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Fatty Acid Biosynthesis in Rabbit Mammary Gland during Pregnancy and Early Lactation

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The pattern of fatty acids synthesized by mammary-gland explants from rabbits during pregnancy and early lactation has been studied. From day 12 to day 18 of pregnancy, long-chain ($C_{14:0}$–$C_{18:1}$) fatty acids were the major products. From day 18 to day 21 of pregnancy there was an increase of up to 12-fold in the rate of fatty acid synthesis per unit wet weight of tissue that was almost exclusively caused by the synthesis of octanoic fatty acid and decanoic fatty acid, which are characteristic of rabbit milk. These medium-chain fatty acids were mainly incorporated into triglycerides. From day 22 to day 27 of pregnancy there was little change in the rate of fatty acid synthesis and the proportions of fatty acids synthesized were essentially the same as those synthesized by the lactating gland, i.e. 80–90% octanoic acid plus decanoic acid. About 2–4 days before parturition a second lipogenic stimulus occurred, although the pattern of fatty acids synthesized did not change.

A striking characteristic of rabbit milk triglyceride is that it contains up to 70% octanoic ($C_{8:0}$) acid plus decanoic ($C_{10:0}$) acids (Smith *et al.*, 1968). These fatty acid are not of dietary origin, but are synthesized within the lactating rabbit mammary gland (Popják *et al.*, 1953; Carey & Dils, 1972). Hall (1971) has reported that the proportions of $C_{8:0}$ and $C_{10:0}$ acids in rabbit milk increase significantly from 2 weeks to 7 weeks *post partum*. In the present paper we report evidence that these acids are present in rabbit milk at parturition, and that they are first synthesized in significant amounts about two-thirds of the way through pregnancy.

Materials and Methods

For studies *in vitro* on fatty acid synthesis in mammary gland, 9–12-month-old virgin New Zealand White rabbits were mated with males of the same strain. The stage of pregnancy was calculated from the day of mating. Animals were obtained from the Joint Animal Breeding Unit, Sutton Bonington, Leics., U.K., and were fed *ad libitum* on a diet of pellets and hay until 3–4h before being killed by cervical dislocation. One animal was used at each stage of pregnancy investigated and at 2 days *post partum*. Small pieces of lobulo-alveolar mammary tissue were immediately removed and placed in Krebs–Henseleit bicarbonate buffer equilibrated in an atmosphere of O_2+CO_2 (95:5) (Krebs & Henseleit, 1932) at room temperature. Explants (about 1 mm^3) were quickly prepared and groups of six to eight explants (5–7mg wet wt.) were incubated in 1.0ml of Krebs–Henseleit bicarbonate buffer at 37°C for 30–180min in an atmosphere of O_2+CO_2 (95:5). Sodium [1-^{14}C]acetate and glucose were present as indicated. The extraction and separation of the ^{14}C-labelled lipids synthesized and the analysis of their constituent ^{14}C-labelled fatty acids by radio-g.l.c. have been described (Strong *et al.*, 1972).

Samples of colostrum were obtained from New Zealand White rabbits at the National Institute for Research in Dairying colony, Shinfield, Reading, U.K. The procedure used has been described (Cowie, 1969). Samples (0.5ml) were saponified for 3h at 85–90°C and fatty acids were extracted into pentane. The acids were methylated and analysed by g.l.c. (Strong *et al.*, 1972).

Results

Fatty acid composition of rabbit colostrum

Samples of colostrum were obtained from rabbits between 0.5h before and 1.0h after parturition. They contained 15.5±1.2 and 10.4±0.7mol at percentage of $C_{8:0}$ acid and $C_{10:0}$ acid respectively (mean± S.E.M. for 10 rabbits). The stage of pregnancy when these fatty acids are first synthesized was therefore investigated (the gestation period for these rabbits was 31–32 days).

Pattern of fatty acids synthesized by mammary explants from pregnant and lactating rabbits

Mammary explants from rabbits between 12 days of pregnancy and 2 days *post partum* were incubated with sodium [^{14}C]acetate plus glucose. The rate of

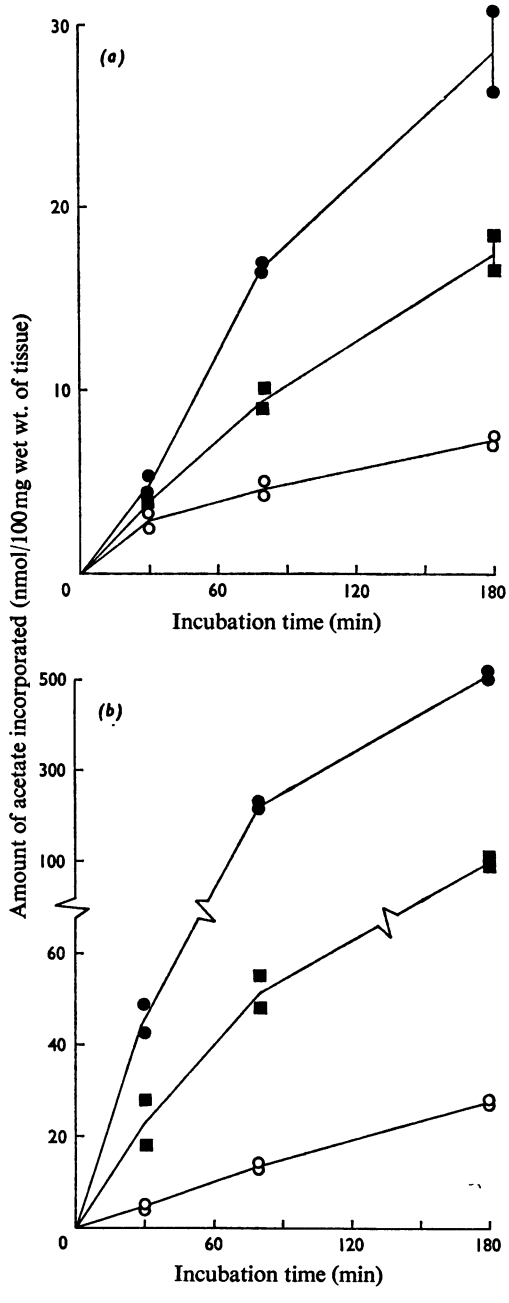

Fig. 1. *Time-course of [^{14}C]acetate incorporation into fatty acids by mammary explants*

Duplicate incubations contained (*a*) 0.1mM-sodium [1-^{14}C]acetate (2μCi) plus 1.0mM-glucose or (*b*) 1.0mM-sodium [1-^{14}C]acetate plus 10mM-glucose. Explants were prepared from rabbit mammary gland at the following times: o, day 16 of pregnancy; ■, day 19 of pregnancy; ●, day 27 of pregnancy.

Table 1. *Effects of substrate concentration and incubation time on the fatty acids synthesized by mammary explants from a rabbit pregnant for 21 days*

Incubations contained sodium [1-^{14}C]acetate (2μCi) and glucose at the concentrations shown. The combined lipid extract from duplicate incubations was analysed. For further details see the text.

Substrates				Percentage incorporation into fatty acids							
Sodium [1-^{14}C]acetate (mM)	Glucose (mM)	Incubation time (min)	Acetate incorporated (nmol/100mg of tissue)	$C_{4:0}$	$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}+C_{16:1}$	$C_{18:0}+C_{18:1}$
0.1	1.0	30	5.9	14	3	42	23	7	4	4	3
0.1	1.0	80	18.8	13	3	40	22	8	4	3	7
0.1	1.0	180	28.6	16	3	37	23	6	5	6	4
1.0	10.0	30	73.4	1	3	38	37	12	4	5	0
1.0	10.0	80	263	0	0	31	40	16	7	6	0
1.0	10.0	180	396	1	1	30	42	16	7	4	2

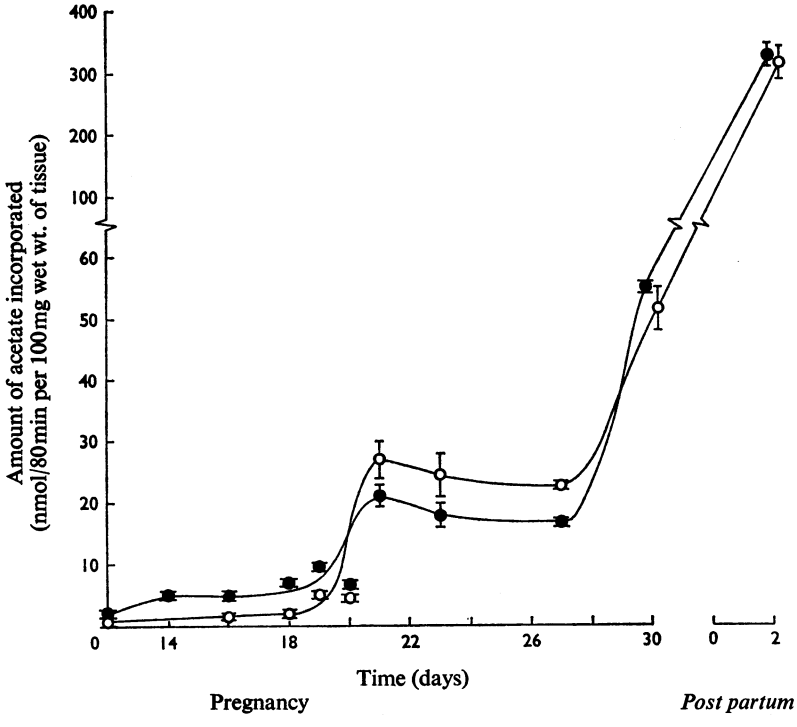

Fig. 2. [^{14}C]*Acetate incorporation into lipids by mammary explants from rabbits at different stages of pregnancy and at early lactation*

Incubations (80min) contained 0.1mM-sodium [1-^{14}C]acetate (1–2μCi) plus 1.0mM-glucose (●). Results from incubations with 1.0mM-sodium [1-^{14}C]acetate (1–2μCi) plus 10mM-glucose (○) have been divided by ten. Duplicate incubations were used, and the mean±half the range between the two values is given.

Table 2. *Fatty acids synthesized by mammary explants*

Incubations contained 1.0mM-sodium [1-^{14}C]acetate (1–2μCi) plus 10mM-glucose and were for 80min. The combined lipid extract from duplicate incubations was analysed. For further details see the text.

	Percentage radioactivity incorporated into fatty acids								
Day of pregnancy	$C_{4:0}$	$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}+C_{16:1}$	$C_{18:0}$	$C_{18:1}$
12	9	1	0	0	8	25	17	18	22
14	9	1	0	0	3	18	43	12	14
16	19	0	7	6	11	21	25	5	6
18	8	1	5	7	12	20	38	4	5
19	3	1	20	31	14	11	15	3	2
20	7	1	18	27	12	14	10	5	6
21	0	0	31	41	16	6	6	0	0
23	1	1	37	39	13	4	3	1	1
27	2	4	62	27	4	1	0	0	0
30	2	2	38	43	11	3	1	0	0
Days *post partum*									
2	0	1	48	43	6	1	1	0	0

Table 3. *Incorporation into lipids of ^{14}C-labelled fatty acids synthesized by mammary explants*

Incubation conditions are as described in Fig. 2. The combined lipid extract from duplicate 80min incubations was analysed (Expt. A) in 0.1mM-sodium [1-^{14}C]acetate plus 1.0mM-glucose, or (Expt. B) in 1.0mM-sodium [1-^{14}C]acetate plus 10mM-glucose.

		Percentage radioactivity incorporated into lipids					
	Day of pregnancy	Triglyceride	1,3-Diglyceride	1,2-Diglyceride	Monoglyceride	Fatty acid	Phospholipid
Expt. A	12	41	5	13	11	18	12
	16	45	6	20	5	5	19
	19	61	4	8	2	11	14
	21	69	4	13	2	6	6
	27	79	4	8	2	3	4
	30	88	1	5	1	3	2
	Days *post partum*						
	2	95	1	2	0	1	1
	Days of pregnancy						
Expt. B	12	39	4	13	4	16	24
	16	51	3	14	3	5	24
	19	71	3	13	1	4	8
	21	92	1	2	0	2	3
	27	92	1	3	0	2	2
	30	92	1	3	1	2	1
	Days *post partum*						
	2	97	1	1	0	1	0

fatty acid synthesis, the proportions of the fatty acids synthesized and their incorporation into glycerolipids were investigated. Fig. 1 illustrates the time-course of [^{14}C]acetate incorporation. With 0.1mM-sodium [1-^{14}C]acetate plus 1.0mM-glucose (Fig. 1*a*) progressive incorporation occurred over the 80min period, but the rate decreased somewhat after 30min with explants from rabbits that had been pregnant for 12, 14 and 16 days. With 1.0mM-sodium [1-^{14}C]-acetate plus 10mM-glucose (Fig. 1*b*) the time-course of acetate incorporation also varied with the stage of pregnancy.

Fig. 2 shows, for each stage of pregnancy and at 2 days *post partum*, the rate of acetate incorporation into lipids for both substrate concentrations during an 80min incubation period. With 1.0mM-acetate plus 10mM-glucose there was a 12-fold increase between day 18 and day 21 of pregnancy and a 3–4-fold increase at the lower substrate concentrations. Between day 27 of pregnancy and day 2 *post partum* a second increase (about 10-fold) occurred in the rate of lipid synthesis.

The fatty acids synthesized in these experiments were then examined. The effects of substrate concentrations and incubation time are illustrated in Table 1. In all experiments a greater proportion of $C_{4:0}$ acid was synthesized with 0.1mM-sodium acetate plus 1.0mM-glucose than with 1.0mM-sodium acetate plus 10mM-glucose. With each of these substrate combinations, the proportions of fatty acids synthesized were independent of incubation time between day 12 and day 18 of pregnancy. However, from day 19 of pregnancy onwards the relative proportions of $C_{8:0}$, $C_{10:0}$ and $C_{12:0}$ acids formed were dependent to some extent on both substrate concentrations and incubation time (see Table 1).

If this small change with incubation time is ignored, the proportions of fatty acids synthesized from 1.0mM-sodium acetate plus 10mM-glucose after an 80min incubation are as shown in Table 2. The increase in acetate incorporation observed between day 18 and day 21 of pregnancy (Fig. 2) was almost exclusively caused by the rapid increase in the rate of synthesis of $C_{8:0}$ and $C_{10:0}$ acids. By day 21 of pregnancy the proportions of $C_{8:0}$ and $C_{10:0}$ acids had increased to 72%. By day 27 of pregnancy the proportion of these fatty acids was the same both as that at 2 days *post partum* (Table 2), and that synthesized *in vivo* at 10 days and 14 days *post partum* (Carey & Dils, 1972). Essentially similar results to those shown in Table 2 were obtained with 0.1mM-sodium acetate plus 1.0mM-glucose as substrate.

Incorporation into glycerides of fatty acids synthesized by mammary explants from pregnant and lactating rabbits

The distribution of the synthesized fatty acids in glycerides was examined at each stage of pregnancy and at 2 days *post partum*. Table 3 shows some of the results obtained. As pregnancy proceeded, the proportion of ^{14}C-labelled fatty acids incorporated into triglycerides increased until at 2 days *post partum* 95–97% was recovered as triglyceride. From day 19 of pregnancy the proportion of triglyceride synthesized (Table 3, Expts. A and B) and the rate of acetate incorporation (Fig. 2) was higher with 1.0mM-sodium acetate plus 10mM-glucose than with 0.1mM-sodium acetate plus 1.0mM-glucose.

Table 4 shows that on day 14 of pregnancy the phospholipid fraction was enriched with $C_{18:0}$ and $C_{18:1}$ fatty acids compared with the triglyceride and 1,2-diglyceride fractions. On day 23 of pregnancy the $C_{8:0}$ and $C_{10:0}$ acids synthesized were concentrated in the triglyceride fraction. The 1,2-diglyceride fraction contained a pattern of fatty acids intermediate between that of the triglyceride and phospholipid fractions; a similar distribution was observed when explants from pseudopregnant rabbit mammary gland were maintained for 1–2 days in organ culture in the presence of insulin, corticosterone and prolactin (Strong *et al.*, 1972).

Table 4. *Esterification of fatty acids synthesized by mammary explants from pregnant rabbits*

Samples of lipid extracts, remaining after fatty acid and lipid analyses had been carried out, were combined irrespective of the substrate concentrations and incubation times used. For further details see the text.

Day of pregnancy	Lipid	Percentage radioactivity incorporated	Percentage radioactivity incorporated into fatty acids							
			$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$
14	Triglyceride	48	1	1	4	22	47	7	7	11
	1,2-Diglyceride	18	2	3	8	20	46	8	10	3
	Phospholipid	17	3	3	2	14	32	9	20	17
23	Triglyceride	80	42	36	10	5	4	1	1	1
	1,2-Diglyceride	7	18	14	5	14	31	5	9	4
	Phospholipid	4	4	5	2	15	34	5	27	8

Discussion

Bousquet *et al.* (1969) have reported that the ultrastructure of epithelial cells of rabbit mammary gland changes markedly between day 18 and day 23 of pregnancy. By day 23 the cytoplasm contains dilated vesicles of endoplasmic reticulum and abundant mitochondria. Lactose becomes detectable in the mammary gland (Denamur, 1963) and the lumen contains protein granules. Our findings support this evidence that lactogenesis is initiated between day 18 and day 23 of pregnancy. There is an increase up to 12-fold in fatty acid synthesis per unit wet weight of tissue during this period (Fig. 2) associated with the rapid synthesis of $C_{8:0}$ and $C_{10:0}$ acids.

Fatty acid synthetase purified from a number of tissues including lactating rabbit mammary gland (Carey & Dils, 1970*a*,*b*) synthesizes long-chain fatty acids by the malonyl-CoA pathway. These are the major products synthesized by mammary explants from rabbits between day 12 and day 18 of pregnancy (Table 2). Between day 18 and day 21 of pregnancy the increased rate of fatty acid synthesis (which is most probably caused by increased activity of the enzymes of the malonyl-CoA pathway) was concomitant with the development of chain termination at $C_{8:0}$ and $C_{10:0}$ acids. With cell-free preparations of lactating rabbit mammary gland synthesis of predominantly medium-chain fatty acids occurs only at very low overall rates of synthesis (Smith & Dils, 1964, 1966). This implies that a degree of cellular organization is required for the rapid synthesis of these acids.

Butyric acid cannot be detected in rabbit milk (Smith *et al.*, 1968). It is, however, synthesized by mammary explants from pregnant rabbits (Tables 1 and 2) and by cell-free extracts (Smith & Dils, 1964, 1966) and by purified fatty acid synthetase (Carey & Dils, 1970*a*,*b*) from lactating rabbit mammary gland. Lin & Kumar (1971) have presented evidence that soluble enzymes in the lactating rabbit mammary gland catalyse the NADH-dependent synthesis of butyrate from acetate by a pathway resembling the reversal of β-oxidation. They suggest that this butyrate may be the preferred 'primer' for the malonyl-CoA pathway rather than acetate. Smith & Dils (1966) could not detect the synthesis of $C_{8:0}$ and $C_{10:0}$ acids by this NADH-dependent pathway with subcellular fractions from lactating rabbit mammary gland. Similarly, less than 4% of the acetyl-CoA incorporated into fatty acids by the partially purified NADH-dependent enzyme system was present in hexanoic fatty acid and longer-chain-length fatty acids (Nandedkar & Kumar, 1969). However, the possibility that the enzymes of the NADH-dependent pathway of butyrate synthesis can interact with the fatty acid synthetase complex of the malonyl-CoA pathway and thereby control chain termination at $C_{8:0}$ and $C_{10:0}$ acids has not been investigated. These pathways may be closely linked in epithelial cells from the mammary gland of rabbits at late pregnancy or during lactation, but not in cell-free preparations of this tissue.

The proportion of ^{14}C-labelled fatty acids synthesized by mammary explants that was incorporated into triglyceride increased from day 12 of pregnancy, whereas the proportion incorporated into phospholipid and 1,2-diglyceride decreased (Table 3). This is consistent with the active role of the mammary gland in synthesizing predominantly triglyceride. The initiation of synthesis of the milk-specific $C_{8:0}$ and $C_{10:0}$ acids after day 18 of pregnancy (Table 2) was associated with an increased incorporation of these acids (Table 4) into triglyceride (and to a lesser extent into 1,2-diglyceride). The small increase in the rate of incorporation of ^{14}C-labelled fatty acids into phospholipid after day 18 of pregnancy is probably a reflection of the proportions of synthesized fatty acids available, since $C_{8:0}$ and $C_{10:0}$ acids are not readily incorporated into phospholipids (Table 4).

Mammary explants from rabbits that had been pregnant for 21 days predominantly synthesized triglyceride that contained a high proportion of $C_{8:0}$ and $C_{10:0}$ acids. Although the rate of fatty acid synthesis per g wet weight of tissue remained almost constant between day 21 and day 27 of pregnancy, the degree of lobule development increased markedly. Hartmann & Jones (1970) observed little change in the DNA content of rabbit mammary gland between day 21 and day 30 of pregnancy. Hence the rate of fatty acid synthesis per cell may well be constant over this period.

A second lipogenic stimulus then occurred 2–4 days before parturition (Fig. 2). This correlates with the increased activities in rabbit mammary gland at the onset of lactation of a number of enzymes involved in fatty acid synthesis, i.e. acetyl-CoA–carbon dioxide ligase (ADP) (EC 6.4.1.2), fatty acid synthetase, ATP–citrate oxaloacetate-lyase (EC 4.1.3.8), D-glucose 6-phosphate–NADP oxidoreductase (EC 1.1.1.49) and 6-phospho-D-gluconate–NAD(P) oxidoreductase (EC 1.1.1.43) (Gul & Dils, 1969; Hartmann & Jones, 1970). It is not clear whether the rate of fatty acid synthesis increases per cell. Hartmann & Jones (1970) found that the DNA content per g wet weight of gland increased 2.5-fold between day 30 of pregnancy and day 2 *post partum*. In contrast, Heitzman (1968) observed no change in DNA content during pregnancy and early lactation, and Denamur (1961) observed a steady increase in DNA content from mid-pregnancy to early lactation.

The nature of these two lipogenic stimuli requires further investigation.

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