Uptake of Substrates for Milk-Fat Synthesis by Lactating-Rabbit Mammary Gland

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1. A cannulation technique is described for measuring arteriovenous differences across the lactating-rabbit mammary gland. 2. Analysis of milk obtained before and after surgery shows no effect of cannulation on milk constituents. 3. Results of blood analysis show significant net changes in the concentrations of glucose, acetate, 3-hydroxybutyrate, triacylglycerols and non-esterified fatty acids across the mammary gland. 4. The molar proportions of individual fatty acids in both the triacylglycerol and non-esterified fatty acid fractions did not alter between the arterial and venous samples. 5. The extraction rates are compared with those obtained from other species.

A characteristic feature of the fatty acid composition of rabbit milk triacylglycerols is the high proportion of octanoic ($C_{8:0}$) acid and decanoic ($C_{10:0}$) acid, which together account for 50% (w/v) of the total milk fatty acids (Hall, 1971). Acetate is an important precursor of both $C_{8:0}$ and $C_{10:0}$ fatty acids in lactating-rabbit mammary gland *in vivo* (Popják *et al.*, 1953; Carey & Dils, 1972). Slices of lactating-rabbit mammary gland also incorporate acetate into these fatty acids, though the rate of synthesis is significantly increased in the presence of glucose (Strong & Dils, 1972). Much lower proportions (less than 10%, w/v) of these acids are found in the milk fat of other monogastric species such as guinea pigs (Smith *et al.*, 1968; Jart *et al.*, 1959) and pigs (DeMan & Bowland, 1963). They are present, however, together with significant proportions of butanoic ($C_{4:0}$) acid and hexanoic ($C_{6:0}$) acid, in the milk triacylglycerols of the cow and the goat (Garton, 1963). In the mammary gland of these ruminants the synthesis of short- and medium-chain fatty acids from acetate and from 3-hydroxybutyrate occurs *de novo* by the malonyl-CoA pathway (Palmquist *et al.*, 1969). By contrast, glucose is the main carbon source for fatty acid synthesis in the lactating mammary gland of monogastric species (Linzell *et al.*, 1969; Bauman *et al.*, 1970).

Quantitative studies on the uptake of precursors for milk-fat synthesis have been carried out in the lactating cow (Bickerstaffe *et al.*, 1974), goat (Annison *et al.*, 1967) and sow (Linzell *et al.*, 1969). In these studies measurements were made of arteriovenous differences across the gland in the conscious animal. Experiments of this type require surgical preparation of the animal to obtain representative blood samples. These techniques have proved difficult with smaller species, although measurements of arteriovenous

differences across the mammary gland have been made on the anaesthetized rat (Hawkins & Williamson, 1972), and mammary-gland uptake of non-esterified fatty acids and of chylomicron glycerides has been studied in the lactating guinea pig (McBride & Korn, 1964).

To quantify the metabolites being used by lactating-rabbit mammary gland, we have developed a cannulation technique that allows the arterial and the venous concentrations of blood metabolites across the lactating mammary gland to be measured in the conscious rabbit. A preliminary report of part of this work has been published (Jones & Parker, 1977).

Experimental

Animals

Sexually mature New Zealand White female rabbits weighing 3–4 kg and that were 14 days *post partum* were used in all experiments. The animals were housed under conditions of 16 h day-length, and water and food (Diet 18, obtained from Dixon and Sons, Ware, Herts., U.K.) were available *ad libitum*. The litter size was on average six.

Surgery

Blood obtained from the carotid artery is representative of arterial blood entering the mammary vascular system, and this vessel was therefore cannulated for collection. A clear vinyl cannula (internal diam. 0.58 mm, outer diam. 0.96 mm; Dural Plastics, Dural, N.S.W., Australia) was inserted approx. 8 cm into the left carotid artery under a general anaesthetic of 3% (v/v) halothane (May and Baker, Dagenham, Essex, U.K.) in a mixture of O_2/N_2O (2:1) by using an open-circuit system. The cannula

was exteriorized at the ear, filled with heparinized [400 units (2.4mg/ml) 0.16M-NaCl and bound to the ear with adhesive tape. Dissection of the mammary blood vessels of the rabbit indicated that mammary-venous blood passes to the lateral thoracic vein, as shown by Linzell (1953). The only other source of venous blood in this vessel is that derived from the skin of the abdomen. A venous cannula (internal diam. 0.5mm, outer diam. 0.8mm) was inserted into a branch of this vessel at a point 3cm dorsal to a line joining the thoracic and first abdominal teats. The tip of the cannula was positioned in the lateral-thoracic vein between the first and second abdominal glands. Once it had been established in the vein, the cannula was exteriorized at the ear and maintained in a similar manner to the carotid preparation. After surgery, the does were returned to their young, which were successfully reared to weaning.

Milk sampling

To ensure that there was no difference in the composition of milk obtained from the glands on the operated and the non-operated sides of the doe, milk was collected before the operation and 24h after all blood samples had been taken. Milk was expressed manually after oxytocin [0.5 USP unit; Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K.] had been injected via the carotid cannula. Milk samples from each side were pooled separately. The pooled milk samples were assayed for lipid, protein and lactose and for the fatty acid composition of the milk triacylglycerols. Milk yield in another group of animals was measured before and after the operation by assessing the gain in weight of the young after suckling.

Treatment of blood samples

Blood sampling was begun 24h after cannulation of the carotid artery and of the lateral thoracic vein. Samples (5ml) were taken from both cannulae twice daily (09:30h and 17:30h) until they blocked or until the haematocrit reached a value of 30%. All samples were obtained within 3 days of the operation. Blood samples were immediately cooled to 4°C and all subsequent centrifugation procedures were carried out at this temperature. A portion (1.5ml) of whole blood was deproteinized with an equal volume of ice-cold 1.0M-HClO₄. The supernatant obtained after centrifugation (3000g_{av} for 10min) was stored at -20°C until analysed for glucose, lactate, 3-hydroxybutyrate and acetoacetate. Plasma was separated from the remainder of the sample by centrifugation (3000g_{av} for 10min) and 1.0ml of plasma was immediately added to methanol (7.5ml) for lipid analysis. A further sample of plasma (0.5ml) was analysed for volatile fatty acids

by g.l.c. (Gardner & Thompson, 1974); n-valeric acid (200µl, 1.25mm) was added as an internal standard.

Analytical methods

The following metabolites were measured enzymically: glucose (Huggett & Nixon, 1957); lactate (Hohorst, 1963); 3-hydroxybutyrate and acetoacetate (Wildenhoff, 1970). Acetate was measured by g.l.c. by using a flame ionization detector. The glass column (2m × 4 mm internal diam; 6.4 mm outer diam.) was packed with 5% (w/w) SP 1000 on Chromosorb G (acid-washed, dimethyldichlorosilane-treated; 80–100 mesh) (Perkin-Elmer, Beaconsfield, Bucks., U.K.) and run isothermally at 140°C. The flow rates of the gases were: N₂ carrier 50ml/min; H₂ 50ml/min; air 600ml/min. Detector response factors were determined by using a standard mixture of volatile fatty acids.

Plasma lipids were extracted by the method of Folch *et al.* (1957) as modified by Storry & Millard (1965). The lipid fractions were separated by t.l.c. on silica gel GF₂₅₄ (E. Merck, Darmstadt, Germany) (Tuckley & Storry, 1974) and the areas corresponding to lipid fractions were scraped from the plates. Triacylglycerol, non-esterified fatty acid, cholesterol and cholesterol ester fractions were eluted with n-heptane, and phospholipid fractions with 10% (v/v) HCl in methanol. The mass of the individual fractions was determined by methods adapted for the Technicon Autoanalyzer (Brumby *et al.*, 1975). The fatty acid composition of the plasma triacylglycerols and of the non-esterified fatty acid fractions were determined by g.l.c. of the n-heptane phase remaining after autoanalysis. Triacylglycerols were hydrolysed and the resulting fatty acids were methylated by adding 20µl of 25mM-sodium methoxide and heating for 1h at 100°C in a sealed ampoule. Non-esterified fatty acids were methylated by adding 20µl of 0.4% (v/v) acetyl chloride in methanol. The individual fatty acids were separated by g.l.c. on a glass column packed with 20% (w/w) diethylene glycol succinate on Chromosorb W (hexamethyldisilazane-treated; 80–100 mesh) (Perkin-Elmer). The sample (10µl) was injected at a column temperature of 70°C and after 5min the temperature was raised at a rate of 10°C/min to a final temperature of 180°C. Gas-flow rates were similar to those used to analyse the volatile fatty acids. Detector response factors were calculated by analysing a standard mixture of methyl esters of fatty acids.

Milk protein and lactose were determined by the method of Cowie *et al.* (1969). Milk lipids were assayed by the enzymic determination of glycerol after saponification with ethanolic 0.5M-KOH (Albrinck, 1959). Their concentration was calculated by assuming a molecular weight for the triacylglycerols of 600 (Smith *et al.*, 1968), and the individual

fatty acids were determined as their methyl esters by g.l.c. as described above.

Results

There were no significant changes associated with the cannulation techniques in the milk composition or fatty acid composition of the milk triacylglycerols. The values for fat, protein and lactose were similar to those reported by Cowie (1969) and those for fatty acid composition of triacylglycerols to those of Smith *et al.* (1968). There was no difference in the milk output, as measured by gain in litter weight, of a similar group of lactating female rabbits between the 5 days preceding surgery (104.3 ± 6.1 g/day) and the same period after cannulation (97.2 ± 9.1 g/day).

The concentrations of glucose, acetate, 3-hydroxybutyrate, acetoacetate and lactate in the arterial and venous (lateral thoracic) blood are shown in Table 1. Apart from acetate, the values for blood metabolites remained constant during the sampling period, despite a decrease in appetite immediately after the operation to implant the cannulae. This lower food intake would decrease microbial fermentation in the caecum (McMillan *et al.*, 1975) and result in the lower acetate concentration in the peripheral blood (0.5 mm) compared with published values (0.8 mm) (Cools & Jeuniaux, 1961) and our own data. The results in Table 1 confirm the role of glucose and of acetate as substrates for rabbit mammary-gland metabolism. They also show that

there is utilization of the ketone bodies 3-hydroxybutyrate and acetoacetate.

The analysis of the lipid fractions of the blood is shown in Table 2. The results show a substantial net decrease across the mammary gland in the concentration of triacylglycerols and a smaller decrease in the concentration of non-esterified fatty acids. There were no significant differences in the arterial and venous concentrations of cholesterol, cholesterol esters or of phospholipids. The molar proportions of the individual fatty acids in the triacylglycerol and in the non-esterified fatty acid fractions did not alter significantly between the arterial and venous samples (Table 3). In addition, there was no significant ($P > 0.05$; Student's *t* test) change in the molar ratio of stearic ($C_{18:0}$) acid to oleic ($C_{18:1}$) acid in the milk triacylglycerols from that observed in the plasma lipid (Table 3).

Discussion

The value of results obtained from surgically prepared animals depends on the animal returning to a normal physiological state after the operation. In the experiments described, daily milk yields recorded before and after surgery showed that milk production was unaffected by cannulation of the blood vessels, and all metabolites assayed, apart from acetate, showed no change over the experimental period. The data can be compared with those obtained from other species by expressing results in terms of the extraction rate (E) of the

Table 1. *Concentration of metabolites in the carotid artery and in the lateral thoracic vein of ten lactating rabbits*
Mean values $\pm$ S.E.M. are given with numbers of blood samples analysed shown in parentheses. Venous concentrations that are significantly different from the arterial concentration are shown as: *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$. The symbols + and - indicate production or uptake of the metabolite.

	Metabolite (mm)				
	Glucose (35)	Acetate (32)	3-Hydroxy- butyrate (35)	Acetoacetate (33)	Lactate (31)
Carotid artery	5.80 ± 0.16	0.51 ± 0.08	0.20 ± 0.02	0.09 ± 0.01	0.82 ± 0.06
Lateral thoracic vein	$4.58 \pm 0.12^{***}$	$0.17 \pm 0.02^{***}$	$0.11 \pm 0.01^{**}$	$0.06 \pm 0.01^*$	0.76 ± 0.06
Difference	-1.22 ± 0.12	-0.34 ± 0.06	-0.09 ± 0.01	-0.03 ± 0.01	-0.06 ± 0.07

Table 2. *Concentration of plasma lipids in the carotid artery and in the lateral thoracic vein of ten lactating rabbits*
Mean values $\pm$ S.E.M. are given with the numbers of blood samples analysed shown in parentheses. Venous concentrations that are significantly different from the arterial concentration are shown as: *** $P < 0.001$, ** $P < 0.01$. The symbols + and - indicate production or uptake of the metabolite.

	Plasma lipid fraction (mg/100 ml)				
	Triacylglycerols (30)	Non-esterified fatty acids (30)	Cholesterol (30)	Cholesterol esters (30)	Phospholipids (30)
Carotid artery	46.3 ± 5.2	19.2 ± 1.1	11.9 ± 0.6	28.4 ± 1.8	74.0 ± 4.6
Lateral thoracic vein	$34.5 \pm 4.8^{***}$	$14.4 \pm 1.0^{**}$	11.3 ± 0.6	29.5 ± 2.1	71.2 ± 3.7
Difference	-11.8 ± 2.4	-4.7 ± 1.5	-0.6 ± 0.6	$+1.1 \pm 1.1$	-2.8 ± 5.3

Table 3. Fatty acid composition of the triacylglycerol and non-esterified fatty acid fractions of plasma from the carotid artery and from the lateral thoracic vein from ten lactating rabbits

Mean values $\pm$ s.e.m. are given with the numbers of samples of plasma analysed shown in parentheses. There were no significant differences ($P > 0.05$) between the arterial and venous samples analysed in either the triacylglycerol or the non-esterified fatty acid fractions studied. Statistical analysis of the results used a paired t test in which the proportions (mol/100mol) of the fatty acids $C_{16:0}$ plus $C_{18:0}$ plus $C_{18:1}$ plus $C_{18:2}$ were compared in the plasma fractions studied. A similar paired t test was done on the proportions (mol/100mol) of the remaining fatty acids.

Plasma	Lipid fraction	Fatty acid composition (mol/100mol)												
		C _{8:0}	C _{10:0}	C _{12:0}	C _{14:0}	C _{16:0}	C _{16:1}	C _{17:0}	C _{18:0}	C _{18:1}	C _{18:2}	C _{18:3}	C _{20:0}	
Arterial (18)	Triacylglycerols	3.7±0.8	2.8±0.6	0.8±0.1	2.8±0.3	35.8±2.0	5.2±0.4	Not detected	7.0±0.8	22.0±1.1	15.7±0.8	4.4±0.7	Not detected	
Venous (17)	Triacylglycerols	3.8±0.8	3.4±0.9	0.9±0.2	3.3±0.5	34.6±2.1	5.1±0.4	Not detected	7.4±0.9	22.4±0.9	14.7±0.9	4.5±0.7	Not detected	
Arterial (8)	Non-esterified fatty acids	1.6±0.5	1.3±0.3	1.2±0.2	3.2±0.3	32.9±2.1	4.6±0.7	7.6±1.7	11.4±0.7	18.0±0.9	11.3±1.4	3.8±0.6	3.2±0.6	
Venous (9)	Non-esterified fatty acids	1.7±0.5	1.9±0.5	1.6±0.2	3.5±0.3	29.1±1.7	4.4±0.5	8.3±2.3	16.4±2.9	13.5±1.2	9.5±0.9	7.1±1.6	4.7±0.9	

metabolite (Table 4). The results show noteworthy differences between species, despite the fact that in some cases samples were obtained from anaesthetized animals (sow and rat). The high extraction rate of acetate by the mammary gland of the ruminant is well established, and the value for the rabbit indicates the importance of this metabolite in mammary metabolism. Preliminary studies (C. S. Jones, unpublished work) on mammary blood flow have been undertaken in the conscious rabbit by combining the thermodilution method for measuring cardiac output (Fegler, 1954) with the indicator fractionation technique (Sapirstein, 1956, 1958) for assessing the distribution of blood to different tissues. The fractionation studies have been undertaken with ^{86}Rb (The Radiochemical Centre, Amersham, Bucks., U.K.), which has been shown to be a suitable indicator of mammary blood flow (Hanwell & Linzell, 1973). Results obtained from non-lactating animals were similar to those previously reported (Neutze *et al.*, 1968), and in lactating does a blood flow for the mammary gland of 60–80 ml/min per 100 g wet wt. of tissue was recorded. It is possible to calculate rate of uptake of acetate by the mammary gland from the arteriovenous difference and blood flow and the result obtained (0.75–0.98 mg/h per g wet wt. of tissue) is similar to published data for the conscious ruminant and the perfused guinea-pig mammary gland (Mepharm *et al.*, 1976).

The present results show that there is a net uptake of plasma non-esterified fatty acids by the lactating-rabbit mammary gland. This is also the case with the lactating rat (Hawkins & Williamson, 1972) and guinea-pig (McBride & Korn, 1964) mammary glands. In the fed lactating goat, fatty acids released from triacylglycerols by the action of lipoprotein lipase at the endothelial cell wall exchange with the non-esterified fatty acids. There is therefore no net change in concentration across the gland (Annison *et al.*, 1967). Arteriovenous differences for non-esterified fatty acids have been observed in both the lactating cow (Kronfeld, 1965) and the lactating goat (Annison *et al.*, 1968), but only in the starved state when the concentrations of arterial non-esterified fatty acids are raised. In our study, the concentrations both of triacylglycerols and of non-esterified fatty acids were similar to those determined in non-lactating rabbits by Moore & Williams (1966). The net change across the gland may indicate a significant utilization of non-esterified fatty acids by the lactating-rabbit mammary gland.

The values for the extraction rate of triacylglycerols shown by the sow and the rabbit are considerably lower than for the other three species listed. This may reflect a balance between the proportion of the milk fat that is synthesized within the gland and that which is taken up from the

Table 4. *Comparison of the extraction rates (E) of blood metabolites by the lactating mammary gland of various species* Extraction rates (%), mean values $\pm$ S.E.M. and *n* (=number of experiments) are shown. Values for the cow were taken from Bickerstaffe *et al.* (1974), for the goat from Linzell (1968) and for the sow from Spincer *et al.* (1969). The values for the rat were determined from the data of Hawkins & Williamson (1972) and for the rabbit from the present work. The metabolite extraction rate $E = (\text{arterial concentration} - \text{venous concentration}) \times 100 / (\text{arterial concentration})$.

Species	Metabolite extraction rate					
	Glucose	Acetate	3-Hydroxy-butyrate	Acetoacetate	Triacylglycerol	Non-esterified fatty acid
Cow <i>n</i> = 6	28 $\pm$ 1.6	61 $\pm$ 2.1	39 $\pm$ 3.8	Not determined	58 $\pm$ 1.9	4 $\pm$ 4.6
Goat <i>n</i> = 12	33 $\pm$ 1.4	63 $\pm$ 2.0	65 $\pm$ 2.0	8 $\pm$ 8.0	45 $\pm$ 5.0	3 $\pm$ 4.0
Sow <i>n</i> = 3	31 $\pm$ 2.0	46 $\pm$ 2.0	11 $\pm$ 2.0	2 $\pm$ 4.0	20 $\pm$ 2.0	Not determined
Rat <i>n</i> = 9	25	Not determined	43	50	45	37
Rabbit <i>n</i> = 10	20 $\pm$ 2.5	60 $\pm$ 3.3	46 $\pm$ 3.1	25 $\pm$ 5.0	23 $\pm$ 4.0	14 $\pm$ 7.0

circulating triacylglycerols. It has been suggested that the synthesis of fatty acids by the lactating mammary gland is quantitatively more important in the sow than the goat (Spincer *et al.*, 1969). This could also be the case with the rabbit, where medium-chain fatty acids synthesized within the mammary tissue contribute at least 50% (w/w) of the total fatty acids secreted as milk triacylglycerols (Hall, 1971).

The observation that there was no change in the molar ratio of stearic acid ($C_{18:0}$) to oleic acid ($C_{18:1}$) in the milk triacylglycerols from that in the plasma lipid suggests the absence of desaturase activity in the lactating mammary gland (Bu'Lock & Smith, 1965). By contrast, there is a marked increase in the proportion of oleic acid observed in corresponding experiments in the cow (Bickerstaffe *et al.*, 1974), goat (Annison *et al.*, 1967) and sow (Linzell *et al.*, 1969). In ruminant mammary tissue the desaturase system decreases the high proportion of stearic acid in the plasma resulting from the hydrogenation of dietary fatty acids in the rumen (Bickerstaffe & Annison, 1970). The incorporation of unsaturated fatty acids lowers the melting point of the milk lipid fraction and improves the digestibility of the long-chain saturated fatty acids (Young & Garrett, 1963). In the lactating rabbit, plasma lipids contain a high proportion of unsaturated fatty acids that can be incorporated into milk triacylglycerols. The melting point and the digestibility of rabbit milk is also modified by the incorporation into triacylglycerols of octanoic acid and decanoic acid synthesized within the mammary gland (Jenness, 1974).

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