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Synthesis of milk specific fatty acids and proteins by dispersed goat mammary-gland epithelial cells

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The method now described for preparation of dispersed lactating goat mammary-gland cells gives a high yield of morphologically and functionally normal mammary cells. The cells synthesize specific goat milk fatty acids in the right proportions, and they respond to hormones by increased protein synthesis. The cells can be frozen and thawed without losing the above properties, which makes them an excellent tool for metabolic and hormonal studies.

INTRODUCTION

Most studies on ruminant mammary-gland metabolism have been carried out on intact mammary gland *in vivo* or *in vitro* and on organ cultures and subcellular fractions (Moore & Christie, 1981; Patton & Jensen, 1976). Only a few metabolic studies have been carried out on dispersed lactating ruminant mammary-gland cells. These studies were limited to investigations of incorporation of acetate, glycerol and longer-chain fatty acids into cellular and secreted lipids by dispersed lactating bovine mammary-gland cells (Kinsella & McCarthy, 1968*a*,*b*; Kinsella, 1968, 1970*a*,*b*, 1971). The synthesis of casein and β-lactoglobulin has been studied in one case (Anderson & Larson, 1970); they showed that dispersed lactating bovine mammary-gland cells responded to addition of a hormone 'cocktail' by increased synthesis of casein and β-lactoglobulin. In addition to the above studies on dispersed cells, a method has been described for preparing functional active acini from bovine mammary gland (Park *et al.*, 1979). Isolated cells and acini offer many advantages over tissue slices and organ cultures in metabolic experiments. Such a preparation could be used in studies of the mechanism and control of milk secretion and in studies on the mechanism of hormone action in mammary gland. One further advantage of dispersed cells is the possibility of making a large batch of a homogeneous cell preparation which can be stored in liquid N_2 for later use. This is especially important in experiments on ruminant mammary metabolism, where the cost of experimental animals is very high.

In preliminary studies with lactating goat mammary tissue, we found that the methods used for cell dispersion in the above-mentioned studies gave a low yield of not very active cells. The studies reported here aimed to develop an improved method for the isolation of lactating mammary epithelial cells for metabolic and hormonal studies. Furthermore, a technique is described which allows storage of the cells in liquid N_2 without damaging the cells.

MATERIALS AND METHODS

Materials

The following chemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.: collagenase, type I (Clostridiopeptidase A, EC 3.4.24.3; 0.15 unit/mg); elastase, type IV (pancreatopeptidase E, EC 3.4.21.36; 60 units/mg); hyaluronidase, type I-S (hyaluronate 4-glycanohydrolase, EC 3.2.1.35; 300 NF-U/mg); trypsin inhibitor, type I-S from soya bean; bovine serum albumin. Deoxyribonuclease I (grade II; EC 3.1.21.1) came from Boehringer, Mannheim, West Germany. Fetal-calf serum, horse serum, Fungizone (250 μg/ml), penicillin (100 units/ml)/streptomycin (100 μg/ml) and powdered medium F12 were obtained from Grand Island Biological Co., Grand Island, NY, U.S.A. Medium 199, powder (with Hanks' salts) and cortisol were from Serva, Heidelberg, West Germany. [4,5-^{3}H]Leucine (10^5 Ci/mol) and sodium [1-^{14}C]acetate (56 Ci/mol) were from The Radiochemical Centre, Amersham, Bucks., U.K. Insulin was purchased from Nordisk Gentofte, Copenhagen, Denmark, and prolactin (NIADDK-oPRL-17) was from the National Hormone and Pituitary Program, Baltimore, MD, U.S.A. Silica gel HR and all other reagents were obtained from E. Merck, Darmstadt, West Germany.

Dissociation of the mammary-gland tissue

Lactating goat mammary-gland tissue was obtained by biopsy under sterile conditions or from whole mammary gland after the animal was killed. The tissue was placed in Krebs–Ringer bicarbonate buffer, pH 7.3 (Krebs, 1950), and freed of some of the connective tissue and fat. This and all the following steps were carried out under sterile conditions in a flow bench.

Tissue pieces (approx. 3–6 g each) were then transferred to Krebs–Ringer bicarbonate buffer (5 ml/g of tissue) containing 14 mM-glucose, 0.1 mg of soya-bean trypsin inhibitor/ml, penicillin (100 units/ml), streptomycin (100 μg/ml) and Fungizone (0.25 μg/ml), collagenase (200 units/ml), hyaluronidase (2 mg/ml), α-chymotrypsin (0.2 mg/ml), elastase (0.1 mg/ml) and deoxyribonuclease I (1 mg/ml). The enzyme solution was filtered through a 0.22 μm-pore Millex filter before use. The digestion mixture was repeatedly injected into the tissue pieces with a 2.0 ml syringe until the tissue had swollen to about double size. The distended gland tissue (one or two pieces) and the digestion solution (approx. 15 ml) were transferred to a glass incubation flask (30 mm × 120 mm) with a glass stopper equipped with a 3 mm-diam. glass tube

leading to the bottom of the incubation flask for inlet of air/CO_2 (19:1).

After about 1 h incubation with continuous gas flow (200 ml/min) in a shaking water bath (90 oscillations/min) at 37 °C, the supernatant was removed and the cells were harvested by centrifugation at 100 *g* for 5 min. The supernatant from the cell harvest containing the digestive enzymes was transferred back to the incubation flask, and the incubation of tissue was continued and the harvest was repeated after 30–60 min. The pellet of dispersed cells and cell clusters was suspended and washed three times in Krebs–Ringer bicarbonate buffer, containing 1% bovine serum albumin, and layered in 5 ml portions over 20 ml cushions of 5% bovine serum albumin in Krebs–Ringer bicarbonate buffer. After centrifugation at 100 *g* for 2 min the intact cells formed a pellet, whereas cell debris and connective-tissue remnants stayed in the supernatant. The pellet from the final wash was suspended in medium 199/F12 (1:1, v/v) containing 20% horse serum and 5% fetal-calf serum, to a final concentration of approx. 5×10^6 cells/ml. Liberation of cells during tissue disintegration was monitored under low magnification in a phase-contrast microscope.

Freezing and thawing of dispersed cells

The cells, suspended in medium 199/F12 (1:1, v/v) containing 20% horse serum and 5% fetal calf serum, to be frozen were made 10% (v/v) with dimethyl sulphoxide and equilibrated at room temperature for 30 min. The cell suspension was then transferred to 2 ml cryo-tubes (1.0–1.5 ml/tube) and cooled to 8 °C in the cold-room. The cooled cryo-tubes were placed in a polystyrene cylinder (26 cm tall, 14.5 cm in diameter, with 3 cm-thick walls) and transferred to a −60 °C freezer for 2 h. By this procedure a cooling rate of approx. 1 °C/min was obtained, and after 2 h the tubes were quickly transferred to liquid N_2. The frozen cell suspensions were thawed by transferring the cryo-tubes directly from liquid N_2 into a 37 °C water bath. Immediately after thawing, the cell suspension was diluted with 1 vol. of medium 199/F12 (1:1, v/v) containing 20% horse serum and 5% fetal-calf serum, to lower the concentration of dimethyl sulphoxide. The cells were allowed to sediment and the supernatant was removed and replaced by medium 199/F12 (1:1) containing 20% horse serum and 50% fetal-calf serum. The cells were then preincubated for 2 h before use for incubation experiments; then the cells were spun down and resuspended in the incubation medium.

Incubation conditions

(*a*) Lipid biosynthesis. Cells (2.5×10^6/ml) were incubated under air/CO_2 (19:1) at 37 °C for the time indicated in medium 199/F12 (1:1, v/v) containing 20 mg of bovine serum albumin/ml and radioactive substrates. The incubation was stopped at the times indicated by addition of chloroform/methanol (1:2, v/v), and the lipids were extracted by the method of Bligh & Dyer (1959). Individual lipid classes were separated by t.l.c. as described by Hansen *et al.* (1984). Plates were scanned for radioactivity on a Berthold thin-layer scanner. The fatty acid compositions of the major lipid classes were analysed by radio-g.l.c. as described by Knudsen *et al.* (1981).

(*b*) Incorporation of [4,5-^{3}H]leucine. Cells (10^6/ml) were incubated at 37 °C in leucine-free medium 199 (200 μl) under air/CO_2 (19:1) containing 1.0 μCi of [4,5-^{3}H]leucine (150 Ci/mmol)/ml, 10 mg of bovine serum albumin/ml and 5% horse serum, and with insulin, cortisol and prolactin added as indicated. At the appropriate times the incubation was stopped by sonication (40 W, 10 s) and 50 μl of the solution was plated on Whatman 3MM filters. After drying, the filters were transferred to 10% trichloroacetic acid and left overnight at 0 °C, followed by heating to 96 °C for 30 min. Finally the filters were washed three times with ice-cold 5% trichloroacetic acid, and once with 70% ethanol. The filters were counted for radioactivity by liquid-scintillation counting.

Transmission electron microscopy

Dispersed cells, suspended in medium 199/F12 (1:1, v/v), were immersion-fixed for 24 h at 4 °C with 2.5% glutaraldehyde buffered to pH 7.2 with 0.1 M-sodium cacodylate buffer. Frozen cell suspension was thawed and preincubated for 2 h. The cells were collected by centrifugation and washed for 3×5 min in 0.1 M-phosphate buffer, pH 7.2. The cell pellets were then suspended in 1.25% agar dissolved in warm distilled water. After cooling in icewater, small cubes of agar were washed in 0.1 M-phosphate buffer, pH 7.2, and post-fixed with 1% OsO_4 in phosphate buffer, pH 7.2, for 90 min at 20 °C. The cells were then pre-stained with 0.5% uranyl acetate in redistilled water for 1 h at room temperature. After stepwise dehydration with ethanol/water solutions containing increasing concentrations of ethanol, the agar cubes containing the cells were embedded in Araldite. Ultra-thin sections were contrasted with 0.4% lead citrate at room temperature for 5 min, and were examined in a Jeol 100 CX electron microscope operating at 80 kV.

RESULTS

The rate of tissue disintegration varied from animal to animal, and with the different enzyme batches used. The most important factor for obtaining a satisfactory yield of free cells and cell clusters was the mode of tissue agitation during the disintegration sequence. In a series of preliminary experiments we found that gentle shaking combined with continuous bubbling with air/CO_2 (19:1) was the only method that gave a substantial amount of free cells and small cell aggregates. The yield of cells in eight different preparations was between 20 and 30%.

A typical picture of the different stages in the disintegration procedure is shown in Fig. 1. The use of hyaluronidase in the digestion medium was absolutely necessary to obtain dispersed cells. Without this enzyme included, the tissue disintegration stopped at the alveolar level, as shown in Fig. 1(*a*). The final cell preparation contained free cells and cell clumps containing from 2 to approx. 50 cells (Fig. 1*c*). The larger cell clumps disintegrated to some extent during freezing and thawing. The intracellular structure of the lactating goat mammary-gland epithelial cells is shown in Fig. 2. The observed structure is typical of what has previously been shown for lactating bovine mammary-gland tissue (Park *et al.*, 1979).

The viability of the cells was tested by their ability to exclude Trypan Blue. More than 95% of the freshly

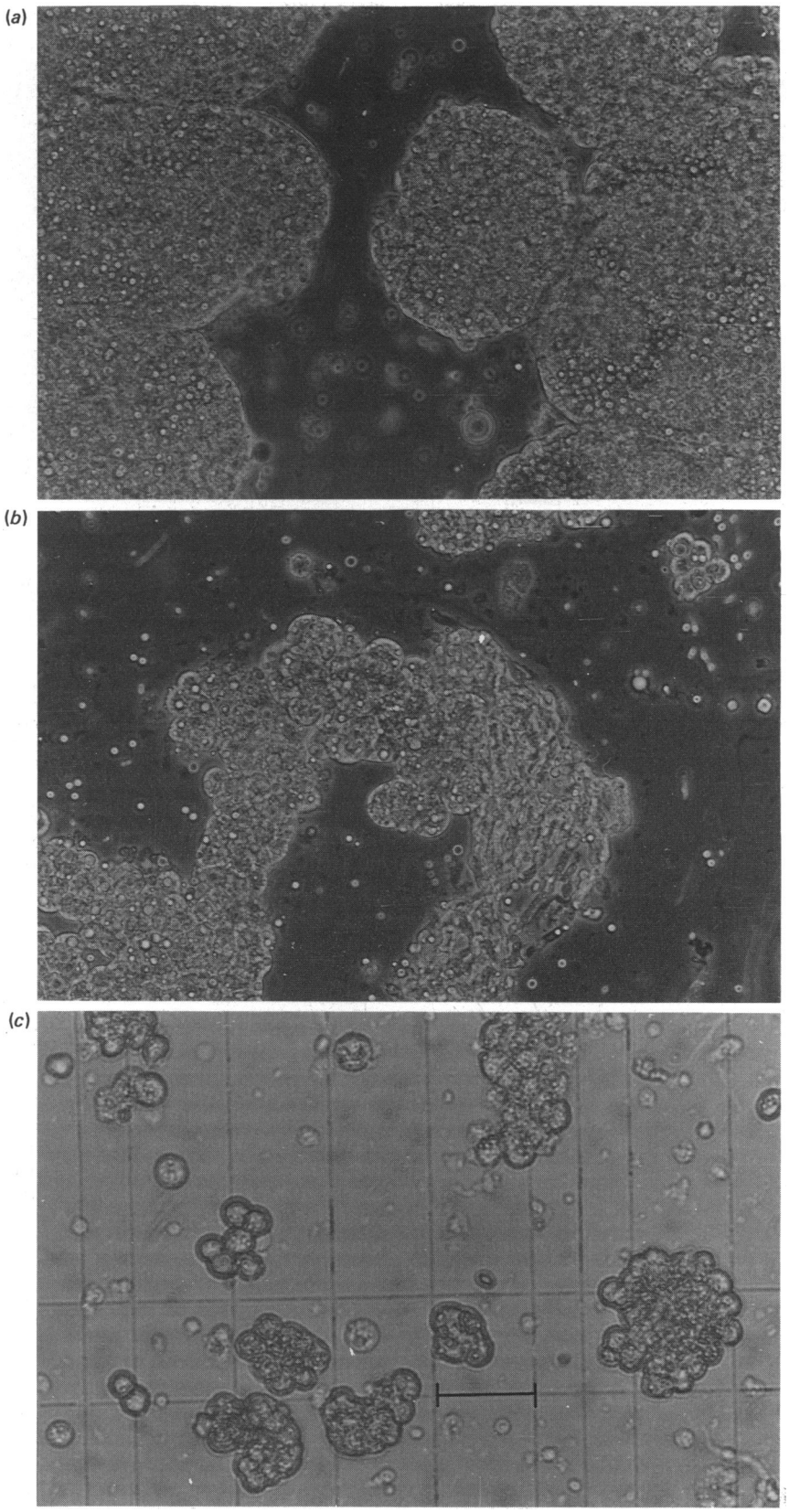

Fig. 1. Disintegration of lactating goat mammary tissue

(*a*) Typical picture from an early stage of digestion, after about 30–40 min of incubation (magnification × 75, phase contrast). (*b*) Picture from the stage of digestion where the tissue has been extensively disintegrated after about 50–60 min of incubation (magnification × 75, phase contrast). (*c*) Typical picture of the final cell preparation (magnification × 75). The bar represents 50 μm.

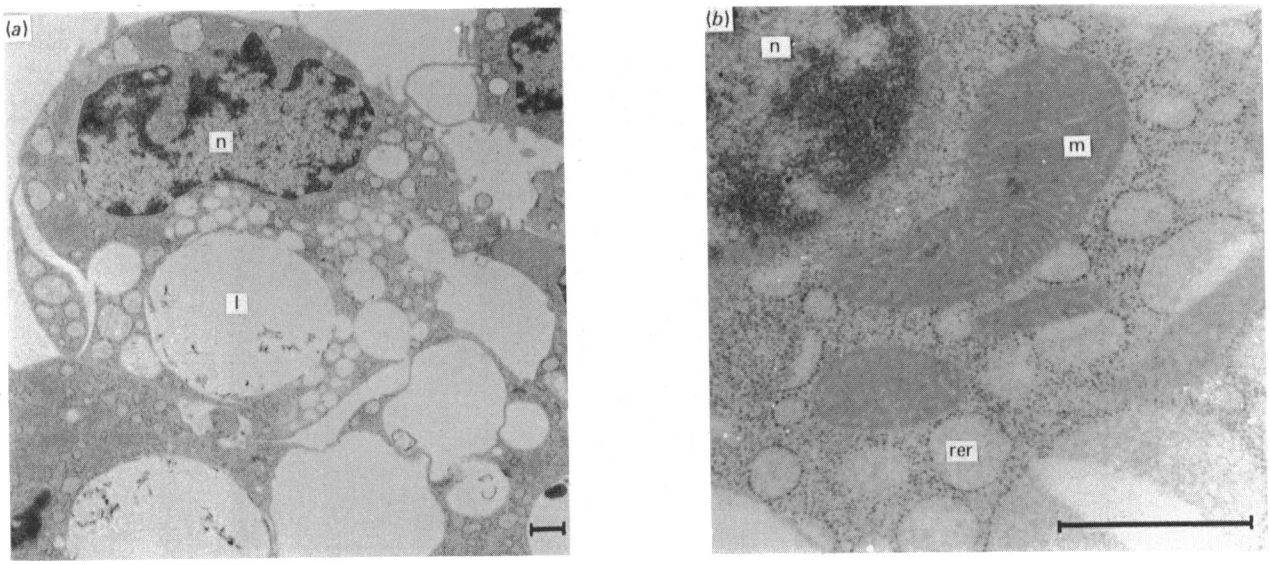

Fig. 2. Electron micrograph of freshly prepared epithelial cells from lactating goat mammary gland

For experimental details see the Materials and methods section. The magnification was (*a*) 4000 and (*b*) 26000. Key: n, nucleus; m, mitochondrion; rer, rough endoplasmic reticulum; l, lipid vacuole. The bar represents 1 μm.

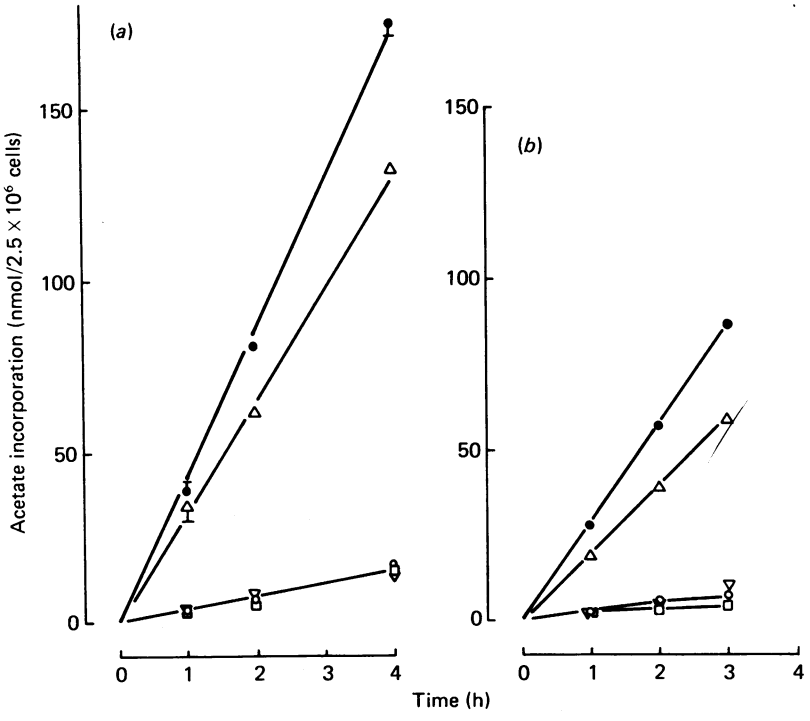

Fig. 3. Incorporation of sodium [1-^{14}C]acetate into lipids of freshly prepared (*a*) and frozen/thawed (*b*) dispersed goat mammary-gland cells

The cells (2.5×10^6/ml) were incubated as described in the Materials and methods section with 2 mM-sodium [1-^{14}C]acetate (sp. radioactivity 2 Ci/mol) in a total volume of 0.4 ml. Samples were extracted at the times indicated, and incorporation of [1-^{14}C]acetate into the various lipid classes (●, total lipids; △ non-esterified fatty acids; □, diacylglycerols; ○, polar lipids) were assayed as described in the Materials and methods section. Frozen cells were stored for 8 months before use. Values are means of duplicates, ±half the difference between duplicates (shown by bars where applicable). The results are typical for incubations with four different cell preparations.

Table 1. Composition of fatty acids synthesized *de novo* and incorporated into triacylglycerols by freshly prepared and frozen dispersed goat mammary-gland epithelial cells

Goat mammary epithelial cells (10^6 cells/ml) were incubated with 1 mM-sodium [1-^{14}C]acetate (sp. radioactivity 5 Ci/mol) as described in the Materials and methods section for 4 h in a total volume of 1 ml. The values are means ±S.E.M. for duplicate incubations with four different cell preparations

Experiment	Percentage distribution (mol/100 mol)							
	$C_{4:0}$	$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$
Goat mammary cells									
Fresh	19.0±2.5	9.5±1.2	8.9±2.3	22.0±3.5	5.8±1.3	14.3±4.1	20.0±2.3	–	–
+500 μM-palmitic acid	29.1±3.0	10.5±0.3	7.7±0.8	18.9±1.8	5.24±1.1	11.3±0.8	17.4±0.2		
Frozen	27.0±4.5	14.0±1.2	10.3±1.8	20.3±2.8	6.5±1.5	12.3±1.6	10.0±2.2	–	–
Goat mammary tissue slices*	3	1	2	14	4	31	40	–	–
Goat milk†	9	6	5	11	4	10	21	12	21

* Data from Grunnet & Knudsen (1979).
† Data from Glass *et al.* (1967).

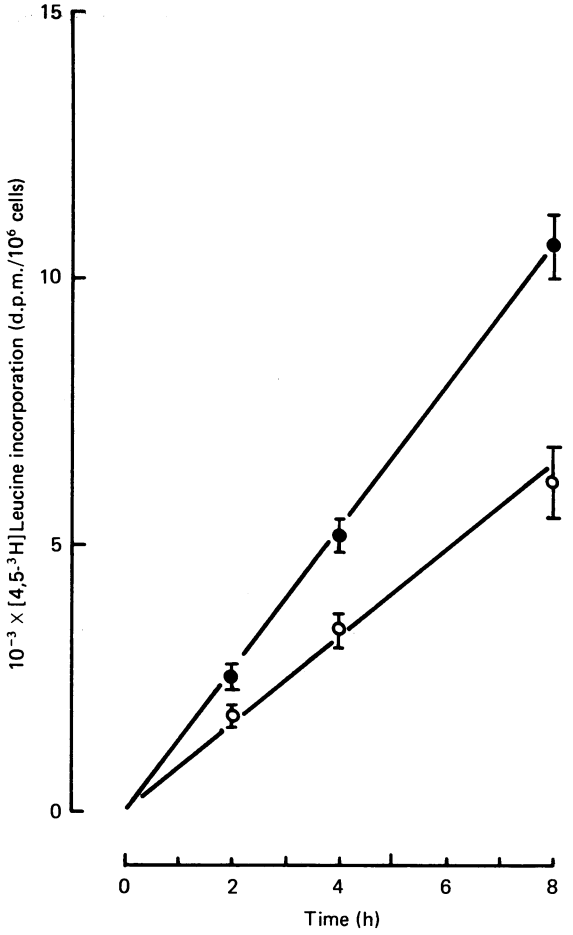

Fig. 4. Incorporation of [4,5-^{3}H]leucine into protein of freshly dispersed goat mammary-gland epithelial cells and frozen/rethawed cells

The cells (10^6/ml) were incubated as described in the Materials and methods section with 1.0 Ci of [4,5-^{3}H]-leucine (sp. radioactivity 150 Ci/mol) in a total volume of 0.4 ml. The incorporation of [4,5-^{3}H]leucine into proteins was measured as described in the Materials and methods section. The data are expressed as means of duplicate determinations, ±half the difference between duplicates (shown with bars). The results are typical for incubations with three different cell preparations: ●, freshly prepared cells; ○, frozen/rethawed cells.

prepared cells in eight different preparations excluded the stain. The survival of cells after freezing and thawing, based on their ability to exclude Trypan Blue, was about 60%. The same survival was calculated from the capacity for lipid biosynthesis of the cells before and after freezing. This survival rate after freezing and thawing is similar to what has been reported for rabbit mammary-gland epithelial cells (Haeuptle *et al.*, 1983).

Synthesis of lipids and protein

The incorporation of [1-^{14}C]acetate into total lipid and various lipid classes was linear with time for at least 3 h for both freshly prepared and frozen/thawed cells (Figs. 3*a* and 3*b*), and the incorporation rate had only decreased slightly after 5 h of incubation.

Approx. 70% of the newly synthesized fatty acids was incorporated into triacylglycerols by freshly dispersed

Table 2. Effect of prolactin on the protein synthesis in frozen/rethawed goat mammary-gland epithelial cells

Incubation conditions were as described in Fig. 4. The hormone concentrations were: insulin, 5 μg/ml; cortisol, 5 μg/ml; prolactin, 0.2 μg/ml. The results are expressed relative to control incubations without prolactin added, as means ± S.E.M. for three independent cell preparations. The 100% (control) values are given in parentheses (d.p.m./10^6 cells).

Time (h)	Relative incorporation with prolactin (%)
2 h	113.2 ± 5.9 (2019 ± 671)
4 h	127.2 ± 6.8 (3204 ± 659)
6 h	121.4 ± 5.9 (5819 ± 218)

and by frozen/thawed cells (Fig. 3). The apparent incorporation of labelled acetate into cellular lipids by the frozen/rethawed cells (Figs. 3*a* and 3*b*) is about 60% of the incorporation by freshly prepared cells. However, if it is taken into account that only about 60% of the cells are viable (see above), the incorporation per viable cell is almost unchanged by storage. The results are similar to those found for dispersed lactating cow mammary-gland cells (Kinsella, 1971). Dispersed cells as well as tissue slices synthesize the same spectrum of fatty acids; however, the cells synthesize a larger proportion of short- and medium-chain fatty acids and a smaller proportion of long-chain fatty acids than do tissue slices. In the mammary gland, approx. 50% of the fatty acids incorporated into triacylglycerols are synthesized *de novo* and the rest is absorbed from the blood stream. In the results presented here, only the distribution among fatty acids synthesized *de novo* is monitored; thus an apparent content of butyric acid of 20% corresponds to a content of approx. 10% in the milk lipid fraction, since butyric acid is exclusively synthesized *de novo*. We have measured the content of long-chain fatty acids in the cells and found that it is sufficient to support lipid synthesis for the incubation periods that we have used. The shift in fatty acid composition after freezing/rethawing is most probably caused by a liberation of fatty acids from cells destroyed by the freezing and thawing procedure. A similar effect can be obtained if freshly prepared cells are incubated with 500 μM-palmitic acid (Table 1). The incorporation of [4,5-^{3}H]leucine into proteins was linear with time for more than 6 h with both fresh and frozen/thawed cells (Fig. 4). Furthermore, frozen/rethawed cells were shown to respond by increased protein synthesis to addition of prolactin in the presence of insulin and cortisol (Table 2).

DISCUSSION

The results presented above show that morphologically and functionally normal dispersed goat mammary-gland cells can be prepared with high yield by the method described. The results also show that the prepared cells respond to addition of prolactin in the presence of insulin and cortisol by increased protein synthesis; they can therefore be regarded as a good model for metabolic and hormonal studies.

The fact that the cells can be frozen and stored in liquid N_2 without losing their ability to synthesize specific milk components and the ability to respond to prolactin addition makes it possible to carry out a large number of experiments on one homogeneous cell preparation. This is a great advantage both experimentally and economically over that of preparing fresh cells for each experiment. The need to make new cells for each experiment would make it impossible for a large number of laboratories to carry out experiments with ruminant mammary-gland cells. The present technique overcomes this problem. In the following paper (Hansen *et al.*, 1986) is given an example of how the cells can with advantage be used to solve a problem concerned with the pathways of triacylglycerol synthesis in ruminant mammary gland.

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