g* for 60min at 2°C. The clear supernatant, containing the soluble enzyme and protein fractions, was aspirated and stored for enzyme assays at 0–4°C. Protein in the supernatant was measured by the biuret method (Gornall, Bardawill & David, 1949).

The preparation of the homogenate from the external abdominal oblique muscle was carried out in a similar manner to that described for the mammary gland.

*Enzymes.* All enzyme assays were performed within 6h of the time of death. The methods used and the precautions taken in the assay of phosphofructokinase (EC 2.7.1.11), 6-phosphogluconate dehydrogenase (EC 1.1.1.44), phosphoglucomutase (EC 2.7.5.1), ATP–citrate lyase (EC 4.1.3.8) and acetyl-CoA carboxylase (EC 6.4.1.2) were similar to those described by Jones (1967). In the assay of ATP–citrate lyase the reaction was started by the addition of CoA rather than ATP because an initial unidentified oxidation of NADH occurred with the addition of ATP, particularly in mammary tissue obtained during colostrum formation and late lactation. With our homogenization procedure we did not repeat the observations of Easter & Dils (1968) that a large proportion of rabbit mammary-gland acetyl-CoA carboxylase activity is particle-bound, and thus the values for the particle-free fractions approximate to the total activity of the homogenate. Acetyl-CoA synthetase (EC 6.2.1.1) was assayed by the method of Kornacker & Lowenstein (1965) except that 2-mercaptoethanol (10mM) was substituted for GSH. Although acetyl-CoA synthetase was assayed at 37°C, its activity is expressed at 25°C, by using the experimentally determined conversion factor 0.55, to conform with the other five enzymes, which were all assayed at 25°C.

*Milk analysis.* Milk samples (3–5ml) were collected immediately before suckling. Milk fat, protein and lactose were determined as described by Cowie *et al.* (1969).

*Chemicals.* Sugar phosphates and coenzymes were obtained either from Boehringer Corp. (London) Ltd., London W.5, U.K. or Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.; herring-sperm DNA was from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.; oxytocin was from Parke, Davis and Co. Ltd., Hounslow, Middx., U.K., as Pitocin. $Na_2{}^{14}CO_3$ was supplied by The Radiochemical Centre, Amersham, Bucks., U.K.

## RESULTS

*Expression of enzymic activity.* Enzyme activities are expressed on the basis of the DNA content of the mammary-tissue homogenates. This provides a measure of enzyme activity per cell though, because of the variety of cell types present, it does not necessarily reflect the activity in those cells synthesizing milk constituents. To allow comparison of the results presented here with those calculated on other bases, the yields of homogenate DNA and nitrogen and particle-free supernatant nitrogen per g wet wt. of mammary tissue are shown in Fig. 1.

The yield of DNA increased significantly ($P<0.05$) at the initiation of lactation, remained at this high value until day 15 and then decreased to values similar to those observed during pregnancy by day 20 of lactation. The yield of homogenate nitrogen increased significantly ($P<0.05$) between day 1 and day 5 of lactation, reached a maximum value at day 15 and declined to low values in late lactation. Although the changes in the concentration of soluble protein were smaller they paralleled those of homogenate nitrogen.

*Enzyme activity.* The activities (expressed as μmol of substrate utilized/min per g of homogenate DNA at 25°C) shows a broadly similar pattern of change for all five enzymes (Fig. 2). Activities remain relatively constant during pregancy, rise between parturition and mid-lactation (day 20) and remain at high values until late lactation (day 45). Phosphofructokinase activity is high in 'dry' rabbits (non-pregnant, non-lactating rabbits) and falls to low values by mid-pregnancy (Fig. 2*a*), whereas ATP–citrate lyase activity is undetectable in 'dry' mammary glands but appears during pregnancy (Fig. 2*d*). The increases after parturition vary from the rapid rise of ATP–citrate lyase activity to the slow change of phosphoglucomutase, the activity of which is not significantly ($P<0.05$) elevated until day 20 (Fig. 2*c*). The increases in activity between days 1 and 20 are significant ($P<0.05$) for all the enzymes except acetyl-CoA carboxylase. There is a significant increase ($P<0.05$) between days 1 and 10 for acetyl-CoA carboxylase but subsequently the activity varies erratically with large standard errors (Fig. 2*e*).

The limited results obtained for acetyl-CoA synthetase showed that its activity fell from 0.96 μmol

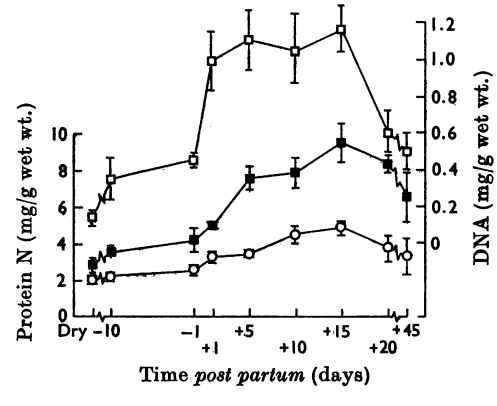


Fig. 1. Yield of DNA (□), homogenate N (■) and soluble protein N (○) from mammary tissue of Dutch rabbits killed at selected times during the lactation cycle. Each point represents the mean ± S.E.M. of results from three animals. 'Dry' means not pregnant, not lactating.

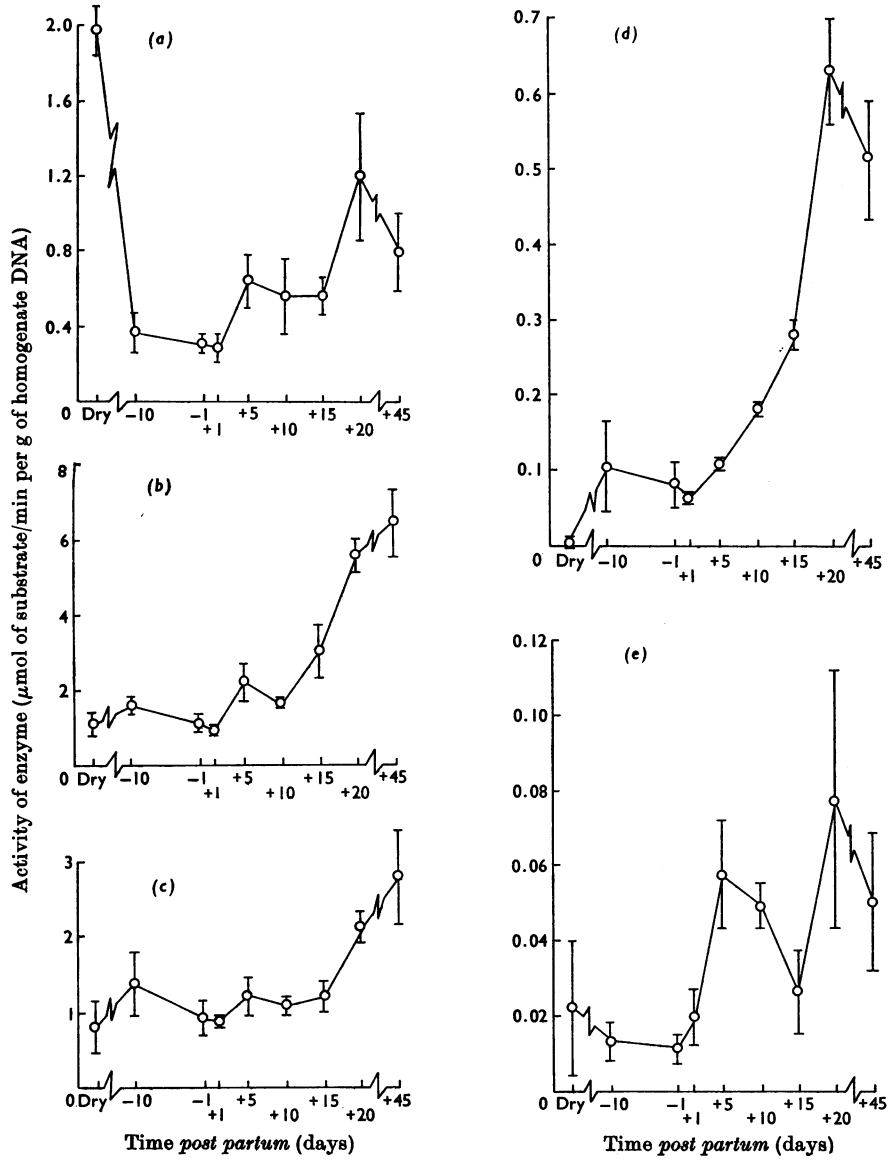


Fig. 2. Activities of enzymes in the mammary tissue from Dutch rabbits killed at selected times during the lactation cycle. Each point represents the mean ± S.E.M. of results from three animals. (*a*) Phosphofructokinase; (*b*) 6-phosphogluconate dehydrogenase; (*c*) phosphoglucomutase; (*d*) ATP–citrate lyase; (*e*) acetyl-CoA carboxylase.

of substrate/min per g of DNA on day 20 to 0.33 on day 45.

*Milk yield and composition.* An indication of the synthetic activity of the mammary glands of the lactating rabbits is illustrated by the mean yields of milk, milk fat, lactose and protein for each group (Fig. 3). The highest yields of milk, milk fat and lactose occurred at 15 days and that of protein at 20 days of lactation.

Rabbit milk is rich in fat (15–25g/100ml) and protein (8–15g/100ml). By contrast with the relatively constant values for fat and protein throughout lactation, the lactose content fell from 2.8g/100ml on the first day of lactation to less than

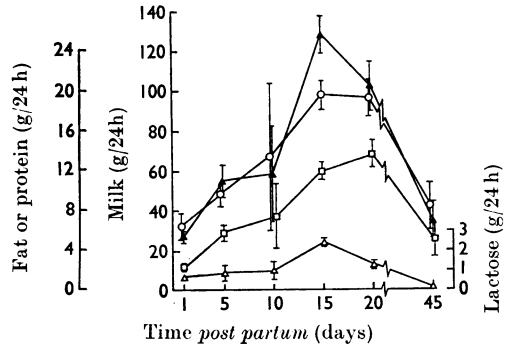


Fig. 3. Yields of milk (▲), milk fat (○), lactose (△) and protein (□) from Dutch rabbits throughout lactation. Each point represents the mean ± S.E.M. of results from three rabbits.

0.2g/100ml in late lactation. These observations are in agreement with the detailed studies on the yield and composition of milk of Dutch and New Zealand White rabbits carried out by Cowie (1969). The changes in milk yield and composition were not closely aligned with the changes in the concentration of DNA in the mammary tissue during lactation (Fig. 1), but the concentration of homogenate nitrogen did appear to bear some relationship to the functional activity of the mammary gland.

## DISCUSSION

After comparatively slow changes in the yield of DNA during pregnancy, a twofold increase was observed between the last day of pregnancy and the first day of lactation (Fig. 1). This finding conflicts with previous observations in the rabbit in which the yield of DNA either increased from mid-pregnancy to early lactation and then decreased abruptly (Denamur, 1961) or did not change significantly throughout this period (Heitzman, 1968). It is possible that these differences may be explained by the diluting effects of unknown quantities of milk retained in the mammary glands. In the present experiment the lactating glands were emptied of milk by the young immediately before the tissue was sampled and the error caused by the presence of residual milk (Caruolo & Mochrie, 1965) would have been small and consistent. The rapid increase in the concentration of DNA therefore does not appear to reflect cellular changes within the tissue (see review by Munford, 1964), but rather the change from a gland in late pregnancy containing colostrum to lactating glandular tissue containing a small quantity of residual milk (Wheelock, Rook & Dodd, 1965; P. E. Hartmann, A. T. Cowie & Z. D. Hosking, unpublished work). However, a change in the cellular structure of the mammary gland between days 15 and 20 of lactation was suggested by the marked fall in the yield of DNA over this period.

The activities of 6-phosphogluconate dehydrogenase, phosphoglucomutase, ATP–citrate lyase and acetyl-CoA carboxylase were low in the mammary glands from 'dry' rabbits. It is possible that the high activity of phosphofructokinase at this stage resulted from contamination of the sample with muscle. This was the only enzyme studied that had a higher activity in muscle (external abdominal oblique) than in 'dry' mammary gland.

Initiation of lactation in the rabbit was followed by an increase in the activities of phosphofructokinase, 6-phosphogluconate dehydrogenase, phosphoglucomutase, ATP–citrate lyase and acetyl-CoA carboxylase relative to the DNA content of the gland. Similar increases in the enzyme activities of rabbit mammary glands have been reported by Heitzman (1968) for two enzymes involved in lactose synthesis and by Gul & Dils (1969) for five enzymes including 6-phosphogluconate dehydrogenase.

Caution must be exercised in time-course comparisons between species because of the considerable differences that exist in the length of both gestation and lactation. However, enzyme activities attained during early lactation relative to those before parturition in the rabbit appear to be intermediate between the marked increases occurring in rats (Glock & McLean, 1958; Baldwin & Milligan, 1966; Kuhn & Lowenstein, 1967), mice (Hershey, Lewis, Johnston & Mason, 1963) and guinea pigs (Baldwin, 1966) and the negligible changes observed in the mammary gland of the cow (Baldwin & Cheng, 1968). By contrast, blood flow through the mammary gland of the cow increases markedly at parturition (Kjaersgaard, 1968), but does not change in the mouse (M. Reynolds, personal communication). Since both blood-flow and enzyme-activity responses could be important in increasing the availability of rate-limiting metabolites within the mammary gland, it is possible that both respond to the control mechanisms synchronizing the initiation of copious milk secretion with the birth of the young. If this is so, the intermediate changes in enzyme activity in the rabbit indicates that both enzyme-activity and blood-flow responses may be important during lactogenesis in this species.

The activities of the enzymes in the pathways utilizing glucose as a precursor (phosphofructokinase, 6-phosphogluconate dehydrogenase and phosphoglucomutase) were of the same order as those observed by Jones (1967) for the mammary glands of rats at a similar stage of lactation, whereas the activity of ATP–citrate lyase was about 20% of this value. The relatively low activity of this enzyme, which is in agreement with the observations made by Baldwin (1966), and the presence of a more

active acetyl-CoA synthetase suggest that fatty acid synthesis in the rabbit may to some extent resemble that in the ruminant, where ATP–citrate lyase activity is very low (Hardwick, 1966) and acetate rather than glucose is the major precursor of the fatty acids synthesized within the mammary gland. In this connexion Popják, Folley & French (1949) demonstrated a significant incorporation of intravenously injected [1-$^{14}$C]acetate into short-chain fatty acids within the mammary gland of intact rabbits, and rabbit milk fat contains a higher proportion of short-chain fatty acids than that of other small mammals (Smith, Watts & Dils, 1968).

By contrast with the findings of Rook & Campling (1965) for the cow and Corbett (1968) for the ewe the concentration of lactose in rabbit milk decreased to almost zero as lactation progressed (Fig. 3). The mechanism of this change is not evident from the present work; one enzyme directly concerned with the synthesis of lactose from glucose, phosphoglucomutase, actually increased in late lactation. Heitzman (1968) also reported steady increases in two other enzymes of the lactose synthesis pathway, UDP-glucose pyrophosphorylase and UDP-glucose 4-epimerase. The factors determining the lactose content of milk (see review by Palmiter, 1969) are far from being fully understood, and it remains to be determined whether the decline in content in the rabbit in late lactation is due to enzymic or other causes.

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