A SCANNING ELECTRON MICROSCOPE STUDY OF THE LACTATING MAMMARY GLAND

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The study of internal soft tissue with the scanning electron microscope poses special technical problems of fixation, cleaning, and dehydration (3, 4, 6, 7). For the dried, prepared tissue to be biologically significant, the surface configurations and interrelationships of its constituent cells must be preserved in a state similar to the way they appear in life. At the same time, the tissue surface, in order to be accessible to scanning, must be washed quite free of any residue of the fluids that normally bathe it. Since tissue fluids contain proteins that are preserved by the same fixatives that preserve cells, these requirements may conflict. The technique to be described here was developed to solve these problems for the study of changes in the natural, internal surfaces of the mouse mammary gland. It has successfully exposed the free surfaces of cells composing the secretory alveoli, revealing some unexpected configurations as well as features predictable from transmission electron microscopy (2, 8, 9).

The mouse mammary gland in the lactating state is spongelike in appearance by light microscopy. It is composed of a dendritic system of ducts ending in clusters of alveoli; lumina of alveoli and ducts are at all times filled with milk containing abundant fat and protein in suspension or solution. Transmission electron microscopy (8, 9) shows that particles of milk protein within Golgi-derived vesicles are discharged from secreting cells by fusion of the vesicle membrane with the cell membrane bordering the lumen. Fat droplets within the cytoplasm lack limiting membranes and are secreted by the pinching off from the cell surface of the droplet enclosed in a thin layer of cytoplasm and cell membrane. Microvilli are irregularly dispersed over the luminal surfaces of alveolar and ductal cells.

MATERIALS AND METHODS

BALB/cCrgl female mice at 13 ± 2 days of lactation were anesthetized by injection of Nembutal 1½ hr after removal of the nursing pups. The glands were surgically removed, rinsed in isotonic saline, and cut with a razor blade into strips 1–2 mm thick. (Living mammary tissue is soft, slippery, and stringy with connective tissue; cutting at this stage invariably macerates the cut surfaces.) The strips were placed in a 2% solution of glutaraldehyde in glass-distilled water for 24 hr; the unbuffered glutaraldehyde (Polysciences, Inc., Rydal, Pa.) solution usually had a pH of 6–7.

After fixation, the tissue strips were bisected longi-

tudinally with a razor blade, exposing two freshly cut faces from each strip (the tissue now being firm enough to slice cleanly). These strips were rinsed in distilled water and bathed in 16% glycerol for 24 hr and then in 20% ethanol for 24 hr. The prolonged glycerol and ethanol washes helped to remove most milk components from exposed lumina. Dehydration in ethanol (50, 75, 95 100, 100%, 15 min each) was followed by replacement of the ethanol with Freon 113 in three steps (50% Freon in ethanol, two washes of 100% Freon, 15 min each). The tissue strips were dried by the Freon critical-point method of Cohen et al. (1) and were then mounted on specimen stubs and rotary coated in a vacuum evaporator with 15 ± 5 nm of (0.9999) pure gold at a vacuum of 0.3 ± 0.2 μm Hg.

Freeze-drying and air-drying were used in some of our early experiments with unsatisfactory results but have not been tested with our current fixation and washing procedures. We have not tried CO_2 critical-point drying, since the Freon dryer operating at relatively low pressure and temperature yields well preserved tissue, with only about 15 min needed to complete one drying run; other advantages of the Freon method are discussed by Cohen et al. (1).

The prepared tissue was examined in a Stereoscan Mark IIa scanning electron microscope. Magnification was read directly from a meter calibrated to an accuracy of $\pm 3\%$.

RESULTS AND DISCUSSION

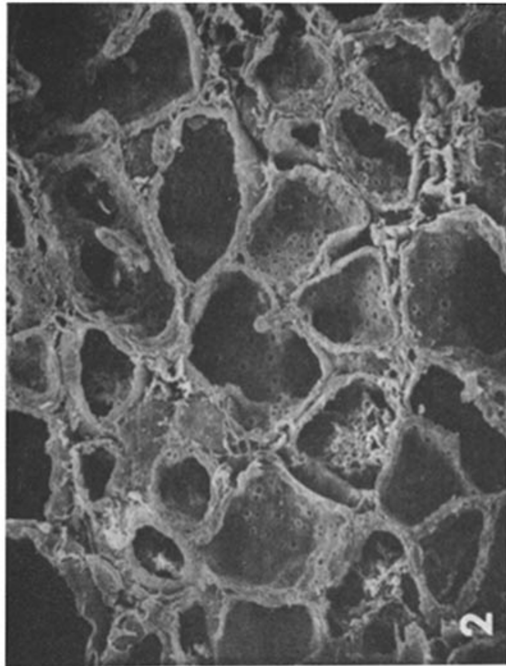
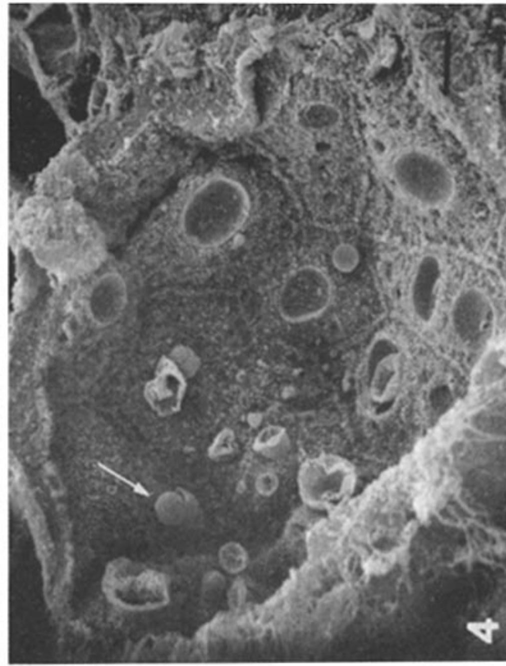
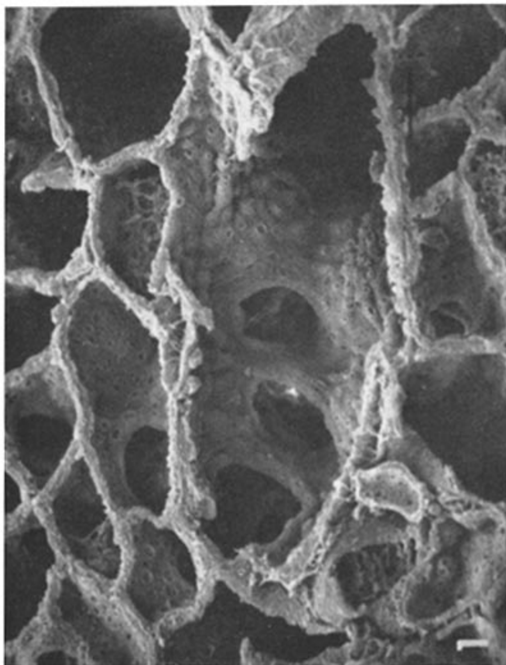
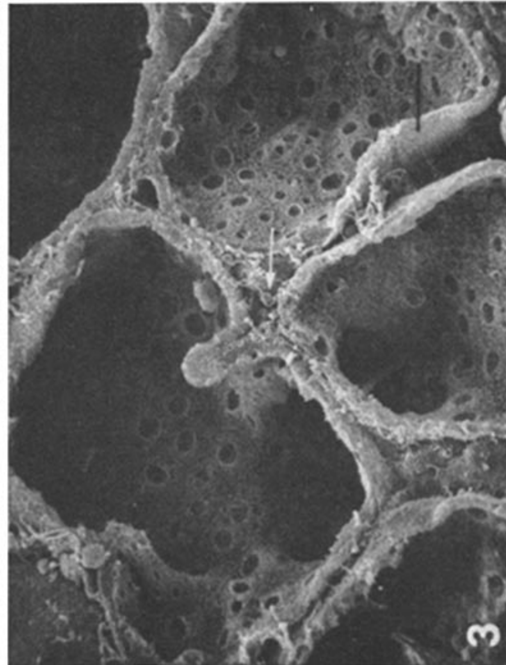
The technique yielded good preservation of the over-all morphology of the mammary gland. The profiles of alveoli and ducts seen in Figs. 1 and 2 are like those familiar from histological sections. The turgor developed in the gland during 1½ hr without suckling is evident from the close packing of inflated alveoli and the thinness of alveolar walls in Fig. 3. Connective tissue fibers are compacted between alveoli and ducts, and capillaries, occasionally still enclosing erythrocytes, may be identified.

Fine structure of cell surfaces, the ultimate test of this preparation technique, appears to be faithfully and cleanly preserved according to all available standards of comparison (Figs. 4–8). Microvilli, abundantly but irregularly scattered over luminal cell surfaces, measure (e.g., in the stereomicrograph in Fig. 7) about 100 nm in diameter, in good agreement with measurements from transmission micrographs of thin sections. Figs. 5–8 and other micrographs show little or no residue of milk other than small, spherical particles within the size range of milk protein granules seen in transmission micrographs. However, we cannot be certain that the background between microvilli represents the natural membrane surface (or glycocalyx) rather than a thin precipitate of milk constituents deposited during drying.

Most of the milk fat was extracted by the alcohol dehydration. The shallow craters present on nearly every cell surface mark the sites of fat droplets that presumably were in the process of secretion but still partly embedded in the superficial cytoplasm at the moment of fixation. Extraction of the lipid apparently caused rupture of the overlying cell membrane, which either collapsed within the empty crater or was torn away (Fig. 5). Some strips of tissue after drying for scanning were immersed in propylene oxide and embedded in epoxy resin for thin sectioning. Transmission micrographs showed craters and ruptured membranes at cell surfaces; fat droplets more deeply embedded in the cytoplasm had not been extracted.

Modified Karnovsky's fixative (8), used in our laboratory for transmission electron microscopy, was tried in place of unbuffered glutaraldehyde, but apparently it fixed the milk too well, leaving an evident residue over luminal surfaces. Osmium tetroxide fixation or postfixation preserved large fat droplets in cells and lumina and resulted in considerable disruption of the tissue during processing.

The classical description of the lactating mammary gland as resembling bunches of grapes does not fit the three-dimensional structure revealed by scanning microscopy. Figs. 1–3 show that alveoli are extensively confluent with each other and that many individual chambers have several exits. At higher magnification in Fig. 4, the mosaic of flattened cells composing an engorged alveolus is visible; borders between cells are distinctly marked by bands of close-set microvilli. Such bands are shown at higher magnifications in Figs. 5 and 6, and stereoscopically in Fig. 7. The consistent occurrence of microvilli on both sides of apical cell junctions is apparent in transmission electron micrographs of sections, but their conspicuously greater density along junctional lines becomes evident only in surface view. Further, since the microvilli stand at various angles to the cell surface, thin sections give the impression of variability in length and shape. Scanning micrographs reveal a much greater consistency in diameter than we had expected. Examination of stereomicrographs

with a mirror stereoscope having a parallax bar[1] indicates that surface microvilli typically are 400–500 nm long, while border microvilli may be 300 nm longer.

The brightness of microvilli in Figs. 5–8 is a beam artifact. When the primary electron beam is incident on a microvillus, it can penetrate through the microvillus and still strike the underlying cell surface. Secondary electrons are emitted where the beam enters the microvillus, where it leaves the microvillus, and from the cell surface. Where the primary beam strikes the cell surface directly, there is only one source of secondary electrons. Hence, the microvilli emit more secondary electrons than the flat surface and appear bright against a duller background.

The three-dimensional view of secreting mammary epithelium provided by the scanning electron microscope thus confirms observations on surface structure from light and transmission electron microscopy and yields new information on the form and arrangement of microvilli, the process of fat secretion, and the shapes and interrelationships of alveoli and ducts. These surface features provide good indices of the physiological state of the gland; normal and pathological changes in such features may prove peculiarly accessible to study by this technique. Our investigation is being extended to comparison of prelactating with suckled and unsuckled lactating gland and to preneoplastic nodules and mammary adenocarcinomas.

[1] The amount of displacement of a point in a pair of micrographs when the sample is tilted through a given angle is the amount of parallax of the point. True lengths can be measured from stereophotographs, using instruments and techniques employed in the analysis of aerial photographs (5).

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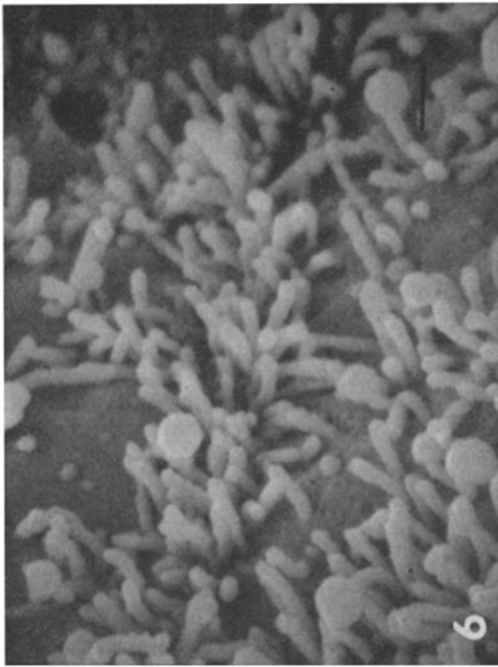
FIGURES 1 and 2 Survey micrographs of two parts of the same block of mammary tissue; the exposed surface was cut with a razor blade after fixation as described in the text. An elongate terminal duct with four visible ports occupies the center of Fig. 1. The close-packed alveoli make up the bulk of the tissue and, especially in Fig. 2 (center and upper right), are seen to communicate extensively with one another and often to have more than one opening. Fig. 1, × 190; Fig. 2, × 180.

FIGURE 3 Higher magnification of part of Fig. 2. On the luminal surfaces of alveolar cells are numerous small craters left by extracted fat droplets. Between alveoli are clumped connective-tissue fibers and occasional red blood cells (arrow). Because the gland was engorged with milk after 1½ hr without suckling, the alveolar walls are thin (during suckling many alveoli contract and the cells change shape, projecting into the lumen). The razorblade cut has sectioned the walls squarely and without gross damage. × 440.

FIGURE 4 Part of an alveolus showing a polygonal pattern of distinct cell boundaries marked by bands of densely set microvilli. The craters left by extracted fat droplets often contain collapsed membrane remnants, and occasionally a small fat droplet persists (arrow). × 1020.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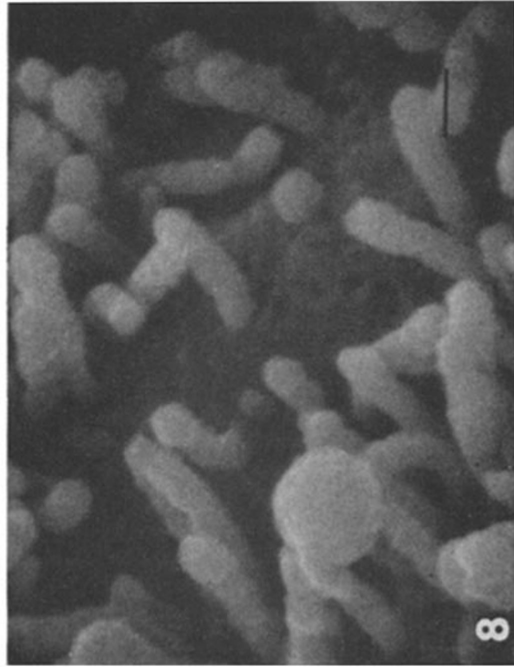
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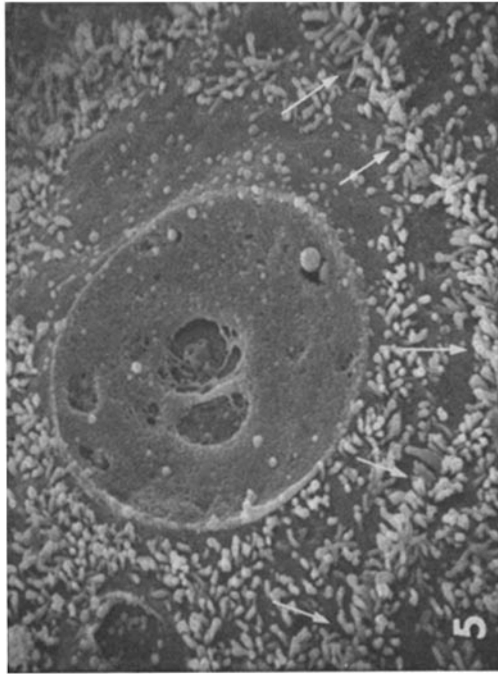
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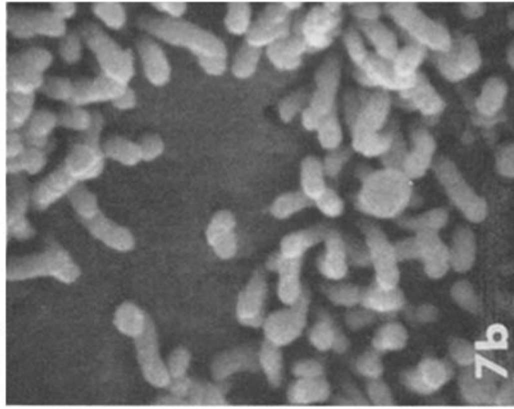
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