CELL CONTACTS IN THE MOUSE MAMMARY GLAND

I. Normal Gland in Postnatal Development and the Secretory Cycle

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ABSTRACT

The nature and distribution of cell contacts have been examined in thin sections and freeze-fracture replicas of mammary gland samples from female C3H/Crgl mice at stages from birth through pregnancy, lactation, and postweaning involution. Epithelial cells of major mammary ducts at all stages examined are linked at their luminal borders by junctional complexes consisting of tight junctions, variable intermediate junctions, occasional small gap junctions, and one or more series of desmosomes. Scattered desmosomes and gap junctions link ductal epithelial and myoepithelial cells in all combinations; hemidesmosomes attach myoepithelial cells to the basal lamina. Freeze-fracture replicas confirm the erratic distribution of gap junctions and reveal a loose, irregular network of ridges comprising the continuous tight-junctional belts. Alveoli develop early in gestation and initially resemble ducts. Later, as alveoli and small ducts become actively secretory, they lose all desmosomes and most intermediate junctions, whereas tight and gap junctions persist, The tight-junctional network becomes compact and orderly, its undulating ridges oriented predominantly parallel to the luminal surface. It is suggested that these changes in junctional morphology, occurring in secretory cells around parturition, may be related to the greatly enhanced rate of movement of milk precursors and products through the lactating epithelium, or to the profound and recurrent changes in shape of secretory cells that occur in relation to myoepithelial cell contraction, or to both.

INTRODUCTION

The mammary gland in lactation is a massively productive organ of secretion. Because its cycle of differentiation, lactation, regression, and inactivity is under the control of hormones that can be experimentally manipulated in laboratory animals, and because the amplitude of this cycle is great, the gland is a particularly useful model of development in secretory epithelia (40, 65). In common with other epithelia, mammary parenchyma shows strong morphological and functional polarity, but the particular expression and significance of polarity differ with the behavior of cells occupying different positions in the gland (secretory alveolus vs. excretory duct) or in the developmental cycle (resting state vs. lactation).

The occurrence of characteristic cell junctions serving to maintain a layer of cells as a coherent sheet and a permeability barrier is essential to the polarized function of epithelia in general, al-

though both the ultrastructure of the junctional complex (15) and the degree of "leakiness" of the transepithelial permeability barrier (18) may differ in different organs. Changes in properties of cell junctions during functional differentiation of an epithelial tissue might be expected, and could be important in a strongly cyclic one. To determine whether the distribution and morphology of cell contacts in the mammary gland vary with the natural sequence of hormonal environments and cellular responses, we have examined thin sections and freeze-fracture replicas of mammary gland samples from female mice at stages from birth through pregnancy, lactation, postweaning involution, and the resting state. The observations to be reported indicate that significant changes do occur, particularly in relation to active secretion and excretion of milk by alveoli.

The growth pattern of mouse mammary gland is well known from light microscope studies (11, 16, 43, 68, 69). Each of the ten glands at birth consists of a primary duct and a system of branches extending a short distance from the nipple into the mammary fat pad. The ducts elongate and branch until, at maturity (around 9 wk after birth), the duct system has ramified through the fat pad, the branches ending in slender or club-shaped twigs; little additional growth occurs in the adult virgin mouse (but there is variation among strains and individuals). Further ductal branching occurs in early pregnancy and alveolar buds grow out from the sides and ends of the ducts. By about 12 days of gestation, clusters of small alveoli cover the duct system, and during the remainder of gestation the alveoli enlarge and become distended with secretion, while the adipose stroma of the gland begins to release its fat stores. Some cell proliferation within alveoli continues (66, 67) both before and after parturition (at about 21 days), and full secretory development of the gland is reached several days *post partum*. Nursing usually stops at about 3 wk *post partum*, and involution of the gland sets in. In this process almost all of the alveoli and presumably some ductules disintegrate and the remains are removed by phagocytic cells.

Thus there appear to be two kinds of tissue, distinct in time and space, in the mammary gland: *ducts*, which ramify through the fat pad before puberty, proliferate further under the influence of hormones in early pregnancy, and for the most part persist when lactational hormone levels fall at weaning; and *alveoli*, which originate from ductal tissue specifically in response to the hormones of pregnancy, produce milk in response to lactogenic hormones, and generally do not survive the withdrawal of these hormones. In addition to the typical epithelial cells, myoepithelial cells lie at the periphery of both ducts and alveoli, within the basement membrane. During suckling, milk ejection is facilitated by myoepithelial cell contraction triggered by the pituitary hormone oxytocin (12, 29, 56).

Recent accounts of mouse or rat mammary ultrastructure (2, 9, 21, 23, 24, 28, 38, 42, 51, 55, 59, 70) provide details of cell morphology at various stages. The classic description of cell junctions in vertebrate epithelia by Farquhar and Palade (15) has been amplified by a number of recent authors (3, 7, 10, 17, 22, 27, 39, 45, 61), who have identified a new entity, the gap junction, and have applied the technique of freeze-fracturing to junctional morphology in several kinds of tissues.

MATERIALS AND METHODS

Source of Tissues

Mammary gland samples for transmission electron microscopy were taken from female C3H/Crgl mice that been either anesthetized with Nembutal or killed by neck fracture. Thick and thin sections or freeze-fracture replicas of one to several tissue samples were examined from the animals listed in Table I. In addition, we have reexamined many micrographs of lactating glands from mice of the same strain or from BALB/cCrgl mice, prepared in the course of other studies.

Samples for scanning electron microscopy were from 13-day lactating BALB/cCrgl female mice; the gland of this strain does not differ detectably in its histologic organization or ultrastructure from that of C3H/Crgl mice, except for the absence from BALB/c and presence in C3H of mammary tumor virus virions.

Preparation of Tissue for Sectioning

Mammary tissues for sectioning were routinely fixed in a dilute (1% paraformaldehyde, 3% glutaraldehyde) Karnovsky's fixative (25) in 0.1 M sodium cacodylate buffer, pH 7.2–7.4, for 2 h at room temperature, postfixed in buffered 1% OsO_4, dehydrated in ethanol, washed in propylene oxide, and embedded in Epon 812 (35). The most frequent modification of this routine was staining in block for 1 h with 0.5% uranyl acetate in either distilled

TABLE I

Animals Sampled for Electron Microscope Examination of Thin Sections or Freeze-Fracture Replicas

Developmental stage	Number of animals sampled for: Thin sections	Number of animals sampled for: Freeze-fracture
Virgin		
Newborn	1	
2 day	1	
3 wk	3	1
12 wk	2	
15 wk	1	
Pregnant		
11–19 days	10	2
Lactating		
1 day	1	1
2–16 days	12	4
21 days	1	
Postweaning		
24–48 h	4	4
15 days	1	

water or Veronal acetate buffer. To trace intercellular spaces, some tissue samples before fixation were incubated with horseradish peroxidase and with substrate following the method of Karnovsky (26), or were fixed in aldehyde and osmium fixatives to which lanthanum nitrate (52) or ruthenium red (36, 48) was added. Legends for the electron micrographs will specify only modifications of the routine fixation procedure.

1–2—μm sections of Epon-embedded tissues were stained with Mallory's Azure II-methylene blue (57) or Paragon (37) and examined by light microscopy. Thin sections were cut with diamond knives on an LKB Ultrotome (LKB Instruments, Inc., Rockville, Md.) or a Sorvall Porter-Blum MT-2 microtome (Ivan Sorvall, Inc., Norwalk, Conn.), stained with 5% uranyl acetate in 50% ethanol and with Reynolds' lead citrate (54), and examined in a Siemens Elmiskop 1 or a Philips EM 300 electron microscope operated at 80 kV.

Freeze-Fracturing

Small (about 2 mm^3) pieces of mammary tissue were fixed in the dilute Karnovsky's fixative for 10–15 min, rinsed, and placed in 20% glycerol in cacodylate buffer for 2 h at room temperature. Freeze-fracture replicas were prepared in a Balzers apparatus at −115°C (Balzers AG, Balzers, Liechtenstein) (41). Removing the tissue from the replica presented severe problems owing to the mixed tissue composition of the mammary gland and especially its high fat content; our best mammary gland replicas were considerably more fragmented or contaminated with adherent organic matter than simultaneously prepared replicas of simpler tissues or cultured cells.

Scanning Electron Microscopy

Tissues from lactating BALB/cCrgl mice were fixed in glutaraldehyde, washed in glycerol and ethanol solutions, dehydrated in ethanol, transferred to Freon, and dried in a Freon critical-point dryer; details of the method are described elsewhere (44). Dried tissues, coated with gold, were examined in a Stereoscan Mark IIa microscope.

RESULTS

Histology

The mouse mammary duct system throughout its development is lined by an epithelium that usually is single layered except in zones of active growth. In the primary duct and its main branches the cells typically are, and remain through lactation, roughly cuboidal, with a central nucleus and relatively scant cytoplasm (Figs. 1–3). During late pregnancy and lactation, cells of more distal branches may be larger (Fig. 4), polarized, and vacuolated, often appearing as actively secretory as alveolar cells. In fact, terminal ducts and alveoli are generally indistinguishable in sections (Fig. 5). A virtually continuous layer of longitudinally oriented myoepithelial cells invests the primary duct and its major branches; distally, the myoepithelium becomes discontinuous and its cells stellate, to form an open basket of cell processes around each ductule and alveolus. Epithelium and myoepithelium together are at all times separated from the surrounding adipose stroma by a continuous basement membrane.

Ultrastructure of Cell Contacts in Ducts

Differentiation of myoepithelial from epithelial cells is detectable in the wake of active growth points in all growing glands (Fig. 6); the former cells when mature are identifiable by their peripheral position, a serrated basal profile, and an abundance of parallel cytoplasmic filaments (4–5 nm in diameter). Once this differentiation has occurred, the ultrastructural organization of the duct remains remarkably constant through the mammary cycle, and the kinds and distribution of cell contacts apparent at this time are characteristic of most of the duct system through-

out life. Epithelial cells bear microvilli on their free apical (luminal) surfaces and are closely associated with epithelial neighbors laterally; in major ducts (Figs. 6, 7), basal surfaces of epithelial cells abut almost entirely on myoepithelial cells, whereas in lesser ducts they adjoin basal lamina as well. Myoepithelial cells have no free surfaces but face epithelial cells above, epithelial or other myoepithelial cells laterally, and the basal lamina basally.

Junctional complexes joining epithelial cells at their luminal edges are present from birth, and even before (9), wherever there is a lumen (Figs. 6–8). They consist of tight junctions, more or less weakly developed intermediate junctions, and one or more series of desmosomes; the complex occupies a zone usually 1–2 μm deep (Figs. 8–10). (Gap junctions are also present between ductal cells but are not necessarily a component of the junctional complex.) The joined cell membranes are almost never straight but instead curve and twist mildly but consistently, so that a cross section of the membranes through the depth of the junctional complex is rarely obtainable. For the same reason the cleavage plane in freeze-fractured specimens rarely follows a junctional zone far without topographic irregularities and interruptions. Freeze-fracture replicas nonetheless provide the clearest information on the morphology and extent of tight and gap junctions, whereas intermediate junctions and desmosomes of this tissue are best identified in sections.

As has been demonstrated for other cell types (5, 50, 64), a freeze-fracture in the plane of a membrane splits it to expose two internal faces: a cytoplasmic one, fracture face A of the cleaved membrane, typically bearing many scattered

Abbreviations used in legends
A, face A of freeze-cleaved membrane
B, face B of freeze-cleaved membrane
BL, basal lamina
C, collagen fibrils
Cy, cytoplasm
D, desmosome
Ep, epithelial cell
G, Golgi sacs
IJ, intermediate junction
IS, intercellular space
JC, junctional complex
Lu, lumen
My, myoepithelial cell
TJ, tight junction

FIGURES 1–5 Photomicrographs of 1–3—μm sections of mammary tissues fixed in paraformaldehyde-glutaraldehyde, postfixed in osmium, and embedded in Epon.

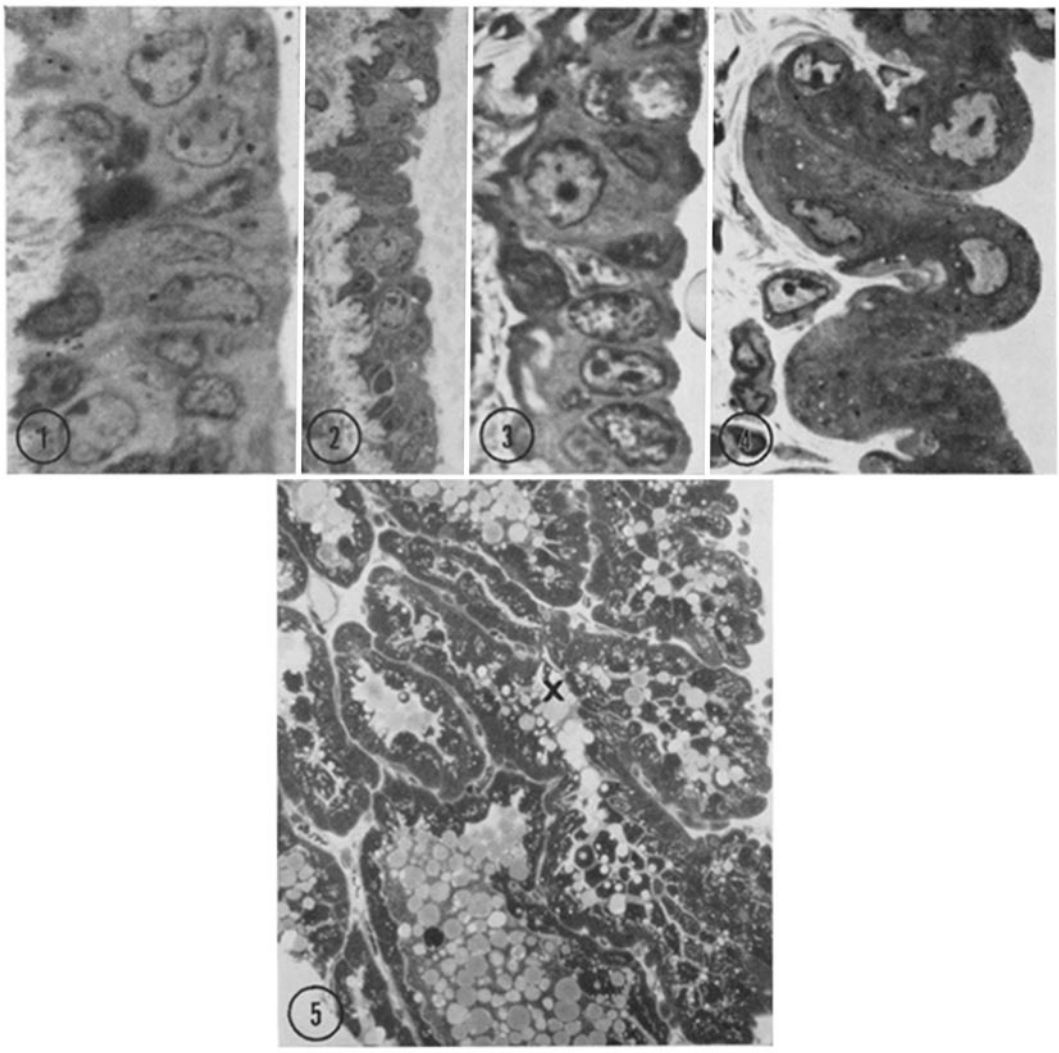
FIGURE 1 Part of a cross section of a primary duct in a mammary gland from a 3-wk old female mouse. The lumen is at the right. The arrangement of nuclei in two tiers is fairly advanced. The dark-stained cells are finely serrated on the surface facing the connective tissue, at the left. Cells bordering the lumen are larger and have larger, central nuclei. Mallory's Azure II-methylene blue. × 1,500.

FIGURE 2 Lower magnification of part of a cross section of a major duct from a 12-wk old virgin female; lumen at right. The basal layer of small, dark, myoepithelial cells is distinct and confers upon the duct a characteristically jagged peripheral profile. Paragon stain. × 750.

FIGURE 3 Part of a cross section of a major duct from a 12-day lactating female; lumen at right. The characteristics and arrangement of cells are much the same as in the 12-wk virgin. Paragon stain. × 1,500.

FIGURE 4 Part of a cross section of a more distal duct branch from a 15-day lactating female; lumen at right. The deeply folded wall consists of a discontinuous myoepithelial layer and epithelial cells that are significantly larger than those in the major ducts but are still not strongly polarized or highly vacuolated. Mallory's Azure II-methylene blue. × 1,300.

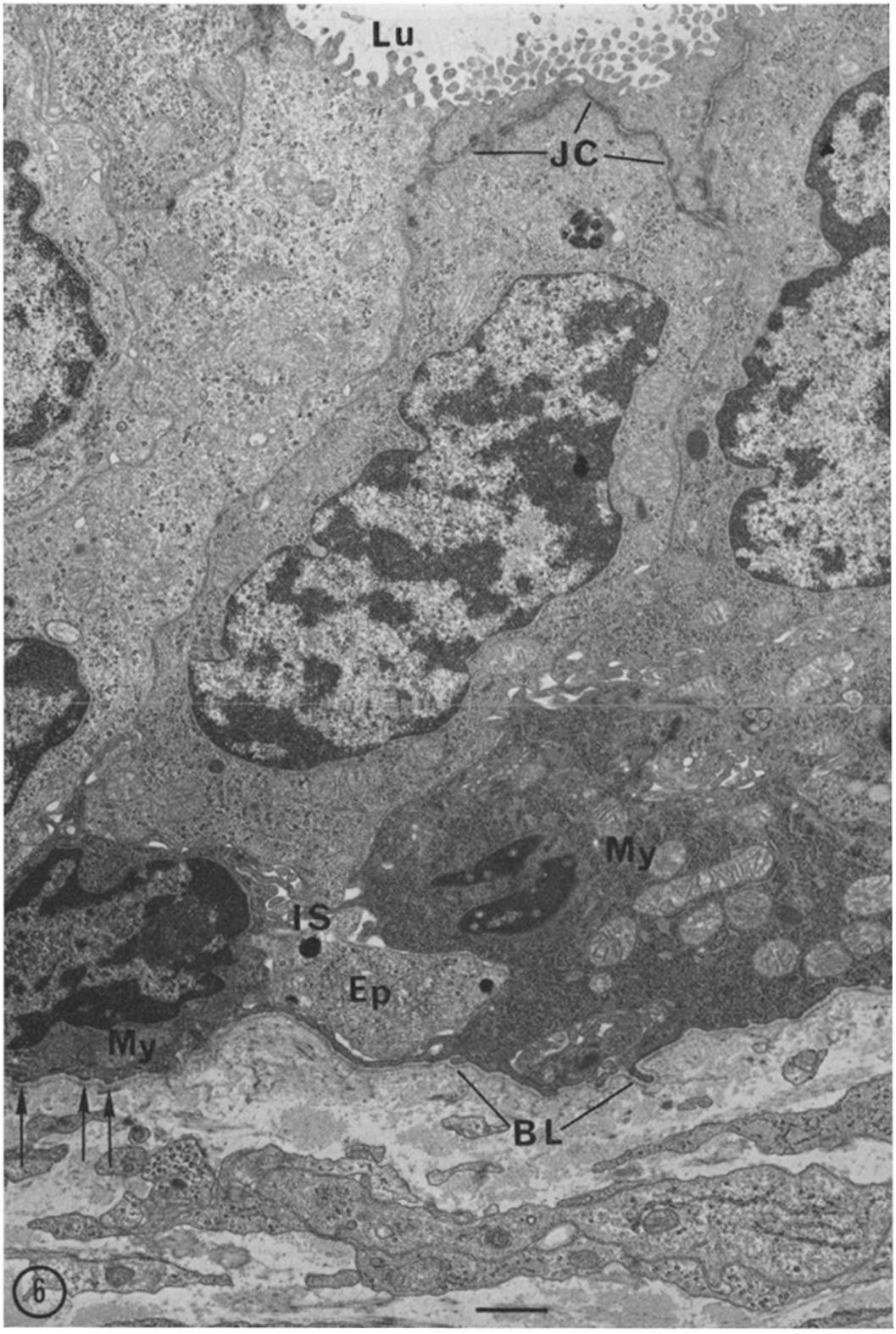
FIGURE 5 Part of a section through a lobule of a 12-day lactating gland. All of the tissue appears secretory: the cells contain spherical fat vacuoles and clusters of tiny vacuoles that represent Golgi elements; the lumina are filled with milk. At *x* several chambers interconnect, suggesting that one of them, not morphologically distinguishable, may serve as a duct. Mallory's Azure II-methylene blue. × 210.



particles about 8 nm in diameter; and a complementary, superficial one, fracture face B of the cleaved membrane, typically bearing fewer particles. Where the cleavage plane passes along cell junctions, it frequently shifts from face A of one cell's membrane to face B of the contiguous cell's membrane. A tight junction appears in freeze-fracture replicas as a series of ridges on face A and complementary grooves on face B of *each* of the two cell membranes involved (10, 17, 22).

The lumen of a mammary duct is recognized in freeze-fracture replicas by the presence of microvilli projecting into it from the apical surfaces of epithelial cells. Where lateral cell membranes are exposed by a fracture along their luminal edges, as in Fig. 11, the ridged or grooved pattern of a tight junction is always visible below the fringe of microvilli. Since the junctional membranes are not necessarily, or even frequently, perpendicular to the luminal surface (see Fig. 8), it is also common to find a fracture plane that follows part of the lateral cell membrane, the tight junction, and then the apical cell membrane of one of the joined cells instead of passing into the luminal cavity (Fig. 12). The apical membrane is interrupted by fractures through the bases of microvilli and the lateral membrane by irregular protrusions or depressions of the cell surface.

The tight junction consists of a network of ridges or grooves in highly irregular patterns. In some areas (Fig. 11) the predominant orienta-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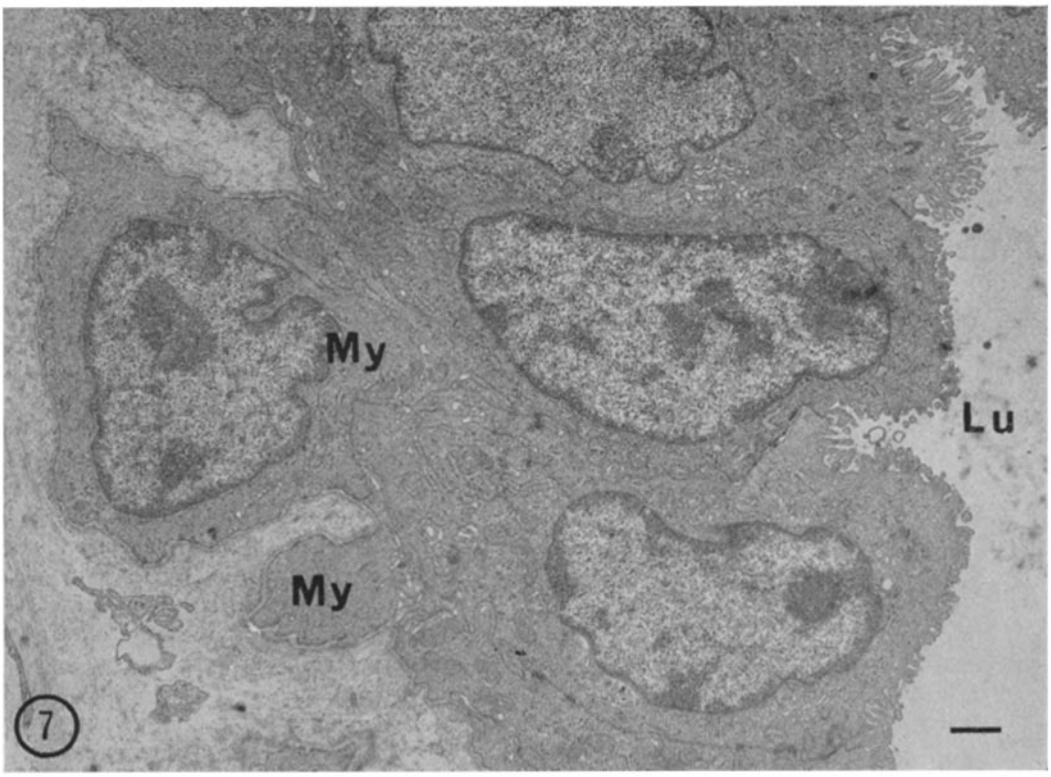


FIGURE 7 Part of a cross section of a major duct from a 10-day lactating female; lumen (*Lu*) at right. The epithelial cells are not notably different from those in the 3-wk gland. Myoepithelial cell (*My*) cytoplasm is packed with parallel filaments, visible at higher magnification. Uranyl acetate in block. Bar = 1 μm; × 7,000.

tion of undulating ridges is parallel with the luminal surface; at least as frequently there is no predominant direction. The luminal edge of the network usually is closed; that is, there is a continuous marginal ridge, without free ends. At the abluminal edge, however, short or long free ends are common. The shapes and sizes of meshes vary within wide limits, as does the width of the tight-junctional zone. In Fig. 12, only two ridges, less than 50 nm apart, separate lateral and apical surfaces in some areas; elsewhere looped ridges or loose ends extend as much as a

FIGURE 6 Thin section of the primary duct shown in Fig. 1, from a 3-wk old female. The luminal (*Lu*) borders of epithelial cells bear an irregular fringe of microvilli. Below this in the apical cytoplasm, the junctional complex of several cells is cut obliquely (*JC*), so that the cytoplasmic density along the intermediate junction clearly outlines the irregular course of the adjoined membranes. Contiguous borders of epithelial cells below the junctional complex are generally closely parallel, with occasional interdigitating folds or intercellular spaces (*IS*) into which cytoplasmic processes extend. Two presumptive myoepithelial cells (*My*) are identifiable by their denser cytoplasmic matrix, more abundant ribosomes, small, dense nucleus, and the hemidesmosomes (arrows) already developed in the cell at left. At higher magnification, small bundles of filaments are visible in this cell. Slender processes extending from the two myoepithelial cells separate the intervening, lighter, epithelial cell cytoplasm (*Ep*) from the basal lamina (*BL*). Cellular and fibrillar elements of the surrounding connective tissue tend to be oriented parallel to the basal surface of the parenchyma. Uranyl acetate in block. Bar = 1 μm; × 11,500.

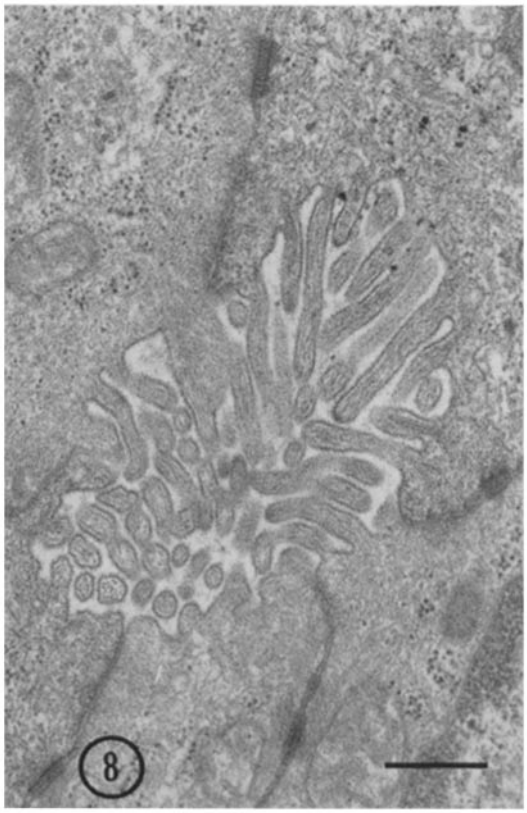


FIGURE 8 The apices of five cells converging on a small lumen in the gland of a 3-wk old female are linked by junctional complexes typical of mammary ducts. Projecting into the lumen are long, slender microvilli with cores of parallel filaments, cut in various planes. An irregular web of cytoplasmic filaments is present under the apical surface of each of the cells. Bar = 0.5 μm; × 27,000.

micrometer from the luminal edge. We have never observed fewer than two ridges between lumen and lateral membrane.

In thin cross sections, the tight junction appears as a zone of intermittent (if delineation of unit membrane structure is clear) fusion of the outer leaflets of adjoining cell membranes (Figs. 9, 10). Although there are many cytoplasmic filaments (about 4–5 nm in diameter) near the luminal surfaces of ductal cells, they do not converge noticeably at the intermediate junction, as they do in some epithelia (15). This junction in mammary ducts, when distinguishable at all, is marked by an increased cytoplasmic density along an area below the tight junction, where opposing cell membranes are separated by a space of about 13 nm (Figs. 8–10). Since there also may sometimes be increased density bordering the membranes of the tight-junctional zone, identification of an intermediate junction is often tenuous.

Typical desmosomes complete the junctional complex in the mammary ducts. Cytoplasmic filaments (about 9 nm in diameter) are often present in small bundles that loop through the dense plaque on the cytoplasmic side of the desmosome. Desmosomes may also be present between epithelial cells at any level below the junctional complex, between epithelial and myoepithelial cells, and between neighboring myoepithelial cells. Myoepithelial (but not epithelial) cells have distinct small hemidesmosomes, lacking associated cytoplasmic filaments, at their basal surfaces. The external contour of ductal myoepithelial cells characteristically is serrated, with hemidesmosomes often at the peaks, and the basal lamina follows this contour closely.

Gap junctions may be found in mammary ducts by both thin-section and freeze-fracture techniques. In freeze-fracture replicas, they appear as plaques of 8-nm particles on the A faces of lateral cell membranes and of complementary depressions on the B faces (17, 39). The largest plaque we found (Fig. 13) is about 0.8 μm in its longer diameter and is not near any other identifiable junction. Other plaques have been near or even within the network of a tight junction. In several instances we found multiple small gap junctions close together; five were present in one lateral membrane area about 3 μm in diameter. All of these gap junctions were found in replicas of tissues from virgin mice; we have been unable thus far to obtain identifiable replicas of ducts in lactating tissue. We have, however, identified occasional gap junctions in thin sections of ducts at all stages examined, joining epithelial cells with each other or with myoepithelial cells; they are recognized in cross section as strictly parallel membranes separated by a gap of about 3 nm.

Contiguous lateral surfaces of epithelial cells in areas in which morphologically specialized junctions are absent usually run roughly parallel, maintaining an average intercellular space of 12–14 nm. Their course is more or less sinuous

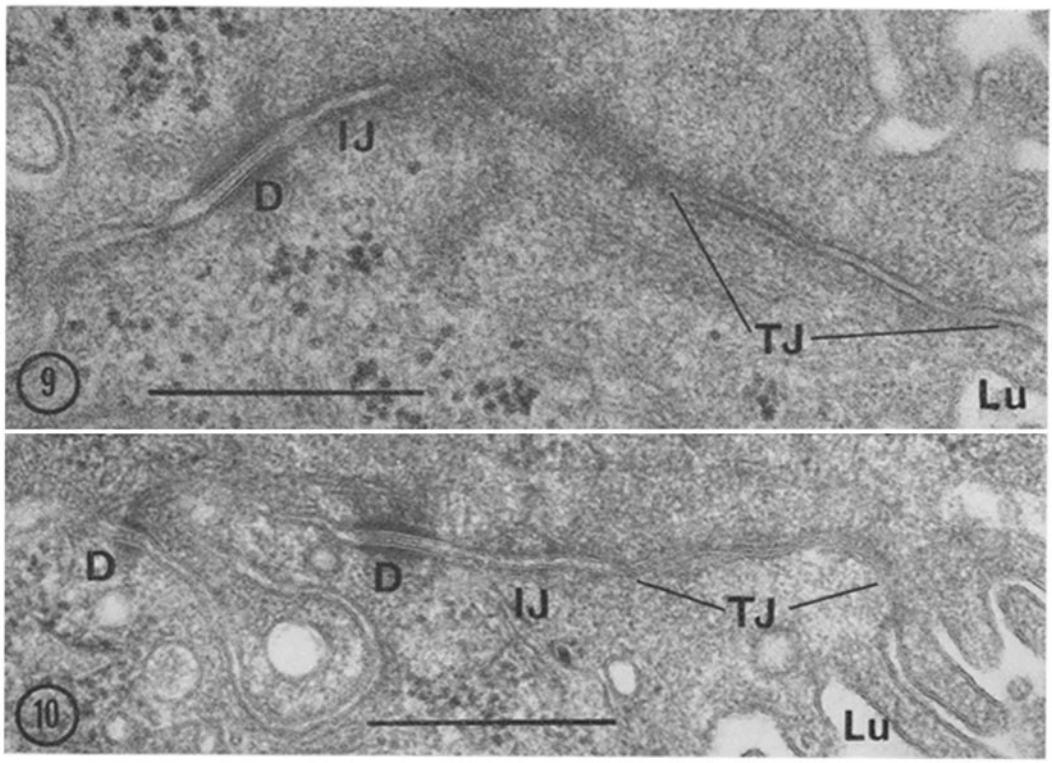


FIGURE 9 Junctional complex in a primary duct from a 3-wk old female; luminal border (*Lu*) at the right. A rather long tight junction (*TJ*) with slightly increased cytoplasmic density on either side is followed by a twist in the membranes that obscures their contours; further increase in the density of adjacent cytoplasm is evident here and in the short, distinct intermediate junction (*IJ*) that follows. Basal to a well-formed desmosome (*D*), with associated filaments, the contiguous membranes are convoluted. Uranyl acetate in block. Bar = 0.5 μm; × 72,000.

FIGURE 10 Junctional complex in a primary duct from a 10-day lactating female; luminal border (*Lu*) at the right. Basal to the tight junction (*TJ*), the cytoplasmic density is increased somewhat along a region that may be considered an intermediate junction (*IJ*). Basal to a desmosome (*D*) are folded membranes and then another, small desmosome. Uranyl acetate in block. Bar = 0.5 μm; × 64,000.

and at intervals is thrown into interdigitating folds (Figs. 6, 7, 9, 10). Widened intercellular spaces are frequent at the meeting of three cells, and this is particularly conspicuous where one of the cells is myoepithelial; elongate cell processes commonly extend into these gaps (Fig. 6).

Except for a possible increase in the number of desmosomes, the major ducts do not display any morphological changes during lactation. Lesser ducts, however, come increasingly to resemble alveoli as they approach their alveolar termini, and these changes affect cell contacts as well as cytoplasmic organelles; they will be discussed after a consideration of typical alveolar junctions.

Ultrastructure of Cell Contacts in Alveoli

When alveoli first appear during pregnancy, their cells are relatively undifferentiated and in every respect resemble those of small ducts. Junctional complexes are present at luminal cell borders, and desmosomes may be found also at other locations. As gestation proceeds and alveolar cells differentiate and begin to produce fat and protein, desmosomes in alveoli become increasingly rare, the few that remain usually lacking associated cytoplasmic filaments. An occasional simple desmosome is still present on day 1 *post partum*. Thereafter, throughout lactation and involution, desmosomes appear to be

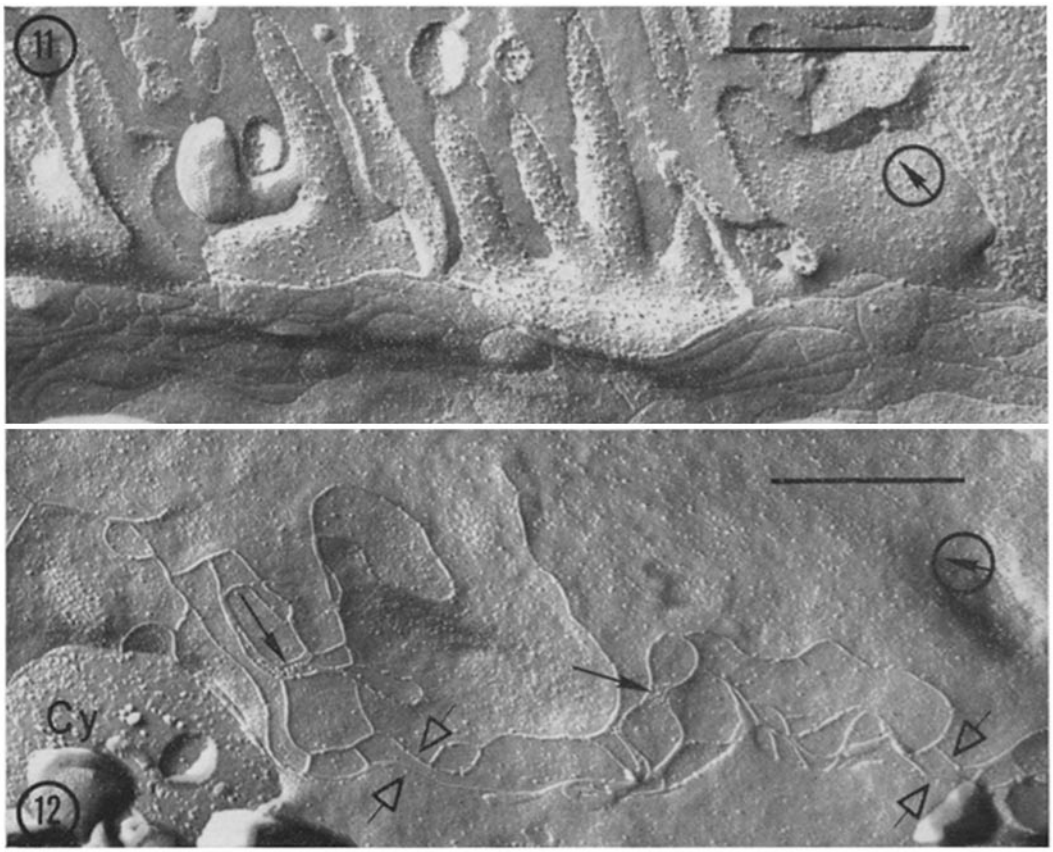


FIGURE 11 Freeze-fracture replica of a small part of a luminal cell border in a gland from a 3-wk old female. Microvilli project from the luminal surface into the smoothly fractured fluid filling the lumen at the top of the figure. Below the luminal border is a network of grooves on the B face of one of the membranes involved in the tight junction. A dip in the replica, probably reflecting a curve in the membrane contour, darkens and distorts part of the network. Circled arrow indicates shadowing direction in this and subsequent figures of freeze-fracture replicas. Bar = 0.5 μm; × 65,000.

FIGURE 12 Freeze-fracture replica of part of a long tight-junctional zone in the gland of a 3-wk old female. The luminal cell membrane is seen in its B face at the bottom of the micrograph, then the cleavage plane shifts to the A face of the adjacent cell's membrane to show the highly irregular network of ridges of the tight junction. Two ridges extend abluminally as free ends. At the paired, open arrows, only two ridges constitute the junction. At the single arrows are small clusters of particles that could represent either disaggregated ridge particles or tiny gap junctions. At the left, the tight junction is interrupted where the fracture plane has passed into the cytoplasm (*Cy*) of one of the cells. Bar = 0.5 μm; × 51,000.

absent from all locations in alveoli. Rarely, a pair of minute dense areas on adjacent epithelial cell membranes is seen, but neither intercellular laminae nor cytoplasmic filaments are involved and the intercellular distance is not modified.

As desmosomes disappear, so also do most indications of the existence of intermediate junctions; thin sections typically show only tight junctions linking the luminal borders of alveolar cells in lactating or involuting glands (Fig. 14). All of the electron-opaque tracers we have employed for definition of intercellular spaces or materials (lanthanum, horseradish peroxidase, ruthenium red) were occluded by the tight junctions, as shown for peroxidase in Fig. 15. These substances diffuse through interalveolar spaces and penetrate the basal lamina of the alveoli to fill spaces between cells up to the tight junction

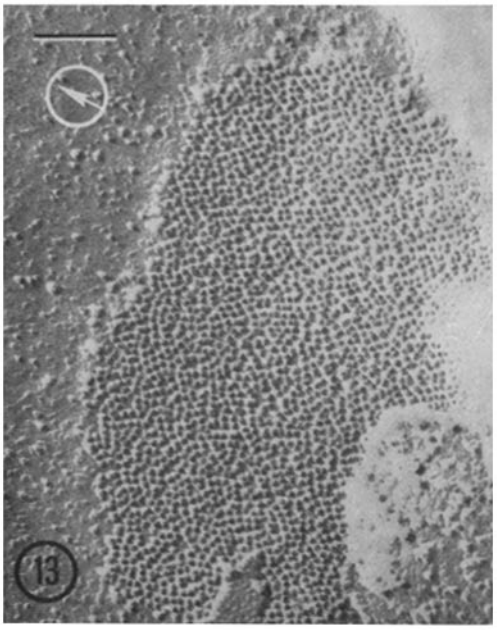


FIGURE 13 A gap junction on face A of an epithelial cell membrane in the gland of a 3-wk old female. The particles are about 8 nm in diameter and are closely but irregularly packed. Bar = 0.1 μm; × 105,000.

They do not diffuse through milk-filled ductules far enough to penetrate the lumina of alveoli in the interior of the tissue block. Our observations are in accord with those of Martinez-Palomo (38), who clearly showed occlusion of lanthanum by tight junctions in mouse mammary gland.

A marked change in the configuration of tight-junctional networks seen in freeze-fracture replicas occurs at or shortly before parturition. In glands sampled at 12 and 16 days of gestation (by which time alveoli are well developed), the ridged network resembles that of ducts in virgin females but is perhaps even less consistent in width, mesh size, and orientation (Fig. 16). On day 1 *post partum* the network has become much more compact (Fig. 17), with undulating ridges oriented in a direction generally paralleling the luminal surface and most meshes narrow and elongate. Careful search of replicas from five females lactating for up to 14 days has revealed no examples of the loose, irregular network so often found at earlier stages. Typical examples are shown in Figs. 17–19. Ridges interconnect, but there are fewer right-angle joinings or straight segments than before, and both luminal and abluminal edges of the network are formed by continuous ridges, with very few loose ends. Less variation is seen now in width of the tight-junction zone: the average is around 250 nm, with 110 nm the narrowest and 500 nm the widest in our samples. Even at the narrowest places there usually are more than three ridges. Undulation of the ridges tends to be shallow at the luminal edge and more marked abluminally; as a result, meshes are long and narrow near the luminal edge and wider and more rounded abluminally. In early involution, the alveolar tight-junction network becomes somewhat less orderly (Fig. 20), but we have found no examples approaching the highly irregular appearance of the junctions in ducts or prelactating alveoli.

Gap junctions are readily observed in freeze-fracture replicas of prelactating alveoli; they resemble those of ducts in size and distribution. In one membrane fracture face, a rectangular area 1.2 × 2.4 μm contained seven gap junctions ranging from 60 to 600 nm in diameter. Relatively larger (often 1 μm or more in diameter) gap junctions are found in most replicas of lactating or involuting alveoli. Occasionally two or more are present on a single membrane face; they may be near (Fig. 17) or remote from a tight-junctional zone but very rarely are found within its network. We have also observed gap junctions in thin sections of alvolar tissue; in at least two instances, a gap junction clearly joined two myoepithelial cell processes end-to-end (Fig. 21).

Below the tight junction, the contours of contiguous lateral cell membranes of alveoli are similar to those in ducts. Relatively straight areas are somewhat more extensive than in major ducts, but interdigitating folds are frequent, and folds and processes are especially pronounced adjacent to myoepithelial cells. Basal surfaces of epithelial cells in lactating alveoli form a labyrinth of extensions and infoldings (Figs. 15, 22); the basal lamina does not follow these convolutions but extends as a smooth blanket over them, and it does not appear to be attached to epithelial membranes by hemidesmosomes.

Between the major ducts at one extreme and the alveoli at the other, successively finer duct branches show gradations in a number of characters. Increasing development of the cytoplasmic apparatus for protein production is accompanied—although not all in parallel—by decrease

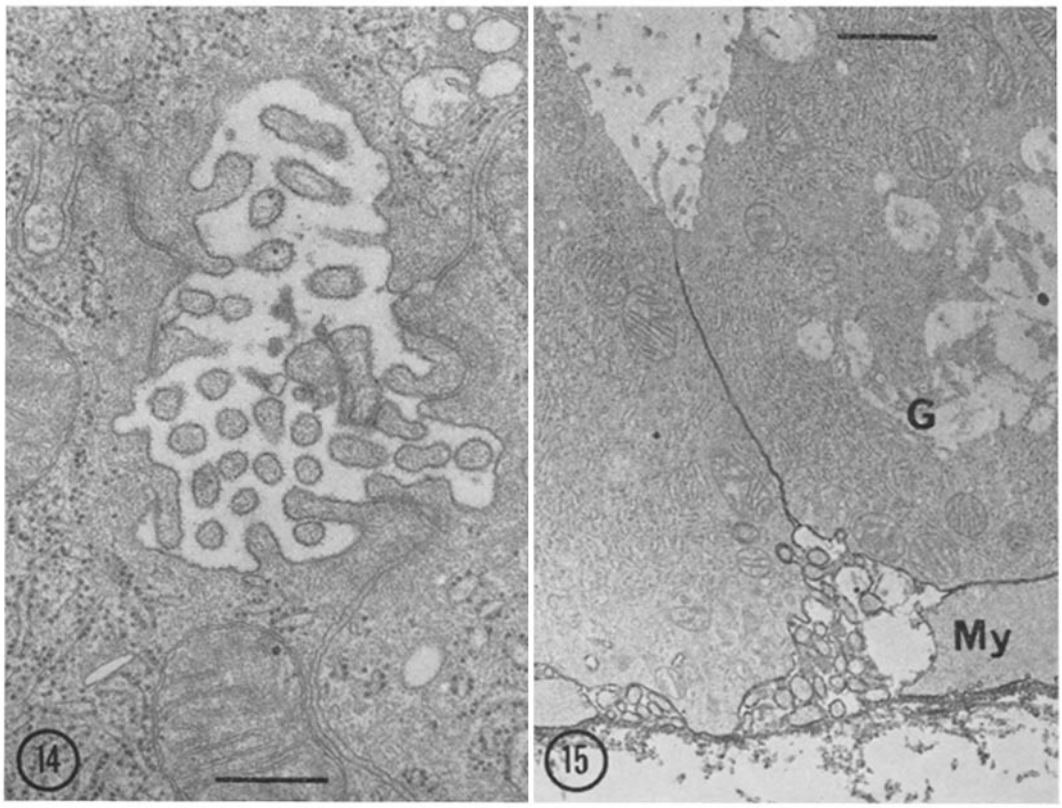


FIGURE 14 The apices of three cells converge on a small bay in the lumen of an alveolus from a 15-day lactating female. At the luminal borders, only tight junctions, with somewhat increased density in the adjacent cytoplasm, can be seen. Compare with Fig. 8. Bar = 0.5 μm; × 30,000.

FIGURE 15 Part of an alveolus from a 14-day lactating female, treated with horseradish peroxidase before fixation; the reaction product may be seen adhering to fibrous elements of the stroma at the bottom, to the basal lamina, to the surfaces of the basal labyrinth, and to lateral cell surfaces as far up as the tight junction at the luminal border. There is no evidence of peroxidase in the lumen. Incubations in peroxidase and substrate have resulted in swelling of basal labyrinth and intercellular spaces and of the Golgi sacs (*G*). Bar = 1 μm; × 13,000.

and disappearance of desmosomes and intermediate junctions, increasing discontinuity of the myoepithelial layer, progressive development of basal labyrinth at epithelial surfaces confronting the basal lamina, and thinning of the external connective tissue sheath.

We have seen no evidence of reformation of desmosomes in degenerating alveoli after weaning. At 15 days after weaning, involution of alveolar structures is almost complete; the surviving small ducts show occasional desmosomes, as they do at other times.

Cell Shape Change in Lactating Alveoli

Variation in the dimensions and shape of alalveoli and alveolar cells is demonstrable in the lactating mammary gland by both light and electron microscopy and is consistent with the evidence of Linzell (29) that in the mouse, as in other mammals, myoepithelial cell contraction results in compression of the alveolar lumen and forces its contents out into the duct system. In lactating mice that have not been nursed for several hours, many alveoli appear inflated with

milk and their epithelial cells are broad and flat, with luminal and basal surfaces approximately parallel. The luminal surface of a part of an inflated alveolus is shown in the scanning electron micrograph of Fig. 23. Cell height, measured on low magnification micrographs of thin sections of similar alveoli, is often as little as 3–4 μm. Glands that have been recently suckled usually contain groups of inflated alveoli, but also contain a large proportion of alveoli in which the lumen is greatly diminished by the protruding apices of epithelial cells; these may extend 6–8 μm above their junctions with neighboring cells. Fig. 24 shows the luminal surface of such an alveolus. To varying extents, the peripheral contours of these alveoli may be scalloped, the cell bases bulging between myoepithelial cell processes into the interstitial space. Cell height, measured in thin sections, often reaches 12–15 μm.

Luminal borders are outlined by rows of closeset microvilli (44), which are distinct at appropriate magnification in scanning electron micrographs; their courses thus can be measured. Perimeters of 16 cells in two engorged alveoli averaged 60 μm. Where alveoli consist of strongly protruding cells, specimens can be tilted for photographing at appropriate angles so that entire borders can be followed. The mean perimeter of 18 cells in two such alveoli was 42 μm.

The observed differences in shape of fixed cells indicate that shapes of individual living cells can change during lactation; if this is the case, such change affects the perimeter of the cell; that is, the length of the girdle of tight junction to neighboring cells. Thus the tight-junctional zone of the lactating cell apparently is capable of stretching and shrinking.

DISCUSSION

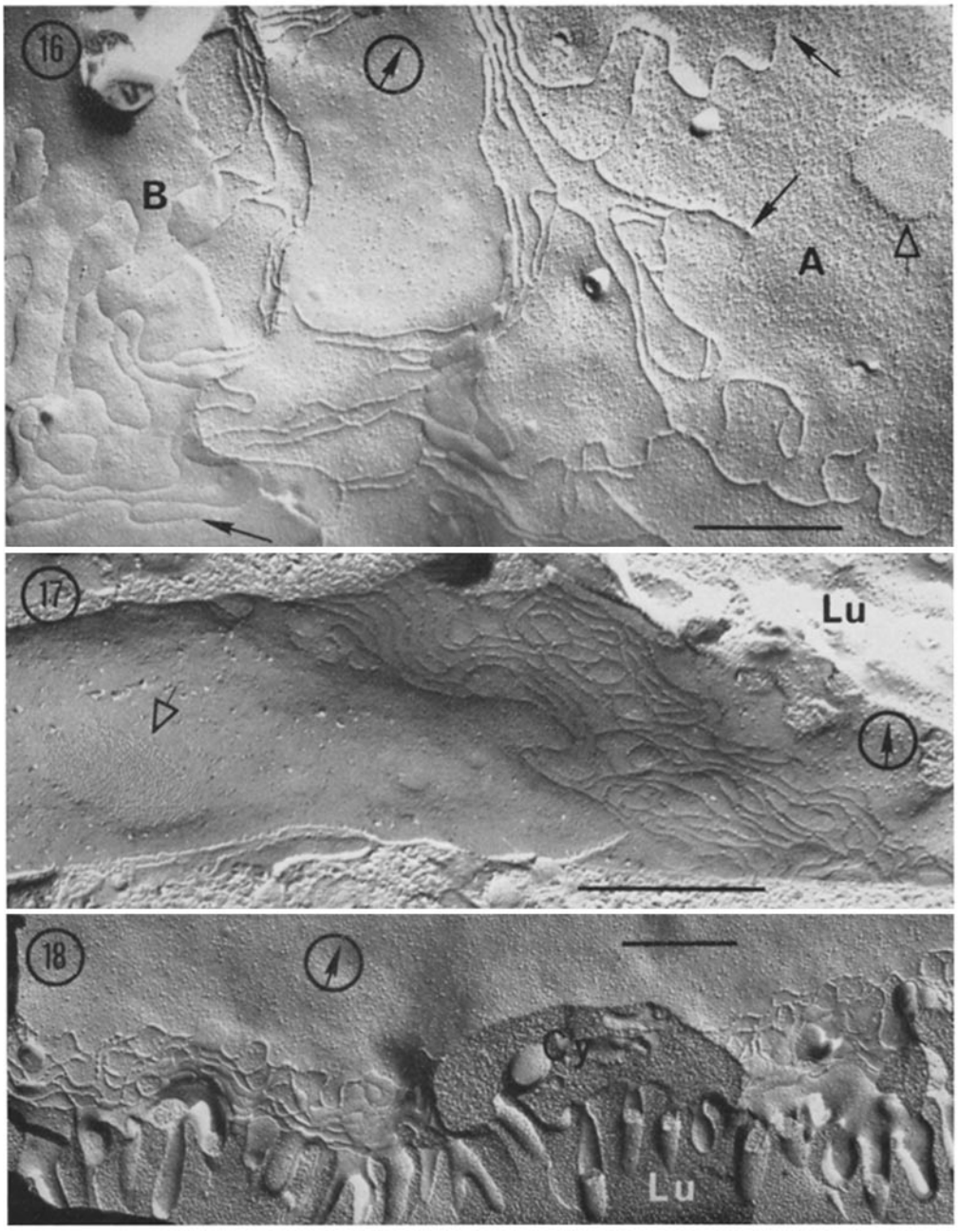
Our studies have demonstrated that cell surfaces in major mammary ducts have characteristic contact specializations that undergo little visible change during the breeding cycle of the mouse. These specializations include belts of tight and (variable) intermediate junctions and desmosomes at luminal borders, scattered gap junctions and scattered desmosomes linking epithelial and/or myoepithelial cells, hemidesmosomes attaching myoepithelial cell bases to basal lamina, and elsewhere more or less parallel, more or less interdigitating cell surfaces. Cells in lesser ducts display the same surface characters until late gestation, when they assume to varying extents both the internal and the surface properties of alveolar cells. Cells of newly formed alveoli in mid-gestation are indistinguishable in surface morphology from duct cells but in late gestation and early lactation they lose all desmosomes and intermediate junctions, differentiate a new tight-junctional configuration, and develop convolutions at epithelial cell surfaces confronting the basal lamina.

The fact that these changes in cell contacts occur in secretory cells at the time of parturition suggests a relationship to some aspect of lactation. The junctional structures involved function in intercellular adhesion and in limiting the passage of ions or molecules across the epithelium via intercellular spaces. We will consider these two functions separately as they appear to operate in ducts and in lactating alveoli.

Adhesive Junctions

Both tight and gap junctions are known to be sites of strong cell-to-cell attachment (4, 7, 15, 51). Intermediate junctions are prominent in some tissues and seem to provide a continuous adhering strip (6, 15), but their usual proximity to tight junctions makes an adhesive function difficult to test. Desmosomes appear to be spots of relatively firm mechanical adhesion, but they are readily disrupted by enzymes used in dissociation procedures (4, 47). Other possible cohesive elements in the mammary tree are the basal lamina, which is liberally attached by hemidesmosomes to myoepithelial cells over the whole periphery of ducts but only along the limited myoepithelial basket of alveoli; and connective tissue fibers, which form a thick sheath around major ducts but only a thin, open network around alveoli.

The nature and magnitude of mechanical stress imposed on ducts and alveoli of the lactating gland are particularly difficult to assess in the mouse. In mammals such as the cow, goat, or rabbit, where milking or suckling ordinarily takes place once or twice a day, milk secretion in the intervals engorges the alveoli. When milking or suckling commences, the neurohormonal milk-ejection reflex is initiated, and oxytocin released by the pituitary causes contraction of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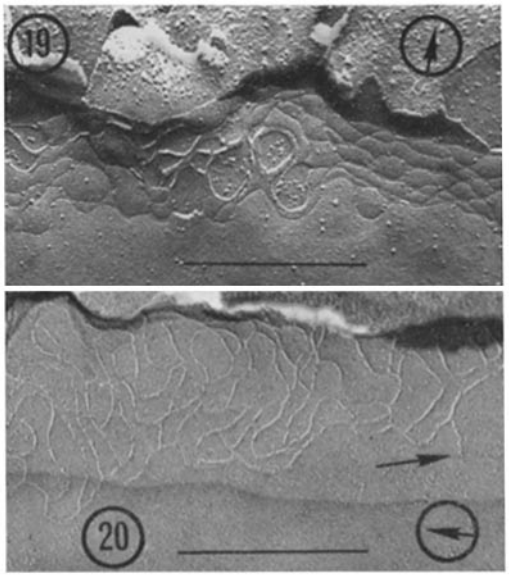

FIGURE 19 B face of a tight junction from the gland of a female at the height of lactation; lumen at top. The network is compact, with the narrowest meshes toward the lumen and more variation albuminally. Bar = 0.5 μm; × 48,000.

FIGURE 20 Part of a tight-junctional zone in a gland from a female 24 h after removal of young (after 21 days of lactation). The network is still compact as compared with prelactating stages, but it has lost the orientation of ridges along the luminal border that is characteristic of lactation, and a free-ended ridge is seen (arrow). The luminal surface is at the top of the figure. Bar = 0.5 μm; × 50,000.

myoepithelial cells, forcing milk from the alveoli into the large ducts or storage cisterns. If release of oxytocin is blocked in these animals, a significant proportion of milk in the gland cannot be removed by suckling (12, 30). Linzell (29) found that topical application of oxytocin to an exposed gland in a living mouse caused visible contraction of alveoli and forced their contents temporarily into the ducts (there are no large cisterns in the mouse gland); where ducts were visible, they became shorter, straighter, and wider. But in the laboratory mouse there is no apparent suckling schedule; the young nurse intermittently for short or long periods throughout the day. How the milk-ejection reflex operates under these circumstances is not known (12). Accurate preservation of alveoli and ducts in natural states of inflation or contraction requires careful fixation by perfusion (29, 56), preferably without previous traumatic handling of the subject. Erratic vasoconstriction during perfusion and other technical problems make fixation of the mouse mammary gland by this means difficult to control, and our attempts have not given reliable results. Our tissues, therefore, were fixed during or after removal from the animal and all of them contain some inflated and some contracted alveoli, representing in part artifacts of handling.

With these qualifications in mind, we suggest that the dramatic differences in cell and alveolar shape in our preparations do reflect states of contraction or extension of myoepithelial cells

FIGURE 16 Freeze-fracture replica of part of an extensive tight-junctional zone in alveolar tissue in a 14-day pregnant female. The luminal surface is out of the picture at the lower left. Most of the network is seen as ridges on face A (*A*) of the cleaved membrane, but at the left of the figure the fracture plane shifts to face B (*B*) of the adjacent cell's membrane, where grooves are seen as complementary continuations of the ridges. The pattern is highly irregular, with no consistency in size or orientation of meshes; several free ends are present, both luminally and abluminally (black arrows). A typical gap junction, consisting of a circular plaque of particles, is at the far right (open arrow). Bar = 0.5 μm; × 41,000.

FIGURE 17 Part of a tight-junctional zone from a replica of a 1-day lactating gland. The luminal surface (*Lu*) is at upper right. The network is compact, with ridges oriented predominantly parallel to each other and to the luminal border; no free ends are visible. At left is a gap junction (open arrow). Bar = 0.5 μm; × 51,000.

FIGURE 18 Replica of a luminal border and part of a lateral cell membrane in an alveolus from a 4-day lactating female. Next to the fringe of microvilli projecting into the lumen (*Lu*) is the tight-junction zone, interrupted at right center where the fracture plane has passed into the cytoplasm (*Cy*) of one of the cells. Ridges of the tight-junctional network undulate mildly near the lumen, more strongly abluminally. The lateral membrane is unusually flat and shows clearly the scattering of particles typical of face A Bar = 0.5 μm; × 32,000.

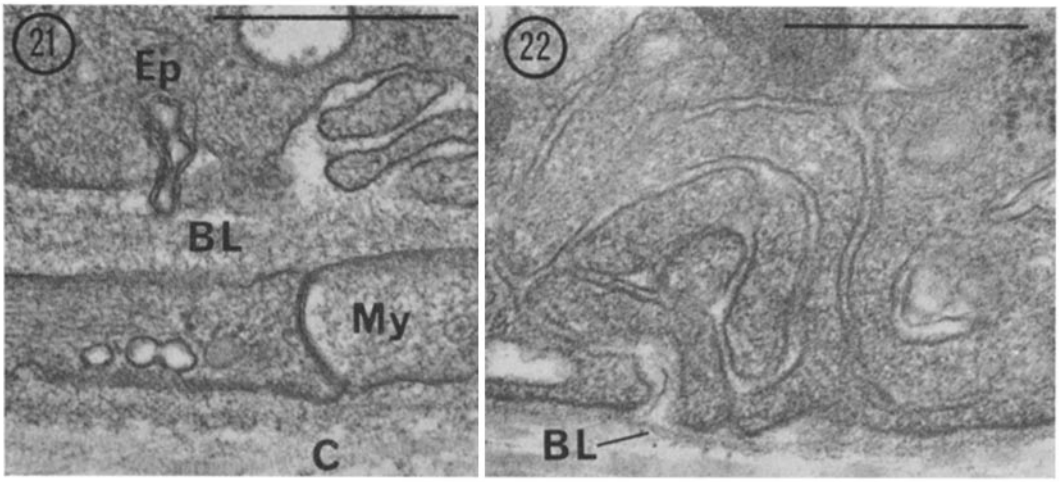

FIGURE 21 Section cut obliquely at the periphery of an alveolus in a 12-day lactating female. The two myoepithelial cell processes (*My*) actually are in the same alveolus as the epithelial cell (*Ep*); the fold of basal lamina (*BL*) that separates them in this area passes out of the section plane a short distance away. The myoepithelial processes are linked end-to-end by a gap junction. Outside of the basal lamina, some collagenous fibrils (*C*) are visible. Bar = 0.5 μm; × 64,000.

FIGURE 22 Section into the basal labyrinth of an alveolar epithelial cell from a 12-day lactating female. The basal lamina (*BL*) forms a smooth sheath over the basal surface and does not follow the contours of the labyrinth. Bar = 0.5 μm; × 64,000.

and are comparable to normally induced changes, although removed from their normal sequence. The shape variations are in fact similar to those shown by Richardson (56) in perfusion-fixed glands of goats.

The perimeters of epithelial cells measured in our scanning electron micrographs of inflated alveoli are about 50% longer than those in contracted alveoli. Evidently the tight junctions (remaining intact as they stretch), the myoepithelial basket, basal lamina, interstitial fiber network, and the pressure of surrounding tissues maintain the integrity of the alveolar epithelium while continuous secretion dilates the lumen and forces the cells slowly to flatten. What happens during the much faster contraction phase is unknown. Our observations indicate that alveolar myoepithelial cells are separate individuals, lacking desmosomal attachments to each other or to overlying epithelial cells. For their contraction to constrict the lumen at least one of two conditions must obtain: they must at some points be fixed to each other or to epithelial cells by gap junctions or unknown other mechanisms; or the basal lamina, to which they are fastened by hemidesmosomes, must have enough tensile strength to transmit the constricting force to the epithelium and its contents.

However it operates, effective contraction of a myoepithelial basket would seem more likely to create folds in the epithelial sheet than directly to cause its cells to change their dimensions. The epithelial cells behave rather as though their own perimeters were elastic, recoiling to their unstretched lengths as intraluminal pressure drops. The tight-junctional belts in this case would act as constricting girdles, guiding cytoplasm into apical and basal protrusions and changing the cell's shape to columnar.

During alveolar shape changes, the tight junctions are the only certain zones of attachment between epithelial cells (gap junctions presumably are present, but we do not know whether they are constant in position). If, as seems probable, membranes do not move rapidly through tight junctions, the area of the apical cell surface should be approximately the same in the flattened and bulging conditions. The height of the bulges suggests that this is generally so, but protein secretion constantly adds bits of Golgi-derived membrane to this surface while the pinching-off of fat droplets subtracts it, so that mem-

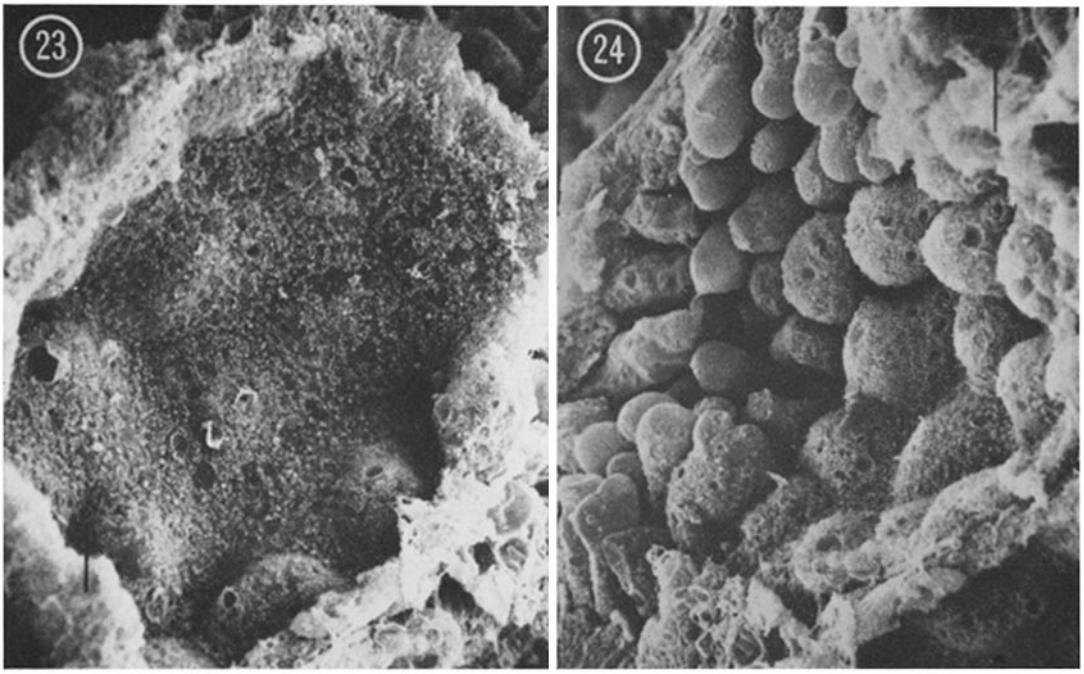

FIGURE 23 Scanning electron micrograph of the luminal surface of part of an alveolus fixed in the inflated state. Cells are flat or only slightly convex. Cell surfaces are irregularly covered with microvilli, and several small craters left by extracted fat droplets are visible. The luminal area seen here includes about 11 cells in full face view. × 1,000.

FIGURE 24 Scanning electron micrograph of the luminal surface of part of an alveolus fixed in the contracted state. Most of the cells visible bulge deeply into the lumen. Most cell apices are irregularly covered with microvilli and small fat craters, but several are smooth where they are distended over large fat droplets. Compare the number and shapes of cells visible here with those in Fig. 23 at the same magnification. × 1,000.

brane flux here is strong. The combined lateral and basal membranes should in theory also remain approximately constant. Basal bulging is not so extreme, and the basal labyrinth does not change detectably with shape change; there is some pinocytotic activity. Lateral cell interdigitations and extensions, if they are labile, could help to accommodate change in cell contour. Foci of cell attachment below the tight-junction belts might inhibit a necessary shifting of lateral epithelial cell surfaces relative to each other and to myoepithelial processes. A need for freedom of movement thus might require the resorption of preexisting desmosomes when lactation begins.

Cell shape changes in ducts are less clear and could not be so drastic since the volume of cytoplasm is much smaller. The walls of major ducts are usually folded in our fixed tissues; moderate heightening and flattening of folds would have more effect on duct volume than any change in shape of the small epithelial cells. Whatever the effect of myoepithelial contraction on the ductal epithelium may be, nonsecreting ducts in the lactating gland function primarily as conduits, and mechanical integrity as an all-important property of their walls is consistent with the abundance of desmosomes and other adhesive junctions found there.

Occluding Junctions

Tight-junctional zones in epithelia are known to occlude passage of electron-opaque substances (15, 17, 22); they vary in their ability to restrict passage of ions (18). Linzell and Peaker (31–34) discuss extensively the relative compositions of blood plasma and milk and the permeability of

alveolar and ductal epithelium. Although milk is isosmotic with plasma, its ionic composition is not the same, common ions being present in different proportions from those in blood. In experiments performed on goats, Linzell and Peaker (33) demonstrated that the major ducts of lactating animals were almost impermeable to several ions and molecules tested, and even labeled water passed slowly. Rapid transport across the epithelium thus appears to be limited to alveolar regions. Passage of indicators from blood into the ducts of a nonbreeding parous goat showed that ducts in the dry gland were more permeable than during lactation, and the fluid in their lumina resembled plasma rather than milk; but the rates of passage of various ions indicated that permeability was still selective.

Linzell and Peaker (31–33) concluded that transport of ions into milk is regulated by membrane pumps at alveolar cell surfaces. When molecules that do not enter the epithelial cells were introduced into either blood or milk they crossed the epithelium in negligible quantities. It appears that, if permeation through tight junctions in the mammary gland normally occurs, it may be very slight as compared with transport through the cells. No similar studies have been reported on fluid in the virgin gland or during the last third of gestation; secretion into alveolar lumina begins at different times in different mammals and is relatively early in the mouse.

The possibility exists that, even though junctional permeability is low throughout the life of the tissue, the condensed and relatively orderly pattern of the tight junction's ridged network in lactation is related to the greatly enhanced rate of polarized transepithelial traffic at this time. The more consistent width of the network and the absence of loose-ended ridges may signify more efficient occlusion; if ridges do coincide with lines of membrane fusion, continuous ridges would prohibit circuitous bypassing of incomplete barriers. More evidence is needed before this question can be seriously discussed.

A second possibility is that the orientation of the network in lactating alveoli is related to cell shape change. Unless all estimates (29, 56, and this report) are in error, tight junctions do undergo significant change in absolute length in a gland that is lactating and suckled: they elongate slowly and shorten fairly rapidly, as discussed above. There are many circumstances in the mammary epithelium as in other tissues in which local membrane distortions are accommodated; for example, microvilli disappear from the apical membrane over a bulging fat droplet (Fig. 24), presumably by flattening into the surrounding membrane continuum. The parallel distortion, *without separation*, of two intimately attached lengths of membrane may require special mechanisms; furthermore, segments of the continuous tight-junctional girdle of each cell must stretch and shrink in coordination with the conjoined membranes of its several neighbors. In fact, failure to maintain complete occlusion may occur under stress. Linzell and Peaker (34) reported that large doses or frequent small doses of oxytocin cause changes in milk composition in goats, suggesting a slight and temporary leakage of tissue fluid into alveoli.

Pinto da Silva (49) has demonstrated rapid, reversible translocation of particles on the A face of human erythrocyte membranes. In mammary alveoli, the initial condensation, at about parturition, of the loose, irregular, tight junction requires redistribution of ridges (which commonly appear to be composed of fused particles [17]). Once the network has acquired its oriented, lactational configuration, length changes involve membranes and ridges together. It may be more than coincidence that the elongate meshwork formed by the undulating ridges is reminiscent of the threads of a loosely knit fabric under varying tension.

Such modest changes as we have observed in junctional morphology in early involuting alveoli may simply reflect the end of active maintenance of functional specialization.

Comparison with Other Epithelia

Other ectodermal glands that excrete intermittently and are equipped with myoepithelial cells are the sweat, salivary, and lacrimal glands. Unlike the mammary parenchyma, the secretory epithelium in all of these is innervated and is maximally active only in response to stimulation that usually is of short duration; continuous accumulation of secretory product in the lumina during long intervals between emptying does not occur, and changes in cell shape do not appear to be profound. All of these glands (1, 14, 46, 62, 63) have conventional junctional complexes in both acini and ducts, and desmosomes appear elsewhere as well.

The epithelium of the urinary tract does

undergo extensive stretching. Changes induced by stretching in the toad bladder (19) show some parallels to those in mammary parenchyma inasmuch as epithelial cell thickness changes markedly; however, regular junctions, including desmosomes, are present in both stretched and contracted states. Luminal surface membranes of transitional epithelium in several mammals are known to have a distinctive structure; this epithelium from rabbit bladders has been studied in freeze-fracture replicas (60). Particle-bearing plaques occupy much of the luminal membrane; they have no counterpart in the mammary epithelium. Apical junctions were not pictured in this study, but earlier reports (e.g., 58) have illustrated in section what appear to be typical junctional complexes and numerous desmosomes, as well as extensive folding of lateral surfaces.

Relatively few different kinds of epithelial tight junctions have been examined in freeze-fracture replicas; Friend and Gilula (17) recently have surveyed several tissues in rats. The loose network of tight-junctional ridges in mammary ducts and prelactating alveoli is somewhat similar to but more irregular than those in glandular epithelia such as pancreas and liver. In its compact and orderly form, the network in secretory alveoli shows resemblance to those illustrated by Friend and Gilula in rat epididymis and duodenum, but it is less extensive than that in the epididymis, less angular than that in the duodenum, and more consistent than either in its parallel orientation and closed border ridges.

Gap Junctions

Recognized as entities separate from tight junctions only recently (27, 52), gap junctions are generally believed to function in ionic and metabolic exchange between linked cells (20, 53) and perhaps only coincidentally in adhesion. Variation in size and location seems to be the rule in all mammalian tissues in which they have been examined (17); unlike tight junctions, they are not limited to epithelia or to chordates. Gap junctions have not to our knowledge been studied in a sequence of developmental states in any other tissue. In the mouse mammary gland, they are moderately common at all stages we have studied, and show no notable differences in ultrastructure. We have the impression that they are less numerous, but on the average larger, in lactating alveoli than in other states; this might simply reflect the need to reduce the number of lateral cell attachments to a minimum. At any stage, however, they occupy too small a proportion of the cell surface to permit significant frequency measurements with the small samples we have used.

General Comments

Of the surface specializations that we examined, the basal labyrinth alone seems to have no significance in cell-to-cell association. It does not unfold when epithelial cells flatten. Several authors (2, 13, 31) have commented that it increases the surface through which absorption of substances from the tissue fluid may take place during lactation.

That a significant difference exists between ductal and alveolar cells is indicated by the extensive development of ducts before gestation and their persistence after weaning, in contrast to the dependence of alveoli for their growth and maintenance upon the hormones of pregnancy and lactation. However, we do not know where the dividing line occurs between hormone-independent and hormone-dependent cells. Highly secretory cells occur during lactation consistently in terminal and subterminal ducts and occasionally in patches in the walls of major ducts (M. K. Nemanic, unpublished data); it is not known whether any of these hormone-sensitive ductal cells are also hormone dependent and succumb to postweaning involution. It seems unlikely that all of them do, as the duct system after involution typically includes more fine twigs than before the first pregnancy. We have been unable to determine the fate of these cells because they cannot be identified at all in freeze-fracture replicas and not reliably in thin sections. Our observations, therefore, do not tell us to what extent the changes in junctional morphology observed during lactation are reversible; nor do they indicate whether these changes represent cellular responses to hormones present during lactation or to mechanical stress associated with lactation.

The occurrence of hyperplastic alveolar nodules in the mammary glands of mammary tumor virus-infected mice permits examination of secretory alveolar epithelium in nonbreeding females. In a continuation of this study[1], we will

[1] Pitelka, D. R., and S. T. Hamamoto. 1972. Cell contacts in the mouse mammary gland. II. Preneoplastic and neoplastic tissue. Manuscript in preparation.

describe epithelial cell contacts in these nodular tissues and in mammary tumors in both resting and lactating females.

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