A Fine Structural Study of Divalent Cation-mediated Epithelial Union with Connective Tissue in Human Oral Mucosa

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ABSTRACT   Following incubation in an isotonic saline solution containing 20 mM EDTA, human oral mucosa may be separated into its epithelial and connective tissue components. Ultrastructural study of the separated tissues reveals that the plane of separation is through the lamina lucida. Hemidesmosomal structure is altered by the separation process: the peripheral density is absent but a fine, generally filamentous material remains associated with the outer membrane leaflet of the hemidesmosome. Desmosome structure is not altered. An intact lamina densa remains attached to the connective tissue fragment. Oral mucosa incubated in EDTA-saline containing calcium, or its return to a divalent cation-supplemented medium after treatment with EDTA, prevents separation. By maintaining the structural integrity of the hemidesmosome, divalent cations appear to play a principal role in uniting oral mucosal epithelium to the lamina propria.

Contact between stratified squamous epithelium and connective tissue occurs at the basal lamina. Adherence of the epithelial sheet to the connective tissue is believed to be mediated *via* specialized attachments (i.e., hemidesmosomes) located on the inferior membrane surface of the basal cell (Porter, '54; Weiss and Ferris, '54; Selby, '56; Odland, '58; Stern, '65; Kelly, '66). Fine hemidesmosomal filaments traverse an electron-lucent zone (lamina lucida) and terminate in an incompletely understood fashion in the lamina densa.[1] These structures are not the equivalent of the light microscopic basement membrane visualized following application of the periodic acid-Schiff procedure. The basement membrane of light microscopy is thought to be due to periodate-engendered aldehydes associated with unit collagen fibrils and protein-polysaccharides immediately beneath the lamina densa (Swift and Saxon, '67).

The region of contact between stratified squamous epithelium and connective tissue has been the subject of considerable attention since it is intimately involved in the process of wound healing (Krawczyk, '71) and in disease processes such as carcinomatous invasion (Frithiof, '69) and severe blistering diseases (Pearson, '67; Wilgram, '67). Additionally, the contact region is of fundamental importance in biological phenomena such as transport of metabolites to the epithelium (Slavkin et al., '71), biogenesis of the basal lamina (Pierce and Nakane, '69; Nadol and Gibbins, '70), and the influence of connective tissue on epithelial behavior (Wessells, '64; Dodson, '67a,b).

Mammalian basal cell connections (via hemidesmosomes) to the lamina densa appear tenacious, since separation does not occur with moderate pulling (Charles and

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[1] In this paper the term lamina lucida applies to the generally electron-lucent zone between the plasma membrane of the basal cell and the electron-dense zone commonly called the basal lamina which separates the epithelium from the connective tissue. The basal lamina will be referred to as the lamina densa. We are aware of at least six names applied to the basal lamina during the evolution of electron microscopic cytology. The first *joint use* of the terms lamina lucida and lamina densa was, as far as we can determine, by Low ('61) who, in the same paper, advocated the term "boundary membrane" be applied to the lamina densa. Stern ('65) reintroduced the terms lamina lucida and lamina densa in a paper concerned with oral mucosal basal cells and the basal lamina. Subsequently, this terminology appears to be used almost exclusively among those studying the ultrastructure of oral mucosa (lamina lucida and lamina densa as described; lamina densa and basal lamina are used interchangeably). The terms lamina lucida and lamina densa are used here for convenience and without prejudice as to the exact interrelationship between the two zones.

Smiddy, ' 57), hypertonic insult (Weiss, '58) or chemically induced irritation (Quasdorf, '62; Listgarten et al., '63). In an analogous situation, mechanical separation of corneal epithelium results in tearing of basal cells, leaving the inferior membrane surface with its hemidesmosomes adhering to the lamina densa (Jakus, '54). Hemidesmosome-like regions of aortic endothelial cells have also been demonstrated to be points of firm cellular attachment to the underlying basal lamina (Stehbens, '60; Ts'ao and Glagov, '70).

Certain preparative procedures allow relatively complete separation of stratified squamous epithelium from the underlying connective tissue (Rosett et al., '67; Neiders and Weiss, '70). One of these methods relies on the removal of divalent cations by chelation and has been employed in avian embryonic systems (Bell et al., '62; Dodson, '67a; Goel and Jurand, '68). The present study extends these observations to the role divalent cations play in maintaining union between stratified squamous epithelium and the lamina propria of human oral mucosa. The fine structural changes occurring when epithelium separates from connective tissue following the removal of divalent cations are described.

MATERIALS AND METHODS

Surgical specimens of human oral mucosa were transferred to the laboratory at room temperature in Earle's balance salt solution [2] (EBS). The time between tissue excision and the beginning of the experiments ranged from 30 to 60 minutes. Specimens were trimmed to approximately $5 \times 2 \times 2$ mm and washed twice in calcium-and magnesium-free phosphate-buffered saline [3] (PBS). Several (3-8) samples were then incubated at 37°C in 5 ml of an isotonic solution of phosphate-buffered saline containing 20 mM of tetrasodium ethylenediamine tetraacetate dihydrate [4] (PBS-EDTA) [NaCl, 6.83 gm; KCl, 0.2 gm; Na$_2$HPO$_4$, 1.15 gm; KH$_2$PO$_4$, 0.2 gm; 1% phenol red, 0.12 ml; Na$_4$EDTA, 7.6 gm; made to 1 liter with distilled water and gassed with 5% CO$_2$ to pH 7.2–7.4]. After incubation in PBS-EDTA at 37°C for 50 to 60 minutes, epithelium could be separated easily from the underlying connective tissue with fine forceps using a dissecting microscope. Duplicate separation experiments were attempted using the chelating agent EGTA [5] [ethylene-bis (oxyethylenenitrilo)-tetraacetic acid] in a similar calcium- and magnesium-free PBS solution.

Specimens from each experiment were fixed directly as a control for the fixation and embedding procedures (untreated controls). Other preparations in which tissue separation was attempted consisted of tissue incubated for one and four hours either in PBS-EDTA, PBS-EDTA containing 20 mM of calcium ion (as CaCl$_2$) or in calcium- and magnesium-free PBS. Other tissue slices were incubated in PBS-EDTA for one hour and then either transferred directly to, or partially separated and then transferred to, divalent cation-supplemented medium (EBS) or to EBS containing only calcium or magnesium. After one hour of incubation at room temperature or at 0°C in the divalent cation-supplemented solutions given above, separation was again attempted.

Tissues from all groups were fixed at room temperature for two hours in 2% paraformaldehyde and 2.5% glutaraldehyde in one-half strength Millonig's ('62) buffer. Specimens were postfixed in Millonig's 2% OsO$_4$, stained *en bloc* in 0.5% or 2% uranyl acetate (Karnovsky, '67), dehydrated in graded concentrations of ethyl alcohol and propylene oxide prior to embedding in a Maraglas-D.E.R. 732 mixture (Erlandson, '64). Sections were collected on carbon-reinforced, formvar-coated grids and subsequently stained with uranyl acetate and lead citrate. Sections were viewed with either a Phillips EM 300 or Siemens Elmiskop 1A electron microscope.

In one experiment (3e, table 1) epithelial samples incubated in PBS-EDTA for five hours were both swirled and the basal surface gently scraped. The resultant dissociated cells were collected on cellulose acetate filters and fixed in the usual manner. The filters were subsequently dehydrated in ascending concentrations of ethyl alcohol, infiltrated in several changes

of a 1:1 mixture of 100% ethyl alcohol and complete Epon 812 followed by several changes of complete Epon 812 over a period of four days prior to embedding.

In initial experiments, tissues were prepared for light microscopic observation using either neutral formalin or alcohol-formalin-acetic acid fixation and paraffin embedding. Seven-micron sections were stained with either hematoxylin and eosin or periodic acid-Schiff (PAS) and hematoxylin. In succeeding experiments, formalin-fixed, paraffin-embedded tissues were occasionally utilized to determine whether any epithelial cells remained attached to the connective tissue. This report is based on data obtained from 25 experiments, the tissues from eight of which were extensively analyzed ultrastructurally.

OBSERVATIONS

Table 1 summarizes the results of experiments conducted to determine under what conditions epithelium would separate from connective tissue and whether tissue processed to the point of separation could be prevented from separating by further treatment.

Light microscopic observations

The appearance of tissue held for one hour in EBS (fig. 1) could not be distinguished from tissue fixed immediately following excision. Separated preparations demonstrated a moderately scalloped free connective tissue surface devoid of cells. The demonstration of a basement membrane by the PAS technique was equivocal. The morphology of separated epithelium appeared the same as in the EBS-incubated specimens, except that the minor pedicled projections on the undersurfaces of basal cells were more evident. Occasional spaces were found between basal cells caused by the "dropping out" of transmigrating white blood cells or, perhaps, melanocytes (fig. 2). In occasional experiments, solitary basal cells remained attached to connective tissue. However, using the techniques described, this has been the exception rather than the rule. Tissues incubated in PBS-EDTA and then returned to EBS did demonstrate noticeable shrinkage of basal cells with concomitant widening of the intercellular spaces (fig. 3).

Electron microscopic observations

Control tissue. The fine structure of human oral mucosa has been dealt with extensively by others (Albright, '60; Listgarten, '64; Schroeder and Theilade, '66; Thilander, '68). Therefore, we shall limit our observations to a brief description of the attachment apparatus of the epithelial basal cell and the region of contact.

The basal cell of oral mucosa presented either a gently wavy undersurface (fig. 4) or numerous hemidesmosome-studded, deep cytoplasmic projections which accompanied the lamina densa into the lamina propria. The tonofilaments extended to both hemidesmosomes and to the relatively few desmosomes found on the lateral and superior aspects of the cell (fig. 5). In our preparations the intercellular space between basal cells was usually about the same or slightly less than the membrane-to-membrane distance found in desmosomes (figs. 4, 5).

The lamina densa was evident as an electron-dense layer of varying width. Subjacent to the lamina densa, unit collagen fibrils and subepithelial fibrils[6] were observed. The presence of the extremely dense periods of the subepithelial fibrils together with a slight change in orientation of unit collagen fibrils from those more distant from the lamina (figs. 4, 14) frequently gave the impression of there being a distinct connective tissue zone immediately subjacent to the lamina (Schroeder and Theilade, '66). This zone was more evident in regions having a lamina densa of relatively smooth contour.

Filaments extended from subepithelial fibrils into the lamina densa but the nature of their terminations could not be determined (figs. 7, 13).

The appearance of the lamina lucida was variable. In some regions it was a distinct electron-lucent, filament-filled region which blended gradually with the lamina

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[6] These fibrils are found beneath the lamina densa and frequently send filamentous projections into it. They are a prominent feature of human oral mucosa and have been described in most papers dealing with the subject since 1966. They are akin, if not identical to the fibrils described in some detail by Palade and Farquhar ('65), Susi et al. ('67), Rowlatt ('69) and Bruns ('69). (See Bruns for an extensive discussion.) It is presumed these fibrils assist in the holding of the lamina densa to the connective tissue; however, that remains to be conclusively demonstrated. In this paper Rowlatt's descriptive term "subepithelial fibril" will be used.

TABLE 1

Procedures used to investigate the nature of epithelial-connective tissue union

Incubating solution		Time	Results	Further treatment of 3.	Time	Results
		hours			*hours*	
1. Earl's Balanced Salt solution (EBS)	pH 7.2–4	1 4 24	— — —	3a. Samples returned to PBS (Ca^{++}, Mg^{++} free)	1	+
2. Phosphate Buffered Saline, Ca^{++} Mg^{++} free (PBS)	pH 7.2–4	1 4 24	— — —	3b. Samples returned to EBS,	1	—
3. 20 mM EDTA in PBS	pH 7.2–4	1	+[1]	EBS, EBS (c̄ Ca^{++}), EBS (c̄ Mg^{++})		
4. 10 mM EDTA in PBS	pH 7.2–4	2	+	3c. Samples returned to EBS at 0°C	1	—
5. 20 mM EDTA-PBS with 20 mM Ca^{++}		2	—[2]	3d. Separated epithelium incubated in EDTA-PBS	4	Desmosomes intact, many membranes lysed, marked cytologic damage
6. 20 mM EGTA in PBS	pH 6–8	2	—	3e. Same as 3d only specimens swirled in EDTA-PBS and scraped; cells collected on filters and fixed		Majority of cells from disjunctive layer, few intact cells with broken desmosomes
7. 20 mM EGTA	pH 9	2	± (adhering basal cells)			
8. 20 mM EGTA	pH 10	2	+ (dispersal of tonofilaments and marked cytologic damage to basal cells)			

[1] +, separation.
[2] —, no separation.

densa (figs. 4, 6). In other regions the lucent zone was less distinct (fig. 7).

Hemidesmosomes were a prominent feature of the basal cell. The intracellular attachment plaque presented one of two appearances. The first was a series of triangular-(cone?)-shaped, electron-dense regions, which usually became confluent opposite the inner leaflet of the plasma membrane (fig. 6). In the second and less frequent form, the attachment plaques appeared separate and lacked the pointed projections (fig. 7). Tonofilaments entered the plaques, where they became unresolvable. The plasma membrane was sporadically asymmetric, the inner leaflet usually being more densely stained (figs. 6, 7). Filaments originating on the outer membrane leaflet (fig. 6) merged with the peripheral density (Stern, '65), a more-or-less continuous electron-dense band 60–100 Å in thickness. The same or other filaments, spanned the interval between the peripheral density and the lamina densa. In both control and treated preparations, occasional hemidesmosomes lacked peripheral densities. In these hemidesmosomes, uninterrupted filaments bridged the space between the plasma membrane and the lamina densa. The following approximate measurements were observed: thickness of the plasma membrane, 70–80 Å; distance between the plasma membrane and the peripheral density, 70–100 Å; width of the peripheral density, 60–100 Å; width of the lamina lucida (when distinct), 350–500 Å; width of the lamina densa, 400–500 Å; width of hemidesmosomal filaments, 30–70 Å.

Transport vesicles (fig. 7) and projections of the basal plasma membrane (fig. 5) were frequently observed on regions of the basal plasma membrane devoid of hemidesmosomes. We believe the projections may be fixation artifacts.

Separated tissue. The epithelium separated from the lamina propria consisted of individual cells joined by desmosomes (fig. 8). A considerable widening of the intercellular space was evident. Desmosomal structure was not altered by EDTA treatment. The inferior plasma membrane of the basal cell was readily identifiable by the presence of hemidesmosomes (fig. 9). Flocculent material or whorls of mem-

brane-like material were frequently observed beneath the separated basal cells. A fine, generally filamentous material emanated from the plasma membrane of the hemidesmosomes into the extracellular space. The peripheral density could not be distinguished (fig. 10). The outer surface along the membrane between hemidesmosomes was devoid of demonstratable material (figs. 9, 10).

An intact lamina densa covered the free surface of the connective tissue portion. In regions where the epithelium-connective tissue interface was highly infolded, occasional discontinuities in the lamina densa were observed (fig. 11). This was not the case where the epithelium-connective tissue interface was smooth (fig. 12). A variable degree of swelling was evident in the connective tissue fragment as evidenced by increased spacing between collagen fibrils. The lamina densa appeared as a flocculent, electron-dense region. No indications of previous sites of hemidesmosomal attachment were detected (fig. 13). Small bits of flocculent, electron-dense material were frequently observed superior to the lamina densa.

Tissue returned to a divalent cation-supplemented medium. When compared with control preparations, several changes were obvious in tissues returned to EBS following PBS-EDTA treatment. The epithelial cells appeared shrunken, with resultant condensation of cytoplasm and widened intercellular spaces (fig. 14). The connective tissue appeared slightly swollen, since unit collagen fibrils were separated by more than the usually observed interfibrillar distance. The region of contact also appeared altered. The lamina densa was approximately two-thirds to three-fourths as thick as in control preparations (fig. 15). The peripheral densities of hemidesmosomes were consistently interrupted at many points, much more so than in control preparations (cf. fig. 15 with figs. 6, 7).

DISCUSSION

Results from this study indicate that divalent cations are essential for the union of epithelial basal cells to the lamina densa. Furthermore, the removal of divalent cations allowed the epithelium to be

lifted from the lamina densa with only the extracellular filamentous portion of the hemidesmosome being altered. Based on these findings, divalent cations may be tentatively assigned a principal role in maintaining the structural integrity of hemidesmosomal filaments and peripheral densities. Unfortunately, we have been unable to either localize the site(s) of cation patricipation or decide with certainty how hemidesmosomes come apart following the removal of cations.

Cation binding to both polyanionic proteins and protein-polysaccharides has been demonstrated (Tanford, '61; Schubert and Hamerman, '68) and divalent cations are believed to form cross-bridges between adjacent proteins (Mildvan, '70). Therefore removal of divalent cations may allow unwinding or sliding past one another of cation-linked proteins or protein-polysaccharides resulting in the abundant, generally filamentous material associated with hemidesmosomes following separation. In tissue separated by proteolytic enzymes, the hemidesmosomes possess short (70–100 Å) filamentous stubs which give the impression of hemidesmosomal filaments having been cleaved (Scaletta and MacCallum, '71, and unpublished data) rather than "unwound" as suggested by their conformation when treated with EDTA. In any event, the exact mechanisms involved remain to be determined, as does the precise chemical architecture of the hemidesmosomal complex.

We cannot with certainty identify calcium as the sole divalent cation responsible for mediating union between the two tissues for several reasons. First is our empirical observation that magnesium supplementation is as effective as calcium in re-establishing the union in EDTA-treated tissues. Second, the observation that EGTA, which has an infinity for calcium six times greater than for magnesium, did not work as well as EDTA for separation of epithelium and connective tissue. The affinity of EDTA for calcium is only two orders of magnitude greater than magnesium (Schmid and Reilley, '57; Kopanica et al., '67). (For complex ionic solutions like the tissue system studied, the relative affinities for calcium over magnesium for *each* chelating agent may

be less than that stated; it is assumed, however, that the reduced affinities would maintain approximately the same proportion). There is the theoretical consideration that EGTA may be less efficient than EDTA near physiologic pH since below pH 8.85 it exists as a diprotonated molecule (Sanui and Pace, '67). However, Millington et al. ('66), Sanui and Pace ('67) and Legato and Langer ('69) have conclusively demonstrated by chemical, physiological and electron microscopic cytochemical methods that EGTA is an effective chelator of calcium in mammalian systems near physiologic pH. Third, the lack of sufficient concentrations of chelating agent did not appear to be a consideration, since each agent was present in at least 16 times the concentration required to remove all the calcium and magnesium in the system. (These calculations are based on 5 cm^3 of a 20 mM chelating agent and 1 gm of tissue containing 3 mM each of calcium and magnesium, which is far in excess of the weight of the tissue used.) Fourth, Manery ('61) reported that defatted skin, the only stratified squamous epithelium-connective tissue complex thus far examined, contains on a wet weight basis 2–3 mM of both calcium and magnesium. This magnesium content is considerably higher than in other connective tissues thus far studied. While these considerations do not necessarily prove that magnesium plays a role in maintaining the union between tissues, it nevertheless makes us reluctant to completely dismiss this divalent cation in favor of calcium, the most plentiful extracellular divalent cation and the one considered as the mediator of cell adhesiveness and other cell membrane phenomena (Manery, '69).

We suspect that divalent cation participation in the union phenomenon is of a physicochemical nature and not due to interference with energy production for the following reasons. (1) An increased adhesiveness develops between apposing surfaces of separated epithelium and connective tissue within minutes when returned to a cation-rich medium, although the hemidesmosome is not reorganized (unpublished). (2) The union is re-established at 0°C, a condition that essentially arrests metabolic processes, including deg-

radation of ATP (Emmelot and Bos, '68; Willis and Li, '69).

In human oral mucosa, removal of divalent cations does not result in the "breaking" of desmosomes as has been reported to occur in the liver (Leeson and Kalant, '61), gastrointestinal tract (Sedar and Forte, '64; Dybing et al., '69) and toad bladder (Hays et al., '65). This difference in desmosome character was so striking that we extended incubation times and collected free cells released into the medium. In each instance the great majority (over 90%) of the free cells originated from the disjunctive layer. This was true even when there was evidence of severe cytological damage to the cells of the epithelial fragment after having been in the EDTA solution for five hours. It appears that the nature of the desmosome bond between epithelial cells of human oral mucosa is different from that present in other tissues. Perhaps this is true of other stratified epithelia of ectodermal origin, for chick ectoderm separates as a sheet from the underlying mesoderm under the action of EDTA (Bell et al., '62; Dodson, '67a; Goel and Jurand, '68).

As observed by Bell et al. ('62) and Dodson ('67a) in light microscopic studies of chick embryo systems, removal of divalent cations permits human stratified squamous epithelium to be separated from the underlying connective tissue in a plane just superior to the basement membrane. Ultrastructurally, the plane of separation in human oral mucosa is just superior to the lamina densa as has also been reported by Goel and Jurand ('68) in a chick embryo system. Since hemidesmosomes are the only distinct cytological entity altered following separation, it appears that divalent cations mediate union of epithelium to the lamina densa (which may itself be derived in great part from epithelial cells, cf. Briggaman et al., '71) by maintaining the structural integrity of the hemidesmosomal filaments. The localized alteration of a structure for cell contact makes the role of divalent cations in the tissue union phenomenon under study distinct from the well established but poorly understood roles divalent cations play in cellular adhesion and hardening of the cell membrane (Manery, '69).

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1 Section of oral mucosa which had been incubated in EBS for one hour. H and E, × 400.

2 Partially separated epithelial-connective tissue preparation following incubation in 20 mM EDTA for one hour. The tissues have shifted relative to one another to the extent that an exact matching is not possible. H and E, × 400.

3 Tissue incubated in PBS-EDTA for one hour followed by one hour in EBS. Except for widened intercellular spaces between melanin-containing basal cells in several regions of the micrograph, the preparation appears similar to the control illustrated in figure 1. The perinuclear halos in this micrograph have been observed in all types of preparations and are not considered a "specific" change. H and E, × 400.

440
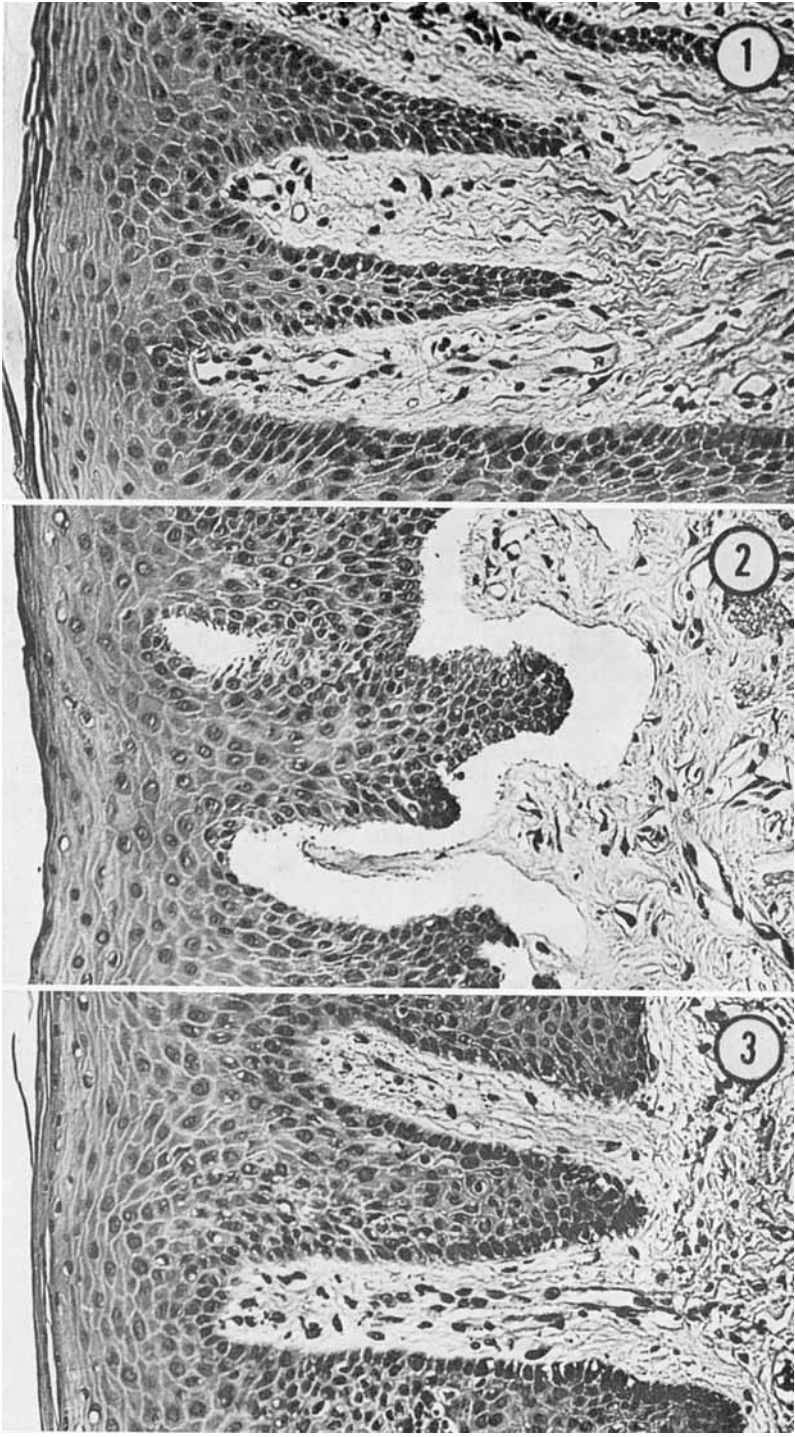
All electron micrographs were taken from sections treated *en bloc* with uranyl acetate and stained after sectioning with uranyl acetate and lead citrate. Magnifications are approximate.

4   Survey micrograph of an epithelial basal cell following a one hour incubation in EBS. A desmosome is indicated (arrow) as are hemidesmosomes (arrows HD). The lamina lucida, lamina densa and subjacent lamina propria are also demonstrated. × 20,700.

5   Portions of three epithelial basal cells near the lamina densa. Note the irregular intercellular distances as compared with the fixed spacing of the desmosome (D) between two of the cells. The small protrusions of the plasma membrane (arrows) are probably engendered during processing. × 48,000.
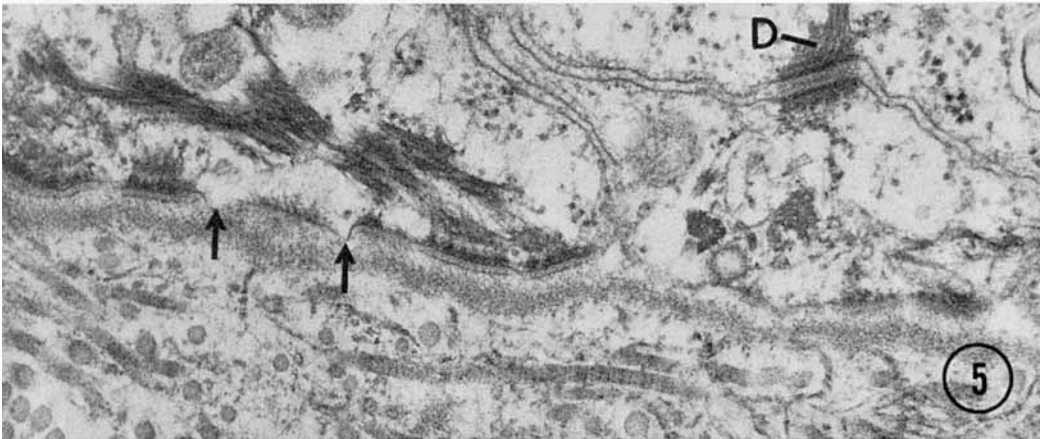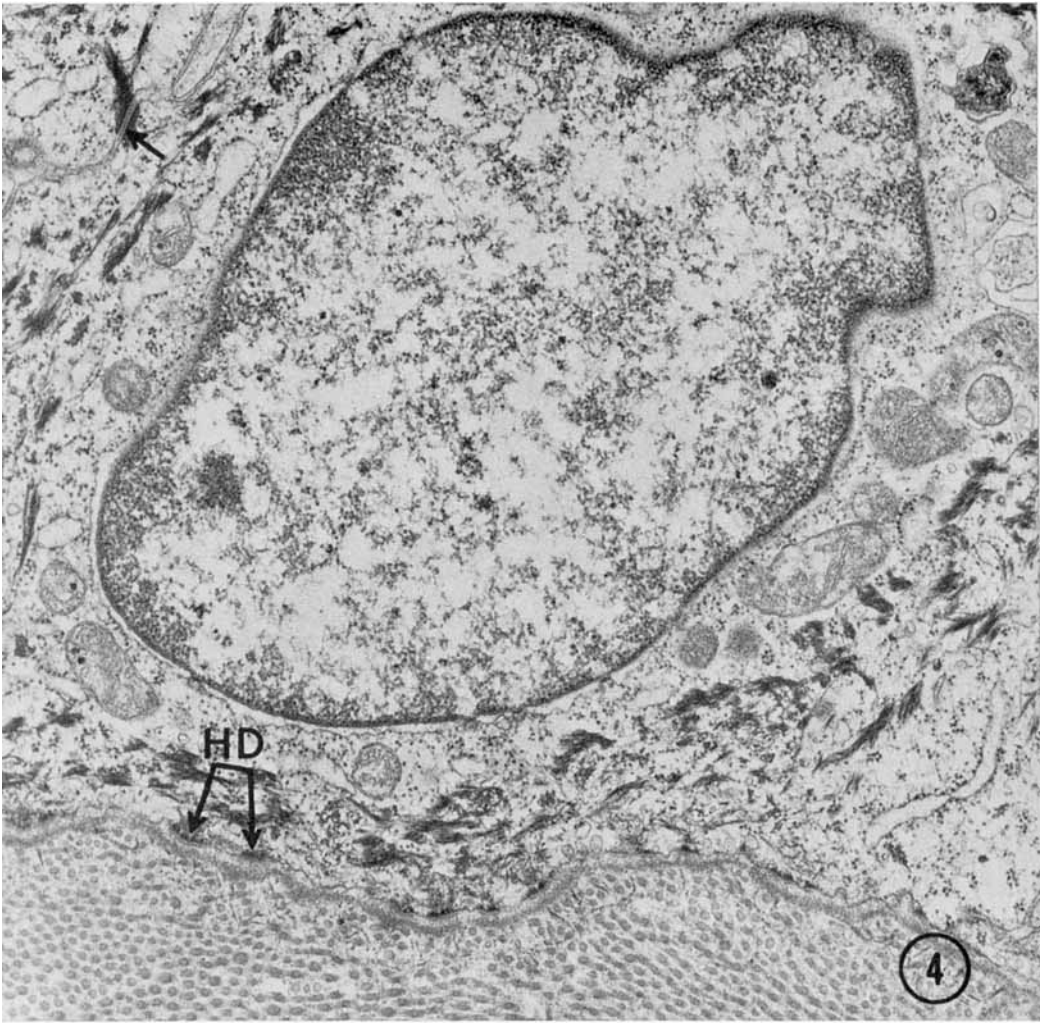
PLATE 3

6   High power micrograph of the more usual hemi-desmosomal configuration. Fine filaments (arrow) originating on the outer leaflet of the plasma membrane merge with the peripheral density (PD). Other filaments from the peripheral density span the lamina lucida (LL) to the lamina densa (LD). Both this specimen and the one illustrated in figure 7 were incubated in EBS for one hour. × 130,000.

7   Hemidesmosomes appear less continuous in this micrograph. Transport vesicles (P) are frequently observed along the undersurface of basal cells. Numerous subepithelial fibrils in various planes of section are present as are unit collagen fibrils (C). One subepithelial fibril (DF) sends its filamentous terminations into the lamina densa. This section is very heavily impregnated with uranium salts. × 129,000.
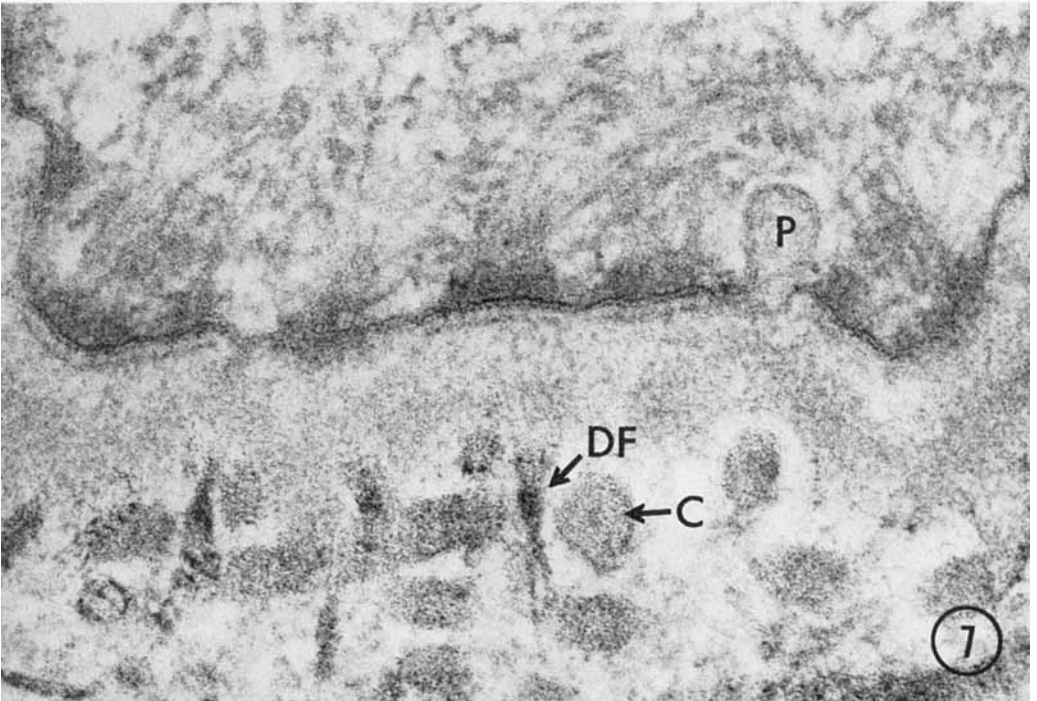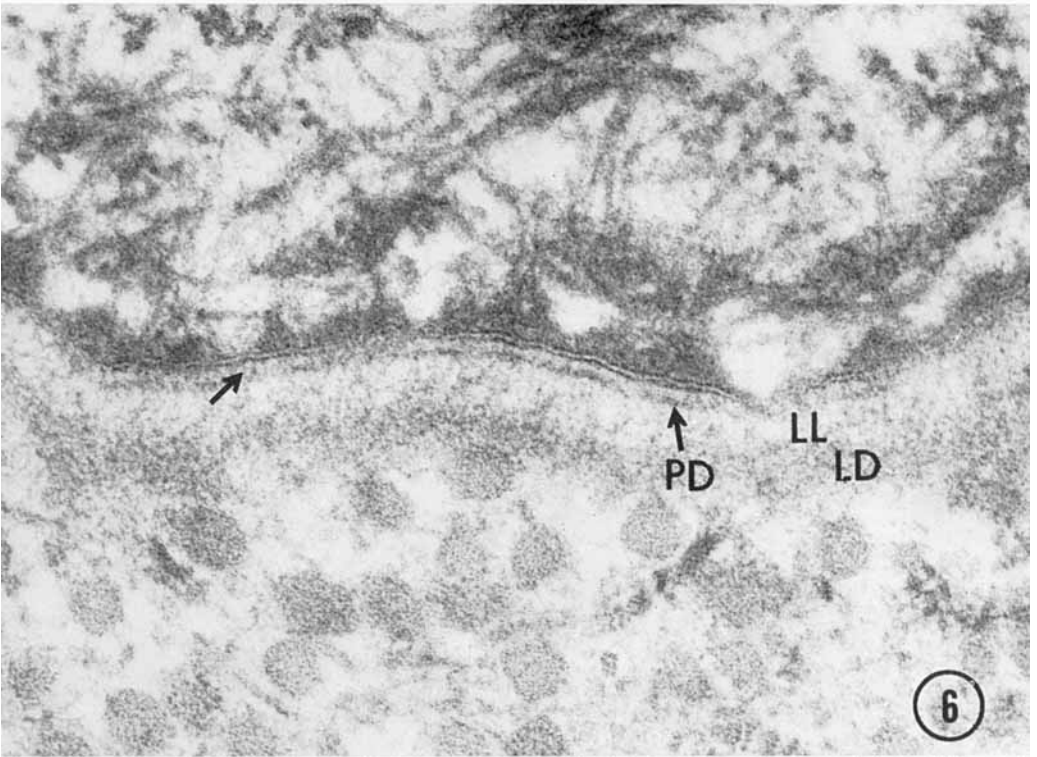
PLATE 4

8 Survey micrograph of epithelium separated from the lamina propria following EDTA treatment. The intercellular spaces are considerably widened, but the desmosomes are still intact. $\times$ 6,900.

446
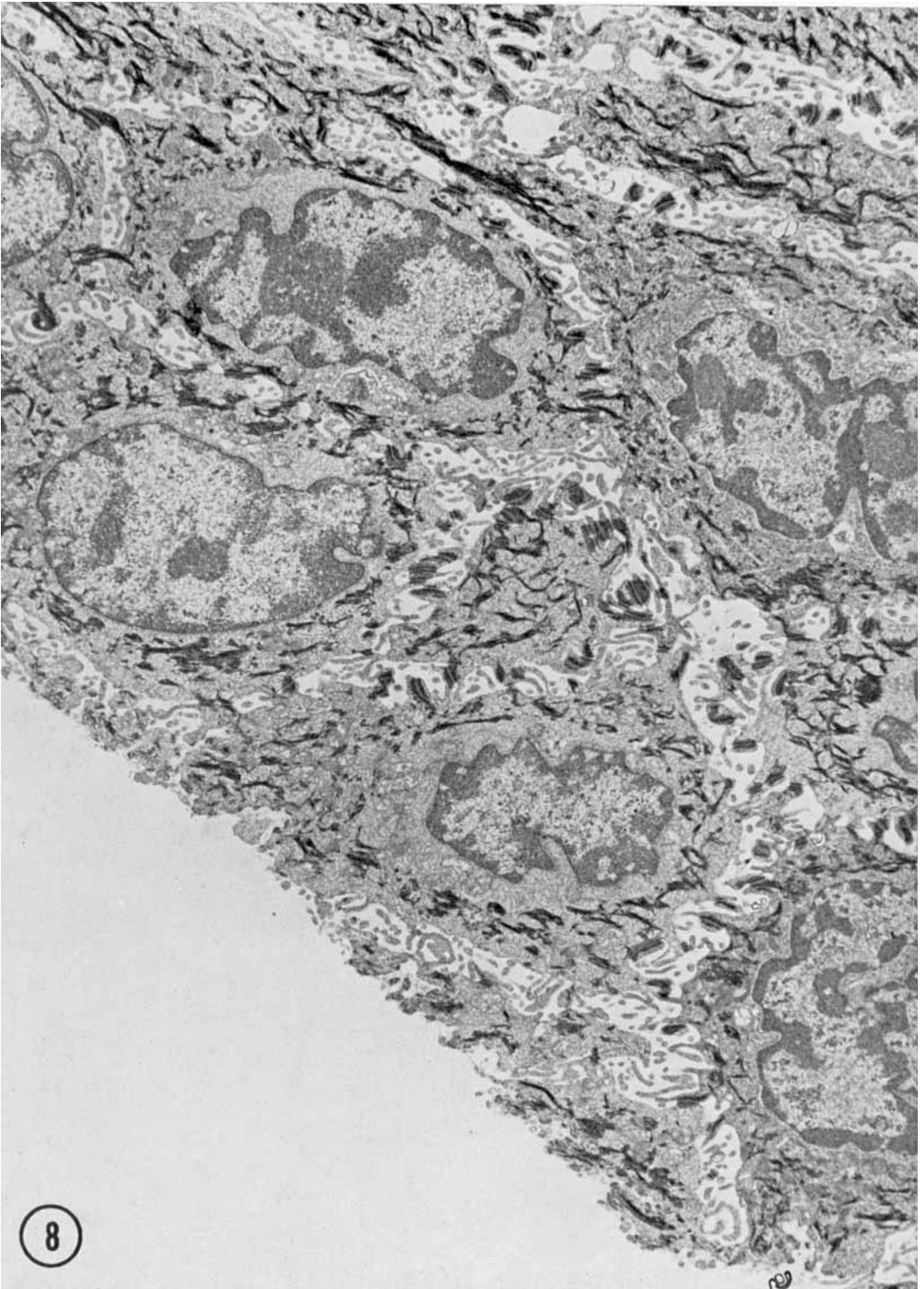
PLATE 5

9　Epithelial basal cell of a separated preparation. Numerous hemi-desmosomes (arrows) are evident along the undersurface of the cell. × 17,800.

10　Hemidesmosomes after separation demonstrate a loss of the peripheral density and the presence of a generally filamentous material radiating from the plasma membrane. × 112,000.

448
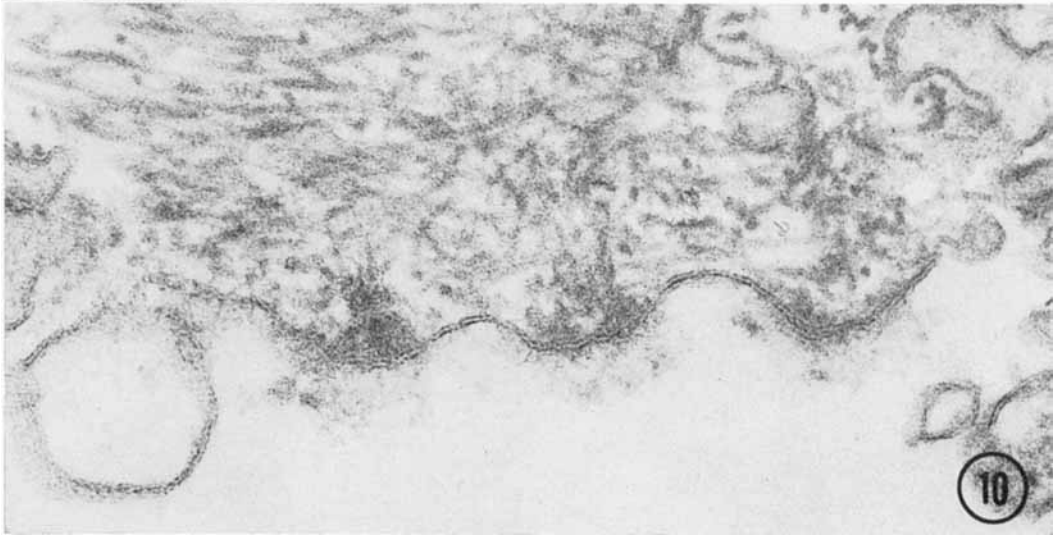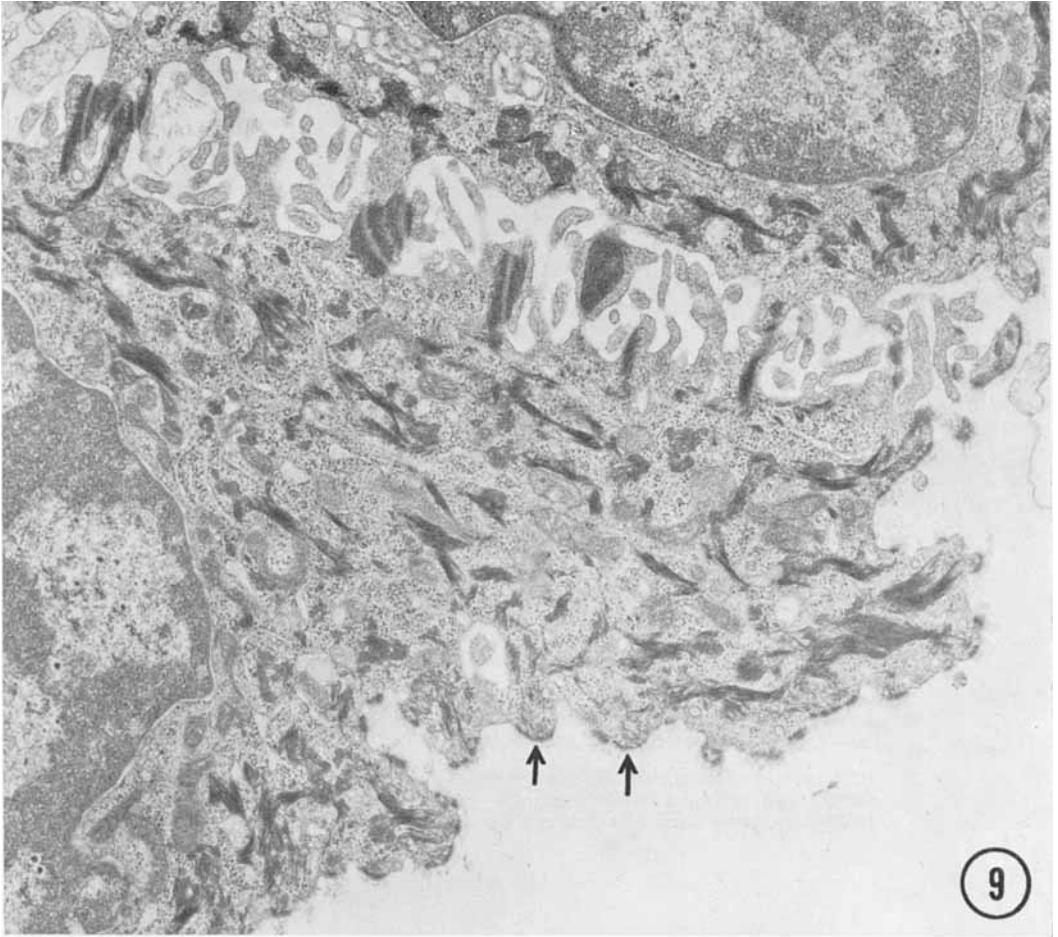
PLATE 6

11 Following separation the lamina densa remains with the connective tissue, as illustrated in this micrograph. Occasional gaps (arrow) in the lamina densa are observed in regions where there had been extensive interdigitation between epithelium and connective tissue. × 16,000.

12 Intact lamina densa in a region where the epithelial connective tissue interface was relatively flat. × 31,600.

13 High power micrograph of the lamina densa and subjacent subepithelial and collagen fibrils from the connective tissue. Sites where hemidesmosome were attached are no longer evident. × 105,000.
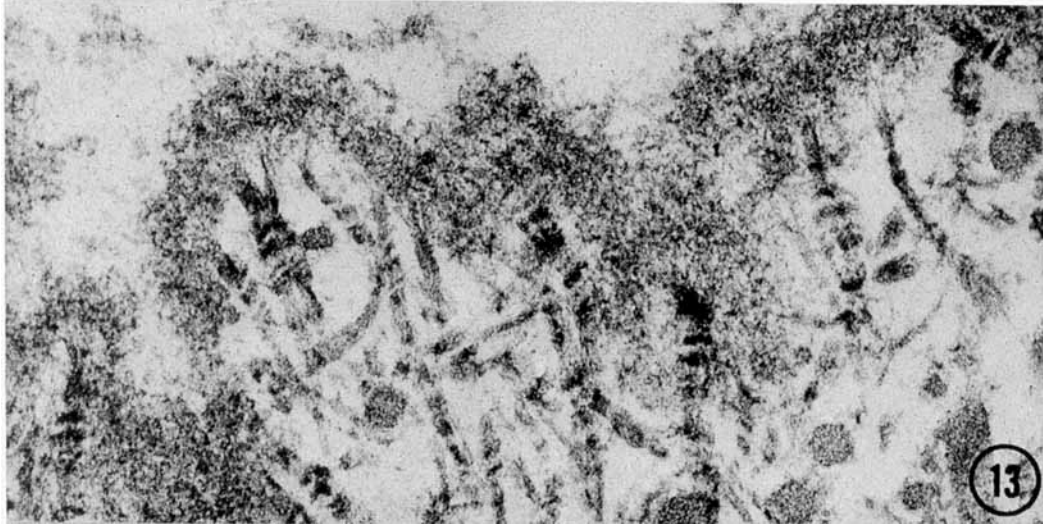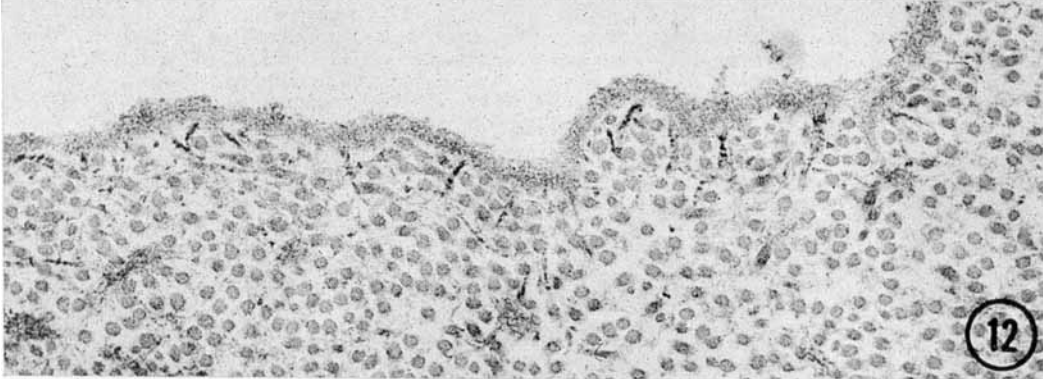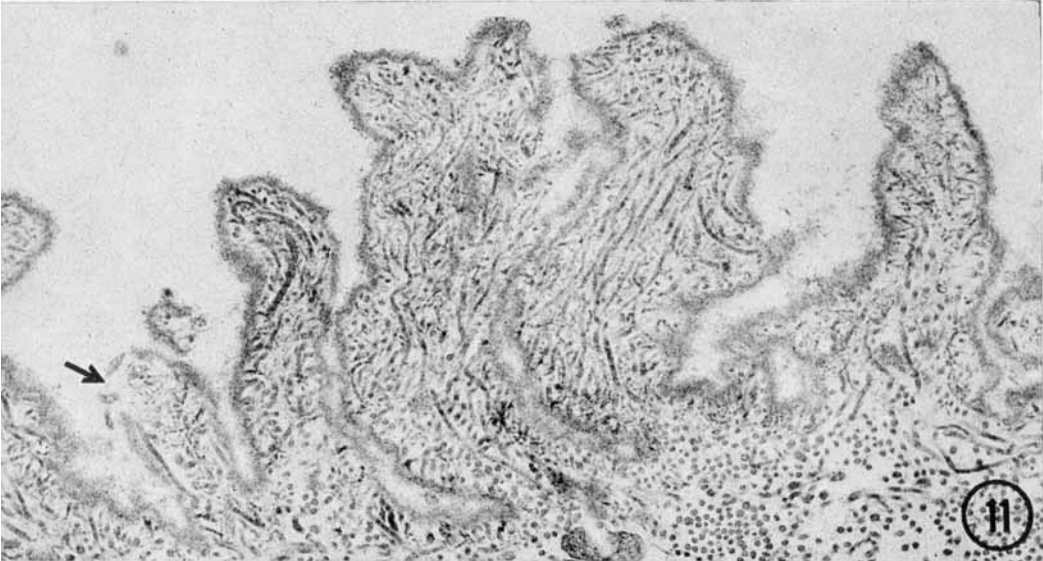
14 Basal cell-lamina propria region which had been incubated in EBS for one hour following a one hour incubation in PBS-EDTA. Widened intercellular spaces, a thinned lamina densa and increased spacing between collagen fibrils are evident. This micrograph depicts maximal changes observed following return to a cation-rich environment. The dark inclusions in the basal cell represent melanin. × 12,600.

15 Contact region of tissue which had been re-exposed to cations. The hemidesmosomal peripheral densities appear discontinuous and the lamina densa is somewhat thinner ($\frac{2}{3}$ to $\frac{3}{4}$) than the control. × 105,000.