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The Effects of Hormones on Milk-Fat Synthesis in Mammary Explants from Pseudopregnant Rabbits

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1. When freshly prepared explants from pseudopregnant-rabbit mammary gland were incubated with sodium [1-^{14}C]acetate plus glucose, they synthesized triglyceride and phospholipid containing long-chain fatty acids. Explants cultured with insulin and corticosterone also synthesized these products. The addition of prolactin to this culture medium increased the rate of fatty acid synthesis up to 40-fold and the explants synthesized predominantly triglyceride enriched with $C_{8:0}$ and $C_{10:0}$ fatty acids characteristic of rabbit milk. 2. The maximum rates of fatty acid synthesis obtained by explants from pseudopregnant-rabbit mammary gland after culture with insulin, corticosterone and prolactin were similar to those observed with freshly prepared explants from lactating-rabbit mammary gland. The time in culture required to attain these maximum rates varied between animals, and did not appear to be connected with the time required (6–7 days) to synthesize the maximum proportions of $C_{8:0}$ and $C_{10:0}$ acids. 3. As the pattern of short- and medium-chain milk fatty acids is characteristic for many species, the techniques described to determine the time-course for the development of this pattern can be used to investigate hormonal response.

The hormonal induction of the synthesis of milk proteins and lactose synthetase in mammary-gland explants has been extensively investigated in the mouse by the technique of organ culture [see Forsyth (1971) for review]. In contrast, the effect of hormones on lipogenic activity in mammary explants has received little attention. Though insulin stimulates fatty acid synthesis from glucose by mammary-gland explants from mid-pregnant mice (Moretti & Abraham, 1966), the rate of synthesis is better maintained over 2 days by culture with insulin, corticosterone and prolactin (Mayne & Barry, 1970). Appearance of lipid droplets in mammary explants can be observed histologically with this hormone combination in a number of species, including the rabbit (Barnawell, 1965; Denamur, 1968; Mills & Topper, 1970). Bolton (1971) has reported that when explants from pseudopregnant-rabbit mammary gland were cultured for 2 days with insulin, corticosterone and prolactin, the incorporation of [1-^{14}C]glucose and [6-^{14}C]glucose into total lipids increased sevenfold over control cultures without prolactin. Lipid synthesis was stimulated to a lesser extent by insulin plus prolactin. In none of these studies, however, was an attempt made to determine whether the pattern of fatty acids synthesized was characteristic of the milk of the species.

Rabbit milk triglycerides contain a characteristic pattern of fatty acids, i.e. up to 70% are octanoic acid plus decanoic acid (Smith *et al.*, 1968). These acids are synthesized within the rabbit mammary gland (Popják *et al.*, 1953; Carey & Dils, 1972), but are not synthesized by cell-free extracts (Smith & Dils, 1964, 1966) or by the purified fatty acid synthetase complex (Carey & Dils, 1970*a*,*b*) from this tissue. It appears, therefore, that a degree of structural integrity of the mammary epithelial cells is required for the efficient chain-length control of fatty acid synthesis to yield medium-chain fatty acids. In the present paper we describe experiments to determine the rate and pattern of the individual chain-length fatty acids synthesized by explants from pseudopregnant-rabbit mammary gland when cultured for up to 14 days with insulin plus corticosterone with and without prolactin. The results are compared with those obtained by using both freshly prepared and cultured explants from lactating tissue. A preliminary report of part of these results has been given (Strong *et al.*, 1971*a*).

Materials and Methods

Animals

Five 9–12-month-old virgin female New Zealand White rabbits (albinos) were used for pseudopregnancy studies. Mature rabbits of the same strain were used on the fourth and seventh day of lactation. All

animals were from the National Institute for Research in Dairying colony, Shinfield, Reading; their standard diet has been described (Forsyth & Myres, 1971). Pseudopregnancy was induced in the virgin rabbits by a single intravenous injection of human chorionic gonadotrophin (Forsyth & Myres, 1971). The stage of pseudopregnancy was measured from the time of injection and animals were killed by cervical dislocation on the 11th. day of pseudopregnancy.

Materials

Some of the materials used have been described (Forsyth & Myres, 1971). Medium 199 and sodium benzylpenicillin (British Pharmacopoeia grade) was obtained from Glaxo Laboratories, Greenford, Middx., U.K. Ox insulin was given by Dr. G. A. Stewart, The Wellcome Foundation Ltd., Dartford, Kent, U.K., and corticosterone was provided by Merck, Sharp and Dohme Ltd., Hoddesdon, Herts., U.K. Sheep prolactin [NIH-P-S-6 (25 i.u./mg) and NIH-P-S-9 (30i.u./mg)] was a gift from the Endocrine Study Section, National Institutes of Health, Bethesda, Md., U.S.A. Sodium [1-^{14}C]acetate and [U-^{14}C]glucose were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Fatty acids, neutral lipids and BF_3–methanol reagent were purchased from Sigma (London) Chemical Co., London S.W.6, U.K. MN-Silica Gel G was obtained from Mackerey, Nagel and Co., Düren, Germany, and diethyleneglycol adipate plus phosphoric acid on Diatomite C from Jones Chromatography Co., Newport, Mon., U.K.

Methods

Preparation and culture of mammary explants. Explants of lobulo-alveolar mammary tissue were prepared (Forsyth & Myres, 1971) and cultured at 37°C on medium 199 (containing 5.5mM-glucose, 5.9mM-sodium acetate, 10mM-$NaHCO_3$ and 50i.u. of penicillin/ml) in an atmosphere of O_2+CO_2 (95:5) for up to 14 days. Insulin (5.0μg/ml), corticosterone (1.0μg/ml) and prolactin (1.0μg/ml) were added as indicated. The medium was changed every 3 to 4 days. Up to six explants were cultured on 1ml of medium (Forsyth & Myres, 1971), or in more extensive experiments, up to 32 explants were cultured on 5ml of medium (Bolton & Bolton, 1970). Because some change in wet weight of tissue occurred during culture with prolactin, results are expressed per five explants (approx. 2.4–2.5mg wet wt.). Histological preparations of explants were examined microscopically after staining with Haematoxylin and Eosin (Forsyth & Myres, 1971). Viability of cells was assessed by the integrity and the staining properties of the nucleus and cell cytoplasm. Unless otherwise stated, more than 75% of the mammary epithelial cells remained viable at the end of the culture periods.

Incubation of cultured explants. After culture, groups of five to ten explants were transferred to 1–5ml of gassed Krebs–Henseleit original bicarbonate buffer (Krebs & Henseleit, 1932) containing 1–10mM-glucose in the presence or absence of 0.1–1.0mM-sodium acetate as indicated. Incubation was for up to 3h at 37°C under O_2+CO_2 (95:5). Explants were removed from the medium by suction filtration on to a glass sinter, rinsed in buffer and weighed in some instances. They were then transferred either to 1.0ml of 5M-KOH in methanol–water (1:1, v/v) containing suitable carrier fatty acids before saponification, or to 2.4ml of chloroform–methanol–water (5:10:4, by vol.) for total lipid analysis. In four separate experiments, only negligible quantities of the ^{14}C-labelled fatty acids synthesized by explants were found in the incubation medium.

Extraction and separation of ^{14}C-labelled lipid classes. Explants in chloroform–methanol–water were homogenized and lipid was extracted by the method of Bligh & Dyer (1959). A portion of the extract was used to determine the total radioactivity of the lipid by liquid-scintillation counting. Another portion was concentrated to 10μl and lipid classes were separated by t.l.c. on silica gel G by using light petroleum (b.p. 40–60°C)–diethyl ether–acetic acid (60:40:1, by vol.) as developing solvent. When further analysis of the individual lipids was not required, the bands of silica were scraped off, and their radioactivity was determined by liquid-scintillation counting. Recovery of radioactivity applied to the plates was >95%. To determine the ^{14}C-labelled fatty acid composition of the lipid classes, triglyceride, diglyceride and fatty acids were extracted three times from the silica scrapings with 1.5ml of diethyl ether. Monoglyceride and phospholipid were extracted three times with 1.0ml of chloroform–methanol–90% (v/v) formic acid (20:10:1, by vol.). In all cases, the solvent was removed and 1.0ml of 5M-KOH in methanol–water (1:1, v/v) was added together with suitable carrier fatty acids before saponification. Where indicated, the phospholipid extract was analysed by t.l.c. on layers of silica gel G (0.3mm thick) with chloroform–methanol–water (65:25:4, by vol.) as developing solvent.

Analysis of ^{14}C-labelled fatty acids by radio-g.l.c. The method is described in detail, since losses of volatile fatty acids can be decreased and more accurately determined than in previous methods (Carey & Dils, 1970*a*). Lipids were saponified for 3h at 80–90°C, cooled to 0°C and 1.5ml of diethyl ether–pentane (3:7, v/v) was added before acidification. Fatty acids were extracted and the sample was re-extracted twice with 1.5ml of the diethyl ether–pentane mixture. The pooled extracts were made up to 5.0ml with pentane and portions were transferred

to vials fitted with silicone-rubber seals. Further carrier fatty acids ($C_{8:0}$ up to and including $C_{18:3}$) were added if necessary to give suitable mass peaks during g.l.c. A mixture of equal weights of butyric acid, hexanoic acid and nonanoic acid (48μg in all) was added and the volume was slowly decreased to 0.2ml under N_2. BF_3–methanol reagent (0.2ml) was added and the vial was stoppered and heated at 65°C for 10min. Quantitative methylation occurred even if two phases remained during heating. The vials were cooled to 0°C and 0.3ml of water was added. The upper layer was transferred to a small tube that had been drawn out at the bottom; the lower layer was extracted with 0.2ml and then 0.1ml of pentane and the combined extracts were slowly evaporated to 10μl under N_2 and were taken up into a microsyringe. The tube was washed with 20–30μl of pentane and the washings were evaporated to 10μl and taken up into a second syringe. The extract and washings were injected from the two syringes on to a 1.52m (5ft) column of 10% (w/w) diethyleneglycol adipate plus 3% (w/w) phosphoric acid on 100–200 mesh Diatomite C. The injection port was maintained at 130°C and the oven temperature increased linearly from 40° to 190°C at 8°C/min. Technical details of the interfacing of the Pye 104 gas–liquid chromatograph and the Panax Radiogas detector system used have been described (Strong *et al.*, 1971*b*).

Losses during these procedures of butyric acid (about 50–60%) and hexanoic acid (about 20%) relative to that of nonanoic acid were calculated from their mass peaks. Negligible proportions of $C_{4:0}$ and $C_{6:0}$ acids were synthesized by explants, except by those freshly prepared from lactating-rabbit mammary gland (see Table 1). The overall recovery of longer-chain fatty acids during the procedures was approx. 80%.

Results

Fatty acid synthesis by freshly prepared explants from lactating-rabbit mammary gland

The rate and chain length of fatty acids synthesized by these explants was first established to provide a baseline for subsequent experiments. Maximum synthesis occurred with 1.0mM-acetate plus 1.0mM-glucose, or with 10mM-glucose (Table 1). With all substrate concentrations used, the proportion of ^{14}C-labelled fatty acids of medium chain length (about 90% $C_{8:0}$ plus $C_{10:0}$) recovered from the explants corresponded very closely with that synthesized by lactating-rabbit mammary gland *in vivo* (Carey & Dils, 1972). Four other experiments with explants or slices from lactating-rabbit mammary gland gave similar patterns of synthesized fatty acids, though the ratio of ^{14}C-labelled $C_{8:0}$ to $C_{10:0}$ acids isolated from the tissue was variable (see Table 1).

Fatty acid synthesis by cultured explants from lactating-rabbit mammary gland

The effect of organ culture with insulin, corticosterone and prolactin on the survival of explants from lactating-rabbit mammary gland and the rate and pattern of fatty acid synthesis was investigated. After culture for 4 days in the absence of hormone or in the presence of prolactin alone, the rate of lipogenesis was too low to enable the pattern of fatty acids synthesized to be determined. Only about 5% of the cells remained viable at the end of the culture period. In all experiments with insulin present in the medium (Table 2), more than 75% of the cells remained viable and the explants maintained their ability to synthesize predominantly medium-chain

Table 1. *Fatty acid synthesis by freshly prepared explants from lactating-rabbit mammary gland*

Mammary explants were prepared from a lactating rabbit 4 days *post partum*. Groups of ten explants were incubated for 3h at 37°C in 5.0ml of Krebs–Henseleit bicarbonate buffer containing the substrates shown.

Substrates		Rate of fatty acid synthesis	Percentage incorporation of radioactivity into fatty acids				
Acetate (mM)	Glucose (mM)	(nmol incorporated/3h per five explants)	$C_{4:0}$	$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$>C_{10:0}$
0.1*	—	0.2	Insufficient for analysis				
1.0*	—	1.6	Insufficient for analysis				
0.1*	1.0	21	7	1	58	31	3
1.0*	1.0	88	0	1	51	42	6
—	1.0*	12	6	2	70	22	0
—	10.0*	90	7	1	56	36	0

* Radioactive substrates {sodium[1-^{14}C]acetate (2μCi) or [U-^{14}C]glucose (1μCi)}.

Table 2. *Fatty acids synthesis by explants from lactating-rabbit mammary gland after culture for 4 days*

Mammary explants prepared from a lactating rabbit 7 days *post partum* were cultured with the hormones indicated (I = insulin; C = corticosterone; P = prolactin). Groups of five explants were then incubated for 1h at 37°C in 1.0ml of Krebs–Henseleit bicarbonate buffer containing 0.1mM-sodium [1-^{14}C]acetate (2μCi) plus 1.0mM-glucose. The values in parentheses indicate the number of results obtained. With uncultured explants, the mean value ±S.E.M. is given. For duplicate values, the mean ± half the range between the two values is given.

Time in culture (days)	Hormones present	Rate of fatty acid synthesis (nmol incorporated/h per five explants)	Percentage incorporation of radioactivity into fatty acids					
			$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}+C_{16:1}$	$C_{18:0}+C_{18:1}$
0	—	3.3 ± 1.2 (4)	65.5 ± 1.0	26.8 ± 1.6	3.9 ± 1.4	2.1 ± 0.5	1.7 ± 0.5	0.0 ± 0.0
4	I	0.1 ± 0.0 (2)	23 ± 5	37 ± 5	20 ± 4	11 ± 6	9 ± 2	0 ± 0
4	I+C	0.9 ± 0.0 (2)	28 ± 0	48 ± 2	15 ± 1	5 ± 0	3 ± 0	1 ± 1
4	I+C+P	2.5 ± 1.0 (2)	33 ± 0	46 ± 0	14 ± 1	5 ± 0	2 ± 0	0 ± 0

fatty acids. The combination of insulin, corticosterone and prolactin gave a rate of synthesis similar to that obtained with freshly prepared explants, though the ratio of $C_{8:0}$ to $C_{10:0}$ acids synthesized was lower and a higher proportion of $C_{12:0}$ acid was formed.

Fatty acid synthesis by cultured explants from pseudopregnant-rabbit mammary gland

Bolton (1971) found that when explants from pseudopregnant-rabbit mammary gland were cultured with insulin and corticosterone, the stimulation by prolactin of glucose incorporation into lipids was greatest after 2 days' culture. For comparison, the effect of prolactin on the rate and pattern of fatty acids synthesized by these explants maintained for 2 days in culture with insulin and corticosterone was examined. Table 3 shows that prolactin markedly increased the rate of incorporation of precursors into fatty acids. Both maximum stimulation (38-fold) and the highest rate of fatty acid synthesis (34nmol of acetate incorporated/3h per five explants) were obtained by using 1.0mM-sodium [1-^{14}C]acetate plus 1.0mM-glucose. This rate was about 40% of that observed with these substrate concentrations with freshly prepared explants from lactating tissue (88nmol of acetate incorporated/3h per five explants; see Table 1). The stimulation of [U-^{14}C]-glucose incorporation by prolactin (three- and ten-fold) was similar to that observed by Bolton (1971). However, the major products synthesized, in both the presence and absence of prolactin in the culture medium, were long-chain fatty acids. Only small proportions of $C_{8:0}$ (2%), $C_{10:0}$ (10%) and $C_{12:0}$ (12%) acids were formed.

The effect of increasing the period of culture to 5 days is shown in Table 4. Without prolactin in the culture medium, incorporation of acetate into fatty acids was low and incorporation of glucose could not be detected. Again prolactin strongly stimulated fatty acid synthesis, and acetate incorporation was stimulated to the same degree as after 2 days in culture (see Table 3). With prolactin omitted from the culture medium, the major products were long-chain fatty acids. Culture with prolactin produced a marked change in the pattern of fatty acids synthesized. A higher proportion (26–51%) of the $C_{8:0}$ and $C_{10:0}$ acids characteristic of rabbit milk were formed, although still a smaller proportion than that synthesized by the lactating tissue after culture for 4 days in the presence of prolactin (Table 2).

With prolonged culture with insulin, corticosterone and prolactin for up to 14 days (Table 5, rabbit 1), the maximum proportion of [^{14}C]acetate incorporated into $C_{8:0}$ and $C_{10:0}$ acids (62%) was observed after 7 days of culture. Beyond this time, the proportion of medium-chain fatty acids synthesized de-

Table 3. *Fatty acid synthesis by explants from pseudopregnant-rabbit mammary gland after culture for 2 days*

Mammary explants prepared on the 11th day of pseudopregnancy were cultured for 2 days with insulin plus corticosterone in the presence or absence of prolactin. Groups of ten explants were then incubated for 3h at 37°C in 5.0ml of Krebs–Henseleit bicarbonate buffer containing the substrates shown.

Substrates			
Acetate (mM)	Glucose (mM)	Prolactin	Rate of fatty acid synthesis (nmol substrate incorporated/3h per five explants)
0.1*	–	+	1.7
0.1*	–	–	0.4
1.0*	–	+	4.1
1.0*	–	–	0.7
0.1*	1.0	+	14
0.1*	1.0	–	0.5
1.0*	1.0	+	34
1.0*	1.0	–	0.9
–	1.0*	+	4.3
–	1.0*	–	0.4
–	10.0*	+	9.7
–	10.0*	–	3.2

* Radioactive substrates {sodium[1-^{14}C]acetate (4μCi) or [U-^{14}C]glucose (4μCi)}.

creased. To determine whether the pattern of fatty acids synthesized was affected by the incubation time of fatty acid synthesis, the time-course of acetate incorporation was investigated in this experiment. To enable duplicate incubations to be used after the various times in culture it was necessary to simplify the experimental design. In this and subsequent experiments 0.1mM-sodium [1-^{14}C]acetate plus 1.0mM-glucose were used as substrates. Culture without prolactin was omitted, since negligible proportions of $C_{8:0}$ and $C_{10:0}$ acids were synthesized in these circumstances (see Tables 3 and 4, and Table 5, rabbit 3). The pattern of fatty acids synthesized by explants after each period in culture was unaffected by the incubation time used (30–180min); incorporation of acetate was essentially linear for up to 80min. (In a subsequent experiment, linearity of acetate incorporation was maintained for 180min after culture for 0, 2, 4 and 6 days in the presence of prolactin.)

Fig. 1 shows the results of an experiment to define more closely the maximum response of fatty acid synthesis over an 11-day period in culture with insulin, corticosterone and prolactin. The maximum rate of fatty acid synthesis (4.9nmol of acetate incorporated/h per five explants) was established after 2–4 days in culture. Synthesis then decreased with continued culture. However, the maximum proportion of medium-chain fatty acids synthesized (44%) was observed only after 7 days in culture (Table 5, rabbit 2). The rate of synthesis of the individual fatty acids during the 11-day period of culture is shown in Fig. 2. The rate of synthesis of all fatty acids (except $C_{18:0}$ plus $C_{18:1}$) was markedly increased after the first day in culture. Between 2 and 4 days in culture, the rate of synthesis of the individual fatty acids changed considerably. During this period the synthesis of $C_{8:0}$ and $C_{10:0}$ acids increased linearly, whereas synthesis of $C_{12:0}$ acid was the same after 2 and 4 days in culture. In contrast, synthesis of $C_{14:0}$ acid decreased after 2 days and that of $C_{16:0}$ plus $C_{16:1}$ acids decreased after 1 day in culture. After 4 days in culture, there was a decreased rate of synthesis of all the C_8–C_{16} fatty acids and a corresponding decrease in the overall rate of synthesis (Fig. 1). (In contrast with C_8–C_{16} acids, the rate of synthesis of C_{18} fatty acids in this and two similar experiments was unaffected by prolactin in the incubation medium for up to 14 days.) The response of fatty acid synthesis to increasing concentrations of prolactin was also investigated in this experiment. For each period of culture examined, no difference in the rate or pattern of fatty acids synthesized was observed with 0.2, 1.0 or 5.0 μg of prolactin/ml of culture medium.

When the lipid classes synthesized in the experiment described in Table 5 (rabbit 2) were examined, the proportion of ^{14}C-labelled fatty acids incorporated into triglycerides increased from 40% on day 0 to 86% on day 7 of culture (Table 6). There was a corresponding decrease in the proportion of phospholipid synthesized. More than 80% of the lipid, which had the same R_F on t.l.c. as cholesterol plus 1,2-diglyceride, was saponifiable and was presumed to be

Table 4. *Fatty acid synthesis by explants from pseudopregnant-rabbit mammary gland after culture for 5 days*

Mammary explants prepared on the 11th day of pseudopregnancy were cultured for 5 days with insulin plus corticosterone in the presence or absence of prolactin. Groups of five explants were then incubated for 3h at 37°C in 2.0ml of Krebs–Henseleit bicarbonate buffer containing the substrates shown.

Substrates			Rate of fatty acid synthesis (nmol incorporated/3h per five explants)	Percentage incorporation of radioactivity into fatty acids						
Acetate (mM)	Glucose (mM)	Prolactin		$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$
0.1*	–	+	6.4	5	26	20	22	18	0	8	3	0
0.1*	–	–	0.9	1	6	13	24	29	5	12	5	3
1.0*	–	+	14	6	20	15	23	22	2	12	0	0
1.0*	–	–	2.1	5	4	12	26	29	5	12	6	1
0.1*	1.0	+	33	14	37	21	15	10	1	2	0	0
0.1*	1.0	–	1.5	2	8	17	25	28	3	11	6	0
1.0*	1.0	+	120	14	29	23	16	15	2	1	0	0
1.0*	1.0	–	3.0	3	8	15	25	29	2	10	8	0
–	1.0*	+	26	15	31	19	20	13	0	1	1	0
–	1.0*	–	0									
–	10.0*	+	130	15	32	22	18	14	0	0	0	0
–	10.0*	–	0									

* Radioactive substrates {sodium[1-^{14}C]acetate (10μCi) or [U-^{14}C]glucose (6μCi)}.

1,2-diglyceride. The distribution of ^{14}C-labelled fatty acids in the triglyceride, 1,2-diglyceride and phospholipid fractions showed a degree of specificity, with relative enrichment of phospholipid with longer-chain fatty acids (Table 7). After 1 and 2 days in culture, the ^{14}C-labelled fatty acid composition of the 1,2-diglyceride was intermediate between that of triglyceride and phospholipid, whereas after 7 days in culture the composition more closely resembled that of the triglyceride. The significance of this is not known. Similar changes were observed when the lipid classes synthesized in the experiment described in Table 5 (rabbit 1) were examined. The proportion of radioactive fatty acids in the triglyceride fraction increased from 79 to 89% between 2 and 7 days in culture, and the proportion of $C_{8:0}$ plus $C_{10:0}$ acids in triglycerides increased from 46 to 71% during this period. After both periods in culture, the phospholipid fraction was enriched with longer-chain fatty acids and 75% of the radioactivity was present in phosphatidylcholine, 7% in phosphatidylethanolamine, 5% in cardiolipin or phosphatidic acid or both and 4% in phosphatidylserine.

Finally, Table 5 (rabbit 3) shows the results of an experiment in which the pattern of fatty acids synthesized by explants cultured with insulin, corticosterone and prolactin was determined after each day in culture for up to 7 days. The maximum proportion of $C_{8:0}$ plus $C_{10:0}$ acids synthesized occurred after 6–7 days, as did the maximum rate of fatty acid synthesis (4.1nmol of acetate incorporated/h per five explants compared with 0.2nmol incorporated/h per five explants on day 0). The pattern of fatty acids synthesized after 7 days in culture without prolactin was essentially the same as that synthesized on day 0.

Noteworthy features of the results in Table 5 are (1) that irrespective of when the maximum rate of overall fatty acid synthesis was achieved, the maximum proportions of $C_{8:0}$ and $C_{10:0}$ acids synthesized occurred after 6–7 days in culture with insulin, corticosterone and prolactin and (2) that after the second day in culture, the proportions of $C_{8:0}$ and $C_{10:0}$ acids increased, the proportion of $C_{12:0}$ acid was relatively constant and that of longer-chain fatty acids decreased.

Histology

Before culture, mammary tissue from pseudopregnant rabbits showed good lobulo-alveolar development. The majority of alveolar lumina were closed and contained little or no secretion. After culture with insulin and corticosterone, there was an enlargement, often very marked, in the size of the alveolar and duct lumina, but these contained no stainable secretory material and secretory vacuoles (lipid droplets) were absent from alveolar cells. The further addition of prolactin at all dosages resulted in

Table 5. *Effect of time in culture on the chain length of fatty acids synthesized by mammary explants from pseudopregnant rabbits*

Explants prepared on the 11th day of pseudopregnancy were cultured with insulin, corticosterone and prolactin for the times shown. Groups of five explants were then incubated at 37°C with 1.0ml of Krebs–Henseleit bicarbonate buffer containing 0.1mM-sodium [1-^{14}C]acetate (2μCi) plus 1.0mM-glucose. Incubation times were 30–180min (rabbit 1), 60min (rabbit 2) and 180min (rabbit 3). The fatty acid patterns shown are the mean values of closely agreeing analyses performed as follows. For rabbit 1, the mean value ± S.E.M. is given of four to six analyses on the combined extracts from incubations carried out for 30–180min after each period of culture. The results for rabbit 2 are the mean values ± half the range between the two values of two analyses carried out on the combined extracts from duplicate incubations with explants cultured with 0.2 and 5.0μg of prolactin/ml of medium respectively. For days 0 and 11 in culture, lipid extracts from duplicate incubations were pooled and analysed; with explants cultured for 11 days, 1.0μg of prolactin/ml of medium was used. (The overall rates of fatty acid synthesis are shown in Fig. 1, and the rate of synthesis of the individual fatty acids in Fig. 2.) With rabbit 3, duplicate incubation extracts were analysed and the average value ± half the range between the two values is shown.

Experiment	Time in culture (days)	Rate of fatty acid synthesis (nmol incorporated/h per five explants)	Percentage incorporation of radioactivity into fatty acids					
			$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}+C_{16:1}$	$C_{18:0}+C_{18:1}$
Rabbit 1	2	2.8	11.3±1.2	29.6±1.9	19.7±0.5	19.4±1.5	18.9±2.2	1.1±0.5
	7	2.5	19.6±0.9	41.6±1.4	20.2±1.2	10.0±0.8	6.2±0.4	2.4±0.7
	9	1.1	14.3±1.9	42.0±3.8	21.5±2.7	10.5±2.4	10.3±1.9	1.8±1.2
	11	0.8	14.0±1.3	42.8±1.5	22.5±1.8	10.3±1.9	9.8±1.4	0.5±0.5
	14	0.3	6.8±1.9	35.8±1.9	25.5±2.7	15.0±0.7	12.8±0.8	4.3±2.0
Rabbit 2	0	0.1	0	0	6	20	24	50
	1	3.8	3±0	13±0	16±1	28±1	37±2	3±0
	2	4.9	5±0	20±0	20±0	26±1	27±0	2±0
	4	4.9	9±1	28±0	21±0	23±2	17±1	2±0
	7	2.6	12±0	32±0	22±0	16±1	15±1	3±0
	11	1.0 (nmol incorporated/3h per five explants)	8	27	21	21	17	6
Rabbit 3	0	0.7	1±1	1±1	3±0	21±5	49±2	25±3
	1	3.5	1±1	5±0	12±1	31±0	46±1	5±0
	2	5.4	2±1	15±3	21±0	28±3	33±1	1±1
	3	6.3	2±0	12±2	16±1	25±0	42±2	3±1
	4	6.5	5±0	21±1	21±1	22±1	28±2	3±1
	5	9.7	9±1	29±1	22±1	20±2	18±1	2±1
	6	9.5	12±1	35±2	21±0	16±2	14±1	2±1
	7	12.3	11±2	33±2	22±1	18±2	15±1	1±0
	7*	0.4	0±0	1±1	3±1	24±2	37±5	35±3

* Prolactin omitted from the culture medium.

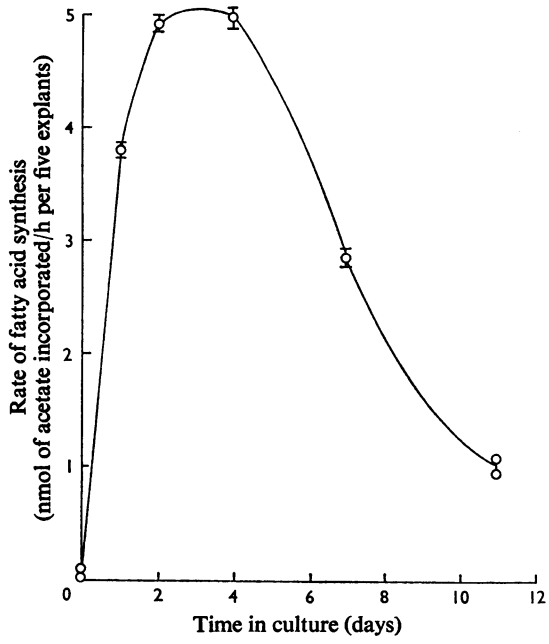

Fig. 1. *Effects of time in culture on the synthesis of fatty acids by mammary gland explants from an 11-day-pseudopregnant rabbit*

Explants were cultured with insulin and corticosterone for the times shown. Prolactin was present at concentrations of 0.2, 1.0 and 5.0 μg/ml of medium. With explants cultured for 11 days, 1.0 μg of prolactin/ml of medium was used. Duplicate groups of five explants were then incubated for 1 h at 37°C in 1.0 ml of Krebs–Henseleit bicarbonate buffer containing 0.1 mM-sodium [1-^{14}C]acetate (2 μCi) plus 1.0 mM-glucose. For each period in culture, altering the concentration of prolactin had no effect on the rate of fatty acid synthesis. The mean value ±S.E.M. of the six determinations of acetate incorporation after each period in culture is therefore quoted. For days 0 and 11 in culture, duplicate results are given. The proportions of the individual fatty acids synthesized after each period in culture are shown in Table 5 (rabbit 2) and the rates of synthesis of individual fatty acids in Fig. 2.

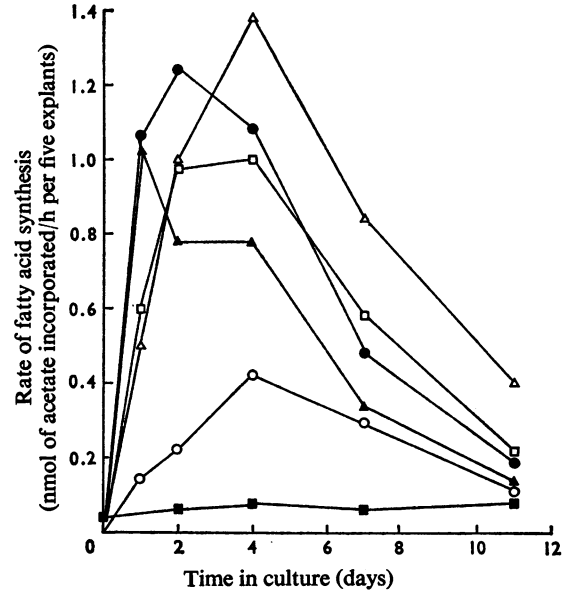

Fig. 2. *Rate of synthesis of individual fatty acids by mammary explants from an 11-day-pseudopregnant rabbit as a function of time in culture*

The rate of synthesis of individual fatty acids was calculated from the overall rate of fatty acid synthesis (see Fig. 1) and the proportions of the fatty acids synthesized (see Table 5, rabbit 2). ^{14}C-labelled fatty acids were extracted from duplicate groups of explants, and the extracts were combined and analysed by radio-g.l.c. In all cases, the two analyses agreed closely (see Table 5) and the average values were used to calculate the rate of synthesis of individual fatty acids. With explants cultured for 11 days, a single radio-g.l.c. analysis was carried out on the combined extracts from duplicate determinations. Fatty acids: ○, $C_{8:0}$; △, $C_{10:0}$; □, $C_{12:0}$; ●, $C_{14:0}$; ▲, $C_{16:0} + C_{16:1}$; ■, $C_{18:0} + C_{18:1}$.

marked stimulation of secretory activity, with lipid droplets prominent in alveolar cells and secretory material in the lumina. Secretion could first be clearly recognized after 2 days in culture, and accumulated progressively thereafter, reaching a maximum in the longer-term experiments by about day 11. Explant survival appeared quite satisfactory up to 1 week and there was no dramatic decline in the second week. However, in the second week there was a tendency for small areas of necrosis to appear in the stroma and an indication that the alveolar epithelium was becoming disorganized and losing its cellular connections, though the cells themselves still appeared mainly viable. Thus the decreased rate of fatty acid synthesis observed between 7 and 14 days in culture may be due to the inhibitory effects of the secreted material which accumulates rather than to cell degeneration. Levy (1964) has suggested that the decrease in fatty acid synthesis by rat mammary gland observed during weaning may be due to the inhibitory effects of milk lipid.

Table 6. *Effect of time in culture on the lipids synthesized by explants from pseudopregnant-rabbit mammary gland*

Details of the experiment are described in Table 5 (rabbit 2). Except for 0 and 11 days in culture, each result represents the mean value ± half the range between the two values of two analyses.

Time in culture (days)	Percentage incorporation of radioactivity into lipid classes					
	Triglyceride	1,3-Diglyceride	Cholesterol plus 1,2-diglyceride	Monoglyceride	Fatty acid	Phospholipid
0	40	6	15	5	8	26
1	74±1	1±0	6±0	1±0	1±0	17±0
2	75±1	1±0	8±1	0±0	1±0	15±0
4	78±2	0±0	7±1	0±0	1±0	14±1
7	86±1	1±0	5±0	0±0	1±0	7±1
11	78	1	5	0	2	14

Table 7. *Effect of time in culture on the distribution between lipid classes of individual fatty acids synthesized by explants from pseudopregnant-rabbit mammary gland*

Details of the experiment are described in Table 5 (rabbit 2) and Table 6. Lipid extracts from duplicate cultures containing 1.0μg of prolactin/ml of medium were pooled and were analysed by t.l.c.

Time in culture (days)	Lipid class	Percentage incorporation of radioactivity into fatty acids							
		$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$
1	Triglyceride	6	21	20	25	24	4	0	0
	1,2-Diglyceride	3	13	19	28	32	5	0	0
	Phospholipid	0	3	6	24	38	14	5	10
2	Triglyceride	7	24	21	21	22	5	0	0
	1,2-Diglyceride	4	14	17	31	24	10	0	0
	Phospholipid	6	8	33	22	21	10	0	0
7	Triglyceride	10	32	22	18	15	2	1	0
	1,2-Diglyceride	8	32	20	19	17	0	3	1
	Phospholipid	2	6	11	27	36	0	15	3

Discussion

The hormonal induction of lipogenic activity in mammary explants has received little attention. Previous studies [see Topper (1970) for review] have shown that when explants of mammary tissue from pregnant or virgin mice are maintained in organ culture in the presence of insulin, a wave of cell division appears to take place within 48–72h. If cortisol is also present in the medium, a marked development of the rough endoplasmic reticulum occurs and the cells are then highly susceptible to induction of the enzymes of casein and lactose synthesis by prolactin.

Rabbit mammary gland was chosen in this study, as it is particularly easy to dissect out explants containing predominantly epithelial cells, and the pattern of fatty acids synthesized by these cells at lactation is very characteristic, i.e. about 70% $C_{8:0}$ plus $C_{10:0}$ acids (Carey & Dils, 1972).

The results show that when mammary-gland explants from pseudopregnant rabbits are cultured with insulin, corticosterone and prolactin for 6–7 days, they synthesize predominantly triglycerides, which are enriched with the medium-chain fatty acids characteristic of rabbit milk. Similarly, about 95% of the fatty acids synthesized by freshly prepared explants from lactating-rabbit mammary gland are esterified as triglyceride (C. R. Strong & R. Dils, unpublished work), and consist almost exclusively of $C_{8:0}$ and $C_{10:0}$ acids (Table 1). Uncultured explants freshly prepared from pseudopregnant-rabbit mammary glands synthesize both triglyceride and phospholipid containing long-chain fatty acids (Tables 6 and 7).

The maximum rates of fatty acid synthesis observed with explants from pseudopregnant-rabbit mammary tissue after culture with prolactin were similar to those with freshly prepared explants from the lactating-rabbit mammary gland. However, the time in

culture required to attain these maximum rates and to develop the synthesis of medium-chain fatty acids varied between animals, and may depend on the composition of the mammary gland's cell population at the time of explantation. The stage in the cell cycle and the hormonal milieu in which cell division actually takes place could affect the synthetic capacities of cells in relation to both the rate of fatty acid synthesis and the control of fatty acid chain-length termination. It could also affect the cell's subsequent ability to respond to hormones *in vitro*, so that some cells may respond over the culture period by increasing the rate of synthesis of only long-chain fatty acids. Other cells may respond initially in this way, but may then develop the capacity to control chain-length termination at $C_{12:0}$ or $C_{14:0}$ acids. A third population of cells may respond to hormones and synthesize $C_{8:0}$ and $C_{10:0}$ acids after only a short period in culture; this is indicated by the marked response observed in one experiment (Fig. 2) in the rate of synthesis of $C_{8:0}$ and $C_{10:0}$ acids after only 1 day in culture with hormones.

There was no apparent correlation between the time in culture required for synthesis of $C_{8:0}$ and $C_{10:0}$ acids and the time and magnitude of the maximum response in the overall rate of fatty acid synthesis. In three experiments, the highest proportions of medium-chain fatty acids were synthesized after 6–7 days in culture, whereas the maximum rate of overall synthesis occurred after 2–4 days in culture. In the experiment described in Figs. 1 and 2, the response over the first 1–2 days in culture is largely due to the synthesis of long-chain fatty acids. These are the major products synthesized by cell-free preparations of lactating-rabbit mammary gland (Smith & Dils, 1964, 1966) and by the purified fatty acid synthetase complex from this tissue (Carey & Dils, 1970*a*,*b*). This initial response is probably due to the induction of the enzymes of the malonyl-CoA pathway. Over the following 4–5 days in culture, the capacity of the explants to terminate chain elongation at $C_{8:0}$ and $C_{10:0}$ acids becomes more evident. The enzyme system(s) involved in this chain termination are as yet unidentified, but are not an inherent part of the purified fatty acid synthetase complex (Carey & Dils, 1970*a*,*b*). This chain termination is very effective in freshly prepared explants from lactating tissue, as less than 7% of the fatty acids synthesized were of chain length greater than C_{10} (Table 1). After culture for 4 days in the presence of insulin, corticosterone and prolactin, such explants synthesized up to 21% of longer-chain fatty acids, i.e. to a certain extent they lost the ability to control chain termination at $C_{8:0}$ and $C_{10:0}$ acids. This may partly explain the fact that explants from pseudopregnant-rabbit mammary gland still synthesized 38–56% of fatty acids of chain length greater than C_{10} even after 6–7 days in culture with hormones (Table 5). In none of the experiments reported was there an increase in the low rate of incorporation of acetate into $C_{18:0}$ plus $C_{18:1}$ acids during culture with hormones (see Fig. 2). If these acids are synthesized by elongation of C_{16} acids, the elongation mechanism appears to be unaffected by culture with hormones.

As the pattern of milk fatty acids is so species-specific (see Morrison, 1970) the development of this pattern can, in principle, be used to assess the response to hormones of non-secretory mammary epithelial cells in organ culture for each species. The method has recently been used to show that explants from virgin-mouse mammary gland respond to culture with insulin, corticosterone and prolactin by synthesizing a pattern of fatty acids approaching that found in mouse milk (Wang *et al.*, 1972). The technique would be of minimum use with those species (e.g. guinea pig; Smith *et al.*, 1968) whose milk contains only long-chain fatty acids.

It might now be interesting to compare these synthesis results with those obtained by using mammary-gland explants from rabbits at different stages of pregnancy, and also to compare the hormonal requirements and timing for the induction of the enzymes for casein, lactose and fatty acids. With the system described in the present paper, the dependence of lipogenesis on the presence of prolactin provides an excellent opportunity to study the synthesis and degradation of the enzymes involved in fatty acid synthesis in developing mammary gland.

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