

DISPERSED MAMMARY GLAND EPITHELIAL CELLS

I. Isolation and Separation Procedures

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ABSTRACT

The mammary gland from midpregnant rabbits has been dissociated into individual cells by enzymatic digestion, divalent cation chelation, and gentle shearing. A heterogeneous cell population is obtained, comprising ~60% parenchymal cells, ~10% myoepithelial cells, and ~30% connective tissue cells, including fibroblasts, plasma cells, and macrophages. The epithelial cells are characterized by the presence of fat droplets, which in 65% of the cells form large supranuclear vacuoles. Their buoyant density is less than 1.045, allowing their separation from myoepithelial cells and connective tissue cells by isopycnic centrifugation in a density gradient. The homogeneity of the epithelial cell fraction has been assessed by light and electron microscopy. The cells are viable and functionally active as indicated by their ability to exclude vital dyes, incorporate labeled precursors, consume oxygen, maintain intracellular Na^+ and K^+ concentrations, and retain their structural integrity. In addition, when cultured in Petri dishes, the cells grow as a monolayer, reestablish junctional complexes and retain cell polarity.

The differentiation of the mammary gland under hormonal influence has been analysed in intact animals (3, 11, 27, 28) as well as at the level of organ explants (review reference 26). In the mouse it has been established that explants cultured in the presence of insulin, steroids, and lactogenic hormones are able to undergo DNA synthesis, develop a secretory apparatus, and synthesize milk proteins. More recently, it has been shown that milk protein synthesis can be maintained in dispersed cells from bovine mammary gland (4) and that casein synthesis can be induced in dispersed cells from normal human breast (9) or from midpregnant mouse mammary gland (7). However, the data from these studies pertain only to average events occurring in heterogeneous populations of parenchymal, myoepithelial, and connective tissue cells.

A homogeneous cell population now provides the means to analyze and characterize the response to known biological stimuli of one given cell type, that is, the parenchymal cell. In addition, accessibility of the plasma membrane allows a topographical analysis of macromolecules of biological interest, such as hormone receptors, as well as a quantitative correlation between the kinetic behavior and the biological activity of a specific ligand. Recognition sites on epithelial cells for the specific uptake of proteins, i.e. immunoglobulin A, have been postulated (6); their identification and characterization in the mammary gland are greatly facilitated in a homogeneous cell population. The procedure of dissociation described in this paper is a modification of the technique of Amsterdam and Jamieson (1) and consists of enzyme digestion, divalent cation chelation, and

mild shearing treatment. A heterogeneous cell population including parenchymal and interstitial cells is obtained which can then be enriched in epithelial cells by equilibrium density centrifugation. As a consequence of the striking increase in the rate of fatty acid synthesis at midpregnancy in the rabbit (18, 25), large lipid droplets accumulate in the cytoplasm of the parenchymal cells (5), endowing the cell with a buoyant density less than that of other cell types isolated. These methods and some of the properties of the dissociated cells are described in the present paper; the results of short-term culture experiments and hormone-induced differentiation of dissociated cells will be reported later (footnote 1).

MATERIALS AND METHODS

Reagents

Reagents were obtained from the following sources: Worthington Biochemical Corp., Freehold, N.J.: purified α -chymotrypsin, 61 U/mg; chromatographically purified collagenase (*Clostridium histolyticum*), ~450 U/mg; crude bovine testis hyaluronidase, 400 USP U/mg (free of proteolytic activity as tested against Azocoll); purified swine pancreas elastase, 60 U/mg; and purified soybean trypsin inhibitor (STI). From Armour Pharmaceutical Co., Chicago, Ill. we obtained bovine serum albumin (BSA), powder Fraction V. Medium 199 (M 199), modified Eagle's medium (MEM) (without leucine), and antibiotic-antimycotic mixture, composed of 10,000 U penicillin, 10 mg streptomycin, and 25 μ g Fungizone per ml, were obtained from Grand Island Biological Co., Grand Island, N.Y. [^3H]Thymidine (9.21 Ci/mmol), L-[^{14}C]leucine (25 Ci/mmol), and [^3H]inulin (1-3 Ci/g) were obtained from New England Nuclear, Boston, Mass. Ilford L-4 emulsion was purchased from Ilford, Ltd., Ilford, Essex, England. All other chemicals were reagent grade.

Animals

Five-month-old white New Zealand rabbits that had been bred for the first time were used on day 18 of gestation. Following induction of anesthesia (Nembutal [Abbott Laboratories, North Chicago, Ill.], 0.5-1 ml, i.v.) the abdominal mammary glands were removed under sterile conditions.

Incubation Media

All dissociation and incubation media consisted of Krebs-Ringer bicarbonate solutions (KRB) equilibrated

to pH 7.3 with 95% O_2 and 5% CO_2 and contained a complete amino acid supplement (13). 14 mM glucose, 0.1 mg/ml soybean trypsin inhibitor, 100 U/ml penicillin, 100 μ g/ml streptomycin, 0.25 μ g Fungizone were also present. Collagenase (200 U/ml), hyaluronidase (2 mg/ml), α -chymotrypsin (0.2 mg/ml), and elastase (0.1 mg/ml) were added as salt-free powders. Ca^{++} and Mg^{++} were adjusted to 0.1 mM and 1.2 mM, respectively, in the enzyme mixture and in the cation replacement solution and adjusted to 2.5 and 1.2 mM, respectively, in the solution containing BSA. All media were filtered through 0.45 μ m Millipore filters (Millipore Corp., Bedford, Mass.) before use.

Dissociation Protocol

The procedure for the dissociation of midpregnant mammary gland consisted of an enzymatic digestion, a divalent cation chelation, and a mechanical disruption as modified from Amsterdam and Jamieson (1). The media, time, and temperature used in each of the 10 steps are summarized in Table I.

Enrichment of Mammary Epithelial Cells

The freshly dissociated mammary gland cells were suspended in 2 ml of 35% BSA solution at a final concentration of 5×10^7 cells/ml and placed in the bottom of a 12-ml cellulose nitrate tube (step 10). A continuous BSA gradient ranging from 35% to 5% BSA was built on top of the cushion. The tubes were spun to equilibrium for 15 min at 10,000 g_{avg} at 24°C (step 11). The light and heavy fractions and the pellet were then harvested and washed twice in M 199 before counting, fixation, or cultivation (steps 12 and 13). BSA solutions were prepared with Fraction V BSA powder dissolved in KRB containing 2.4 mg/ml of HEPES (acid), 2.5 mM Ca^{++} , 1.2 mM Mg^{++} , and antibiotics as mentioned above. The solutions were equilibrated with 5% CO_2 and 95% O_2 , the pH was adjusted to pH 7.4 by addition of 1 N sodium hydroxide, and the concentration of Na^+ was maintained at 140 mM. The densities were adjusted with KRB, using a refractometer and a standard curve relating refractive index to density.

Analytical Procedures

DNA was measured according to the fluorometric method of Kissane and Robins (12) and general protease activity was assayed against Azocoll (17).

Morphological Methods

Pieces of intact mammary gland were fixed in 2% formaldehyde-2% glutaraldehyde in 0.1 M Na cacodylate, pH 7.4, for 2 h at room temperature. Dissociated cells were fixed by mixing 1 vol of cell suspension with 2 vol of the above fixative. After fixing the cells for 1 h, they were transferred to a polyethylene microfuge tube and centrifuged at ~10,000 g for 2 min (Microfuge 152,

¹ Kraehenbuhl, J. P. Dispersed mammary gland epithelial cells. II. Short term culture and hormone induced differentiation. Manuscript in preparation.

TABLE I
Sequence of Dissociation of the Mammary Gland into Individual Cell and Enrichment in Parenchymal Cells

Steps	Treatment	Medium composition	Time <i>min</i>	Temperature °C
1	Collection of the gland	KRB, 0.1 mM Ca ⁺⁺ , 1.2 mM Mg ⁺⁺	5	24
2	Injection of enzyme to the gland interstitial space	KRB, 0.1 mM Ca ⁺⁺ , 1.2 mM Mg ⁺⁺ , collagenase (200 U/ml), elastase (0.1 mg/ml), α -chymotrypsin (0.2 mg/ml), hyaluronidase (2 mg/ml)		
	Dissection of fat and connective tissue		5	24
3	First enzymatic digestion	as in step 2	60	37
4	Chelation of divalent cations	KRB, 1 mM EDTA (no Ca ⁺⁺ , no Mg ⁺⁺)	2 × 5	37
5	Replacement of divalent cations	KRB, 0.1 mM Ca ⁺⁺ , 1.2 mM Mg ⁺⁺	2 × 5	37
6	Second enzymatic digestion	as in step 2	30	37
7	Pipetting	as in step 2	5	24
8	Filtering (nylon gauze)	as in step 2	5	24
9	Washes	KRB, 2.5 mM Ca ⁺⁺ , 1.2 mM Mg ⁺⁺ , 2.4 mg/ml HEPES	10	24
10	Cell suspension (5 × 10 ⁷ cells/ml)	KRB, 2.5 mM Ca ⁺⁺ , 1.2 mM Mg ⁺⁺ , 2.4 mg/ml HEPES, 35% BSA	2	24
11	Isopycnic centrifugation 10,000 × g_{avg}	as in step 10	15	24
12	Washes	Medium 199	10	24
13	Final cell suspension (5 × 10 ⁶ cells/ml)	Medium 199		37

Steps 3–6 were carried out in a water bath at 37°C under 95% O₂ and 5% CO₂ with agitation at 130 oscillations per min.

Beckman Instruments, Inc., Fullerton, Calif.). The resulting pellet was cut into thin disks with a razor blade. Tissue blocks and disks of cells were postfixed in 1% OsO₄ buffered with 0.1 M Na cacodylate to pH 7.4 for 1 h at 4°C, washed once with Veronal-acetate buffer, pH 7.4, and stained in block for 1 h at room temperature with 0.5% uranyl acetate in Veronal-acetate buffer, pH 5.0 (8). The tissue or cells were dehydrated in ethanol and propylene oxide and embedded in Epon. For light microscopy, 0.5- μ m Epon sections were stained with 1% methylene blue in 1% Na borate, examined and photographed with a Zeiss Photomicroscope II. Phase contrast and differential interference microscopy of living cells were performed on the same microscope to monitor the different steps in the dissociation and purification procedure.

[¹⁴C]Leucine Incorporation

Mammary gland epithelial cells (10⁶ cells/ml) were incubated at 37°C in MEM without leucine containing 2 μ Ci/ml [¹⁴C]leucine (8 × 10⁻⁴ mM). At 60, 120, and 210 min, cells and media were harvested, the cells were spun at 100 g for 2.5 min and then sonicated for 30 s with a Sonifier sonicator (Branson Instrument Corporation, Danbury, Conn.). Media and cells were precipitated with 10% TCA, and the precipitate was washed twice with cold 5% TCA before preparation for liquid scintillation counting (13).

[³H]Thymidine Labeling

Mammary gland epithelial cells (16⁶ cells/ml) were incubated for 2 or 4 h in M 199 containing 1 μ Ci/ml

[³H]thymidine (10⁻⁴ mM) and processed for liquid scintillation counting as described above. Cells were also processed for autoradiography (2), using Ilford L₄ emulsion. The same fixation and embedding procedures were followed, but staining in block was omitted.

Oxygen Consumption

Oxygen depletion rates were determined for cell suspensions with a Clark oxygen probe (Yellow Springs Instrument Co., Yellow Springs, Ohio) in a 2-ml glass and stainless steel chamber maintained at 25°C (19). The incubation medium for the oxygen depletion rate was M 199 equilibrated with 95% air (5% CO₂). 5–15 × 10⁶ cells were introduced into the chamber. Rates of oxygen depletion were related to μg DNA and expressed as nmol O₂/min/10⁶ cells, assuming 7 μg DNA per 10⁶ cells.

Measurements of Intracellular Monovalent Cations

Na⁺ and K⁺ content and concentration were measured both in nonincubated mammary gland tissue pieces 40–100 mg wet weight (WW) and in dissociated cells incubated at 37°C for 60–180 min (2.5–8 × 10⁶ cells per sample). At the end of the incubation, the dissociated cells were washed rapidly twice in isotonic sucrose (2.8 g/100 ml). This was sufficient to remove more than 99% of the extracellular Na⁺ and K⁺ content. Water content was determined by overnight drying to a constant weight in a hot air oven at 100°C and equating weight loss with the water content of the samples. The dried samples were then extracted at 60°C for 90 min in 50–100 μl of 10 N nitric acid. The acid extracts were diluted with an appropriate amount of 0.1 N hydrochloric acid, and Na⁺ and K⁺ were measured with a flame photometer using external standards prepared in 0.1 N HCl. The extracellular space (ECS) was determined by incubating tissue pieces or dissociated cells for 1 h in the presence of [¹⁴C]inulin, assuming that the concentration of labeled inulin in the ECS was the same as in the bathing medium. For a given ion (*i*), intracellular concentration was calculated as follows:

$$I_{(i)} = \frac{[T_{(i)} \times \overline{DW/WW}] - (\overline{ECS} \times [i]_{\text{ECS}})}{1 - (\overline{ECS} + \overline{DW/WW})}$$

where

- $I_{(i)}$ = intracellular concentration (mmol/l intracellular water);
 $T_{(i)}$ = ionic tissue content (mmol/kg DW);
 \overline{ECS} = mean fractional ECS. Whole tissue: 0.355 ± 0.015 (SEM), *n* = 22. Dissociated cells: 0.438 ± 0.014 (SEM), *n* = 9;
 $[i]_{\text{ECS}}$ = extracellular concentration of *i* for dissociated cells, this value was considered as negligible (equal to zero) since measurements of ECS Na⁺ and K⁺ concentrations

after two washes in isotonic sucrose had shown that they were less than 1% of the normal value,

$\overline{DW/WW}$ = mean ratio of dry over wet weight. Whole tissue: 0.244 ± 0.012 (SEM), *n* = 30. Dissociated cells: 0.166 ± 0.011 (SEM), *n* = 9.

Cell culture conditions

5 × 10⁶ dispersed parenchymal cells in 1 ml of M 199 complemented with 10% autologous midpregnant rabbit serum were cultured in plastic Petri dishes (5 cm²) for up to 5 days. The medium was changed every other day. The cells were fixed and embedded in the dish when prepared for electron microscopy.

RESULTS

Morphology of the Intact Mammary Gland

At midpregnancy (day 18 of gestation) the mammary gland is characterized by a well developed lobuloalveolar system and narrow glandular lumina filled with homogeneous secretion product (Figs. 1 and 2). Of all nucleated cells, 55% are epithelial and 65% of these contain a large supranuclear fat droplet (Table II). A report on the fine structure of the rabbit mammary gland at midpregnancy has been published (5). The secretory apparatus of the parenchymal cells is poorly developed with a scattered rough endoplasmic reticulum but abundant free ribosomes, a small Golgi complex and a few dense granules in Golgi vesicles and apical vacuoles. Some cells have several small lipid droplets and a more elaborated secretory apparatus.

Dissociation sequence

The overall procedure has been summarized in Table I, and the different steps have been monitored under low magnification phase contrast or Nomarski optics. After collection of the gland in KRB (step 1), 5 ml of enzyme mixture was injected into the interstitium of the gland (1 g WW), and the distended gland with the enzyme mixture was incubated at 37°C under 95% O₂ and 5% CO₂ for 1 h (step 2). During this first enzyme digestion (step 3), the gland loosened due to the digestion of tissue stroma. Adipose cells were liberated and formed a fat layer, which could be easily removed from the incubation medium. The tissue was then incubated in KRB containing 1 mM EDTA for 10 min at 37°C (step 4). The divalent cations were reintroduced by an incubation for 10 min in KRB containing 0.1 mM

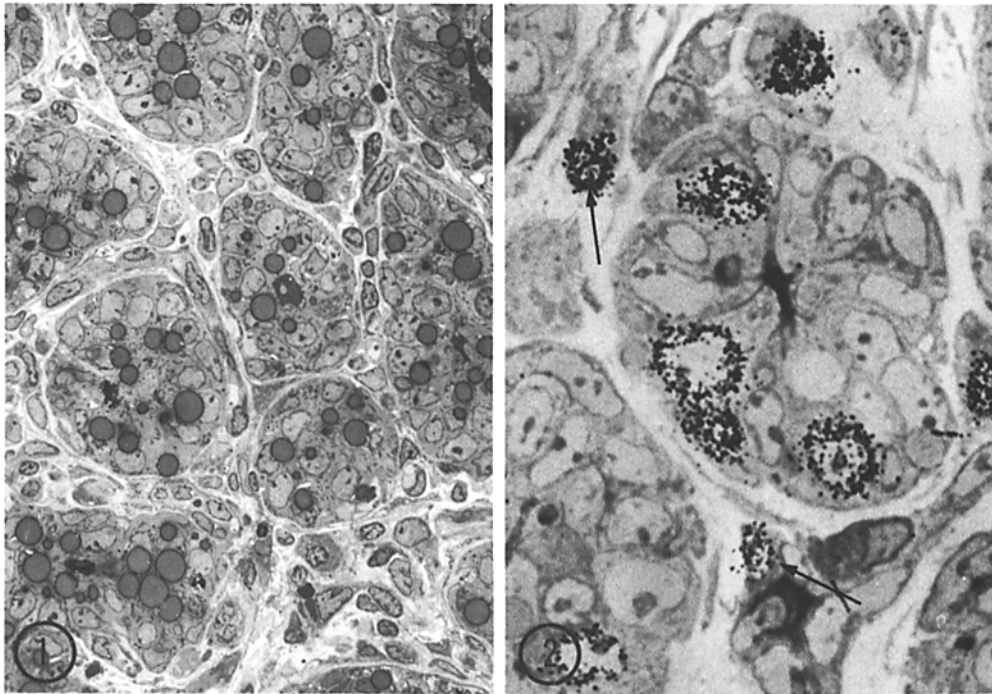


FIGURE 1 Light micrograph of a 0.5 μm section of the mammary gland from a midpregnant rabbit (day 18 of gestation). Note the presence of numerous acini, the lumina of which are filled with dense secretion product. Many epithelial cells contain large fat droplets. $\times 450$.

FIGURE 2 Light microscope autoradiogram of a 0.5 μm section through the mammary gland (day 18 of gestation) labeled for 4 h with [^3H]thymidine as described in Materials and Methods. The silver grains are associated with several nuclei of parenchymal cells. Labeled connective tissue cells are indicated by arrows. $\times 1,300$.

TABLE II
Distribution of Cell Types in the Mammary Gland of Midpregnant Rabbits

	Total nucleated cells	
	%	
Epithelial cells	56.0 \pm 3.0	
Epithelial cells containing large fat droplets	36.0 \pm 1.5	
Myoepithelial cells	12.0 \pm 1.5	
Interstitial cells	32.0 \pm 3.0	
Plasma cells	2.5 \pm 0.8	

1,000 nucleated cells were counted in sections prepared from the mammary glands of five different rabbits (day 18 of pregnancy). Values represent the mean \pm SEM.

Ca^{++} and 1.2 mM Mg^{++} (step 5). Vacuolization appeared in some cells after EDTA treatment and persisted after the reintroduction of divalent cations. After the second enzymatic digestion (step 6), groups of acini and dispersed cells

formed a fine suspension which was pipetted up and down in a siliconized, fire-polished Pasteur pipette (step 7). The cell suspension was filtered through a 20- μm mesh nylon gauze to remove any undissociated tissue, cell clumps and connective tissue (step 8). The cell suspension (5 ml) was then layered over 8-ml cushions of 4% BSA in KRB containing 2.5 mM Ca^{++} and 1.2 mM Mg^{++} . After centrifugation at 100 g for 5 min, the intact cells formed a packed pellet, whereas cell debris, fat cells, and enzymes were found in the supernate, which was discarded. The cells were washed twice by resuspension in the same solution and centrifuged (step 9). The cell suspension consisted of epithelial and interstitial cells, and the distribution of the different cell types reflected that of intact tissue. Over 95% of the cells excluded Trypan blue. The yield of washed, freshly dissociated cells was 30–40% based on DNA content as compared to that in intact tis-

sue. From 1 g of tissue, 10^8 cells were recovered. Recoveries were monitored at each step of the dissociation procedure and are summarized in Table III. The cells were finally suspended in 35% BSA in KRB containing 2.5 mM Ca^{++} and 1.2 mM Mg^{++} at a concentration of 5×10^7 cells/ml (step 10).

Enrichment of Epithelial Cells by Isopycnic Centrifugation

Dissociated cell suspensions can be enriched in epithelial cells by a relatively simple equilibrium density procedure adapted from Steinman and Cohn (24) (Table I, steps 11–13). The cells, after centrifugation to equilibrium, can be roughly separated into three main fractions: a light fraction banding at $\rho = 1.045$ consisting of epithelial cells rich in fat droplets, a heavy fraction ($\rho = 1.075$) containing some epithelial cells without large lipid droplets and connective tissue cells, and a pellet formed by erythrocytes, interstitial cells and a few epithelial cells. For each of the three fractions, the total number of nucleated cells, the percentage of Trypan blue-positive cells, the cell types present

as observed in 0.5- μm Epon sections (light microscopy), and thin sections (electron microscopy) were determined, and the results are summarized in Table IV.

Structural Aspects of the Cell Fractions

The three cell fractions have been examined by light microscopy and the different cell types of each fraction identified by electron microscopy. The light fraction consists almost exclusively of epithelial cells, 70% of which contain large lipid droplets (Fig. 3 a and b), whereas the heavy fraction (Fig. 3 c) and the pellet (Fig. 3 d) contain mainly myoepithelial cells, connective tissue cells, including plasma cells, fibroblasts, and a few parenchymal cells. Erythrocytes and Trypan blue-positive cells were recovered from the pellet. Although 95% of the cells in the light fraction excluded Trypan blue, some cytoplasmic blebs and vacuoles were observed. At the electron microscope level, cells in the light fraction present the same cell organization when compared to the parenchymal cells *in situ*. Most cells, however, are rounded up but retain their polarity with regard to

TABLE III
Recoveries Based on DNA Content

Steps	Treatment	DNA content		%
		μg	μg	
1	Starting material 1 g (wet weight) of gland	2,000 \pm 100 ($n = 16$)	0	
2	Dissection of fat and connective tissue	1,760	240 \pm 17 ($n = 5$)	12
3	1st enzyme digestion	1,703	57 \pm 5 ($n = 5$)	3
4	EDTA treatment	1,506	197 \pm 13 ($n = 5$)	10
5	Ca^{++} replacement	1,430	76 \pm 6 ($n = 4$)	4
6	2nd enzyme digestion	783	647 \pm 26 ($n = 5$)	32
7	Pipetting		Total loss	61
8	Filtering			
9	BSA wash			
10	Cells recovered after the BSA wash	705 \pm 7 ($n = 5$)		

Recoveries of a typical dissociation protocol. Results are expressed in mean values \pm SEM. In steps 1–4, DNA has been measured in the incubation media after the lobules and the cells have been removed by centrifugation. To obtain a homogeneous solution, the tissue fragments were digested with crude collagenase for 5 h. The overall yield is 35% when determined from the recovered cells (step 10) and 39% when calculated from the balance sheet. The difference is explained by an incomplete recovery in step 8, some material remaining bound to the filter.

TABLE IV
Enrichment of Parenchymal Cells by Isopycnic Centrifugation

Fraction <i>s</i>	Total cell applied	Total epithelial cells
	%	%
Total	100	100
Light $\rho = 1.045$	51.0 ± 1.5	80-95
Heavy $\rho = 1.075$	23.0 ± 3.5	15-20
Pellet $\rho = 1.100$	25.0 ± 5.0	<5

Mean values \pm SEM of five experiments.

the distribution of cytoplasmic organelles. Two different types of parenchymal cells are present in the light fraction. The majority of the cells (>70%) have a large fat droplet and a poorly developed secretory apparatus (Fig. 4). A few elements of rough endoplasmic reticulum are found around the nucleus in the basal pole of the cell. Parenchymal cells (25%) with smaller and numerous lipid vacuoles present a more developed rough endoplasmic reticulum occupying the same position in the cell when compared to the large droplet-containing cells. The Golgi complex is developed and contains protein granules which can also be recognized in small apical vacuoles (Fig. 5). In most cells, especially those filled with large lipid droplets, no specializations of the apical plasma membrane, including microvilli or remnants of tight junctions, are observed.

Cells present in the heavy fraction and in the pellet were identified by electron microscopy. The heavy fraction contained a few parenchymal cells with a developed secretory apparatus and small lipid vacuoles. It was enriched in myoepithelial cells identified by the presence of typical thin and thick filaments (Fig. 6). Plasma cells (Fig. 7) were recovered in the pellet.

Incorporation of [^{14}C]Leucine

Isolated parenchymal cells (light fraction) incorporated [^{14}C]leucine into TCA-insoluble proteins at a rate of 10^4 dpm per μg DNA per 210 min.

The rate of incorporation was linear between 30 and 210 min (Fig. 8).

[^3H]Thymidine incorporation

When freshly dissociated epithelial cells were exposed to [^3H]thymidine for 4 h, 15% of the cells exhibited nuclear labeling as determined by autoradiography. This percentage of labeled cells did not significantly differ from the labeling in intact tissue (Table V). Approximately 90% of the labeled cells are epithelial both in freshly dissociated cell preparations and in intact tissue (Fig. 2). This is reflected in the high labeling index in the light fraction, as compared to the heavy fraction. These results are in good agreement with data from [^3H]thymidine incorporation into TCA-insoluble material.

Oxygen Consumption

The analysis of oxygen depletion rates of freshly dissociated cells or enriched parenchymal cells (light fraction) indicated that the cells were viable, although a 4-h incubation period was required for the cells to achieve optimal oxygen consumption (Fig. 9). The depletion rate was 0.58 ± 0.05 nmol $\text{O}_2/\text{min}/10^6$ cells 1 h after dissociation, and increased to 1.83 ± 0.29 nmol $\text{O}_2/\text{min}/10^6$ cells and remained steady after 4-h incubation in M 199 at 37°C with 95% air and 5% CO_2 . The oxygen consumption of parenchymal cells (light fraction) and interstitial cells (heavy fraction) was not significantly different from that of freshly dissociated cells.

Composition of Intracellular Cations

Table VI shows the results for intact tissue, freshly dissociated mammary gland cells and rabbit erythrocytes. The cells were incubated for up to 180 min at 37°C in M 199 containing 10% normal rabbit serum and remained in steady state with respect to water and ions. The content and concentration of Na^+ and K^+ ions were also measured in the light and heavy fractions obtained after isopycnic centrifugation and did not differ significantly from the values for freshly dissociated cells. The values for rabbit erythrocytes served as a control.

Culture of Dispersed Parenchymal Cells

Cultures established in Petri dishes at a density of 10^6 cells/ cm^2 were maintained for up to 5 days. At day five, the parenchymal cells formed a mono-

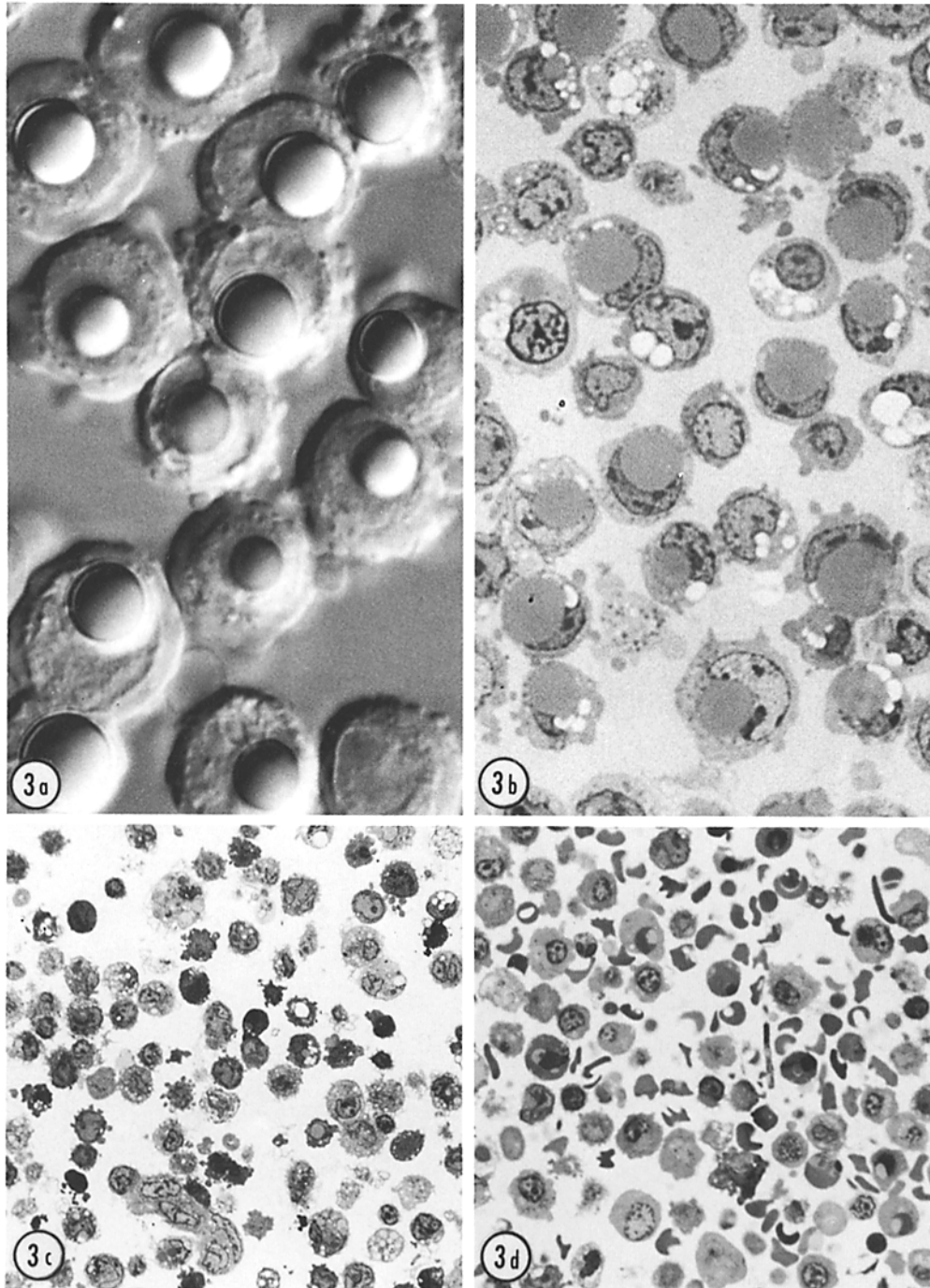


FIGURE 3 Light micrographs of different cell fractions obtained after isopycnic centrifugation of dispersed mammary gland cells. (a) Light fraction ($\rho = 1.045$). Note the presence of numerous fat-containing cells. Differential interference microscopy of living cells. $\times 2,000$. (b) Light fraction. $0.5 \mu\text{m}$ -thick section of cells fixed and processed immediately after the end of the centrifugation. Some vacuoles in the parenchymal cells appear empty, probably a consequence of lipid extraction during dehydration and embedding. $\times 1,300$. (c) Heavy fraction ($\rho = 1.075$). This fraction consists of a few epithelial cells with large fat droplets, parenchymal cells containing several small lipid vacuoles, and cells identified as connective tissue cells, including myoepithelial cells and fibroblasts. $\times 550$. (d) Pellet containing erythrocytes, plasma cells, interstitial cells and a few fat-containing cells. $\times 550$.

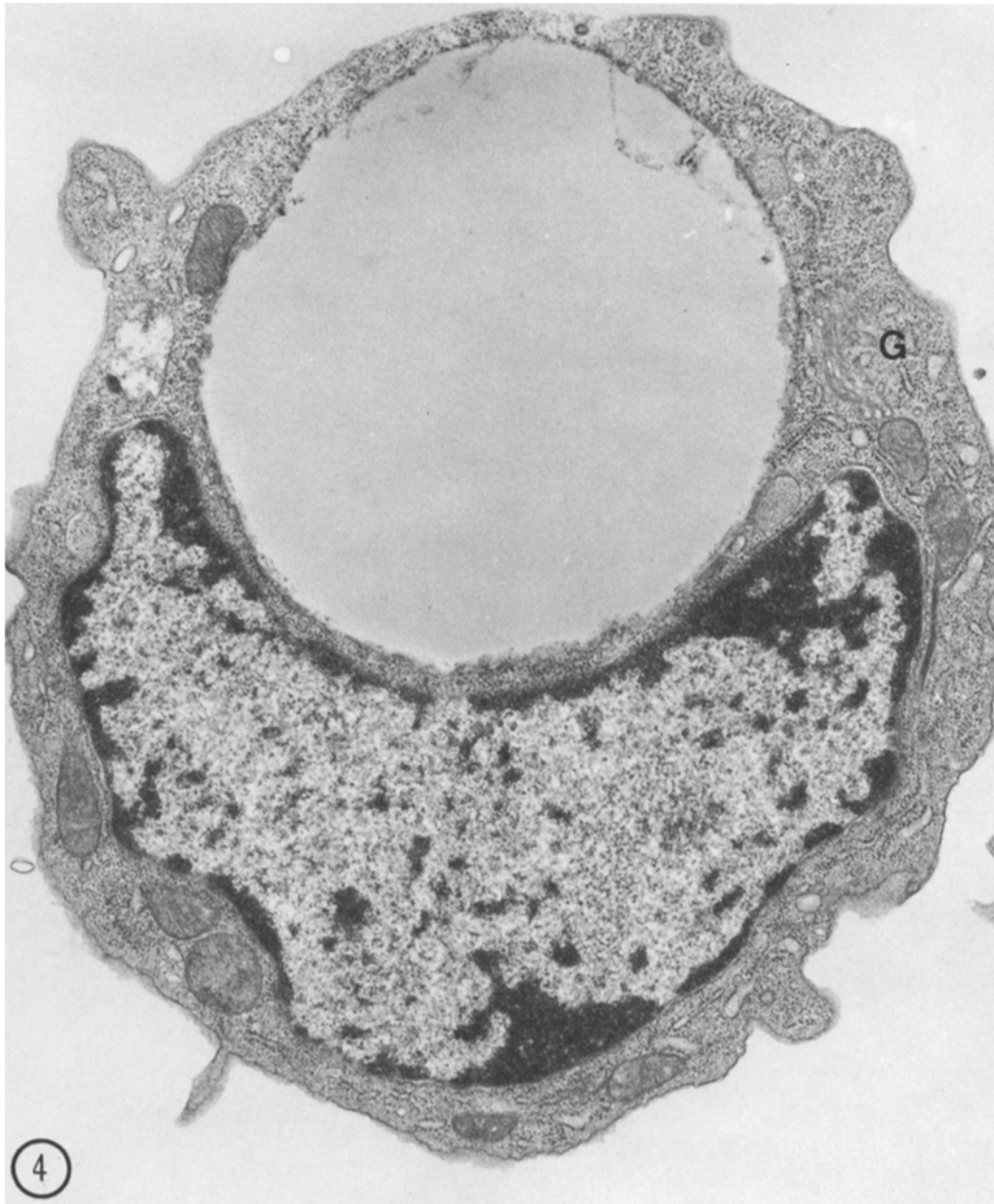


FIGURE 4 Electron micrograph of a section through a cell from a light fraction. A typical cell containing a large fat droplet is shown. The secretory apparatus is poorly developed: the rough endoplasmic reticulum is scattered through the cytoplasm, and the Golgi complex (G) is small. Stained with uranyl acetate and lead citrate. $\times 14,000$.

layer. Junctional complexes including desmosomes and tight junctions were clearly identified in the electron microscope (Fig. 10). The cells contain lipid vacuoles and dense granules. In sections

perpendicular to the plane of the monolayer, microvilli were observed on the surface facing the incubation medium indicating that cell polarity is retained in culture (Fig. 11).

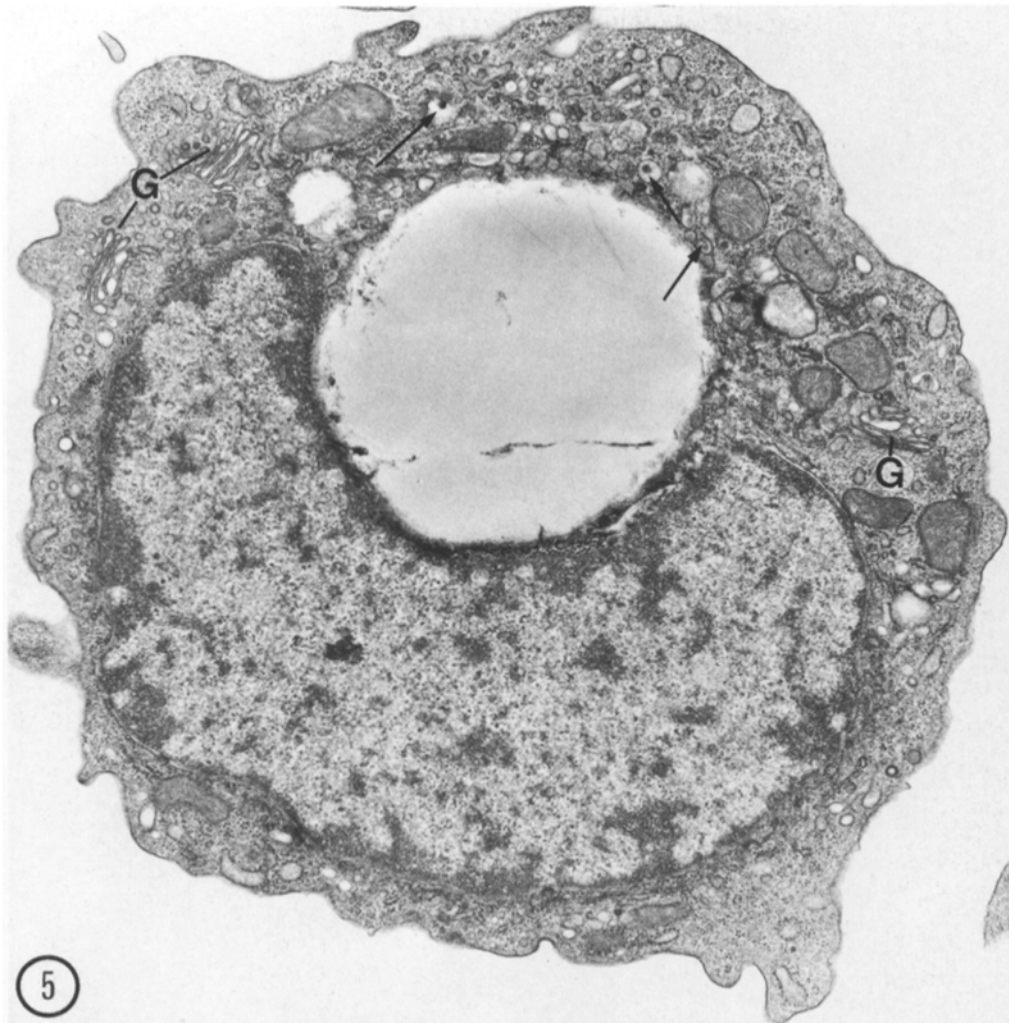


FIGURE 5 Electron micrograph of a section cut through a more mature epithelial cell present in the light fraction. Several Golgi complexes G are present, and dense granules similar to those seen *in vivo* are enclosed in apical vesicles (arrows). $\times 12,500$.

DISCUSSION

The results suggest that the mammary gland of the midpregnant rabbit can be dissociated into individual cells by a combination of enzyme digestion, chelation of divalent cations, and mechanical shear forces generated by flask shaking and pipetting. The dissociation procedure of Amsterdam and Jamieson was followed since the different steps in the dissociation procedure had been carefully examined (1) and a population of viable cells was obtained in good yield (2). Modifications, however, had to be introduced in order to digest the extensive connective tissue of the rabbit mam-

mary gland. Therefore, the first digestion time was increased from 10 min to 60 min and elastase was added to the enzyme mixture. With this modification, the procedure yields a suspension of heterogeneous cells that have a high degree of viability as assessed by biochemical, physiological, and morphological means. The yield is 30–40% based on DNA determinations as compared to intact tissue. The major losses occur during EDTA treatment and mechanical disruption (Table III). These can be minimized by lowering the concentration of EDTA from 2 mM to 1 mM and by shortening the incubation time. The distribution of cell types

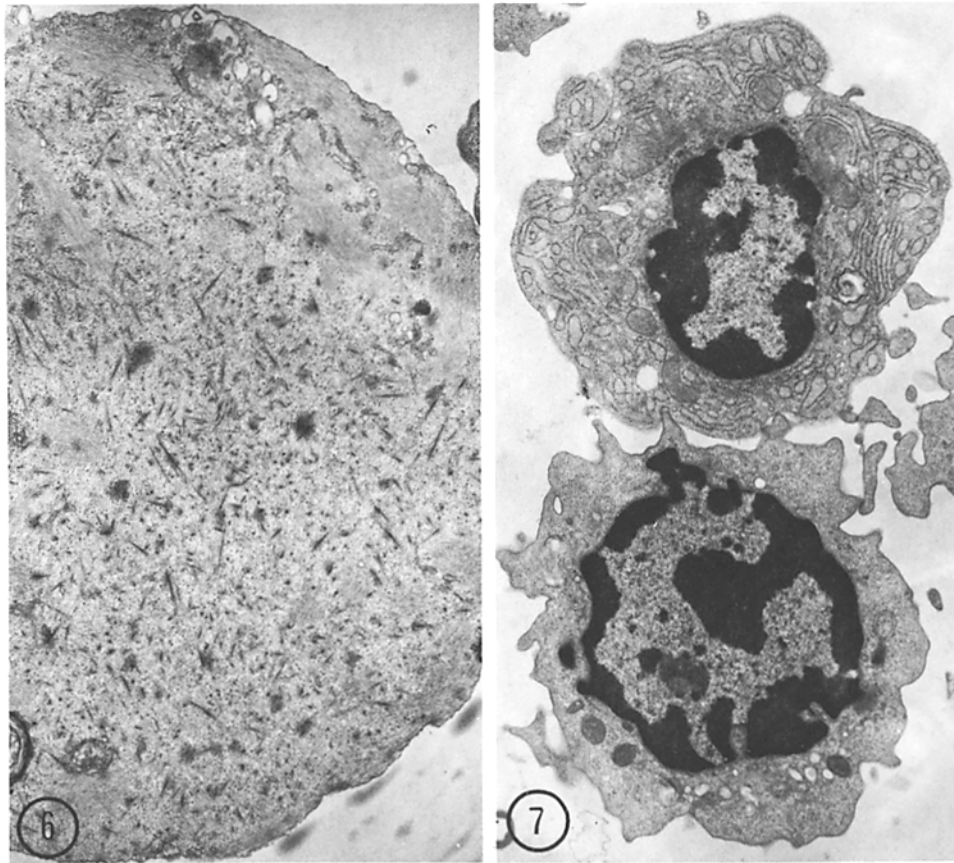


FIGURE 6 Electron micrograph of a section through a myoepithelial cell from the heavy fraction. Typical thick and thin microfilaments cut in different planes of section are present in the cytoplasm. Stained with uranyl acetate and lead citrate. $\times 13,000$.

FIGURE 7 Electron micrograph of a section through cells from the pellet. A plasma cell (top) and a lymphoid cell (bottom) are shown. Stained with uranyl acetate and lead citrate. $\times 3,200$.

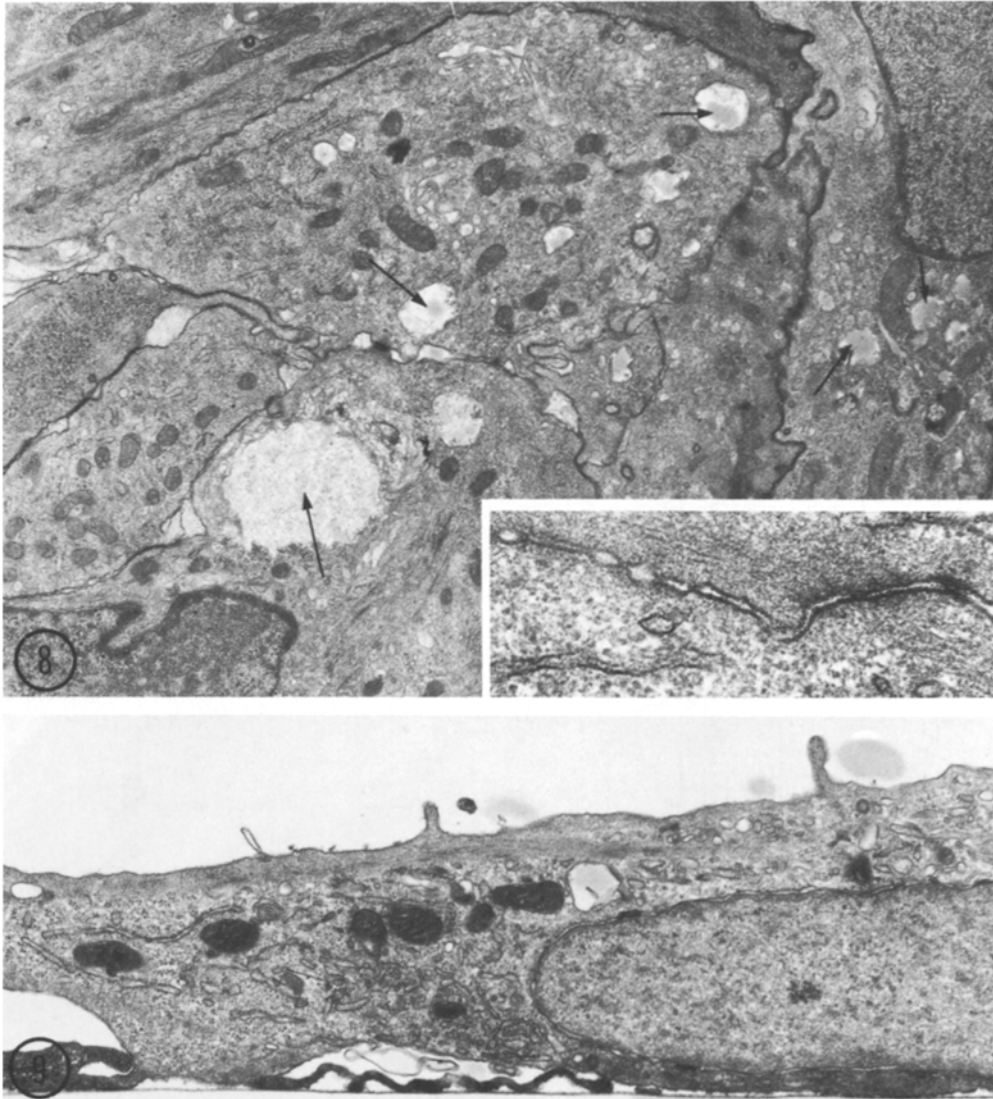
after dissociation parallels that of intact tissue, indicating no selective loss of a given cell type.

A variety of techniques used to dissociate mammary gland tissue into cell suspension have been reported (21, 29) and have recently been reviewed by Kerkof and Abraham (14). In most studies, the heterogeneity of the cell population obtained has not been analyzed and no attempts have been made to enrich the cell suspension in parenchymal cells. Such an enrichment is highly desirable for metabolic investigations or for hormone receptor analysis. In all stages of differentiation of the mammary gland (virgin, midpregnant, lactating, and regressing), the myoepithelial cells and the connective tissue cells represent a significant part of the cell population. Thus, at midpreg-

nancy in the rabbit, only $\sim 60\%$ of the cells are epithelial.

Two specific methods for enriching the cell suspension in parenchymal cells obtained from lactating mammary gland have been published (14, 22). In the procedure of Kerkof and Abraham (14) the cell suspension is freed of fat cells, whereas Pretlow et al. (22) achieved an enrichment in acinar cells exhibiting alkaline phosphatase activity. The yield, however, obtained with velocity sedimentation in an isokinetic gradient of Ficoll is one order of magnitude lower than that of the present procedure.

To achieve an enrichment in epithelial cells, we took advantage of their low buoyant density and separated them from the myoepithelial cells and



FIGURES 8 and 9 Electron micrographs of parenchymal cells cultured at a density of 10^6 cells per cm^2 for 5 days in M 199 complemented with 10% autologous midpregnant rabbit serum. Stained with uranyl acetate and lead citrate.

FIGURE 8 Section parallel to the plane of the dish. The cells form a monolayer with junctional complexes. Lipid vacuoles are scattered through their cytoplasm (arrows). At higher magnification (insert, $\times 40,000$), a desmosome and a tight junction are identified. $\times 10,000$.

FIGURE 9 Section perpendicular to the plane of the monolayer. Microvilli are present on the cell surface facing the culture medium. $\times 15,000$.

the connective tissue cells by isopycnic centrifugation. In the rabbit, at midpregnancy (day 18-22), there is a striking increase in the rate of fatty acid synthesis, due almost entirely to medium-chain fatty acids which are characteristic of rabbit milk

(18, 25). This intense synthesis leads to marked changes in the morphological structure of the parenchymal cells, reflected by the formation of large lipid droplets in the supranuclear region (5). The onset of lipogenesis in the rabbit precedes

protein synthesis by 4–5 days. When protein synthesis starts, the parenchymal cells have already secreted the large fat droplets which, in intact tissue, can be recognized in the acinar lumen. At that stage, numerous smaller fat droplets are present in the cytoplasm of the epithelial cells. The large lipid droplets confer on the cells their low buoyant density and facilitate their separation from connective tissue cells by isopycnic centrifugation.

When freshly dissociated cells are centrifuged to equilibrium on a continuous BSA gradient, two main bands in addition to a pellet are observed: a light fraction ($\rho = 1.045$) and a heavy fraction ($\rho = 1.075$). Following this procedure the total cell recovery based on the count of the nucleated cells or DNA determinations is between 85–100%. The light fractions contain more than 90% morphologically identified parenchymal cells. The other cells represent some interstitial cells, i.e. myoepithelial cells, remaining bound to parenchymal cells during the separation procedure.

The functional integrity of the epithelial cells (light fraction) was assessed by biochemical and morphological tests. The isolated parenchymal cells retain the characteristic morphological features of their counterparts *in situ* including the

distribution of the cytoplasmic organelles and fat vacuoles. The separation procedure, however, selects for cells with a high content of fat vacuoles which is reflected by the higher proportion of cells containing large lipid droplets in the light fraction when compared to the intact tissue or to freshly dissociated cell suspensions. As pointed out above, the cells in the light fraction might be at an earlier stage of differentiation as compared to the epithelial cells of the heavy fraction.

The incorporation of [^{14}C]leucine into the light-fraction cells is linear over a period of hours and parallels that of intact tissue. The rate of DNA synthesis, as reflected by [^3H]thymidine incorporation and nuclear labeling, is high both in intact tissue and in dissociated cells (Table IV). In intact

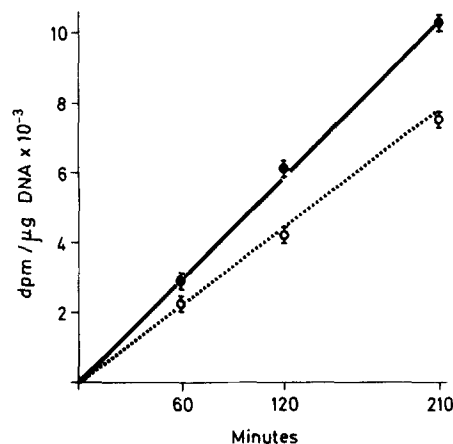


FIGURE 10 Incorporation of [^{14}C]leucine into protein of purified mammary gland parenchymal cells (light fraction). The cells ($10^6/\text{ml}$) were incubated in M 199 or normal rabbit serum (●—●) or insulin (10^{-7} M) (○- -○) and $1 \mu\text{Ci}/\text{ml}$ [^{14}C]leucine. The incorporation of [^{14}C]leucine into TCA-insoluble proteins of the parenchymal cells was measured as described in Material and Methods. The rate of incorporation into both incubation media is linear over 210 min. The data of 3 experiments are expressed as mean values \pm SEM.

TABLE V
Distribution of Labeled Cells after [^3H]Thymidine Incorporation

	Epithelial cells/total nucleated cells	Labeled cells/total nucleated cells	Labeled epithelial cells/total nucleated cells
	%	%	%
Intact mammary gland tissue	55.5 ± 2.8	14.4 ± 1.0	12.9 ± 1.0
Freshly dissociated cells	51.4 ± 4.2	15.4 ± 2.1	12.5 ± 0.8
Light fraction	95.2 ± 5.3	21.0 ± 3.7	20.3 ± 3.8
Heavy fraction	18.4 ± 1.8	7.1 ± 0.1	5.0 ± 0.1

Mean of three experiments \pm SEM. In each experiment, 500 nucleated cells were counted.

TABLE VI
Cellular Composition of Intracellular Monovalent Cations

	Intracellular ion contents		Intracellular ion concentrations	
	Na ⁺	K ⁺	Na ⁺	K ⁺
	mmol/kg DW		mmol/liter intracellular H ₂ O	
Intact tissue ($n = 5$)	309 ± 6	190 ± 10	80 ± 4	127 ± 7
Freshly dissociated cells ($n = 6$)	110 ± 8	257 ± 4	55 ± 4	129 ± 6
Rabbit erythrocytes ($n = 5$)	44 ± 1	285 ± 6	25 ± 1	166 ± 4

Values represent the mean \pm SEM; n = number of experiments.

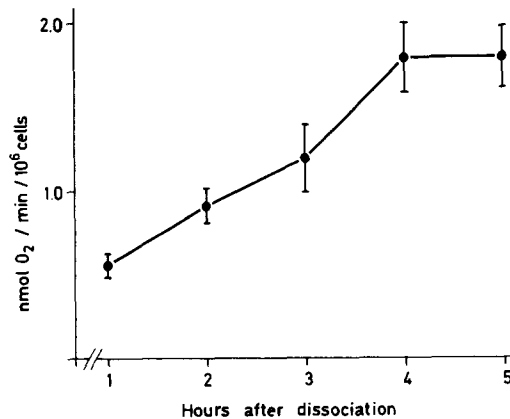


FIGURE 11 Oxygen consumption rates for dissociated mammary gland cells. $5\text{--}15 \times 10^6$ cells in protein free M 199 were introduced into the electrode chamber. Oxygen depletion rates were measured at each time point during a 15-min period. The rates were related to the DNA content in each sample and expressed as $\text{nmol O}_2/\text{min}/10^6$ cells, assuming $7 \mu\text{g DNA per } 10^6$ cells. Rates in light and heavy fractions did not differ from those of total cell population represented in the figure. The results of three experiments are expressed in mean values \pm SEM.

tissue, over 90% of the labeling is associated with parenchymal cells, a further indication of the high proliferation rate of the tubulo-alveolar system of the rabbit mammary gland at midpregnancy. Parenchymal cells in freshly dissociated cell preparations and in both purified fractions also represent the major labeled cell population.

The results of the study of oxygen consumption rates for isolated cells indicate that cells incubated for a 4-h period consume 3.5 times more oxygen than cells incubated immediately after the end of the dissociation procedure. The maximal rates are maintained for a period of hours. The rates for parenchymal cells (light fraction) or for interstitial cells (heavy fraction) do not differ significantly from rates reported for the total cell population. The consumption rate for mammary gland parenchymal cells is $1.8 \text{ nmol}/\text{min}/10^6$ cells and compares well with rates reported for mouse gastric chief and mucous cells, i.e., $2.8 \text{ nmol}/\text{min}/10^6$ cells (23). The temperature (25°C) used in our incubation conditions could explain the lower rate observed in mammary gland cells. These rates are probably typical of secretory cells, which are relatively poor in mitochondria, and contrast with high values reported for gastric parietal cells, $15.2 \text{ nmol}/\text{O}_2/\text{min}/10^6$ cells (23), or hepatocytes, $22.3 \text{ nmol O}_2/\text{min}/10^6$ cells (10).

The intracellular concentration of monovalent cations in dispersed cells remains constant during a 180-min incubation period, although the oxygen consumption rate increases during the same period, an indication of metabolic recovery. The sodium concentration is $55 \text{ mmol}/\text{l}$ intracellular water, and the potassium concentration $129 \text{ mmol}/\text{l}$ iter. The latter compares well with potassium concentrations in intact tissue (Table VI), whereas the sodium concentration differs significantly. The accuracy of the calculated intracellular cation concentrations in intact tissue depends mainly on the determination of extracellular space, including the interstitial, blood and milk spaces, which may not be freely permeable to [^{14}C]inulin even after prolonged incubation periods. Their ion content, especially Na^+ , may contribute to alter the intracellular profile. In the midpregnant rabbit, the ion composition of the milk space is not known and is difficult to measure: attempts by Peaker and Taylor (20) to measure in vivo the milk space in lactating rabbits have failed, since, in contrast to those in other species (15, 16), the ducts of one gland empty to the exterior independently and [^{14}C]lactose, a marker of the milk space, diffuses backwards. Consequently, the exact degree of contamination of intracellular Na^+ content by extracellular Na^+ cannot be determined. Therefore, the monovalent cation concentration that we have measured in dispersed cells probably better reflects the true intracellular composition, since the milk space does not have to be taken into account.

Finally, in short term culture, the cells grow, reestablish junctional complexes characteristic of the mammary epithelium, and maintain polarity. In addition, preliminary results indicate that the cells respond to insulin and steroids by proliferating and to lactogenic hormones by initiating milk protein synthesis and secretion (see footnote 1, page 391).

I thank Drs. J. D. Jamieson and G. E. Palade for their criticism, encouragement, and support during the course of this work, and Miss L. Racine and J. Pfeiffer for their excellent technical assistance. I am very grateful to Dr. M. Rossier who did the intracellular ion concentration measurements and to Dr. H. Engers for revising the manuscript.

This study was supported by a U.S. Public Health Service research grant A1 21 166 and by the Fonds National de la Recherche Scientifique Suisse, requête 3.514-0.75.

Received for publication 8 July 1976, and in revised form 22 October 1976.

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