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Studies on the Mode of Uptake of Blood Triglycerides by the Mammary Gland of the Lactating Goat

THE UPTAKE AND INCORPORATION INTO MILK FAT AND MAMMARY LYMPH OF LABELLED GLYCEROL, FATTY ACIDS AND TRIGLYCERIDES

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1. The mode of uptake of the precursors of milk fat by the mammary gland of the lactating goat has been examined by infusing radioactive fatty acids, glycerol or doubly labelled triglycerides into the mammary artery or jugular vein of animals surgically prepared to permit samples of arterial and venous blood to be withdrawn without disturbance to the animal. 2. Acetate was taken up by the mammary gland and incorporated into milk fat. The decrease in the specific radioactivity of blood acetate across the gland was evidence of acetate production, but there was no significant release of labelled lipid from the mammary gland. 3. When labelled long-chain fatty acids or glycerol were infused into the lactating goat, there was extensive transfer of radioactivity into milk in spite of the absence of net uptake of substrate by the mammary gland. The decrease in the specific radioactivity of each substrate across the mammary gland, however, showed that both fatty acids and glycerol were simultaneously taken up and released by mammary tissue. 4. The infusion of chylomicra and triglyceride emulsions labelled with ^{3}H and ^{14}C revealed that both glycerol and fatty acids were released during triglyceride uptake by mammary tissue. Changes in the $^{3}H/^{14}C$ ratio during the transfer of triglyceride from blood into milk showed that at least 80% of the triglyceride was hydrolysed during uptake, but the potential re-utilization of both products of hydrolysis for triglyceride synthesis in mammary tissue implied that only a minimum value could be obtained from the change in the ratio. 5. The time-course of the transfer of ^{3}H and ^{14}C into milk and lymph were closely similar after the infusion of [2-^{3}H]glycerol tri[1-^{14}C]oleate or of a mixture of [2-^{3}H]glycerol and [1-^{14}C]oleate. 6. The results were consistent with the hypothesis that plasma triglycerides are extensively or completely hydrolysed during mammary uptake.

The major precursors of ruminant milk fat are known to be the chylomicron, low-density lipoprotein and free fatty acid fractions of blood lipids, which supply preformed fatty acids, and acetate and 2-hydroxybutyrate, which give rise to most of the fatty acids of chain length C_4–C_{16}. Quantitative information on the uptake and metabolism of these substrates has been obtained by simultaneous measurement of arteriovenous differences and plasma flow, and, in some instances, by combining this technique with isotope-dilution studies (see Linzell, 1968).

The uptake of plasma triglyceride by the mammary gland of the lactating goat may account for up to 60% of milk-fat production (Annison *et al.*, 1967), but little is known of the mechanism of transfer of triglycerides across the capillary wall of the gland. The increased activities of lipoprotein lipase in mammary venous plasma observed by Barry *et al.* (1963) and the release of free fatty acids by mammary tissue shown by the decrease in the specific radioactivity of plasma free fatty acids during passage through the gland (Annison *et al.*, 1967) suggest that lipolysis might accompany triglyceride uptake. McBride & Korn (1964) investigated the uptake by guinea-pig mammary tissue of chylomicra in which the triglyceride was labelled with [^{14}C]glycerol and [^{3}H]palmitic acid, and showed that changes in the $^{14}C/^{3}H$ ratio in the mammary tissue lipid relative to that of the injected chylomicra were consistent with appreciable hydrolysis of the chylomicron triglyceride during uptake, but the site of hydrolysis was not determined.

We have investigated the mechanism of uptake of labelled fatty acids, glycerol and chylomicron triglyceride labelled with [^{14}C]glycerol and [^{3}H]palmitic

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acid by infusing these isotopically labelled precursors of milk fat into the mammary artery or jugular vein of lactating goats. These studies were extended to include emulsions of doubly labelled triglycerides. In both series of experiments changes in the $^{14}C/^{3}H$ ratio during the transfer of infused triglycerides into milk were consistent with appreciable hydrolysis of triglyceride in mammary capillaries during uptake.

In several experiments the transfer of radioactivity into mammary lymph was measured during the infusion of the radioactive materials. No evidence was obtained for the transfer of intact or partial glycerides, but small amounts of labelled glycerol and labelled fatty acids were detected in the lymph draining the mammary gland.

Preliminary accounts of this work have been presented (West *et al.*, 1967*a*,*b*).

Materials and Methods

Experimental animals

The infusion procedures and measurement of mammary blood flow were as described previously (Annison *et al.*, 1967) using similar surgically prepared and trained lactating goats. In some experiments animals were used that had been surgically prepared for mammary intra-arterial infusions by autotransplanting one mammary gland to the neck (Linzell, 1963). The animals were given hay *ad lib.*, and a concentrate–cereal mixture (about 1 kg/day) was given in roughly two equal parts at the time of milking (09:30 and 16:00h). The infusions of radioactive substrates (without added carrier) were done on fed animals during the period 11:00–16:00h. These were milked hourly until 5h after the end of the infusion, an intravenous injection of oxytocin (200munits) being used to aid milk ejection.

The passage of radioactivity from infused substrates into mammary extracellular fluid and lymph was studied in seven goats, by cannulating and collecting lymph from the main ducts draining one mammary gland beyond the mammary lymph node (efferent duct) (Linzell, 1960) and in three experiments by simultaneously collecting lymph from one of the several afferent lymph ducts carrying lymph to the node. This was done because, in the lymph node the sinuses carrying lymph lie in intimate relationship with the blood capillaries and exchange of some soluble components has been demonstrated in the mammary lymph node (Rasmussen & Linzell, 1964). In most experiments these operations were done 24–48h before the main experiment, which was performed only when the animal had fully recovered from the operation and when lymph was flowing normally. In other experiments the operation for cannulation of the lymph ducts and other vessels was done under long-acting spinal anaesthesia (Smith, Kline and French anaesthetic no. 90054, injected epidurally in the lumbosacral space) and the experiment was then done immediately while the animal's hindquarters were still anaesthetized. The animals usually sat contentedly eating under these conditions.

Radioactive materials

^{14}C-labelled stearic acid, palmitic acid and acetic acid, [1-^{14}C]glycerol, [1-^{14}C]glycerol tripalmitate, [2-^{3}H]glycerol trioleate, glycerol tri[1-^{14}C]oleate and glycerol tri[1-^{14}C]palmitate were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The long-chain acids were bound to albumin and infused as described previously (Annison *et al.*, 1967).

Methods

Collection and preparation of labelled chylomicra. [1-^{14}C]Glycerol tripalmitate (200μCi/16mg) and [9,10-^{3}H]palmitic acid (2.5mCi/6.4mg) were dispersed in aq. 10% (v/v) ethanol (5ml) and injected into the duodenum of a donor goat (age 4 months, body weight 11kg) prepared with duodenal and intestinal lymphatic catheters (Lascelles & Morris, 1961*a*,*b*). Intestinal lymph was collected continuously, and monitored at frequent intervals for radioactivity (^{14}C and ^{3}H). The lymph samples containing most of the radioactivity were pooled and centrifuged in 20ml portions layered with 10ml of 0.15M-NaCl (containing 0.1g of disodium EDTA/l and adjusted to pH7.0$\pm$0.2 with 1M-NaOH) at 63000*g* for 30min. The top layers (8–10ml) from each tube were then transferred to fresh tubes by using a Pasteur pipette and the tubes were again filled to within 1cm of the top of the tube with the 0.1M-NaCl, and the centrifugation repeated. The top 8–10ml from each tube was again removed and diluted with approx. 30ml of sterile 0.15M-NaCl. Samples (0.2ml) of this material were taken for scintillation counting and the remainder infused intra-arterially into one mammary gland of a lactating goat. The material infused contained 10μCi/ml.

Preparation of triglyceride emulsions. Two triglyceride emulsions were prepared by adding 2.0mCi of [2-^{3}H]glycerol trioleate (143mCi/mmol) and either 0.2mCi of glycerol tri[1-^{14}C]oleate (38mCi/mmol) or 0.2mCi of glycerol tri[1-^{14}C]palmitate (87mCi/mmol) to sterile saline (0.15M-NaCl, 100ml) containing Tween 60 (0.235%, w/v) and Span 60 (0.015%, w/v). The mixtures were warmed to 60°C and passed through a piston emulsifier (Gallenkamp, London) to yield a stable emulsion containing particles in the size range 0.5–3.0μm. The emulsions were diluted when necessary by adding sterile saline.

Determination of plasma glycerol concentration. The method described by Wieland (1965) that makes use of glycerokinase from rabbit muscle and glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) from *Aerobacter aerogenes* was modified to increase the sensitivity 8-fold by using ethanol instead of perchloric acid as the protein precipitant. Plasma (2ml) was pipetted into 6ml of ethanol in a glass-stoppered centrifuge tube. After thorough mixing the precipitated protein was removed by centrifugation (15min, 3000*g*). The supernatant layer was transferred to a glass-stoppered graduated centrifuge tube and the precipitate was re-extracted with 2ml of 75% ethanol, which was added to the original supernatant layer. The volume of the combined supernatant layers was reduced to 0.5–1.0ml under a stream of N_2 at 60°C and the volume was then adjusted to 2.0ml with water. Lipid was extracted by thorough mixing with chloroform (0.5ml). After centrifugation at 3000*g* for 30min, the upper phase was transferred to a 50ml glass-stoppered flask and the dissolved chloroform was removed by bubbling N_2 saturated with water vapour through the extract for 2min. The following reagents were added to a quartz spectrophotometer cuvette of 1cm light-path: 0.86ml of buffer (0.315M-glycine, 1.575M-hydrazine, 0.007M-$MgCl_2$, pH9.8), 0.02ml of glycerol 3-phosphate dehydrogenase suspension [18i.u./0.5mg per ml of 2M-$(NH_4)_2SO_4$; Boehringer Corporation (London), London W.5, U.K.], 0.05ml of ATP (0.05M), 0.05ml of NAD^+ (0.02M) and 1ml of (*a*) water or (*b*) glycerol standard (0.2μmol/ml) or (*c*) deproteinized samples. The extinction at 340nm was measured in a Hilger–Gilford reaction-kinetics spectrometer (model RK2; Hilger and Watts, London N.W.1, U.K.) against air until it was constant. The reaction was started by the addition of 0.02ml of glycerokinase (EC 2.7.1.30) suspension (85i.u./mg per ml) in 2M-$(NH_4)_2SO_4$, and the extinction was measured until it was constant. The amount of glycerol was then calculated from the increase in extinction corrected for the change in the blank. Thus a change in extinction of 1 corresponded to a concentration of 0.322μmol of glycerol/cuvette (Horecker & Kornberg, 1948) and /ml of plasma with the method described. With pure solutions of glycerol prepared from anhydrous glycerol (Vogel, 1956) the observed increases in extinction were approx. 90% of the calculated theoretical values and the results were corrected accordingly. If sufficient spectrophotometer cells were available, 12 or more determinations could be done simultaneously.

Determination of plasma glucose concentration. The glucose oxidase method of Huggett & Nixon (1957) was used.

Measurement of glycerol and glucose specific radioactivities. The specific radioactivities of glycerol and of glucose were measured after their isolation from deproteinized plasma and separation by t.l.c. on cellulose layers. The deproteinization was done as for the estimation of glycerol concentration except that carrier glucose and glycerol (40mg of each) were added initially and the volume of ethanol was increased accordingly.

After the extraction of lipid with chloroform, the aqueous solution was added to an ion-exchange column (16cm×0.75cm) consisting of two parts of Amberlite IRC-50, activated by washing with 1M-HCl, and one part of Amberlite R IR-45, activated by washing with 2M-NaOH. The glucose and glycerol were completely eluted by 150ml of water. The eluate was concentrated to 0.5ml on a rotary evaporator under reduced pressure at 60°C before application to the thin-layer plate.

Microgranular cellulose powder (Whatman Chromedia CC41) (60g), was mixed with 140ml of water, then shaken for 2min and spread immediately on to glass plates (20cm×20cm) to produce a layer 0.4mm thick. The adsorbent was activated for 30min at 105°C before use. A marker solution containing glycerol and glucose was applied to two outside lanes, each 3cm wide, and the plasma extract to the centre lane. The origin was 3cm from the bottom edge. The chromatograms were developed for about 40min in tanks saturated with solvent vapour (butan-1-ol–acetic acid–water, 4:1:1, by vol.). After evaporation of the solvent, the marker lanes were sprayed with an aqueous solution containing $KMnO_4$ (1%, w/v) and Na_2CO_3 (2%, w/v); the compounds were seen as yellow spots on a purple background and changed later to grey spots on a brown background. Glucose and glycerol had R_F values of 0.3 and 0.6 respectively. The glucose and glycerol fractions in the centre lane were transferred to 5cm sintered-glass funnels (porosity 3) fitted with B19 glass cones. The funnels were seated on 25ml glass-stoppered test-tubes with side arms and the cellulose was packed down on to the sinter under reduced pressure. The glucose and glycerol were each eluted with water (approx. 15ml). A sample of the eluate (usually 10ml) was transferred to a scintillation vial, then was evaporated to dryness and the radioactivity measured by liquid-scintillation counting. A portion of the remainder of the eluate was also taken for determination of the concentration of either glycerol or glucose by using the methods described above.

Determination of the concentrations and specific radioactivities of plasma lipids and plasma lipoproteins. The plasma lipoproteins were separated essentially by the gradient centrifugation methods described by Cornwall *et al.* (1961).

Blood samples (50ml) were immediately cooled in ice and centrifuged at 3000*g* for 1h at 4°C. Plasma (2×4ml) from each sample was layered under 4.5ml 0.15M-NaCl–EDTA in 13.5ml tubes. The tubes were then filled to capacity with 0.15M-NaCl–EDTA, and centrifuged at 9300$g_{av.}$ for 30min. The chylomicron

band in the top 5mm of saline was removed together with a further 5mm of saline. The remaining solution was layered over 5.5ml of 2.0M-NaCl–EDTA, and the tubes filled with 0.15M-NaCl. After the caps were in place, the tubes were centrifuged in the same head at $105000g_{av.}$ (40000rev./min) for 18–22h at 4°C. The low-density β-lipoproteins were observed as a haze in the top 3 or 4mm of the tube and this haze diffused on standing. The top 3ml was removed as the β-lipoprotein fraction and a further 3ml, which extended to the 2M-NaCl, was removed as a separate fraction since it contained the high-density β-lipoproteins. The remainder (approx. 6ml) containing the high-density or α-lipoprotein and albumin fractions, was also removed as a separate fraction.

The amounts of free fatty acids and triglyceride in the plasma and of triglyceride in the separated lipoprotein fractions were determined by using internal standards (heptadecanoic acid and glycerol triheptadecanoate) that can be assayed accurately by g.l.c. (West & Rowbotham, 1967). The plasma (5ml) and lipoprotein fractions were extracted by the method of Folch *et al.* (1957) with 0.1M-Na_2HPO_4–NaH_2PO_4 buffer, pH 6.0, instead of water and washed with chloroform–methanol–0.1M-sodium phosphate buffer (3:48:47, by vol.). Glycerol triheptadecanoate was added to the various lipoprotein fractions equivalent to 20mg/ml of plasma for all fractions except the chylomicron fraction to which marker equivalent to 40mg/ml of plasma was added. The lipids were separated on thin layers of silica gel G (West & Rowbotham, 1967). Portions (one-fifth) of the free fatty acid fractions from the original plasma samples and of the triglycerides from the original plasma and the separated lipoprotein fractions were transferred to a scintillation vial for the assay of radioactivity and the fatty acids in the remainder of each sample were methylated for the subsequent determination of the composition and concentration of the fatty acids by g.l.c. (West & Rowbotham, 1967).

Extraction and analysis of milk lipids. The extraction and subsequent determination of the percentage composition and specific radioactivity of the milk lipid was determined as described by Annison *et al.*, (1968).

Measurement of blood acetate specific radioactivity. Acetate was isolated from blood by steam distillation as described by Annison & Linzell (1964) and after titration the dried samples were assayed for radioactivity as described below.

Measurement of radioactivity. All substrates were assayed by liquid-scintillation counting as described by Annison *et al.* (1968).

Results

Uptake of free fatty acids

When [1-^{14}C]stearic acid bound to albumin was infused into the arterial blood supply of an autotransplanted mammary gland (65.8nCi/min for 76min) of a starved goat the specific radioactivity of stearic acid in mammary arterial, general arterial (from the pulmonary artery in this goat only) and mammary venous plasma reached a constant value within 40min (Table 1). The specific radioactivity of stearic acid in mammary venous plasma was 55% of that of the mammary arterial plasma, indicating that 45% of the mammary venous stearic acid was added during passage through the gland, in agreement with previous results (Annison *et al.*, 1967, 1968). However, as in the previous studies, there was considerable transfer of labelled stearic acid into milk-triglyceride stearic acid and oleic acid (Table 1). During the infusion oleic acid in circulating plasma free fatty acids became labelled, but part of this conversion occurred in mammary tissue, as the specific radioactivity of oleic acid in mammary venous plasma fatty acid was threefold higher than that in arterial plasma (Table 1).

The mammary uptake of stearic acid from plasma was investigated further by infusing [1-^{14}C]stearic

Table 1. *Specific radioactivities and concentrations of plasma free stearate and oleate and the transfer of radioactivity into milk triglyceride fatty acids during the infusion of* [1-^{14}C]*stearic acid* (65.8*nCi/min for* 76*min*) *into the artery of a transplanted mammary gland of a starved lactating goat*

Steady-state values at the end of the infusion are shown with the numbers of determinations in parentheses.

	Stearate		Oleate	
	Concn. (mequiv./l)	Sp. radioactivity (μCi/g)	Concn. (mequiv./l)	Sp. radioactivity (μCi/g)
Pulmonary artery, free acid	0.231 (6)	18.3 (3)	0.228 (6)	0.43 (2)
Transplant artery, free acid	0.274 (2)	106.0 (1)	0.260 (2)	0.43 (2)
Transplant vein, free acid	0.191 (6)	58.3 (2)	0.280 (6)	1.49 (3)
Milk, triglyceride*	—	22.8	—	4.80

*Maximum values were obtained in milk secreted 360 min after the start of infusion.

Table 2. *Specific radioactivities and concentrations of arterial and mammary venous plasma free stearate during the infusion of* [1-^{14}*C*]*stearic acid* (63 *nCi/min for* 240 *min*) *into the mammary artery of a fed lactating goat*

The mean specific radioactivity of general arterial plasma free stearate during the infusion was 16.4 μCi/g and the concentration was 0.046 mequiv./l (mean of three determinations).

	Mammary arterial plasma		Mammary venous plasma		Mammary lymph	
Time (min)	Concn. (mequiv./l)	Sp. radioactivity (μCi/g)	Concn. (mequiv./l)	Sp. radioactivity (μCi/g)	Concn. (mequiv./l)	Sp. radioactivity (μCi/g)
125	0.046	83.7	0.057	63.3	0.064	14.9
290	0.053	7.3	0.067	3.2	0.092	6.9
325	0.056	4.4	0.050	3.0	0.094	2.3
355	0.038	2.6	0.056	1.2	0.050	2.5

Table 3. *Specific radioactivities and concentrations of plasma constituents during the infusion of sodium* [*U*-^{14}*C*]-*acetate into the mammary artery of an autotransplanted mammary gland of a fed lactating goat*

The specific radioactivity of the acetate entering the gland was calculated from the blood flow (500 ml/min), infusion rate (8.33 μCi/min) and the arterial acetate concentration.

	Mammary artery		Mammary vein	
	Concn. (mg/100 ml)	Sp. radioactivity (μCi/g)	Concn. (mg/100 ml)	Sp. radioactivity (μCi/g)
Acetic acid	10.8	155.0	4.2	92.0
Free fatty acids	3.66	0.50	3.66	1.29
Triglyceride	15.4	0.032	10.9	0.039
Cholesterol ester	} 151.0	0.004	} 142.0	0.004
Cholesterol		0.004		0.004
Fatty acid		<0.001		<0.001
Free cholesterol	49.0	0.11	45.0	0.08

acid intra-arterially into a fed lactating goat (63 nCi/min for 240 min) and monitoring the concentration and specific radioactivity of lipids in arterial and mammary venous plasma and in mammary lymph for 355 min (Table 2). At most times there was a net release of stearate into venous plasma, and at all times the lower specific radioactivity of stearate in mammary venous plasma indicated the production of free stearate in the mammary gland (Table 2). Examination of mammary lymph showed that the specific radioactivity of free stearate reached only 18% of the same fraction in arterial plasma: the corresponding value for mammary venous plasma was 76% (Table 2). The specific radioactivity of lymph stearate decreased much more slowly than that of plasma, showing that the two pools of free fatty acids were not in equilibrium over the time-course of the experiment.

The concentration of free fatty acids in lymph (2.7–5.4 mg/100 ml) was within the range of plasma free fatty acid concentration reported previously (Annison *et al.*, 1967).

Uptake of acetate

When [U-^{14}C]acetate was infused directly into the artery supplying the gland, the free fatty acid leaving the gland was labelled to a very limited extent (Table 3). No evidence was obtained of the release of short-chain or medium-chain length free fatty acid from mammary tissue. In this experiment approx. 29% of the mammary arterial plasma triglyceride was taken up by the gland and there was no significant release of partial glycerides into the mammary venous plasma as both arterial and venous concentrations of these substances were less than 0.05 mg/100 ml. Although there was a slight increase in the specific radioactivity of plasma triglycerides across the gland (from 0.032 to 0.039 μCi/g), this difference indicates that there was not an appreciable release of newly synthesized fat into the venous drainage of the mammary gland. Acetate was not incorporated into blood cholesterol or cholesterol esters to any appreciable extent in the mammary gland (Table 3).

Table 4. *Metabolism of glycerol and free fatty acids by the mammary glands of lactating goats by the infusion of [1-^{14}C]glycerol and [9,10-^{3}H]palmitic acid into the jugular vein*

The results were used to construct Fig. 1.

	Goat no. 1.		Goat no. 2.	
Blood flow through mammary gland (ml/min)	1030		1360	
Packed erythrocyte volume (%)	27		26	
[^{14}C]Glycerol infusion rate (μCi/min)	0.533		0.588	
[^{3}H]Palmitic acid infusion rate (μCi/min)	2.68		2.94	
Milk triglyceride glycerol output (mg/min)	1.45		1.41	
Milk triglyceride glycerol specific radioactivity (μCi/g of C)	8.04		10.09	
Milk triglyceride palmitate output (mg/min)	17.5		18.8	
Milk triglyceride palmitate specific radioactivity (μCi/g of C)	17.2		24.8	
CO_2 produced by mammary gland (mg/min)	179		202	
Specific radioactivity of mammary gland CO_2 (μCi/g of C)	1.00		1.42	
O_2 uptake by mammary gland (mg/min)	97.7		111.5	
Respiratory quotient of mammary gland	1.45		1.41	
	Artery	Mammary vein	Artery	Mammary vein
Blood glycerol (mg/100ml)	0.23	0.22	0.32	0.29
Blood glycerol specific radioactivity (μCi/g of C)	514	361	466	358
Plasma glucose (mg/100ml)	63	41	67	43
Plasma glucose specific radioactivity (μCi/g of C)	4.0	4.0	6.3	6.3
Blood CO_2 specific radioactivity (μCi/g of C)	0.42	0.51	0.38	0.53
Plasma palmitate (mg/100ml)	1.0	1.0	2.1	1.9
Plasma palmitate specific radioactivity (μCi/g of C)	145	99	112	84
Plasma triglyceride palmitate (mg/100ml)	1.9	1.1	3.1	1.9
Plasma triglyceride palmitate specific radioactivity (μCi/g of C)	16.2	16.8	25.0	25.3

Uptake of glycerol

The mammary metabolism of glycerol was investigated during the intravenous infusion of [1-^{14}C]glycerol into two lactating goats, and further information on plasma free fatty acid metabolism were obtained by including [9,10-^{3}H]palmitic acid in the infusion solution. The concentrations of glycerol in arterial and mammary venous plasma show that there was little net uptake of glycerol by the mammary glands, but the specific radioactivity in mammary venous plasma decreased to 70 and 77% respectively of the arterial values, indicating that glycerol was released by the mammary gland (Table 4). The transfer of radioactivity into milk triglyceride showed that free glycerol was taken up by the gland (Table 4), the simultaneous uptake and release of glycerol providing an explanation for the absence of a marked arteriovenous difference across the mammary gland. The information obtained about [^{3}H]palmitic acid in these experiments confirmed the results of Annison *et al.* (1967) on the simultaneous release and uptake of free fatty acids by mammary tissue (Table 4).

The extent of incorporation of blood glycerol into milk-fat glycerol calculated from the ratio of their specific radioactivities and the output of milk-fat glycerol differed by only 0.01 mg/min from the measured uptake of glycerol from arterial plasma. Palmitic acid contributed 11.9 and 16.8% respectively of the milk triglyceride palmitate in the two goats.

The contribution of glycerol to overall glucose synthesis was 0.78 and 1.35% respectively, but the transfer of radioactivity into glucose made it impossible to interpret the extent of $^{14}CO_2$ production by the mammary gland.

In further experiments the transfer of [^{3}H] glycerol and [^{14}C]palmitate from plasma into mammary lymph and milk was examined (Fig. 1). The continuing relatively high amounts of [^{3}H]glycerol in lymph after the end of the infusion was unexpected, and possibly reflected the diffusion of some glycerol from secretory tissue in addition to the inflow from plasma. It is well established that there is a considerable hold-up of labelled triglyceride in the mammary gland, as shown by the time-interval between the uptake of labelled lipid and its appearance in milk (Hardwick *et al.*, 1963). The high amount of labelling of the triglycerides in secretory tissue achieved by the back-diffusion of only a very small fraction of the tissue glycerol would account for the labelling of lymph. The radioactivity (μCi/min) appearing in lymph during the experiment was only 1.6 and 0.2% respectively of the ^{3}H and ^{14}C appearing in milk during the same period. Glycerol appeared in lymph and milk fat more quickly than the fatty acid, and as in previous experiments (Table 2), relatively small

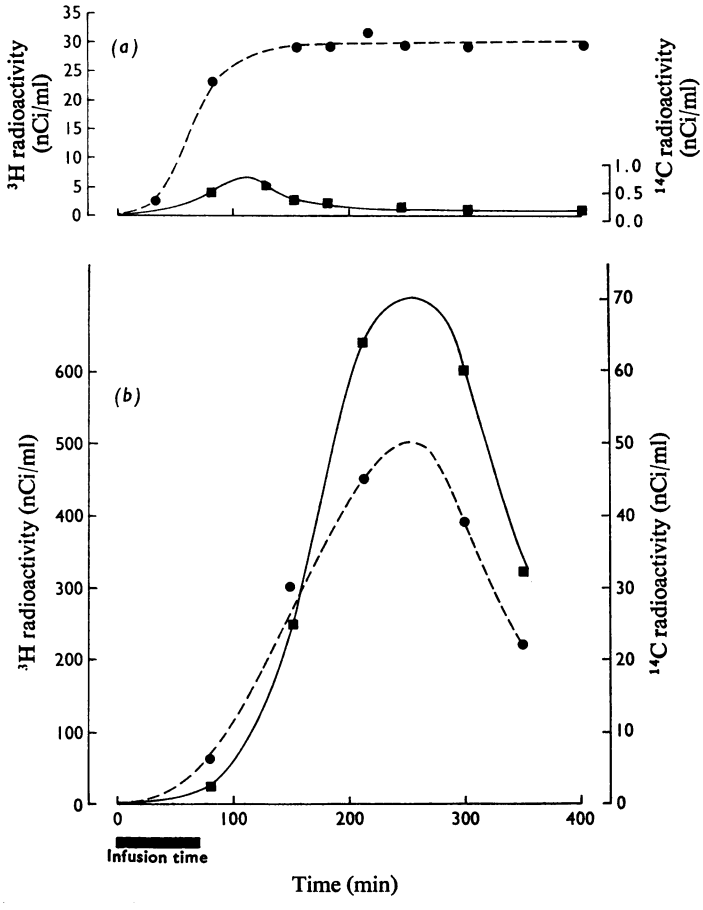

Fig. 1. *Rate of appearance of ^3H radioactivity and ^{14}C radioactivity into (a) mammary lymph and (b) milk after infusion of [2-^3H]glycerol and [1-^{14}C]palmitic acid into the jugular vein of a fed lactating goat*

[2-^{3}H]Glycerol was infused at 13.62μCi/min and [1-^{14}C]palmitic acid at 1.09μCi/min for 78min. ●, ^{3}H radioactivity; ■, ^{14}C radioactivity.

amounts of the fatty acid were transferred into lymph.

Uptake of chylomicron triglyceride

To investigate the uptake of triglyceride, doubly labelled chylomicra were prepared in a donor goat by injecting [^{14}C]glyceryl tripalmitate and [^{3}H]palmitic acid into the duodenum and collecting intestinal lymph for 6h. Some 43% of the ^{14}C and 38% of the ^{3}H was collected (Fig. 2) and at least 90% of the activity was associated with the chylomicra. Lymph produced during the period 48–90min was used in the preparation of washed chylomicra, which were infused over a period of 60min into the mammary arteries of two goats, one with an autotransplanted mammary gland and the other into a normal mammary gland *in situ*. Blood samples were taken from the artery downstream from the point of infusion, from the mammary vein and from a carotid artery. The arteriovenous differences of the blood lipids showed that there was a considerable uptake of chylomicra and low-density β-lipoproteins by the mammary gland (Table 5). There was little change in the specific radioactivity of either the glycerol or fatty acid moieties of the chylomicron and low-density β-lipoprotein triglyceride across the mammary gland (Table 6) indicating that the mammary gland removed triglyceride that was representative of the triglyceride of these fractions and that there was no release of triglyceride by the gland into mammary venous plasma. However, the specific radioactivity of mammary venous plasma palmitate was greater than that in the artery, showing the release, as free fatty

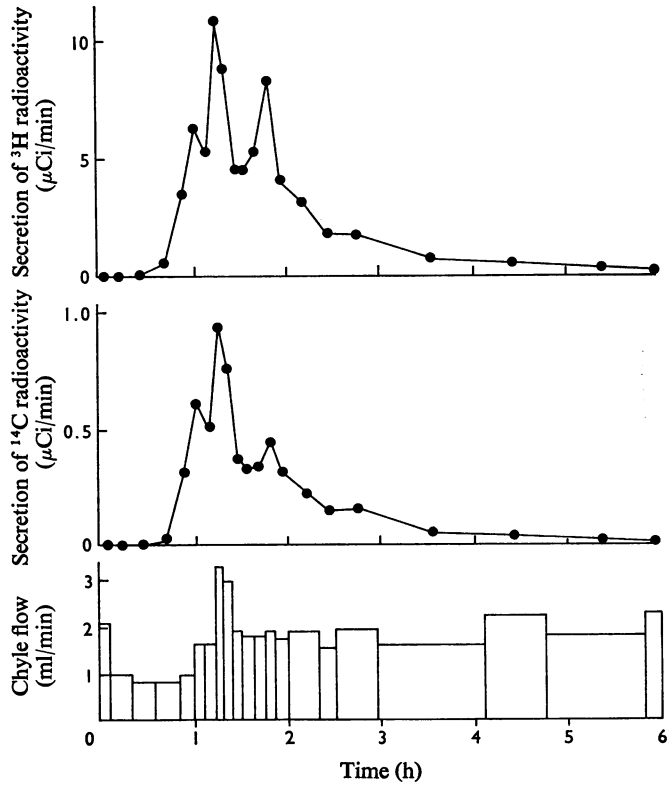

Fig. 2. *Secretion of labelled chyle in the intestinal lymph of a donor goat after the duodenal infusion of* [^{14}*C*]*glycerol tripalmitate* (200 μ*Ci*) *and* [9,10-^{3}*H*]*palmitic acid* (2.5 *mCi*)

acid, of palmitic acid derived mainly from chylomicron triglyceride and to a much lesser extent from the β-lipoprotein triglyceride. From the specific radioactivity values, it was calculated that at least 30% of the chylomicron triglyceride uptake by the gland was hydrolysed to plasma free palmitic acid, but this value considerably underestimated the free fatty acid release, as free fatty acid is known to be taken up by the gland (see above).

Some of the triglyceride of the β-lipoprotein fraction might have been derived from the chylomicron triglyceride but since the ^{14}C/^{3}H ratio was only 35% of that of the chylomicron triglyceride, and the specific radioactivity of the palmitic acid was only 34% of that of the general circulating free fatty acid, much of the β-lipoprotein triglyceride was probably synthesized in the liver from plasma free fatty acids and ^{14}C-labelled materials derived from the glycerol moiety of chylomicron triglyceride.

The change in ^{14}C/^{3}H ratio from 0.086 in the plasma chylomicron triglyceride to 0.052 in the milk triglyceride (at the peak of milk-fat specific radioactivity) indicated that there was a greater loss of [^{14}C]glycerol than of [^{3}H]palmitic acid during the synthesis of milk fat from labelled chylomicron triglyceride. This result showed that at least about half of the chylomicron triglyceride taken up by the gland was hydrolysed before incorporation into milk fat. The specific radioactivity of the milk palmitic acid reached 9.2% of that of the chylomicron triglyceride palmitate indicating that at least this proportion of milk-fat palmitate was derived from this source, but this was a minimum value as the specific radioactivity of the milk fat had not reached a plateau value.

Uptake of tripalmitate emulsions

An emulsion of [2-^{3}H]glycerol tripalmitate and glycerol tri[1-^{14}C]palmitate was infused into the jugular vein of a lactating goat and the transfer of [^{3}H]glycerol and [^{14}C]palmitate from plasma into mammary lymph and milk was examined. Glycerol appeared in lymph and milk fat more quickly than the fatty acid (Fig. 3), which is in agreement with the

Table 5. *Arteriovenous differences of triglycerides of individual plasma lipoprotein fractions and of plasma free fatty acids across the mammary glands of two lactating goats*

All values are the means of three determinations. See also Table 6. Goat no. 3 and goat no. 4 had the mammary gland transplanted to the neck.

	Goat no. 3			Goat no. 4		
	Artery	Mammary vein	Arteriovenous difference	Artery	Mammary vein	Arteriovenous difference
Plasma triglyceride (mg/100ml)	6.6	4.5	2.1	4.4	2.3	2.1
Chylomicron						
β-Lipoprotein						
Low-density	3.5	1.5	2.0	5.9	5.3	0.6
High-density	3.7	3.6	0.1	5.0	5.0	0.0
High-density α-lipoprotein	6.7	6.5	0.2	9.9	9.7	0.2
Total	20.5	16.1	4.4	25.2	22.3	2.9
Plasma free fatty acids (mequiv./l)	0.25	0.24	0.01	—	—	—
Plasma volatile fatty acids (mequiv./l)	1.13	0.59	0.54	1.76	0.82	0.94
Plasma flow (ml/min)		600			520	

Table 6. *Specific radioactivities of palmitate in plasma triglyceride and plasma free palmitate compared with that of milk-fat triglyceride palmitate during the infusion of doubly labelled chylomicra into the artery of the right mammary gland of a lactating goat*

Goat no. 3 was used. The infusion rate of chylomicron triglyceride was 0.271 μCi of ^{14}C and 2.90 μCi of ^{3}H/min. See also Table 5.

		Concentration (mg/100ml)	Specific radioactivity (μCi/g) ^{14}C	Specific radioactivity (μCi/g) ^{3}H	$^{14}C/^{3}H$ ratio
Plasma lipids					
Chylomicron triglyceride palmitate	Artery	1.52	7.34	85.7	0.086
	Mammary vein	1.04	7.40	87.0	0.084
Low density β-lipoprotein triglyceride palmitate	Artery	0.70	0.108	3.60	0.030
	Mammary vein	0.31	0.104	3.56	0.029
Free palmitic acid	Artery	1.43	—	12.3	—
	Mammary vein	1.36	—	22.0	—
	Sample time (min)				
Milk	44		0.007	0.07	0.102
Triglyceride palmitate	77	—	0.028	0.43	0.064
	137	—	0.110	1.94	0.056
	193	—	0.281	5.23	0.054
	253	—	0.422	7.88	0.052
	415	—	0.355	7.41	0.048

results obtained by infusing free [^{3}H]glycerol and free [^{14}C]palmitic acid (Fig. 1). Small amounts of [^{14}C]-palmitic acid were transferred into lymph. The $^{3}H/^{14}C$ ratio in milk triglyceride, collected at intervals, varied considerably. The ratio in the first milk sample (160min after the infusion) was about twice that of the infused tripalmitate, indicating a more rapid transfer of glycerol into milk, but the ratio quickly fell to about 20% of that of the infused tripalmitate (Fig. 4). This value was lower than that obtained with the doubly labelled chylomicra (34%) and showed that at least 80% of the infused tripalmitate was hydrolysed during mammary uptake and transferred into milk. Similar changes in $^{3}H/^{14}C$

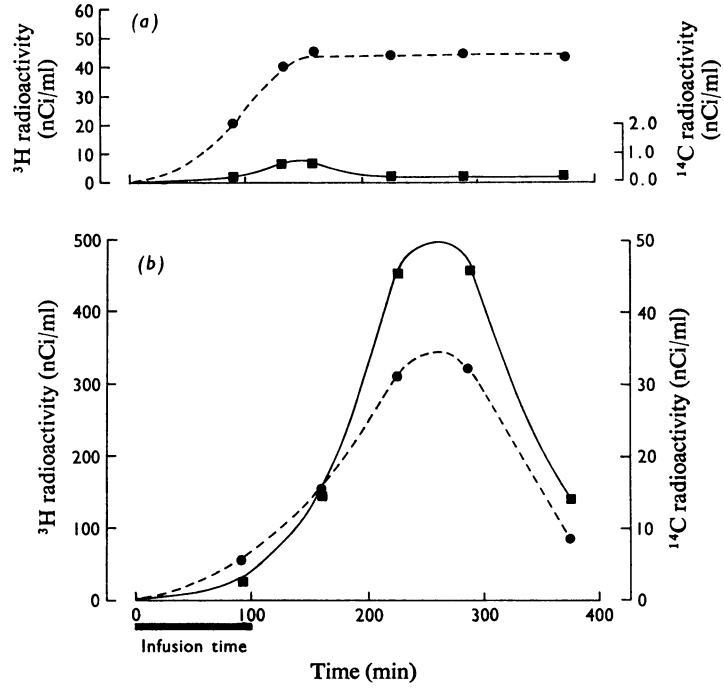

Fig. 3. *Rate of appearance of ^{3}H radioactivity and ^{14}C radioactivity into (a) mammary lymph and (b) milk after the infusion of an emulsion of [2-^{3}H]glycerol trioleate and glycerol tri[1-^{14}C]palmitate into the jugular vein of a fed lactating goat*

[2-^{3}H]Glycerol trioleate was infused at 11.58 μCi/min and glycerol tri[1-^{14}C]palmitate at 1.18 μCi/min for 100 min. ●, ^{3}H radioactivity; ■, ^{14}C radioactivity.

ratio in milk triglycerides were obtained during the infusion of free [^{3}H]glycerol and [^{14}C]palmitic acid (Fig. 4) and an emulsion of [2-^{3}H]glycerol trioleate and glycerol tri[1-^{14}C]oleate.

Composition of mammary lymph

Lymph collected during the infusion of labelled materials into the lactating goat was analysed for glycerol, acetate and lipids. The concentrations of glycerol, acetate and plasma free fatty acids were similar to those in arterial plasma (Table 7), but the concentrations of the complex lipids (triglycerides, phospholipids and cholesterol esters) were up to five times lower than the corresponding concentrations in arterial plasma (Table 7). There were only slight differences in the lipid content of afferent and efferent mammary lymph. Only traces of monoglyceride were detected in lymph, and diglycerides were not present in the samples examined.

The fatty acid composition of the triglyceride and free fatty acid fractions of mammary lymph were compared with those of samples of arterial and mammary venous plasma, and of milk taken at the same time. The lymph and plasma lipids were characterized by the absence of the short-chain and medium-chain length fatty acids found in goat milk and by the presence of much greater proportions of stearic acid than are found in milk fat (Table 8). Examination of the lipoproteins in lymph, showed that the triglycerides were present in all the lipoprotein fractions and the concentration was about five times less than the circulating amounts in plasma (Table 9).

Discussion

Radioactive materials have been used to investigate the mode of uptake by the goat mammary gland of fatty acids, glycerol and triglycerides, their release from mammary tissue and their transfer into milk and mammary lymph. These studies were made possible by the use of goats surgically prepared to permit samples of arterial and mammary venous blood to be

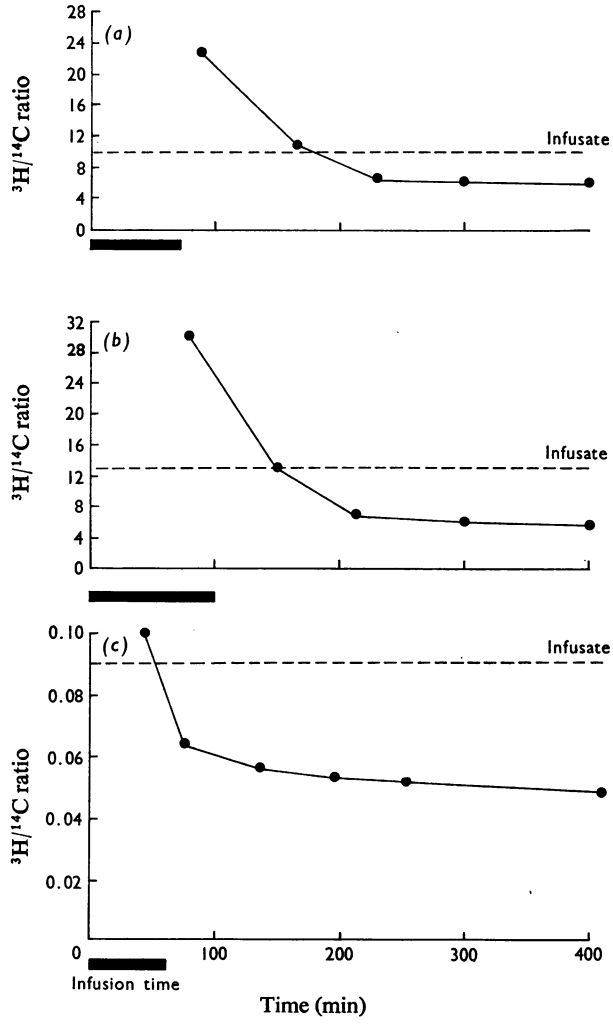

Fig. 4. *Change in $^3H/^{14}C$ ratios with respect to time in milk from a lactating goat infused with (a) [2-^3H]glycerol and [1-^{14}C]palmitic acid, (b) an emulsion of [2-^3H]glycerol trioleate and glycerol tri[1-^{14}C]palmitate and (c) chylomicra labelled with [1-^{14}C]palmitic acid and [2-^3H]glycerol*

withdrawn from unanaesthetized, undisturbed animals under conditions previously shown to be without effect on mammary metabolism (Linzell, 1963). When combined with isotope-dilution techniques, the measurement of changes in the concentration and specific radioactivity of materials in blood, lymph and milk allowed their rates of uptake and release into venous blood, lymph and milk to be calculated, as the rates of flow of blood, lymph and milk were known. Time-course studies on the appearance of labelled metabolites in mammary lymph and milk were facilitated by the use of goats prepared with an autotransplanted mammary gland, which allowed materials of high specific radioactivity to be supplied directly to the gland via the mammary artery.

The quantitative importance of acetate in milk-fat synthesis established by Annison & Linzell (1964) was confirmed by the present results (Table 3), which also showed that this substrate makes little contribution to the lipids that appear in mammary venous plasma. A detailed examination of the free fatty acid fraction of mammary venous plasma failed to detect short-chain length or medium-chain length fatty acids, and the long-chain fatty acids present were of

Table 7. *Compositions of arterial blood, mammary venous blood and mammary lymph from a lactating goat*

	Concentration (mg/100ml)		
Substance	Arterial blood	Mammary venous blood	Mammary lymph
Acetate*	5.4	2.5	5.9
Glycerol*	0.19	0.12	0.28
Triglyceride†	7.5	6.3	1.7
Free fatty acid†	3.1	2.5	5.4
Phospholipid†	22.5	23.3	6.0
Cholesterol ester†	55.0	46.3	40.0

* As mg/100ml of blood.
† As mg/100ml of plasma.

Table 8. *Fatty acid compositions of triglyceride and free fatty acid fractions from arterial blood, mammary venous blood, lymph and milk fat*

	Fatty acid composition (mol/100mol)						
	Free fatty acid			Triglyceride			
Lipid source ...	Arterial blood	Mammary venous blood	Lymph	Arterial blood	Mammary venous blood	Lymph	Milk fat
Fatty acid							
$C_{4:0}$	—	—	—	—	—	—	2.9
$C_{6:0}$	—	—	—	—	—	—	2.1
$C_{8:0}$	—	—	—	—	—	—	2.7
$C_{10:0}$	—	—	—	—	—	—	12.0
$C_{12:0}$	—	—	—	—	—	—	7.6
$C_{14:0}$	3.3	4.9	3.6	2.5	2.5	5.4	12.8
$C_{15:0}$	—	—	—	—	—	—	0.5
$C_{16:0}$	20.9	26.2	25.5	22.4	21.2	29.4	25.3
$C_{16:1}$	3.1	4.2	4.2	5.4	5.5	8.7	1.4
$C_{18:0}$	28.7	24.0	29.1	23.3	21.5	18.9	4.7
$C_{18:1}$	33.0	29.3	31.1	36.1	40.2	29.8	25.2
$C_{18:2}$	5.5	6.1	6.2	6.0	5.5	3.3	2.7
$C_{18:3}$	3.3	3.7	—	1.8	1.7	—	—
$C_{20:3}$	2.1	1.5	—	0.3	0.3	4.2	—
$C_{20:4}$	—	—	—	2.1	1.6	—	—

very low specific radioactivity relative to blood acetate. These results show that fatty acids synthesized in the mammary gland from acetate are not released into mammary venous blood. The decrease in the specific radioactivity of acetate across the mammary gland was consistent with results demonstrating acetate release by mammary tissue (Annison & Linzell, 1964).

When labelled free fatty acids and glycerol were infused into the mammary artery of the lactating gland, there was a significant transfer of radioactivity into milk fat, and a decrease in the specific radioactivity of each component during its passage through the gland (Table 4). The absence of a consistent arteriovenous difference in the concentrations of plasma free fatty acids and glycerol suggested that the uptake of each component was roughly balanced by the release of unlabelled material. Labelled palmitate and glycerol both appeared in lymph, although the transfer of glycerol was more rapid and more extensive (Fig. 1).

When triglyceride differentially labelled with ^{3}H and ^{14}C in the glycerol and fatty acid moieties was supplied to the mammary gland as chylomicra or as

Table 9. *Triglyceride contents of lipoprotein fractions from the mammary lymph of a lactating goat*

Lipoprotein fraction	Triglyceride concentration (mg/100ml)
'Chylomicron'	0.50
Low-density β-lipoprotein	0.21
High-density β-lipoprotein	0.43
High-density α-lipoprotein	0.53

triglyceride emulsions, labelled free glycerol and free fatty acids were detected in the mammary venous blood and in lymph. The radioactivities from both glycerol and fatty acid were also incorporated into milk triglycerides, although the proportion derived from each was different. The change in the $^3H/^{14}C$ ratio that occurs during the transfer of triglyceride from blood to milk would provide a valid measure of the extent of triglyceride hydrolysis during uptake if either of the products of hydrolysis was not re-utilized for triglyceride synthesis. Both fatty acids and glycerol have been shown to be incorporated into milk triglyceride, however, and the change in $^3H/^{14}C$ ratio gives only a minimum value. With the triglyceride emulsion, the change in $^3H/^{14}C$ ratio showed that at least 80% of the triglyceride had been hydrolysed during uptake: a somewhat lower value was obtained with doubly labelled chylomicra. The smaller change in $^3H/^{14}C$ ratio observed with chylomicra may reflect the fact that in this material palmitate was concentrated at the 2-position of glyceride glycerol (Garton & Duncan, 1965), but the infused tripalmitate had palmitate distributed uniformly at the 1-, 2- and 3-positions. Glycerol was transferred into lymph and milk more quickly than labelled fatty acid, and the time-course of the appearance of these materials in lymph and milk (Fig. 3) was closely similar to that observed when a mixture of free fatty acid and glycerol was supplied to the mammary gland (Fig. 1). These results provided further evidence that plasma triglycerides are largely or completely hydrolysed during their uptake by the mammary gland.

The fatty acid composition of the plasma free fatty acid and triglyceride fractions of mammary lymph clearly showed that these materials were not synthesized at the sites of milk fatty acid synthesis. The marked differences in the $^3H/^{14}C$ ratio of the lymph triglycerides relative to that of plasma triglyceride during the infusion of doubly labelled chylomicra or triglyceride emulsions was evidence against the transfer of intact blood triglycerides into lymph.

The present results are in agreement with the hypothesis that the mechanism by which plasma triglycerides are taken up and incorporated into milk fat involves hydrolysis and re-esterification within the mammary tissue (McBride & Korn, 1964; Annison *et al.*, 1967; West *et al.*, 1967*a*). The evidence is consistent with the hypothesis that hydrolysis occurs on the luminal surface of the capillary endothelial cells, and that the liberated glycerol and free fatty acids are in part taken up by mammary tissue, the remainder appearing in mammary venous blood. Important supporting evidence for this hypothesis was provided by Schoefl & French (1968) who, in an electron-microscopical study, detected injected chylomicra attached to the endothelial cells in the mammary glands of lactating mice, but no sign of the lipid particles outside the capillaries in the extracellular space.

There is evidence to show that lipoprotein lipase is involved in the hydrolysis of triglycerides before uptake by various tissues. The enzyme has been found in heart and lung (Anfinsen *et al.*, 1952), adipose tissue (Hollenberg, 1959; Cherkes & Gordon, 1959), muscle (Hollenberg, 1960) and in lactating mammary tissue (McBride & Korn, 1963; Robinson, 1963*a*) but not in liver (Robinson, 1965; Olson & Alaupovic, 1966), although Mayes & Felts (1968) considered lipoprotein lipase to be present in liver in an inactive state. The presence of the enzyme has been associated with the hypothesis that the hydrolysis of triglycerides of chylomicra and lipoproteins to free fatty acids and glycerol precedes their uptake by tissues (Robinson, 1963*b*). It has also been suggested that the action of the enzyme takes place at the luminal surface of the capillary endothelium because the enzyme is released into the venous circulation of organs involved in triglyceride uptake such as the lactating goat mammary gland (Barry *et al.*, 1963) and the perfused rabbit hind limb (Robinson & French, 1960). This is supported by electron-microscope studies of mammary (Schoefl & French, 1968) and adipose tissue (Wasserman & McDonald, 1963; Moskowitz & Moskowitz, 1965).

In the experiments in which [^{14}C]glycerol was infused into lactating goats, the glycerol was also incorporated into glucose, but because of the specific radioactivities of arterial and mammary venous plasma were equal, this must have occurred in tissues outside the mammary gland, (e.g. liver). Further, it is impossible to determine the contribution of glycerol to CO_2 production in the whole animal and in the mammary gland, because one cannot distinguish between $^{14}CO_2$ derived directly from glycerol and indirectly via glucose. The release of glycerol into mammary venous plasma was somewhat greater than the hydrolysis of triglyceride by the mammary gland suggesting that glycerol synthesized *de novo* by the gland was released into the venous circulation.

The possible uptake of intact or partial glycerides, not excluded by the present findings, was studied by using glycerol ethers (Bickerstaffe *et al.*, 1970).

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