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Initiation of lipogenic enzyme activities in rat mammary glands

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The activities of acetyl-CoA carboxylase, ATP citrate-lyase and fatty acid synthetase remained low until parturition at 22 days of gestation and increased significantly within 1 day *post partum*. Administration of progesterone on days 20 and 21 and at parturition abolished the increases for at least 48h after parturition. Removal of the pups of normal rats prevented the increases in activities of acetyl-CoA carboxylase and ATP citrate-lyase, but not of fatty acid synthetase, and administration of prolactin, corticosterone or insulin did not stimulate activity. Tissue from suckled glands in which the ducts had been ligated at parturition showed no increase in the activities of acetyl-CoA carboxylase and ATP citrate-lyase within 24h, whereas fatty acid synthetase activity was similar to that in the sham-operated contralateral glands. Foetoplacentectomy on day 18 increased the activity of fatty acid synthetase but not of acetyl-CoA carboxylase and ATP citrate-lyase; suckling of these dams by foster pups increased both acetyl-CoA carboxylase and ATP citrate-lyase.

It is well recognized that the decline in plasma progesterone concentration during the last 2 days of pregnancy (Nicholas & Hartmann, 1975; Bartholomeusz *et al.*, 1976) acts as a trigger for the initiation of lactose synthesis in rat mammary glands (for reviews, see Kuhn, 1971, 1977). However, enzymes for the synthesis of milk constituents do not all develop simultaneously: casein synthesis is initiated before (Nardacci *et al.*, 1978), and fatty acid synthesis after, lactose synthesis (Martyn & Hansen, 1980).

Changes in the activity of various enzymes connected in some way with lipogenesis in the rat have been reported many times (e.g. Baldwin & Milligan, 1966; Gumaa *et al.*, 1973). A number of these enzymes appear to increase from the time of parturition, whereas others, such as thioesterase II, which terminates the chain in the synthesis of medium-chain fatty acids (Smith & Ryan, 1979), and lipoprotein lipase, which is concerned with the provision of fatty acid from plasma chylomicrons and very-low-density lipoproteins (Otway & Robinson, 1968; Hamosh *et al.*, 1970), are developed before this time.

Our demonstration that initiation of fatty acid synthesis does not depend solely on withdrawal of progesterone but in addition requires removal of milk from the gland by suckling (Martyn & Hansen, 1980), raises the question of which steps of synthesis are stimulated by milk removal.

In the present study we have used treatments *in vivo* that alter the normal changes in lipogenic rate and have measured the activities of acetyl-CoA carboxylase, ATP citrate-lyase and fatty acid synthetase. The results indicate that milk removal by suckling, together with the hormonal alterations that occur during lactogenesis, is an important stimulus for the increase in the first two of these enzymes.

Materials and methods

$Na_2{}^{14}CO_3$ (sp. radioactivity 2.18GBq/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Ovine prolactin (N.I.H. PS10, 30i.u./mg) was a gift from the Endocrinology Study Section, National Institutes of Health, Bethesda, MD, U.S.A. Protamine/zinc/bovine insulin (40 units/ml) was from the Boots Co. Ltd., Nottingham, U.K. Corticosterone, progesterone, calf thymus DNA type 1, ATP, reduced glutathione, bovine serum albumin (fraction V), CoA, malonyl-CoA, β-NADH, β-NADPH and malate dehydrogenase (EC 1.1.1.37) were from Sigma Chemical Co. Ltd., St. Louis, MO, U.S.A. Acetyl-CoA was prepared by the method of Simon & Shemin (1953).

Female Wistar rats from our colony were used as described previously (Martyn & Hansen, 1980). Parturition, except in those rats given progesterone, occurred on day 22, taken as day 0 of lactation.

Mammary-tissue extracts were prepared by the

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procedure of Mackall & Lane (1977), except that tissue was homogenized in 3 vol. of buffer and the pellet obtained after centrifugation of the homogenate for 10 min at 2000 *g* was stored in 0.5 M-$HClO_4$ at −15°C and used for the determination of DNA. Enzyme activities were measured in the final supernatant obtained by centrifugation at 73000 *g* for 45 min.

Measurement of enzyme activities

Acetyl-CoA carboxylase (EC 6.4.1.2) activity was determined by measuring fixation of [^{14}C]-bicarbonate in the presence of acetyl-CoA (Mackall & Lane, 1977).

Activity of ATP citrate-lyase (EC 4.1.3.8) was measured spectrophotometrically at 340 nm, essentially by the method of Srere (1959). The reaction mixture contained 50 mM Tris/HCl buffer, pH 8.3, 3 mM-$MgCl_2$, 3 mM-ATP, 0.1 mM-CoA, 10 mM-glutathione, 20 mM-citrate, about 0.1 mM-NADH, 55 μg (55 units) of malate dehydrogenase and high-speed supernatant in a final volume of 3.0 ml. The reaction was started by the addition of ATP. Controls omitting either citrate or enzyme preparation gave negligible rates.

The activity of fatty acid synthetase was measured spectrophotometrically at 340 nm by a modification of the assay described by Lynen (1962). The assay system contained 0.2 M-potassium phosphate buffer, pH 6.6, 0.08 mM-acetyl-CoA, 0.08 mM-malonyl-CoA, 6 mM-glutathione, 1 mM-EDTA and 0.075 mM-NADPH in a final volume of 3.0 ml. The reaction was started by the addition of enzyme. Controls in which the enzyme was omitted produced negligible reaction rates.

Expression of results

All three enzymes were assayed under conditions where the activity was linearly related to the amount of protein and to the time of incubation. Enzyme activities are expressed in units/mg of cellular DNA or munits/mg of cytosol protein, where 1 unit transforms 1 μmol of HCO_3^-, NADH or NADPH/min at 37, 30 and 30°C respectively. Both methods of expression have been used, as the ratio of DNA to tissue protein varies within the period studied (Baldwin & Milligan, 1966). Protein was determined by the method of Lowry *et al.*, (1951), with bovine serum albumin as a standard. DNA was measured by the method of Burton (1956), with calf thymus DNA as a standard. The values of enzyme activities reported represent the mean ± S.E.M., with the numbers of rats used to obtain the means in parentheses.

Results

Inhibition of normal changes by progesterone

The activities of acetyl-CoA carboxylase, ATP citrate-lyase and fatty acid synthetase remained low until parturition and increased significantly (2–4-fold) by day 1 of lactation with suckling (Fig. 1). By day 2, under normal circumstances, these increases were even more noticeable. The plasma progesterone concentration begins to decrease about 2 days before parturition (Nicholas & Hartmann, 1975; Bartholomeusz *et al.*, 1976), but we have shown that injection of small amounts of progesterone at this time inhibits the normal increase in fatty acid synthesis from glucose (Martyn & Hansen, 1980). As Table 1 shows, injection of progesterone prevents enzyme activities from increasing by day 2 of lactation.

Effects of premature withdrawal of progesterone

When 18-day-pregnant rats are foetoplacentectomized, the concentration of circulating progesterone decreases quickly and lactose synthesis begins, but fatty acid synthesis does not increase within 96 h of surgery (Martyn & Hansen, 1980). As Table 2 shows, we found a slight, transient increase ($P < 0.2$) in the activity of fatty acid synthetase, but no increase in the activities of acetyl-CoA carboxylase and ATP citrate-lyase within 96 h. When prematurely delivered rats were given foster pups (aged 1–2 days) about 2 h after surgery, the activities of acetyl-CoA carboxylase,

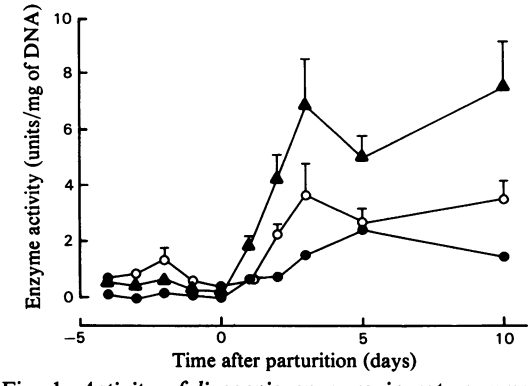

Fig. 1. *Activity of lipogenic enzymes in rat mammary tissue before and after parturition*

Extracts of tissue were prepared in 3 vol. of buffer by the method of Mackall & Lane (1977). Enzyme activities were measured in the cytosol fraction as described in the Materials and methods section and related to DNA concentration in the extract: ●, acetyl-CoA carboxylase; ▲, ATP citrate-lyase; ○, fatty acid synthetase. Each value plotted represents the mean ± S.E.M. (except where the S.E.M. is too small to show) obtained from the 3 and 13 rats. For each enzyme the values on day 1 were significantly higher than on day 0 [$P < 0.001$, 0.002 and 0.05 respectively].

Table 1. *Changes in enzyme activity by day 2 of lactation in control and progesterone-treated rats*
Four rats were injected subcutaneously with 8mg of progesterone in peanut oil on days 20 and 21 and again at parturition (delayed to day 23 in treated rats) before suckling had commenced. Four control rats received oil only. At parturition the size of each litter was adjusted to 12. Enzyme activities were measured as in Fig. 1. Significance of the difference between progesterone-injected rats and control rats: $*P<0.05$, $**P<0.001$.

	Enzyme activity (units/mg of DNA)			Specific activity (munits/mg of protein)		
		Day 2			Day 2	
Enzyme	Day 0 (untreated)	Oil-injected	Progesterone-injected	Day 0 (untreated)	Oil-injected	Progesterone-injected
Acetyl-CoA carboxylase	0.16 ± 0.02 (10)	0.89 ± 0.23	0.17 ± 0.04**	2.0 ± 0.3 (12)	6.7 ± 2.0	1.2 ± 0.2*
ATP citrate-lyase	0.44 ± 0.07 (10)	9.61 ± 1.29	0.44 ± 0.22**	5.3 ± 1.2 (10)	54.4 ± 2.0	3.3 ± 1.0**
Fatty acid synthetase	0.31 ± 0.08 (5)	3.60 ± 0.37	0.68 ± 0.05**	4.3 ± 0.8 (7)	20.7 ± 1.9	4.9 ± 1.1**

Table 2. *Activity of enzymes in mammary tissue of 18-day-pregnant rats after foetoplacentectomy*
At zero time, animals were foetoplacentectomized and some were given 12–15 foster pups (aged 1–2 days) about 2h afterwards. Each value represents the mean ± S.E.M.; (*n*) represents the number of rats used to obtain each value and is the same for each enzyme activity expressed on similar basis. Significance of the difference between suckled and unsuckled glands: $*P<0.05$, $**P<0.02$, $***P<0.001$.

	Activity (units/mg of DNA)					
Time after surgery	Acetyl-CoA carboxylase		ATP citrate-lyase		Fatty acid synthetase	
(h)	Unsuckled	Suckled	Unsuckled	Suckled	Unsuckled	Suckled
0	0.22 ± 0.01 (3)		0.55 ± 0.04		0.63 ± 0.05	
24	0.12 ± 0.02 (5)	0.09 ± 0.01 (4)	0.75 ± 0.09	0.78 ± 0.18	0.92 ± 0.14	0.59 ± 0.09
48	0.20 ± 0.03 (7)	0.27 ± 0.16 (4)	0.44 ± 0.12	1.25 ± 0.13**	1.52 ± 0.38	1.91 ± 0.38
72	0.05 ± 0.01 (6)	0.33 ± 0.10 (5)*	0.27 ± 0.07	2.37 ± 0.47***	0.85 ± 0.15	2.00 ± 0.25**
96	0.07 ± 0.02 (5)	0.95 ± 0.36 (5)*	0.66 ± 0.23	5.02 ± 1.41**	0.88 ± 0.21	4.84 ± 3.07
	Specific activity (munits/mg of protein)					
0	4.8 ± 1.2 (3)		9.4 ± 2.0		11.2 ± 2.7	
24	1.7 ± 0.3 (5)	2.1 ± 0.3 (4)	10.0 ± 1.5	16.1 ± 3.3	12.9 ± 2.2	14.3 ± 3.4
48	2.3 ± 0.3 (9)	5.0 ± 3.1 (4)	6.0 ± 1.0	16.9 ± 1.7***	18.9 ± 4.1	26.0 ± 2.7
72	0.6 ± 0.2 (6)	4.0 ± 0.4 (5)***	6.3 ± 2.5	28.5 ± 4.7**	13.5 ± 2.6	23.7 ± 2.6*
96	0.7 ± 0.1 (4)	10.7 ± 2.0 (5)**	6.2 ± 2.2	61.3 ± 13.6**	10.2 ± 3.5	40.2 ± 14.5

ATP citrate-lyase and fatty acid synthetase increased progressively over 96h (Table 2), although, as with fatty acid synthesis from glucose, the differences between suckled and unsuckled glands were significant only after about 48h.

Effects of suckling after normal parturition

When the whole litter is removed at parturition before suckling can commence, the rate of fatty acid synthesis does not increase as it does when the glands are suckled (Martyn & Hansen, 1980). Table 3 shows that unsuckled glands have significantly lower activities of acetyl-CoA carboxylase and ATP citrate-lyase than suckled glands, but that the activity of fatty acid synthetase appears to increase within 48h even in the absence of suckling. To test the effects of hormones associated with suckling or lipogenesis, unsuckled dams were injected with either prolactin, corticosterone or insulin as described in Table 3. The activities of acetyl-CoA carboxylase and ATP citrate-lyase were similar to, or even less than, the activities observed before parturition (Table 3, cf. Table 1). The activity of fatty acid synthetase was similar to that in unsuckled glands from animals not treated with hormones, except that prolactin-treated rats had lower activity.

As suckling without milk removal does not increase the rate of glucose incorporation into fatty acids (Martyn & Hansen, 1980), enzyme activities were measured under similar conditions. Ligation of the gland ducts prevented the activities of only acetyl-CoA carboxylase and ATP citrate-lyase from increasing as they did in sham-operated contralateral glands in 24h (Table 4). However, in these sham-operated controls the values were

Table 3. *Enzyme activities in suckled glands and in unsuckled glands with hormone treatment after parturition*

Suckling of glands was prevented by removing the litter immediately at parturition. Hormones were given as follows: three injections of 50 i.u. of prolactin in 1.0ml of 0.15M-NaCl intramuscularly at parturiton and 10h and 20h later; or three injections of 2.4 i.u. of insulin intramuscularly at the same times; or two injections of 1mg of corticosterone in 0.5ml of peanut oil at parturition and 20h. These rats were killed 4h after the last injection. Significance of the difference between suckled and unsuckled glands on day 1 after parturition: $*P<0.02$, $**P<0.01$, $***P<0.001$.

	Activity (units/mg of DNA)							
	Day 1		Day 2		Day 1 unsuckled			
Enzyme	Unsuckled	Suckled	Unsuckled ($n=4$)	Suckled	With prolactin ($n=3$)	With corticosterone ($n=4$)	With insulin ($n=3$)
	Specific activity (munits/mg of protein)						
Acetyl-CoA carboxylase	0.10±0.04 (8)	0.61±0.10 (7)***	0.06±0.03	0.72±0.09 (13)	0.03±0.03	0.10±0.02	0.11±0.04
ATP citrate-lyase	0.70±0.16 (8)	1.82±0.37 (7)*	0.50±0.13	4.24±0.80 (11)	0.13±0.05	0.59±0.12	0.66±0.32
Fatty acid synthetase	1.08±0.39 (8)	0.65±0.08 (3)	2.04±0.41	2.25±0.38 (10)	0.34±0.06	1.12±0.18	0.95±0.41
	0.9±0.3 (8)	5.7±0.9 (13)**	0.3±0.1	7.7±1.0 (12)	0.2±0.2	0.2±0.1	0.8±0.1
Acetyl-CoA carboxylase	6.0±3.0 (8)	14.6±7.0 (9)**	3.6±0.5	45.2±9.0 (11)	1.2±0.4	5.6±0.2	4.2±0.8
ATP citrate-lyase	8.0±6.0 (7)	9.7±3.3 (7)	12.2±2.5	23.0±3.0 (7)	3.2±0.6	10.7±2.1	6.0±0.8
Fatty acid synthetase							

Table 4. *Enzyme activities in suckled ligated or sham-operated glands*

The ducts of the right-hand side abdominal and inguinal glands of four unsuckled parturient rats were ligated through an incision in the adjacent skin, and the contralateral glands were sham-operated. The rats were then allowed to suckle 12–14 pups for 24h. Significance: $*P<0.05$.

	Activity (units/mg of DNA)	
Enzyme	Sham-operated	Ligated
Acetyl-CoA carboxylase	0.21±0.04	0.05±0.04*
ATP citrate-lyase	0.98±0.24	0.10±0.03*
Fatty acid synthetase	0.94±0.11	0.88±0.02

lower than in normal rats (cf. Table 3), perhaps because rats after operation suckled less than normal.

Discussion

The development of enzymes involved in provision of the carbon skeleton and reducing equivalents for fatty acid synthesis in rat mammary glands has been studied by a number of authors and reviewed many times. In the present work we have restricted our study to the three enzymes most directly involved in synthesis of the carbon chain. Although the reported activities of these enzymes relative to either DNA or protein, measured under various assay conditions (Howanitz & Levy, 1965; Baldwin & Milligan, 1966; Kuhn & Lowenstein, 1967; Mackall & Lane, 1977) are not strictly comparable, the general pattern found is similar to that described here: all three activities normally remain low until parturition and then increase as lactation progresses.

The increase in these activities coincides with the sudden increase in incorporation of glucose into fatty acids that occurs after parturition (Martyn & Hansen, 1980). In the first 24h the activity of the enzymes relative to DNA or protein increases 2–4-fold (the present paper; Baldwin & Milligan, 1966; Mackall & Lane, 1977). During the perinatal period there is a 2–3-fold increase in the number of secretory cells, observed histologically (Jeffers, 1935; Greenbaum & Slater, 1957), and a similar increase in the amount of DNA and protein (Baldwin & Milligan, 1966), whereas the weight of the whole gland remains constant. The 10-fold increase in the rate of fatty acid synthesis per g of tissue, which we reported previously to occur at 1 day *post partum*, appears to be the product of these two changes.

When progesterone is injected during late pregnancy and at parturition to counteract the normal decrease in plasma progesterone concentration, the

normal stimulation of fatty acid synthesis is abolished and the activity of the three lipogenic enzymes does not increase for at least until day 2 after parturition. However, after either normal parturition or foetoplacentectomy, when the progesterone concentration is low, the activity of fatty acid synthetase increases over the 48h period whether suckling occurs or not, although fatty acid synthesis is not stimulated unless milk is removed from the gland by suckling. Since a decline in progesterone concentration is followed by release of prolactin and other anterior-pituitary hormones (Kuhn, 1969), and since prolactin promotes the synthesis of fatty acid synthetase and inhibits its degradation in organ cultures of rabbit mammary gland in the presence of insulin and glucocorticoids (Speake *et al.*, 1976), the increased activity of fatty acid synthetase can probably be explained by these factors alone. The increase in activity, which is the result of increased enzyme concentration (Smith & Ryan, 1979), was apparently inhibited by injection of ovine prolactin in our experiments, but this effect has also been seen on lactose synthesis and ascribed to the luteotrophic action of the hormone preparation (Kuhn, 1969). Furthermore, administration of ovine prolactin to rats in advanced lactation, unsuckled for 24h, has been shown to suppress fatty acid synthesis in liver and adipose tissue as well as in the mammary gland (Agius *et al.*, 1979).

The rate of fatty acid synthesis does not increase in unsuckled glands because of other circumstances, such as the low activities of acetyl-CoA carboxylase and ATP citrate-lyase, neither of which is apparently stimulated by prolactin or other factors enumerated above. Injection of prolactin, insulin or corticosterone failed to increase the activities of these two enzymes, although these hormones are apparently necessary for mammary-gland function (Nicholas & Topper, 1980). Insulin has been found to stimulate synthesis of milk fatty acids (Balmain *et al.*, 1954; Abraham *et al.*, 1957; Robinson & Williamson, 1977); cortisol therapy restores activity of ATP citrate-lyase in mammary glands in hypophysectomized rats (Baldwin, 1969) and acetyl-CoA carboxylase and ATP citrate-lyase in adrenalectomized rats (Korsrud & Baldwin, 1969; Plucinski & Baldwin, 1976). The failure of endogenous prolactin to increase the activities of acetyl-CoA carboxylase and ATP citrate-lyase was shown by ligating the ducts of the glands on one side of the body and then allowing suckling, which brings about release of prolactin (Subramaniam & Reece, 1975), corticotropin and adrenocorticoids (Meites, 1959; Cowie & Folley, 1961) into the circulation.

The only effective stimulus for enzyme activity that we have found is milk removal, but how this functions is not clear. After weaning during established lactation the activities of acetyl-CoA carboxylase and ATP citrate-lyase decrease rapidly, owing to accumulation of milk (Howanitz & Levy, 1965; Jones, 1967). The concentration of long-chain fatty acyl-CoA increases (Gumaa *et al.*, 1971), and this can inhibit both enzymes to a similar extent (Howanitz & Levy, 1965); but this does not adequately explain the decrease in the amount of enzyme present, and the operation of some other 'local factor' has been invoked (Jones, 1967). Similarly, in the initiation phase, the increase in activity of acetyl-CoA carboxylase after parturition is due to an increase in the concentration of the enzyme rather than to activation of existing inactive enzyme (Mackall & Lane, 1977), and a local factor that causes this must be sought.

It has been shown that synthesis of thioesterase II, which terminates the chain in the synthesis of milk-specific fatty acids in the rat, is induced early in pregnancy, whereas fatty acid synthetase develops only after parturition (Smith & Ryan, 1979) or after foetoplacentectomy (Table 2). Initiation of acetyl-CoA carboxylase and of ATP citrate-lyase are perhaps the last and most critical events necessary to complete the lipogenic sequence. Although acetyl-CoA carboxylase is recognized as an important regulatory site in this pathway (Howanitz & Levy, 1965; Mackall & Lane, 1977), it can only function as such when the supply of acetyl-CoA is sufficient, and this requires an active ATP citrate-lyase. Although citrate synthetase activity changes very little with weaning and suckling after lactation is established, ATP citrate-lyase responds very rapidly (Howanitz & Levy, 1965; Gumaa *et al.*, 1973). At initiation of lactation, although the activities of acetyl-CoA carboxylase and ATP citrate-lyase are both very low before they are stimulated by suckling, the specific activity of the latter enzyme increases much more rapidly (Kuhn & Lowenstein, 1967; the present paper). Under such circumstances, ATP citrate-lyase activity may be an important factor in determining the flux through the lipogenic pathway.

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