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Regulation of lipogenic capacity in lactating rats

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1. The rate of mammary-gland lipogenesis measured *in vivo* from ^3H_2O was suppressed after decreasing the milk demand by decreasing the number of pups from ten to two or three, as well as by giving diets containing lipid [Grigor & Warren (1980) *Biochem. J.* **188**, 61–65]. 2. The specific activities of the lipogenic enzymes fatty acid synthase, glucose 6-phosphate dehydrogenase and 'malic' enzyme increased between 6- and 10-fold in the mammary gland and between 2- and 3-fold in the livers during the first 10 days of lactation. The increases in specific activity coupled with the doubling of liver mass which occurred during pregnancy and lactation resulted in considerable differences in total liver activities when compared with virgin animals. 3. Although consumption of a diet containing 20% peanut oil suppressed the activities of the three lipogenic enzymes in the livers, only the 'malic' enzyme was affected in the mammary glands. 4. In contrast, decreased milk demand did not affect the specific activities of any of the liver enzymes, whereas it resulted in suppression of all three lipogenic enzymes of the mammary glands. There was no effect on either the cytoplasmic malate dehydrogenase or the lactate dehydrogenase of the mammary gland. 5. In all the experiments performed, the activity of the fatty acid synthase correlated with the amount of material precipitated by the rabbit antibody raised against rat fatty acid synthase.

The fatty acids of milk triacylglycerols have three sources: those synthesized within the mammary gland, those synthesized in the liver and transported to the mammary gland, and those derived from the diet. We have previously shown that the presence of fat in the diet suppresses the rates of lipogenesis in both the mammary gland and livers of lactating rats as determined *in vivo* by using ^3H_2O (Grigor & Warren, 1980). This confirmed a similar observation for the lactating mouse (Romsos *et al.*, 1978), but appeared to be contrary to an earlier report by Smith *et al.* (1969) showing that the conversion of acetate and glucose into lipid in mammary-gland slices from lactating mice was not dependent on the dietary history of the mice. In comparable experiments with liver slices from lactating or virgin mice, lipogenesis was indeed suppressed by previous consumption of diets containing lipid. This tissue-specific response to fat feeding was also demonstrated by the activities of a number of lipogenic enzymes. Although the activities were suppressed by fat feeding in the livers, no consistent differences were noted for the mammary gland (Smith *et al.*, 1969).

The apparent discrepancy between the results obtained *in vivo* and *in vitro* prompted us to investigate the effect of fat feeding on the activities of some of these enzymes in the mammary glands and livers of lactating rats. We have also used a second method for manipulating the rates of lipogenesis in studies performed both *in vivo* and *in vitro*. This involved altering the number of pups to be suckled. The results show that the activities of the mammary-gland and liver enzymes are regulated independently, with the mammary-gland enzymes being specifically affected by changes in milk demand whereas the liver enzymes are affected specifically by dietary changes.

Experimental

Animals

Female rats of the Wistar strain were purchased from the University of Otago Animal Breeding Station and mated at between 3 and 4 months of age. Except where stated, the litter sizes were standardized at ten pups. Age-matched virgin animals were used in some experiments. The rats were fed on a commercial pelleted 'breeder' diet (A. E. Reeves, Dunedin, New Zealand) or either the fat-free or 20%-peanut-oil diets used previously

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(Grigor & Warren, 1980). Except where stated, all animals were killed between days 12 and 15 of lactation. A 12h-light/12h-dark cycle was used and animals were killed at 10:00h, midway through the dark period. Animals were housed at a temperature of 20°C.

Chemicals

Acetyl-CoA, malonyl-CoA, $NADP^+$, NADPH, NADH, glucose 6-phosphate, DL-malic acid and agarose were all purchased from Sigma Chemical Co., St Louis, MO, U.S.A., Sodium pyruvate and oxaloacetic acid were supplied by Merck, Darmstadt, West Germany, and Fisons Scientific, Loughborough, Leics., U.K., respectively.

Assays of enzyme activities

All enzyme activities were assayed in 100000 $g \times$ 60min supernatants obtained from 1:5 (w/v) homogenates of inguinal mammary glands and livers prepared in a medium containing 0.25M-sucrose, 1mM-EDTA, 15mM-2-mercaptoethanol and 10mM-Tris/HCl buffer, pH 7.4. For each enzyme, assays were performed spectrophotometrically at 22°C.

Fatty acid synthase activity was assayed in 0.5ml of a mixture containing 0.15mM-NADPH, 0.5mM-acetyl-CoA, 0.1mM-malonyl-CoA, 1mM-EDTA, 1mM-dithiothreitol and 0.1M-potassium phosphate buffer, pH 7.0. The reactions were started by the addition of malonyl-CoA and the absorbance at 340nm was monitored for 1–3min. Glucose 6-phosphate dehydrogenase and 'malic' enzyme activities were assayed in 1ml mixtures containing 0.05mM-$NADP^+$, 0.1M-Tris/HCl buffer, pH 7.4, and 1mM-glucose 6-phosphate plus 8mM-$MgCl_2$ or 2mM-DL-malate plus 1mM-$MnCl_2$ respectively. Reactions were started by the addition of substrate, and the absorbance at 340nm was monitored for 2–5min. Malate dehydrogenase and lactate dehydrogenase activities were assayed in 1ml mixtures containing 0.75mM-NADH and either 1.25mM-oxaloacetate or 2mM-pyruvate. The reactions were started by the addition of substrate, and the maximum rate of absorbance change at 340nm was measured after an initial lag period. All activities were proportional to the amount of protein used. Fatty acid synthase activity was assayed in fresh samples, whereas the other enzyme activities were assayed in samples that had been frozen. No decrease in the specific activities could be detected in samples frozen for up to 2 months. Activities of fatty acid synthase are expressed as half the rate of NADPH oxidation (μmol/min), and those of other enzymes are expressed as μmol of $NADP^+$ reduced or NADH oxidized/min.

Fatty acid synthase from both liver and mammary-gland tissue was purified as described by Smith & Abraham (1975). Antibodies against each enzyme were raised in rabbits and used to quantify the amount of fatty acid synthase protein by using 'rocket' immunoelectrophoresis (Laurell, 1966). The liver fatty acid synthase standard and the antibody to the liver enzyme were used as a routine in these assays.

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

The rates of lipogenesis *in vivo* were determined after injection of ^3H_2O as described previously (Grigor & Warren, 1980).

Results

Our initial experiments showed that the rate of lipogenesis *in vivo* in the mammary gland could be significantly lowered by decreasing the number of pups from ten to three either shortly after parturition or at day 12 of lactation, 2 days before the experiment was performed (Table 1). Similar estimations for the liver lipogenesis were suggestive of a decrease, but were not statistically significant, owing to the large variation in the rates for the control rats. Thus in the mammary gland and probably the liver the rates of lipogenesis are affected both by the inclusion of fat in the diet (Grigor & Warren, 1980) and by modifying the milk demand by changing the number of pups to be suckled.

Table 1 shows also that decreasing the number of pups shortly after parturition causes a significant decrease in the maternal liver mass. This was not observed when the litter size was modified at mid-lactation. The livers of lactating rats are significantly larger than those of virgin animals

Table 1. *Effect of litter size on rates of lipogenesis in mammary gland and liver of lactating rats*
Rates of lipogenesis were determined after injection of ^3H_2O on day 14 of lactation. For full experimental details see the text. Values are means ± S.D. for five rats each with ten pups, or four rats each in groups with decreased litters.

Litter size ...	10	3 (from day 2)	3 (from day 12)
Rate of lipogenesis in mammary gland (μmol of H_2O/h per g)	70 ± 15	45 ± 8*	45 ± 8*
Rate of lipogenesis in liver (μmol of H_2O/h per g)	14 ± 7	8 ± 3	10 ± 4
Liver mass (g)	14.4 ± 2.6	10.9 ± 1.5*	14.1 ± 3.1

* Difference from value for ten pups statistically significant (t test) at $P < 0.01$.

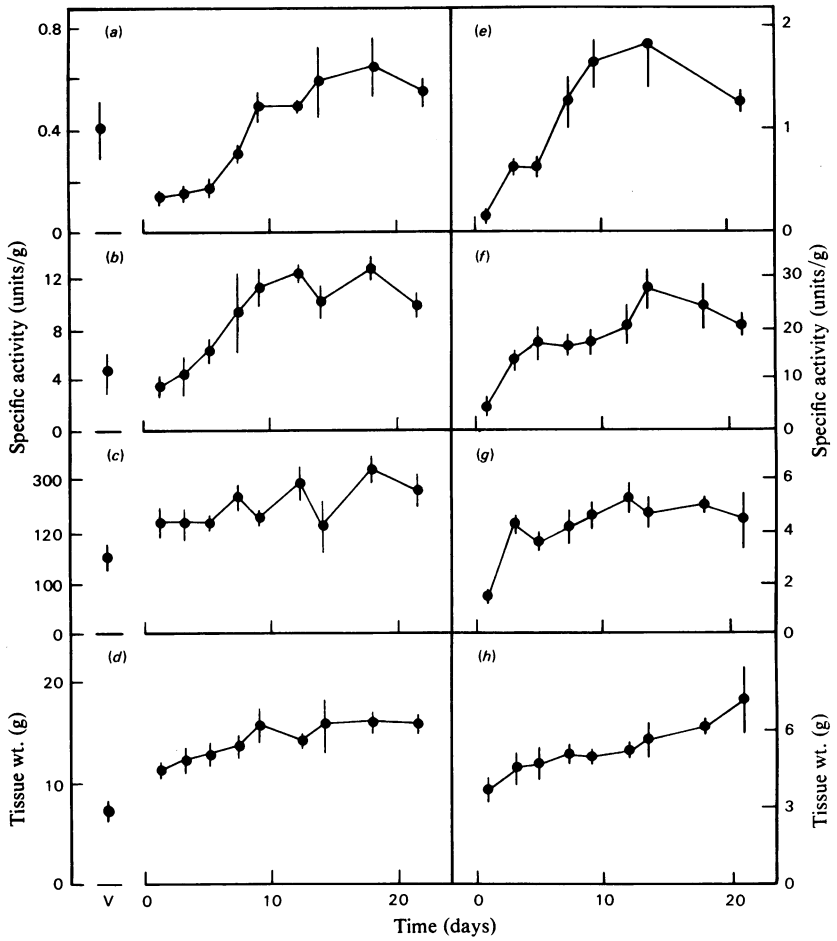

Fig. 1. *Liver and mammary-gland enzyme activities during the lactation period*

Enzyme activities were assayed as described in the text in extracts from livers of virgin rats (V) and livers and mammary glands of rats lactating for the number of days indicated. Values plotted are specific activities (units/g wet wt.) of liver fatty acid synthase (*a*), liver glucose 6-phosphate dehydrogenase (*b*), liver cytoplasmic malate dehydrogenase (*c*), mammary-gland fatty acid synthase (*e*), mammary-gland glucose 6-phosphate dehydrogenase (*f*) and mammary-gland 'malic' enzyme (*g*), and are the means ± S.D. for five rats for virgin rats (V) and rats at 14 days of lactation. At other time points the means ± half the range for at least two rats have been plotted. Also included are the wet weights (g) of liver (*d*) and inguinal mammary glands (*h*).

(Souders & Morgan, 1957; Kennedy *et al.*, 1958), and after an increase in mass during pregnancy further growth occurs up to around day 10 of lactation (Fig. 1*d*).

Fig. 1 shows the specific activities of several enzymes in the mammary glands and livers of lactating and virgin rats. We have chosen to quote our data here in terms of tissue wet weight. The mammary gland is a heterogeneous tissue containing adipose cells, connective tissue as well as mammary epithelial cells, all of which will contribute to the mass of the tissue. The presence of unsecreted milk could also affect the tissue weights obtained. Mammary DNA has been used as a basis for expressing enzyme activities and, although it eliminates the problem of the retained milk, will still include contributions from other cell types. However, Coore & Field (1974) quote data showing that between days 3 and 12 of lactation the mammary DNA content/g wet wt. does not vary, and at the end of lactation on day 21 it had fallen by only 30%.

Different patterns were obtained for the activities of the fatty acid synthase, glucose 6-phosphate dehydrogenase and the cytoplasmic malate dehydrogenase in the livers during the lactation cycle. The fatty acid synthase specific activity was sig-

Table 2. *Effect of diet on enzyme activities in livers and mammary glands of rats*

Fat-free or high-fat (20% peanut oil) diets were fed to lactating rats for 7 days from day 7 of lactation, or for 7 days to virgin rats. Enzyme activities were assayed in 100000 *g* supernatants prepared from extracts of livers and inguinal mammary glands as described in the text. Values listed are means ± S.D. for numbers of rats in parentheses. —, Not determined.

	Lactating rats				Virgin rats	
	Mammary glands		Livers		Livers	
Diet ...	Fat-free	High-fat	Fat-free	High-fat	Fat-free	High-fat
Tissue mass (g)	5.8 ± 0.7 (15)	6.8 ± 1.5 (12)	18.5 ± 2.9 (15)	13.8 ± 2.6 (12)**	7.7 ± 0.7 (6)	6.9 ± 0.6 (6)
Fatty acid synthase						
(units/g)	2.18 ± 0.49 (13)	1.70 ± 0.54 (8)	1.91 ± 0.39 (13)	1.05 ± 0.24 (7)**	0.77 ± 0.10 (5)	0.34 ± 0.12 (5)**
(units/tissue)	13.4 ± 2.6 (13)	11.6 ± 3.0 (8)	30 ± 8 (13)	14 ± 4 (7)**	6.0 ± 1.2 (5)	2.3 ± 0.8 (5)**
(mg of immunoprecipitable protein/g)	22.1 ± 2.9 (7)	18.3 ± 2.9 (6)*	14.3 ± 2.5 (7)	8.2 ± 1.2 (4)**	7.1 ± 1.7 (5)	2.1 ± 0.8 (5)**
Glucose 6-phosphate dehydrogenase						
(units/g)	30 ± 5 (6)	25 ± 4 (7)	15.0 ± 3.0 (8)	6.8 ± 1.6 (8)**	15.1 ± 2.4 (6)	5.3 ± 1.3 (6)**
(units/tissue)	173 ± 26 (6)	161 ± 46 (6)	287 ± 56 (8)	87 ± 28 (7)**	114 ± 29 (6)	36 ± 8 (6)**
'Malic' enzyme						
(units/g)	9.5 ± 1.6 (6)	5.0 ± 1.1 (7)**	5.7 ± 1.6 (8)	2.7 ± 0.7 (7)**	6.0 ± 0.7 (6)	2.4 ± 0.6 (6)**
(units/tissue)	55 ± 9 (6)	37 ± 7 (7)**	108 ± 35 (8)	34 ± 10 (7)**	45 ± 9 (6)	16 ± 4 (6)**
Malate dehydrogenase						
(units/g)	—	—	207 ± 34 (5)	212 ± 29 (6)	192 ± 13 (6)	173 ± 151 (6)*
(units/tissue)	—	—	3560 ± 550 (5)	2670 ± 600 (6)*	1430 ± 110 (6)	1150 ± 150 (6)*

* Difference from value for fat-free diet statistically significant (*t* test), $P < 0.05$.
** Difference from value for fat-free diet statistically significant (*t* test), $P < 0.01$.

nificantly lower during the early stages of lactation than for virgin livers, but increased about 4-fold between days 5 and 12 of lactation (Fig. 1*a*). Glucose 6-phosphate dehydrogenase (Fig. 1*b*) and 'malic' enzyme (results not shown) both had specific activities at the early stages of lactation similar to those in virgin livers and increased approximately 3-fold during the first 10 days of lactation. Taking into account the differences in liver mass, the activities per liver of these enzymes at peak lactation are some 4 times those of livers at parturition and some 5 times those of livers from virgin rats. In contrast, the specific activity of the cytoplasmic malate dehydrogenase changed only slightly during lactation (Fig. 1*c*), suggesting that this enzyme is constitutive and the only changes in activity of this enzyme are related to increases in liver mass.

Fig. 1 also shows the activities of the mammary enzymes. Here a 10-fold increase in the fatty acid synthase specific activity (Fig. 1*e*) and approximately 6-fold increases in those of glucose 6-phosphate dehydrogenase (Fig. 1*f*) and 'malic' enzyme (Fig. 1*g*) occurred during the first 10 days of lactation. These changes are similar to earlier data published, where activities have been related to gland mass (Gumaa *et al.*, 1973) or gland DNA (Baldwin & Milligan, 1966; Gul & Dils, 1969). The mass of the inguinal mammary glands also increases during this period, from 3.5 g to around 6 g at peak lactation (Fig. 1*h*). Values for mammary glands of virgin animals have not been included, because these glands contain mainly non-epithelial tissue (Rees & Eversole, 1964).

Table 2 lists the enzyme activities in the mammary glands and livers of rats fed on fat-free or high-fat (20% peanut oil) diets for 7 days until day 14 of lactation. The specific activities of the fatty acid synthase, glucose 6-phosphate dehydrogenase and 'malic' enzyme in the livers from rats fed on the fat-free diet were approximately double those for the rats fed on the high-fat diet. Furthermore a difference in the liver mass meant that the differences in activities per liver were nearer 3-fold. Similar differences were observed in specific activities of these enzymes in livers from virgin animals, confirming previous observations. The specific activity of the liver malate dehydrogenase did not alter with fat feeding.

In contrast with the liver enzymes, the specific activities of the mammary-gland fatty acid synthase and glucose 6-phosphate dehydrogenase were not significantly altered by fat feeding, although 'malic' enzyme did appear to be suppressed. The relative insensitivity of the mammary-gland enzymes to dietary manipulation confirms the observations made by Smith *et al.* (1969) working with mice.

In both the liver and mammary gland the activity of the fatty acid synthase correlated with the amount of the synthase enzyme determined immunochemically. The values for mg of fatty acid synthase/g of tissue are approximately double those expected from the observations by Smith & Ryan (1979) for the rat mammary gland. The reason for this is not clear. The antibody used in our experiments gave only a single peak when analysed by two-dimensional immunoelectrophoresis. The possibility of some non-immunoreactive material in our fatty acid synthase standard remains to be verified, although for each of four purified synthase preparations only a single band could be detected after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

The effect of different litter sizes on the mammary-gland and liver enzymes was then investigated. The number of pups was decreased from the standard ten to either two or five within the first 2 days after parturition. The total pup weights at day 14 were 46, 132 and 280 g for litters of two, five and ten respectively. Assuming a mean pup birth weight of 5 g, the overall weight gains of 36, 97 and 230 g represent widely different amounts of milk produced. Table 3 shows that there were no differences in the specific activities of the fatty acid synthase, glucose 6-phosphate dehydrogenase and 'malic' enzyme from the livers of these rats, although smaller liver masses meant a decrease in total activities per liver in the rats with decreased litters. In contrast, the activities of all three enzymes in the mammary-gland extracts from the rats with decreased litters were markedly depressed, although again 'malic' enzyme did not fit the pattern for the fatty acid synthase and glucose 6-phosphate dehydrogenase. The specific activities of the mammary-gland lactate dehydrogenase and malate dehydrogenase were, however, unaffected by decreasing the litter sizes to two. This suggests that the changes in activity of the lipogenic enzymes were quite specific. Furthermore, it also suggests that the differences in specific activities for the lipogenic enzymes are not artifacts due to increased retention of milk where the milk demand is low. A small decrease in the mass of the inguinal glands for the rats with decreased litters was observed, but this was insufficient to make the changes in the total activities of these two enzymes statistically significant. Again in this experiment the fatty acid synthase activity correlated closely with the amount of immunoprecipitable enzyme in both the mammary-gland and liver extracts.

Discussion

Our observations have shown that, although the absolute rates of lipogenesis in both the liver and mammary gland of lactating rats are suppressed by fat feeding (Grigor & Warren, 1980) and by

Table 3. *Effect of litter number on mammary-gland and liver enzymes in lactating rats*

Litter numbers were adjusted to ten, five or two pups on day 2 of lactation and animals were killed at day 14. Rats were fed on pelleted diet throughout. Enzyme activities were assayed in 100000 *g* supernatants prepared from extracts of livers and inguinal mammary glands as described in the text. Values listed are means ± S.D. for five rats with two or ten pups or for four rats with five pups. —, Not determined.

	Mammary gland			Liver		
No. of pups	2	5	10	2	5	10
Tissue mass (g)	4.7 ± 0.6*	6.0 ± 1.4	5.7 ± 0.7	11.6 ± 0.9	14.8 ± 2.3	16.4 ± 2.8
Fatty acid synthase						
(units/g)	0.50 ± 0.10**	0.93 ± 0.60 (4)*	1.89 ± 0.39	0.71 ± 0.13	0.73 ± 0.26	0.58 ± 0.13
(units/tissue)	2.12 ± 0.56**	5.39 ± 3.24*	10.4 ± 2.9	8.3 ± 1.9	11.0 ± 2.0	9.4 ± 2.1
(mg of immunochemically precipitable protein/g)	3.55 ± 1.70**	—	20.0 ± 5.6	—	—	4.5 ± 1.0
Glucose 6-phosphate dehydrogenase						
(units/g)	7.2 ± 1.5**	11.3 ± 5.8**	30 ± 3.0	7.9 ± 3.2	10.9 ± 1.8	8.8 ± 2.3
(units/tissue)	32 ± 4**	64 ± 31**	162 ± 8	91 ± 34	165 ± 54	145 ± 45
'Malic' enzyme						
(units/g)	2.5 ± 0.5**	4.8 ± 2.8	4.7 ± 0.6	2.2 ± 0.5	3.1 ± 1.2	2.0 ± 0.5
(units/tissue)	12.0 ± 3.9**	28 ± 17	26 ± 4	26 ± 6	40 ± 26	32 ± 8
Lactate dehydrogenase						
(units/g)	40 ± 9	—	39 ± 6	—	—	—
(units/tissue)	189 ± 60	—	220 ± 31	—	—	—
Malate dehydrogenase						
(units/g)	32 ± 7	—	34 ± 14	—	—	—
(units/tissue)	150 ± 34	—	185 ± 61	—	—	—

* Difference from value for fat-free diet statistically significant (*t* test), $P < 0.05$.
** Difference from value for fat-free diet statistically significant (*t* test), $P < 0.01$.

decreasing the milk demand (the present paper), the activities of three different lipogenic enzymes are regulated in a tissue-specific manner by these two manipulations. The inclusion of lipid in the diet specifically affects the activities of the liver enzymes, whereas the decreased milk demand after change in the number of pups to be suckled specifically affects the mammary-gland enzymes.

The absolute rates of lipogenesis are determined by a variety of factors, of which the concentrations of lipogenic enzymes is only one. The availability of substrate and the short-term modulation of key enzyme activities are more likely to determine the final rate. In particular, pyruvate dehydrogenase appears to have a critical role in the conversion of glucose into fatty acid in both liver and adipose tissue (Coore *et al.*, 1971; Wieland *et al.*, 1972) as well as the mammary gland (Baxter & Coore, 1978; Munday & Williamson, 1981), with acetyl-CoA carboxylase being of secondary importance.

The changes in activities of the other lipogenic enzymes reflect rather changes in the overall lipogenic capacity of any tissue. A large literature exists describing changes in the activities of fatty acid synthase, glucose 6-phosphate dehydrogenase, 'malic' enzyme and the ATP citrate lyase in livers of rats subjected to dietary manipulation (for references see Romsos & Leveille, 1974). Differences in the activities of each of these enzymes have been shown to correlate with the amounts of enzyme protein (Gozukara *et al.*, 1971; Craig *et al.*, 1972; Gibson *et al.*, 1972; Peavy & Hansen, 1975). This is true also for acetyl-CoA carboxylase, which, although subject to short-term regulation, does show adaptive changes in content in response to dietary stimuli (Majerus & Kilburn, 1969; Nakanishi & Numa, 1970).

Smith & Ryan (1979) have shown that a close correlation between the fatty acid synthase activity and amount of enzyme protein exists in the rat mammary gland during the early stages of lactation. Similarly, Mackall & Lane (1977) have shown that the amount of acetyl-CoA carboxylase protein increases with the induction of lactation. In the present study we have also shown that the fatty acid synthase activity correlates with the amount of immunoprecipitable protein in each of the experiments that we have done. It is likely that the same will be true for both the glucose 6-phosphate dehydrogenase and 'malic' enzyme.

Our results also show that not only are there tissue-specific differences in the responses of the three lipogenic enzymes to the manipulations that we have used, but in addition within either the mammary gland or the liver there are differences for each enzyme. For example, of the liver enzymes the fatty acid synthase activity is significantly lower during the early stages of lactation than for virgin animals. This was not observed for glucose 6-phosphate dehydrogenase. In the mammary gland 'malic' enzyme differs from both the fatty acid synthase and the glucose 6-phosphate dehydrogenase in being responsive to fat feeding on the one hand and less responsive to changes in milk demand on the other (Tables 2 and 3). 'Malic' enzyme has been shown to exist in two isoenzymic forms in rat tissues, only one of which is responsive to dietary stimuli (Saito & Tomita, 1973). The distribution of isoenzymes of 'malic' enzyme in the mammary gland does not appear to have been described.

Despite considerable efforts that have been made, the immediate factors controlling the amounts of lipogenic enzymes in rat liver are not well understood. Certainly the hormones insulin and glucagon appear to have a role in regulating the rates of synthesis of these enzymes (Weber & Convery, 1966; Garcia & Holten, 1975; Geisler *et al.*, 1978), but other factors could be involved in the response to dietary lipid (Gozukara *et al.*, 1972; Hizi & Yagil, 1974; Kelley *et al.*, 1975). The situation in the mammary gland is no more clear. From studies with explants, at least three hormones, namely insulin, prolactin and a glucocorticoid, are required for the synthesis of the fatty acid synthase and the induction of fatty acid synthesis (Speake *et al.*, 1976). A study by Martyn & Hansen (1981) suggests that suckling is also required for the induction of acetyl-CoA carboxylase and the ATP citrate lyase, but not the fatty acid synthase. It has been known for some time that suckling is required for the activity of several mammary-gland enzymes to be maintained, and the rise in intramammary pressure at weaning has been implicated as a factor in the decrease of mammary-gland enzymes (Jones, 1967).

In conclusion, we believe that the lactating rat provides an excellent model for studying the regulation of enzyme amounts and enzyme synthesis. Not only do both the liver and mammary gland undergo a prescribed cyclical sequence of events during the lactation cycle, but, superimposed on this, we have shown that the activities and presumably the amounts of several lipogenic enzymes can be manipulated in the liver and mammary gland independently either by dietary means or by altering the milk demand.

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