Enzymic Changes in Rabbit and Rat Mammary Gland during the Lactation Cycle

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1. Activities of glucose 6-phosphate dehydrogenase (EC 1.1.1.49), 6-phosphogluconate dehydrogenase (EC 1.1.1.44), isocitrate dehydrogenase (EC 1.1.1.42), malate dehydrogenase (EC 1.1.1.37), malate dehydrogenase (decarboxylating) (EC 1.1.1.40), and pyruvate carboxylase (EC 6.4.1.1) were determined in subcellular fractions of mammary gland from rabbits during pregnancy, at different stages of lactation and during weaning. The results were compared with those obtained in similar experiments with rat mammary gland. 2. Three bases of expression of the activity of enzymes in the particle-free supernatant fraction of mammary gland were compared. During lactation, activity expressed per mg. of particle-free supernatant protein (uncorrected for milk protein) correlated well with that expressed per μ g. of DNA phosphorus. The disadvantages of expressing activities per g. wet wt. are discussed. 3. The major differences between the two tissues were: (a) neither malate dehydrogenase (decarboxylating) nor a soluble form of pyruvate carboxylase could be detected in rabbit mammary gland at any stage of the lactation cycle; (b) isocitrate dehydrogenase increased in activity during lactation in rabbit mammary gland, but not in that of the rat. 4. Pyruvate carboxylase in the mitochondrial fraction of rabbit mammary gland, and in both the mitochondrial and the soluble fractions of rat mammary gland, did not change in activity during lactation. 5. For each tissue, the NADP-dependent dehydrogenases studied had a high activity at all stages of the lactation cycle compared with the rate of fatty acid synthesis at mid-lactation. The significance of these results is discussed with respect to the supply of NADPH via NADH.

Changes in the specific activities of a number of enzymes in the PFS* fraction of rat mammary gland during the lactation cycle are well documented (McLean, 1958a; Rees & Huggins, 1960; Baldwin & Milligan, 1966). However, comparable data are not available for rabbit mammary gland. Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) have only been assayed in this tissue at 17 days *post partum* (McLean, 1958a) and a number of other enzymes have only been assayed at 30 days *post partum* (Baldwin, 1966). We have therefore examined the activities of a number of dehydrogenases, and also pyruvate carboxylase (EC 6.4.1.1), in subcellular fractions of mammary gland from rabbits during pregnancy, at different stages of lactation and during weaning. The results are compared with those obtained with rat mammary gland.

* Abbreviation: PFS, particle-free supernatant.

MATERIALS

Chemicals. Most of the chemicals used were described by Gul & Dils (1969). Glucose 6-phosphate, IDP and phosphoenolpyruvate (sodium salts), GSH and malate dehydrogenase (EC 1.1.1.37) were purchased from Sigma (London) Chemical Co., London S.W.6. Glycylglycine, orcinol, lactose, diphenylamine and tris were obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex. DNA (sodium salt, from calf thymus) was a product of L. Light and Co. Ltd., Colnbrook, Bucks., and oxytocin was obtained from Sandoz Ltd., Basle, Switzerland.

Animals. The strain and the diet of the animals used were described by Gul & Dils (1969). To determine changes in the specific activities of enzymes in rabbit mammary gland the following groups of animals were used (unless otherwise stated): 25–30 days pregnant and 4–5 days, 10–14 days, 18 days and 30 days *post partum*. Rabbits that had had their litters removed for 2 days or 9–10 days at 30 days *post partum* were also used. In all cases, litter size varied from two to eight. For similar experiments with rat mammary gland, the groups of animals used were: virgin, 20–22 days pregnant and 2 days, 5 days, 14 days, 21 days

Bioch. 1969, 112

and 27 days *post partum*. Rats that had had litters removed for 3 days or 7 days at 21 days *post partum* were also used. Rat litters were reduced to six to eight young at birth, though for studies on pyruvate carboxylase litter size varied from eight to 13. To determine the time of gestation, the foetal crown-rump length tables of Stonsberg (1915) were used for both rabbits and rats. Foetal age thus calculated agreed well with that predicted from the time of mating.

Milk. Rabbit milk was obtained directly from the dissected mammary gland. Milk was obtained from rats by intraperitoneal injection of 2 i.u. of oxytocin into lightly anaesthetized animals. Samples (0.2–0.5 ml.) were collected by amputating the nipples after 10–15 min. Milk could not be obtained from pregnant rats or from rats with mammary involution after weaning.

METHODS

Lactose was determined by the method of Slater (1957) and DNA by the method of Greenbaum & Slater (1957a). DNA results are expressed as μg . of DNA P, as recommended by Leslie (1955). Protein determination, radioactivity measurements, the preparation and assay of acetyl-CoA and the preparation of subcellular fractions from rabbit and rat mammary gland (with 0.3 M-sucrose as homogenization medium) were described by Gul & Dils (1969); 0.3 M-mannitol was used as homogenization medium when lactose determinations were carried out. For studies on mitochondrial pyruvate carboxylase, the nuclear fraction was washed twice and the washings were added back to the material still to be fractionated. The heavy-mitochondrial fraction was then sedimented, washed twice and the washings were again added back to the material still to be fractionated. For rat mammary gland, the PFS fraction was then prepared, whereas for rabbit mammary gland the 'microsomal + PFS' fraction was normally not further fractionated. For studies on dehydrogenases, the PFS fraction of both rabbit and rat mammary gland was obtained by centrifuging the nuclei-free homogenate at 103000g for 60 min.

Assay of enzymes. All enzyme determinations were performed in duplicate. Dehydrogenases were assayed with freshly prepared PFS fractions to avoid the erratic changes in specific activity found on storing this fraction at -20° for 24 or 48 hr. In all cases 80–120% of the activity in the nuclei-free homogenate was recovered in the fresh PFS fraction of rabbit and rat mammary gland at all stages of the lactation cycle investigated. Pyruvate carboxylase was assayed with mitochondrial and PFS fractions that had been stored at -20° for 24 hr. This ensured that the enzyme was assayed at maximum activity in these fractions (Gul & Dils, 1969). At all stages of the lactation cycle the recovery of pyruvate carboxylase in subcellular fractions from full homogenates of rabbit and rat mammary gland varied with the preparation. Only recoveries of 50–140% are included here.

Dehydrogenases were assayed spectrophotometrically at 37° by measuring the oxidation or reduction of nicotinamide nucleotides at 340nm. in a Unicam SP.700 recording spectrophotometer in a total volume of 3 ml. In assays with NADP⁺, incubation mixtures without added substrate were also used. In assays with NADH, incubations containing no added nucleotide were included since, with the enzyme preparations used, there was no oxidation of added

NADH in the absence of added substrate. All enzymes were assayed under conditions in which the reaction rate was linearly related to the incubation time and the amount of enzyme added.

Malate dehydrogenase (decarboxylating) [L-malate-NADP oxidoreductase (decarboxylating), EC 1.1.1.40] was assayed by a modification of the method of Ochoa (1955c). The incubation mixture contained 1.0 mM-DL-malate, 5.0 mM-MnCl₂, 0.17 mM-NADP⁺, 25 mM-glycylglycine-NaOH buffer, pH 7.4, and up to 0.3 mg. of PFS protein.

The malate dehydrogenase assay was based on that of Ochoa (1955b). The incubation mixture contained 0.6 mM-oxaloacetate, 0.14 mM-NADH, 25 mM-glycylglycine-NaOH buffer, pH 7.4, and up to 0.03 mg. of PFS protein.

A slight modification of the method of Kornberg (1955) was used to assay lactate dehydrogenase (EC 1.1.1.27). The incubation mixture contained 1.0 mM-sodium pyruvate, 0.14 mM-NADH, 33 mM-potassium phosphate buffer, pH 7.4, and up to 0.06 mg. of PFS protein.

6-Phosphogluconate dehydrogenase and succinate dehydrogenase (EC 1.3.99.1) were assayed as described previously (Gul & Dils, 1969).

The glucose 6-phosphate dehydrogenase assay was a modification (Huggins & Yao, 1959) of the double-substrate method of Glock & McLean (1953). The incubation mixture contained 1.7 mM-glucose 6-phosphate, 0.085 mM-6-phosphogluconate, 0.17 mM-NADP⁺, 17 mM-MgCl₂, 75 mM-glycylglycine-NaOH buffer, pH 7.4, and up to 0.05 mg. of PFS protein.

The isocitrate dehydrogenase (*threo*-D₅-isocitrate-NADP oxidoreductase, EC 1.1.1.42) assay used was based on the method of Ochoa (1955a). The incubation mixture contained 0.2 mM-DL-isocitrate, 0.6 mM-MnCl₂, 0.17 mM-NADP⁺, 25 mM-glycylglycine-NaOH buffer, pH 7.4, and up to 0.85 mg. of PFS protein.

The phosphoenolpyruvate carboxylase (EC 4.1.1.32) assay was based on that of Chang & Lane (1966). The incubation mixture contained, in a total volume of 1 ml., 50 mM-NaH¹⁴CO₃ (0.1 $\mu\text{Ci}/\mu\text{mole}$), 1.25 mM-phosphoenolpyruvate, 1.25 mM-IDP, 1.0 mM-MnCl₂, 20 mM-GSH, 2.5 mM-NADH, malate dehydrogenase (approx. 6 units) and enzyme protein. Incubations were for 15 min. at 37° . The reaction was stopped with 0.1 ml. of 5M-HClO₄; residual ¹⁴CO₂ was removed as described for the assay of pyruvate carboxylase (Gul & Dils, 1969), and a portion of the reaction mixture was used for radioactivity measurements. Incubation mixtures without added substrate were used as blanks.

ATP citrate lyase (EC 4.1.3.8) was assayed by the method of Baldwin & Milligan (1966).

The units of specific activity of the dehydrogenases assayed are μmoles of nicotinamide nucleotide transformed/min./ μg . of DNA P, and that for pyruvate carboxylase is $\text{m}\mu\text{moles}$ of ¹⁴CO₂ incorporated/min./ μg . of DNA P.

RESULTS

Changes in enzyme activity expressed on a DNA basis. DNA determinations provide an estimate of total numbers of cells in mammary gland at a particular stage of the lactation cycle (Munford, 1963). The expression of enzyme activity on the basis of DNA content should give an estimate of the amount of enzyme per cell. All enzyme activities

Fig. 1. Changes in activities of some dehydrogenases in the PFS fraction of rabbit mammary gland. Vertical lines represent the s.d. of the enzyme activities and numbers in parentheses are the numbers of animals in each group. The graphs show activity in glands of pregnant and lactating animals (○), and activity in glands of animals after weaning (Δ). (a) Glucose 6-phosphate dehydrogenase; (b) 6-phosphogluconate dehydrogenase; (c) isocitrate dehydrogenase; (d) lactate dehydrogenase; (e) malate dehydrogenase. The broken line represents enzyme activity during involution and the solid line represents enzyme activity during lactation.

are therefore expressed on this basis and the s.d. has been calculated throughout.

Fig. 1 shows changes in the activity of a number of dehydrogenases in the PFS fraction of rabbit mammary gland throughout the lactation cycle and Fig. 2 shows comparable results for rat mammary gland. The spread in the values of s.d. recorded in Fig. 1 could well be due to variation in the litter size, or the small number of animals used, or both. However, similar variations were occasionally observed in enzyme activities in rat mammary gland (Fig. 2), where litter size was more controlled

Fig. 2. Changes in activity of some dehydrogenases in the PFS fraction of rat mammary gland. Vertical lines represent the s.d. of the enzyme activities and numbers in parentheses are the numbers of animals in each group. Activity in glands of virgin (V), pregnant and lactating animals (○), and activity in glands of animals after weaning (Δ). (a) Glucose 6-phosphate dehydrogenase; (b) 6-phosphogluconate dehydrogenase; (c) isocitrate dehydrogenase; (d) malate dehydrogenase (decarboxylating); (e) lactate dehydrogenase; (f) malate dehydrogenase. The broken line represents enzyme activity during involution and the solid line represents enzyme activity during lactation.

and the number of animals used was greater. Of the NADP-dependent dehydrogenases studied, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase reached maximum activities at mid-lactation to late lactation in both species. A notable difference between the two

tissues was that isocitrate dehydrogenase increased in activity from late pregnancy to mid-lactation in rabbit mammary gland, but not in that of the rat. During involution there was an initial decrease in activity of this enzyme at early involution in tissues of both species and a rise in activity at late involution

Table 1. *Changes in the activities of pyruvate carboxylase, succinate dehydrogenase and 6-phosphogluconate dehydrogenase in subcellular fractions of rabbit and rat mammary gland*

The numbers in parentheses are the numbers of animals in each group. Results are expressed as means $\pm$ s.d. Succinate dehydrogenase and 6-phosphogluconate dehydrogenase were assayed in freshly prepared fractions. Specific activity is expressed as μ moles of substrate/min./ μ g. of DNA P.

	Specific activity			
	Mitochondrial fraction		PFS	
	Pyruvate carboxylase	Succinate dehydrogenase	Pyruvate carboxylase	6-Phosphogluconate dehydrogenase
Rabbit mammary gland				
25-30 days pregnant (3)	0.06 $\pm$ 0.04	0.6 $\pm$ 0.45	—	—
12-16 days <i>post partum</i> (1)	0.46	—	—	—
18-20 days <i>post partum</i> (3)	0.49 $\pm$ 0.33	2.2 $\pm$ 0.90	—	—
27-30 days <i>post partum</i> (4)	0.84 $\pm$ 0.70	1.7 $\pm$ 0.32	—	—
7 days of weaning at 30 days <i>post partum</i> (3)	0.06 $\pm$ 0.01	0.60 $\pm$ 0.28	—	—
Rat mammary gland				
5-10 days pregnant (5)	0.34 $\pm$ 0.42	0.64 $\pm$ 0.14	1.5 $\pm$ 0.6	13.3 $\pm$ 7.0
20-22 days pregnant (6)	0.71 $\pm$ 0.23	0.73 $\pm$ 0.19	3.5 $\pm$ 2.2	19.5 $\pm$ 3.8
12-14 days <i>post partum</i> (9)	1.6 $\pm$ 1.3	4.0 $\pm$ 1.4	3.0 $\pm$ 1.4	90.4 $\pm$ 20.6
21 days <i>post partum</i> (7)	2.7 $\pm$ 3.4	3.9 $\pm$ 2.6	2.4 $\pm$ 1.9	80.9 $\pm$ 35.0
4 days of weaning at 21 days <i>post partum</i> (6)	0.30 $\pm$ 0.10	0.62 $\pm$ 0.13	0.5 $\pm$ 0.2	12.7 $\pm$ 3.3

in rat mammary gland, but not in that of rabbit. The most marked difference between the two tissues was that the NADP-dependent malate dehydrogenase (decarboxylating) could not be detected in rabbit mammary gland at any stage of the lactation cycle. In rat mammary gland this enzyme increased in activity from pregnancy and reached a maximum at mid-lactation.

In both rabbit and rat mammary glands the NAD-dependent malate dehydrogenase showed an increase in activity from late pregnancy to early lactation. At late involution the enzyme showed an increase in activity in rat, but not in rabbit, mammary gland. Lactate dehydrogenase reached its maximum activity earlier in lactation in rabbit than in rat mammary gland.

Gul & Dils (1969) reported that pyruvate carboxylase is confined to the mitochondrial fraction of rabbit mammary gland at 10-14 days *post partum*, but is present in both the mitochondrial and PFS fractions of rat mammary gland at 12-14 days *post partum*. The activity of pyruvate carboxylase in these subcellular fractions was therefore assayed throughout the lactation cycle (Table 1). Great variations in specific activity were observed at each stage of the lactation cycle in these subcellular fractions. This also applied to the activity of the enzyme in homogenates of these tissues. In rabbit mammary gland, mitochondrial pyruvate carboxylase increased in activity from late pregnancy to lactation and decreased in activity during weaning. Succinate dehydrogenase

activity showed parallel changes. At no stage of the lactation cycle did the activity of pyruvate carboxylase in the unfractionated 'microsomal + PFS fraction' of rabbit mammary gland exceed 0.05 μ mole of substrate/min./ μ g. of DNA P. In rat mammary gland, no significant changes in the activity of pyruvate carboxylase in the mitochondrial or the PFS fractions were observed. In contrast, the marker enzymes used (succinate dehydrogenase and 6-phosphogluconate dehydrogenase) increased in activity from late pregnancy to mid-lactation and decreased in activity during weaning.

Comparison of three bases for the expression of enzyme activities. Enzymic activities in mammary gland have usually been expressed per g. wet wt. of tissue, corrected for retained milk (Greenbaum & Slater, 1957b; McLean, 1958b; Baldwin & Milligan, 1966). This estimation of retained milk in mammary gland homogenates has been based on the percentage of lactose in milk obtained from lactating animals. In the present work, milk was obtained from rabbit mammary gland at all stages of lactation investigated. In addition, retained fluid could be obtained from glands of rabbits at pregnancy and during mammary involution after weaning. Hence corrections for the content of milk or retained fluid were made on the basis of the lactose content of the material obtained at each stage of the lactation cycle. The lactose content of retained fluid was found to be low (0.1-0.5%) compared with that of milk obtained during lactation (2.0%). When this

Table 2. *Effect of retained milk on the apparent specific activity of a number of enzymes in the PFS fraction of rabbit and rat mammary gland*

Specific activities are expressed as μ moles of nicotinamide nucleotide/min./mg. of PFS protein. 'Retained milk' refers to the percentage (w/w) of milk in the glands of individual animals.

	Retained milk (% of wt. of gland)	Specific activity					
		Malate dehydro- genase	Lactate dehydro- genase	6-Phospho- gluconate dehydro- genase	Glucose 6- phosphate dehydro- genase	Isocitrate dehydro- genase (NADP)	Malate de- hydrogenase (decarboxy- lating)
Rabbit mammary gland 4-5 days <i>post partum</i>	13	2.7	3.4	0.20	0.32	0.05	0
	14	2.5	2.9	0.15	0.18	0.06	0
	54	2.9	2.7	0.17	0.28	0.05	0
Rat mammary gland 5 days <i>post partum</i>	5	3.3	1.9	0.17	0.57	0.03	0.19
	8	3.7	2.2	0.22	0.69	0.03	0.20
	10	3.1	1.7	0.18	0.54	0.04	0.17
	26	3.5	1.7	0.20	0.49	0.02	0.22

lactose content of retained fluid was used to calculate the amount of fluid in the gland at pregnancy and at involution, the amount calculated exceeded the total weight of the gland. These results throw doubt on the validity of correcting the weight of rabbit mammary gland at all stages of lactation by determining the lactose content of milk or retained fluid. We also found lactose in homogenates and in the PFS fractions of mammary glands of virgin rats and of rats from which the litter had been removed for 7 days at 21 days *post partum*. Many of these results might be explained by the unspecific nature of the lactose determination used. However, Kuhn & Lowenstein (1967), with a more specific method, could find no correlation between the presence of lactose and of visible milk in mammary glands of parturient rats. Other limitations of the expression of enzyme activities in mammary gland per g. wet wt. of tissue are that present techniques lead to incomplete homogenization of the tissue (for which there are no correction factors) and that an apparent increase in enzyme activity could result from a general increase in cell size (Richardson, 1947; Mayer & Klein, 1961) or glandular tissue (Munford, 1963), and hence in extractable protein.

An alternative way to express enzyme activities in mammary gland is per mg. of protein. It was observed that for mammary gland in both rabbit and rat the percentage of nuclei-free homogenate protein in the PFS fraction did not change significantly from pregnancy through to late lactation. It is therefore possible to compare enzyme activities expressed per mg. of PFS protein at these periods of lactation. For both tissues the percentage of full-homogenate protein in the PFS fraction

decreased at early involution and increased again at late involution. For rat mammary gland the percentage of PFS protein at late involution was comparable with that in the gland of virgin animals.

It would be expected that the protein content of the PFS fraction needs correction for protein derived from milk retained in the gland. However, when activities were expressed per mg. of PFS protein uncorrected for milk protein, they were not affected by the amount of milk in the gland. Examples of the results obtained are given in Table 2. In rabbit mammary gland changes in enzyme activity throughout the lactation cycle followed similar patterns, whether expressed per mg. of PFS protein or per μ g. of DNA P. In rat mammary gland, differences in the pattern of changes in enzyme activity were only observed from late pregnancy to early lactation (5 days *post partum*). Lactate dehydrogenase and malate dehydrogenase did not increase in activity in this period when expressed per mg. of PFS protein, but did increase when expressed per μ g. of DNA P. The assumption that DNA content/cell is constant in rat mammary gland may not be true during this period, since Greenbaum & Slater (1957a) reported that a wave of cell division occurs on the first day of lactation. It is noteworthy that these enzymes do not greatly change in activity throughout the lactation cycle. In many cases, changes in enzyme activity expressed per mg. of protein seem to correlate well with changes expressed per μ g. of DNA P, and the apparent activities measured are not affected by the amount of milk in the gland preparation.

The activity of malate dehydrogenase in rat mammary gland during the lactation cycle was

Fig. 3. Specific activity of malate dehydrogenase in the PFS fraction of rat mammary gland. (a) Specific activity expressed per g. wet wt. of tissue (corrected for the milk of the gland preparation); (b) specific activity expressed per mg. of PFS protein. Vertical lines represent the s.d. of the enzyme activities, and numbers in parentheses are the numbers of animals in each group. Other details are as in Fig. 2.

expressed per g. wet wt. (Fig. 3a) and per mg. of PFS protein (Fig. 3b) for comparison with that per $\mu\text{g. of DNA P}$ (Fig. 2f).

DISCUSSION

When changes in the activities of the enzymes (per $\mu\text{g. of DNA P}$) studied in rabbit and rat mammary gland were compared there were no striking differences between the two species. Although comparable data are not available for rabbit mammary gland, the ratios of maximum activities observed to activities during pregnancy for rat mammary gland, when calculated per g. wet wt. of tissue, were similar to those previously reported (McLean, 1958a; Rees & Huggins, 1960; Baldwin & Milligan, 1966). It is noteworthy that, in rat mammary gland, only those enzymes that did not show a significant increase in activity during lactation (isocitrate dehydrogenase and malate dehydrogenase; Figs. 2c and 2f) showed an

initial decrease in activity 3 days after weaning and a further increase (to that in the gland of the pregnant animal) 7 days after weaning, although the reason for this is not clear. The increase in activity of isocitrate dehydrogenase noted in the present paper in rat mammary gland at 7 days after weaning was observed by Matthes, Abraham & Chaikoff (1963) 1 day after weaning.

Malate dehydrogenase (decarboxylating) can provide NADPH for lipogenesis from NADH in rat adipose tissue and liver (Young, Shrago & Lardy, 1964; Wise & Ball, 1964; Pande, Khan & Venkitasubramanian, 1964; for review see also McLean, Brown & Greenbaum, 1968) by the following sequence of reactions:

This transhydrogenation requires a continual supply of oxaloacetate, and mitochondrial pyruvate carboxylase is a major source of oxaloacetate in a number of tissues (Keech & Utter, 1963; Utter & Keech, 1963). Intramitochondrial oxaloacetate is also needed to synthesize citrate, which is subsequently cleaved to extramitochondrial oxaloacetate and acetyl-CoA by ATP citrate lyase. Both of these products can then be used for lipogenesis, the former for NADPH formation via transhydrogenation (see above) and the latter as C_2 units for fatty acid biosynthesis. Another function of this transhydrogenation might be the reoxidation of NADH produced during glucose breakdown (for review see McLean *et al.* 1968).

In rabbit mammary gland, mitochondrial pyruvate carboxylase increased in activity from late pregnancy to mid-lactation, and then decreased in activity at involution (Table 1). We found that ATP citrate lyase was present in the PFS fraction of this tissue at 10–14 days *post partum* (specific activity $40 \pm 14 \mu\text{moles/min./mg. of PFS protein}$, mean $\pm$ s.d. of four preparations), and that malate dehydrogenase had a high activity throughout lactation (Fig. 1e). However, the absence of malate dehydrogenase (decarboxylating) in this tissue implies that a block in such a transhydrogenation reaction would occur after malate formation, causing diminished NADPH formation or utilization or both. The observed increase in activity of mitochondrial pyruvate carboxylase might, for example, be linked with increased provision of extramitochondrial acetyl-CoA for lipogenesis.

In contrast, malate dehydrogenase (decarboxylating) in rat mammary gland showed a marked increase in activity during lactation (Fig. 2d), and the NAD-dependent lactate dehydrogenase (Fig. 2e) and malate dehydrogenase (Fig. 2f) had a high activity throughout the lactation cycle. Further, ATP citrate lyase is known to increase in activity in rat mammary gland during lactation (Howanitz & Levy, 1965). The presence of both mitochondrial and soluble forms of pyruvate carboxylase in this tissue implies that the transhydrogenation reactions discussed above could be associated with the citrate-cleavage pathway, or could be a separate sequence of reactions not requiring citrate cleavage, but requiring soluble pyruvate carboxylase as a source of oxaloacetate. It might therefore be expected that if transhydrogenation occurred in this tissue and was important for NADPH formation, the enzymes involved would increase in activity as lactation proceeded. However, both the mitochondrial and soluble forms of pyruvate carboxylase had a much lower activity than had any of the other enzymes of the sequence, and the activity did not increase as lactation proceeded. For both rabbit and rat mammary gland, each of the NADP-dependent dehydrogenases studied had a very high activity at all stages of the lactation cycle compared with the rate of fatty acid synthesis during lactation [2 μ moles of acetate incorporated/min./mg. of protein for rabbit mammary-gland 'microsomal + PFS fraction' at 5–21 days *post partum* (Smith & Dils, 1966); 1 μ mole of acetate incorporated/min./mg. of protein for rat mammary-gland 'microsomal + PFS fraction' at 10–15 days *post partum* (Abraham, Matthes & Chaikoff, 1963)]. Since the NADPH/NADP⁺ concentration ratio is high in lactating rat mammary gland (McLean, 1958b), the availability of NADP⁺ probably limits the activity of NADP-dependent dehydrogenases in this tissue (McLean, 1960; Matthes *et al.* 1963). Though superficially there appears to be no need of a pyruvate carboxylase-linked transhydrogenation, only a comparison of NADPH formation via the hexose monophosphate pathway and transhydrogenation in rat mammary gland throughout lactation will determine whether this is in fact the case. The need for this comparison is illustrated by results obtained with rat adipose tissue, where NADP-dependent dehydrogenases have a high activity (Pande *et al.* 1964), yet under conditions of hyperlipogenesis, 30–40% of the NADPH required is supplied by transhydrogenation reactions (Katz, Landau & Bartsch, 1966).

Finally, we found phosphoenolpyruvate carboxylase to be present in (but confined to the mitochondrial fractions of) mammary gland in rat and rabbit at 10–14 days *post partum* (specific activities in rat and rabbit mammary gland

respectively: 8.8 ± 2.4 μ moles/min./mg. of mitochondrial protein, mean $\pm$ S.D. of three preparations; and 0.9 ± 0.3 μ mole/min./mg. of mitochondrial protein, mean $\pm$ absolute deviation of two preparations). Therefore in these tissues pyruvate carboxylase might play a role in gluconeogenesis, as it does in rat liver and kidney (Henning, Stumpf, Ohly & Seubert, 1966; Mehlman, Walter & Lardy, 1967), or in providing glyceride glycerol, as has been suggested for rat and mouse adipose tissue (Ballard, Hanson & Leveille, 1967; Chakrabarty & Leveille, 1968), or in both processes. However, the low activities of pyruvate carboxylase and phosphoenolpyruvate carboxylase that we have observed cast doubt on the importance of this role in rat and rabbit mammary gland.

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