Scanning Electron Microscopy of Cell Surfaces Following Removal of Extracellular Material

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ABSTRACT The application of scanning electron microscopy to the study of cell surfaces is limited in intact tissues, because extracellular material may often obscure the details of nonluminal surfaces. To remove connective tissue elements we have treated human skin and both kidney, and an autonomic ganglion of the rat with hydrochloric acid and collagenase. Regional variations in the basal surface of the nephron are noted following removal of the basement membrane. The basilar interdigitations of the cells of the proximal tubule appeared as parallel ridges encircling the tubule. Ridges on the parietal epithelium of Bowman's capsule were randomly arranged and alternated with smooth surfaces. The dermal surface of the human epidermis has an alveolar or honeycomb appearance due to the elevation of the epidermal ridges and numerous pits for the dermal pegs. At higher magnifications the basal surface of cells of the stratum germinativum possessed numerous and irregular projections. Neurons with their processes are evident in the autonomic ganglion. The soma of the neurons are enclosed by flattened satellite cells. Irregular spaces between opposed satellite cells are interpreted as regions for the passage of processes related to the ganglion cells. Nodes of Ranvier were clearly seen along nerve fibers. Some pitting of the nerve fibers was also noted. The HCl-collagenase method has the advantage of the removal of collagen and basement membrane while preserving the structural integrity of the cell surface.

The majority of scanning electron microscopic studies have been restricted to the apical and cut surfaces of epithelia (Blumcke and Morgenroth, '67; Turner and Green, '72; Waterman, '72; Armstrong and Parent, '73; Goldman and Leif, '73) since the basal surfaces of many cells are obscured by extracellular material. To overcome this problem, collagen has been frequently removed by dissection or enzymatic digestion. Of the several tissues in which we have interest, a variety of technical problems have already been identified in the literature. For example, in peripheral nerves Spencer and Lieberman ('71) showed that endoneural collagen was not completely removed, while enzymatic digestion with collagenase has been reported to result in disturbing artifacts (Dyck and Lais, '70). Similarly, hyaluronidase, α amylase or sodium bromide when applied to the study of the epidermis do not allow observation of the fine surface detail (Sommerlad and Creasey, '70; Finlay et al., '71; Papa and Farber, '71). Parenchymal cells of the liver have been successfully separated by continuous perfusion of the liver with a buffered solution of collagenase and hyaluronidase (Howard and Pesch, '68; Berry and Friend, '69). In these studies, the ultrastructure of isolated liver cells is comparable to normal hepatic cells. Miller and Revel ('75) have shown that the basement membrane may occasionally be removed by blunt dissection; unfortunately this method lacks specificity, is inconsistent in reproducibility and is apparently limited to epithelia.

In this report a method is introduced to selectively and consistently remove the basement membrane and collagen while preserving the integrity of cell urfaces. This procedure, which employs a simple combination of hydrochloric acid (HCl) and

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collagenase, can be used on a variety of tissues. This study describes the steps in the procedure and the results obtained when applied to the kidney, skin, and neural tissue.

MATERIALS AND METHODS

Preservation

Fixed and fresh kidneys, an autonomic ganglion of the pelvis of the rat, and human skin were studied. Tissues were fixed by the following methods: (1) immersion in 10% buffered formalin or (2) immersion or perfusion with 2.5% glutaraldehyde in 0.075 M cacodylate/HCl buffer (pH 7.4) at room temperature. All tissue was fixed for at least three hours.

Digestion

A number of experiments were tried on fixed and fresh tissues. Complete digestion of extracellular material was obtained only on fixed tissues which had been treated with HCl followed by collagenase. One centimeter cubed pieces of tissue were rinsed several times in 0.1 M phosphate buffer (pH 7.4) to remove the fixative and then placed in 8 N HCl for 50–70 minutes at 60°C. The kidney and pelvic ganglion were left in HCl until they could be microdissected in the phosphate buffer (approximately 55 minutes) while the skin was digested until only a sheet of epidermis remained (65 minutes). Longer periods of digestion resulted in dissolution of the tissue.

After HCl digestion the tissue was rinsed three times (5 minutes each) in 30 cc of phosphate buffer to remove the acid. Single nephrons were microdissected free using sharpened needles while the pelvic ganglion was gently teased. All tissue was then placed in 0.1 M phosphate buffered collagenase, pH 6.8, (Worthington, type II) at a concentration of 10mg/10cc of buffer for 3–8 hours at 37°C. For complete digestion of the basement membrane, Worthington type II collagenase was superior to types III and IV.

Mounting and dehydration

Following several rinses in phosphate buffer the samples were placed on a coverslip previously coated with gelatin (Vial and Porter, '74). The tissue was firmly secured to the gelatin by immersion in 2.5% glutaraldehyde in 0.075 M cacodylate/HCl buffer for 15 minutes at room temperature, subsequent to dehydration through a graded series of alcohols to 100% ethanol.

Critical point drying and coating for scanning electron microscopy (SEM)

The coverslips were transferred in fresh 100% ethanol to a Samdri critical point dryer and dried using liquid CO_2 as described by Anderson ('51). The coverslips with attached tissue were secured to aluminum slugs by double stick tape and placed in a vacuum-evaporator with a rotating stage for coating with gold-palladium. Specimens were examined and photographed in an ETEC Autoscan operating at an accelerating voltage of 20 kv.

Transmission electron microscopy (TEM)

Previously scanned specimens were removed from the coverslip with a razor blade, passed through two changes of propylene oxide and embedded in Epon 812. One micron sections were stained with toluidine blue. Thin sections were doubly stained with uranyl acetate and lead citrate and viewed with a Philips 200 EM.

Normal fine structure of the three tissues used was also studied by routine transmission electron microscopic techniques. The tissues were fixed either by immersion or perfusion in 2.5% glutaraldehyde in 0.15 M cacodylate/HCl buffer, dehydrated through a series of graded ethanols and embedded in Epon 812.

RESULTS

Kidney

Treatment of the kidney with HCl alone removes the collagen and thus reveals a smooth basal surface of the tubules (fig. 1). The basilar interdigitations of cells of the proximal tubule, seen in tissue prepared for routine TEM (fig. 2), are covered by the basement membrane. The basement membrane is largely unaffected by the HCl treatment, which accounts for the lack of surface detail seen in figure 1. When the nephron is treated with HCl and collagenase the basement membrane is removed revealing complex folds or ridges of the

tubule (figs. 3, 4). These folds correspond to the basilar interdigitation seen both in TEM (fig. 2) and in light microscopy (fig. 5). The ridges, which vary in length and width, impart an accordion-like appearance to the proximal convoluted tubule since they are arranged perpendicular to the long axis of the tubule. Bumps, known to occur along the proximal tubule, (Fetterman, '70) may be seen as well as areas devoid of prominent ridges. Fewer ridges are seen distally towards the pars recta (not illustrated).

HCl and collagenase caused some damage to cell cytoarchitecture, especially the nucleus. However, as illustrated in figures 6 and 7 of a scanned specimen sectioned for light and TEM, surface features are remarkably preserved. In this example (figs. 6, 7), the apical brush border and basilar interdigitations remain intact while the basement membrane has been removed. The plasmalemma and the mitochondrial membranes retained their normal trilaminar appearance (fig. 7). In addition, the longitudinal orientation of the mitochondria was preserved (fig. 7). Pitting artifacts were sometimes seen in glutaraldehyde-fixed tissues but those were much more frequent after fixation with formalin.

Elevations or ridges were also noted on the surface of the glomerulus after removal of the basement membrane (figs. 8, 9). In contrast to the proximal tubules, the groups of parallel ridges of the parietal epithelium appeared randomly arranged and alternated with smooth surfaces (fig. 8). At the urinary pole, there was a gradual transition to the parallel arrangement of ridges of the proximal tubule.

Autonomic ganglion

The cell bodies of neurons, nestled amid nerve processes of varying size, are evident following treatment of the pelvic autonomic ganglion with HCl and collagenase (fig. 10). One such neuron with its process is shown in figure 11. The normal ganglion is very compact with extracellular space largely occupied by collagen bundles. The loose appearance of the ganglion (figs. 10, 11, 12) following HCl and collagenase is, in part, due to the removal of collagen by acid and enzymatic digestion. At higher magnification the surface of the ganglion cell is irregular (figs. 11, 12). This appearance is due to satellite cells and neurites which normally invest the soma of ganglion cells. Only minimal distortion of the ganglion cell and surrounding neural elements occurs after the HCl and collagenase method (fig. 13). The large nucleus with prominent nucleolus and processes of the ganglion cells are relatively unchanged. However, the capsule of the ganglion cells, consisting of neurites and overlapping processes of satellite cells, appears loosened by the process of digestion. The extracellular space remains largely unstained due to the absence of connective tissue (fig. 13). Many nerve fibers, some of which are enclosed by a perineural sheath, are noted throughout the autonomic ganglion (fig. 14). Nodes of Ranvier are easily visible along nerve fibers (figs. 11, 14). When the nerve fibers are examined with TEM, holes are seen in the myelin sheath (fig. 15). The axon is unaffected and the remaining myelin retains its lamellar arrangement.

Skin

HCl and collagenase treatment effectively removes the dermis leaving a honeycomb or alveolar appearance to the basal surface of the epidermis (fig. 16). The large, branching pits seen on the basal surface formerly held the "dermal pegs" (fig. 16, inset). The cells of the stratum germinativum possess numerous irregular projections at their base (fig. 17), which accounts for a villous appearance of this surface in SEM. A distribution of bulbous protuberances (about 7 μ in diameter) also occurs on the basal surface of the epidermis (arrows in fig. 16).

DISCUSSION

Scanning electron microscopy has aided the morphologist in obtaining a three-dimensional image of many cells. However, some cells of intact tissues are covered by collagen and basement membrane and are not normally viewed by SEM. The present study has detailed a method which unmasks these cell surfaces by removing collagen and basement membrane with HCl and collagenase. With this method, surfaces not ordinarily visible after routine preparative procedures, are now accessible

for study with the SEM. Moreover, there is good preservation of the cell surfaces despite the rather rigorous treatment involved in the preparation of the tissue.

The critical steps in this method are: (1) use of fixed tissue, (2) time of treatment with HCl, (3) sequence of digestion, i.e., HCl must precede collagenase and (4) type of collagenase. HCL treatment of unfixed tissue resulted in digestion of both collagen and cell membranes which confirms an observation by Spencer and Lieberman ('71). Cell membranes were stabilized by formalin or glutaraldehyde fixation. Length of treatment with HCl proved very critical and had to be determined by trial and error for each tissue. Insufficient time resulted in incomplete digestion of the collagen, while prolonged exposure to HCl led to disruption of cells. In general, tissues with large amounts of collagen such as skin required longer periods of digestion than did the kidney in which collagen is sparse. Formalin-fixed tissue required less time in HCl than tissue fixed with glutaraldehyde, but the former produced more fixation artifacts. It seems necessary to remove collagen to expose the basement membrane to the action of collagenase since collagenase alone or collagenase followed by HCl resulted in incomplete digestion. The mechanism by which HCl digests collagen is not completely understood, but may involve acid hydrolysis of several peptide bonds of collagen (Loftfield, '76). The type of collagenase is important. Types III and IV collagenase, relatively pure preparations of the enzyme, were not as effective as Type II. The higher content of impurities in Type II collagenase may be responsible for complete digestion of the basement membrane.

The HCl-collagenase method provides a new and interesting view of the basilar interdigitations of cells of the proximal convoluted tubule. The basilar interdigitations were not seen by Siew ('74) in an earlier SEM study of the nephron. It is significant that this author used HCl; a method which removed collagen but not the basement membrane. In the present study, the basilar interdigitations were uncovered by the removal of the basement membrane with HCl followed by collagenase. The basilar interdigitations of single cells are parallel and oriented perpendicular to the long axis of the tubule. This arrangement of the basilar interdigitations that we observed may however, not conform to the accepted view of the orientation of these processes. From Bulger's ('65) TEM study on serial sections of cells of the proximal convoluted tubule, it would appear that the processes of single cells project in a radial direction from the base of the cells. This image more closely corresponds to our findings on the glomerulus. Our results on the proximal convoluted tubule conform more to those of Maunsbach ('73) who depicts by TEM that the basilar interdigitations of cells of the proximal tubules are aligned perpendicular to the long axis of the tubule.

A clearer picture of the relationship of satellite cells to ganglion cells has also been obtained. Crevices between adjacent satellite cells may represent sites where neurites may pass since it is known that mammalian autonomic ganglion cells are multipolar. Somer finer processes of ganglion cells, or nerve terminals ending on them, may have been removed by teasing and the HCl-collagenase treatment. Spencer and Lieberman ('71) has also studied nerve fibers with scanning electron microscopy. Artifacts such as numerous pits and longitudinal folds were often noted by these authors. With the method used in the present study, pitting of nerve fibers was not as severe as that seen by other authors. The exact nature of the pitting is not known, but may be due to lipid extraction during tissue preparation.

Methods used in the past for epidermal-dermal separation include physical stretching of the skin (Gilbert et al., '63), heat (Stoughton, '56), chemical separations by neutral inorganic salts, weak acids and bases (Stoughton, '56; Felsher, '47; Hambrick and Blank, '54) and enzyme digestion (Hambrick and Blank, '54; Brooks and Godefroi, '63). All have disadvantages in preparation of tissue for ultrastructural study, in that absence of prior fixation of tissue usually renders the methods ineffective.

The use of the SEM in dermatology has centered primarily on the study of hair

(Dawber and Comaish, '70; Carteaud, '70), replications of the superficial stratum corneum (Bernstein and Jones, '69; Barnes, '73), the superficial keratinocytes (Harris, Papa and Stanton, '74; Tring and Jolly, '73) and dermal collagen (Kischer, '74; Dawber and Shuster, '71). An SEM study of the basal layer has utilized the sodium bromide stripping technique and air drying of the specimen (Papa and Farber, '71). In that study, the honeycomb appearance produced by rete ridges was demonstrated, and melanocytes were contrasted with basal cells by incubation of the epidermis with dihydroxyphenylalanine (DOPA) after stripping. However, the methods used resulted in poor demonstration of fine surface detail of basal cells and severe shrinkage artifact. The present method overcomes both these difficulties by prior fixation of the specimen, coupled with critical point drying following HCl and collagenase digestion. Collagenase specifically removes basement membrane material, revealing the fine foldings of the plasma membrane of the basal keratinocytes seen with TEM; critical point drying prevents artifactual separation of basal cells. Further evidence of the specific action of collagenase is shown by loss of only the basement membrane when collagenase is injected at the dermal-epidermal interface in living skin (Kahl and Pearson, '67).

The ovoid, shrunken cells scattered among the basal cells may represent melanocytes. It is well known that melanocytes shrink following formalin fixation, and the relative numbers of shrunken cells in this study roughly corresponds with that seen in the previous SEM study in which melanocytes were identified by the DOPA reaction (Papa and Farber, '71). It should be noted, however, that the basal keratinocytes may shrink during formalin fixation and resemble melanocytes in paraffin embedded sections (Clark et al., '61). Selective staining of melanocytes with the DOPA reaction after fixation but prior to digestion should aid in the identification of these cells.

With the advent of this new technique, it is now possible to determine with SEM the three-dimensional structure of cell surfaces usually obscured by connective tissue elements. Hopefully, this technique will permit a further appreciation of conformational changes of cell surfaces in different functional and pathological states.

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PLATE 1

EXPLANATION OF FIGURES

1 Scanning electron micrograph of a proximal convoluted tubule which has been treated with hydrochloric acid. Note the smooth appearance of the basal surface of the tubule. × 1,800.

2 Transmission electron micrograph of the basal surface of cells of the proximal convoluted tubule. This surface of the cell is characterized by numerous basilar interdigitations (BI) which rest on a thick basement membrane (BM). × 17,000.

3 HCl and collagenase-treated proximal convoluted tubule. The removal of the basement membrane by collagenase reveals parallel folds or ridges (compare to fig. 1). These ridges correspond to the basilar interdigitations. × 3,700.

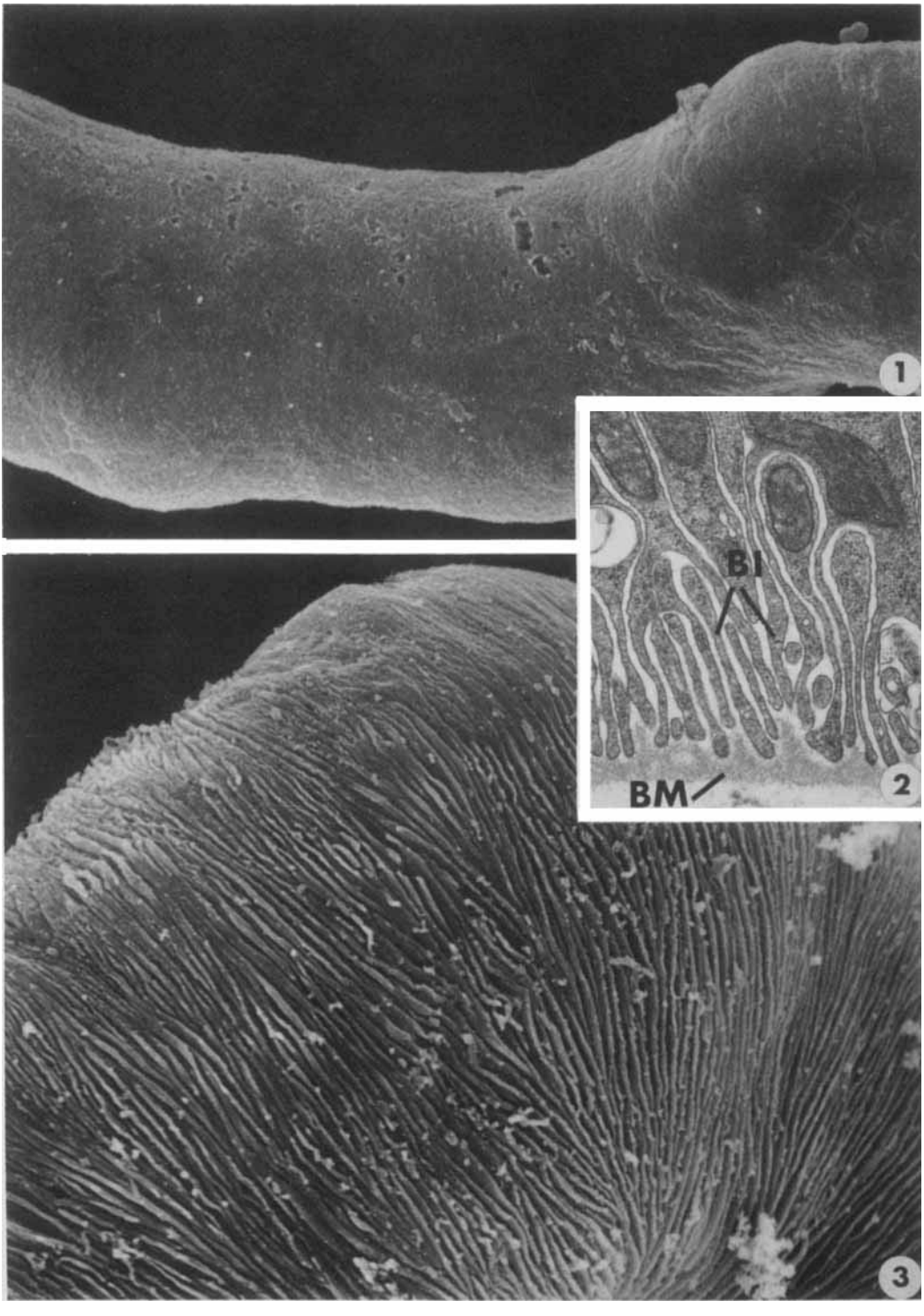

1
BI
BM
2
3

PLATE 2

EXPLANATION OF FIGURES

4 HCl and collagenase-treated proximal convoluted tubule. Elevations or bumps (asterisk) and areas devoid of prominent ridges (arrows) occur along the proximal tubule. × 3,100.

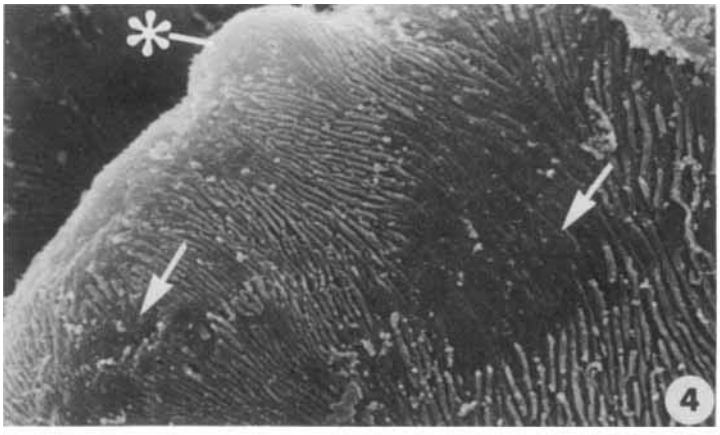
5 Light micrograph of a PAS-stained proximal convoluted tubule. The basal surface of the proximal tubule is striated (arrow) in this tangential section. The striations are largely parallel and correspond to the ridges seen in figure 3. BM; basement membrane. × 1,000.

6 A one micron section of the HCl and collagenase-treated proximal tubule seen in figure 3. Note the intact brush border (BB) and the gold-palladium coat (arrow). × 500.

7 Transmission electron micrograph showing the basal surface of the tubule seen in figure 6. Note that the basement membrane is absent. BI, basilar interdigitations. × 22,000.

8 Low magnification of a renal corpuscle (RC) and associated proximal convoluted tubule (PT) which has been treated with HCl and collagenase. × 700.

9 Basal surface of parietal cells of Bowman's capsule. Note the random arrangement of the groups of ridges. × 3,200.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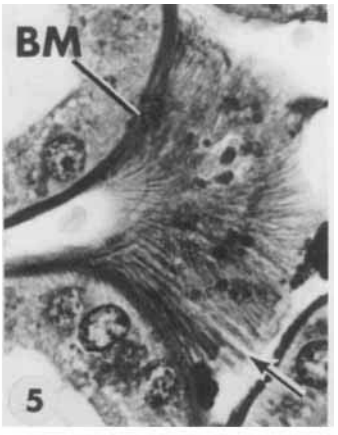
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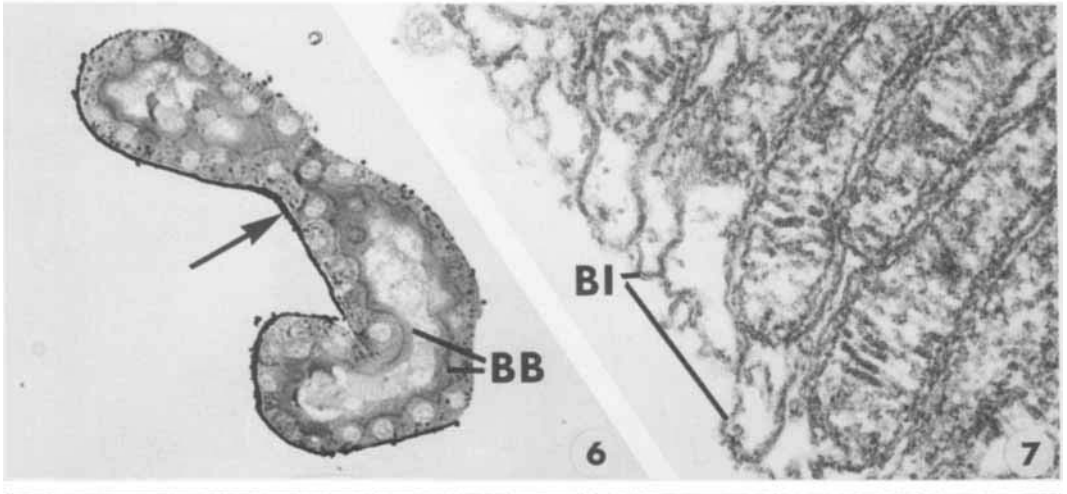
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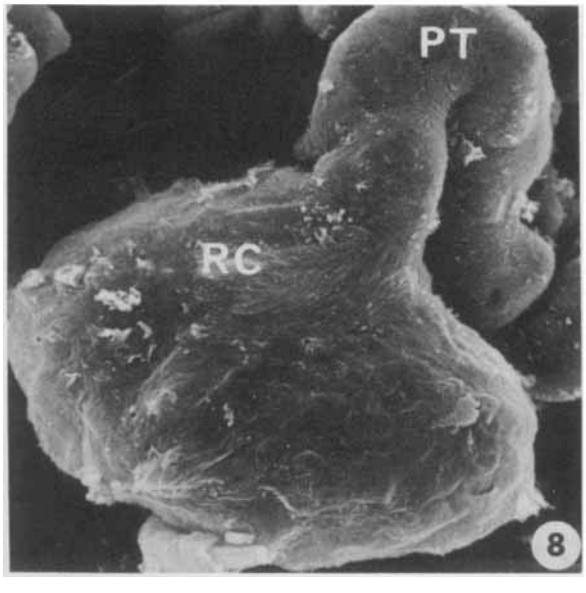
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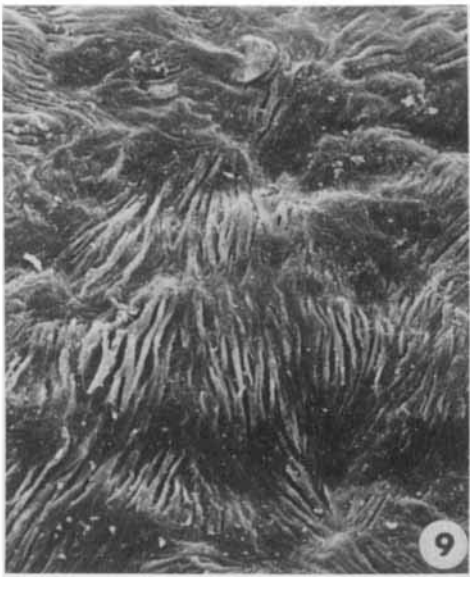
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PLATE 3

EXPLANATION OF FIGURES

10 Scanning electron micrograph of an autonomic ganglion treated with HCl and collagenase. Bundles of collagen would normally obscure identification of single ganglion cells. However, after removal of collagen in this specimen, cells are readily seen. × 500.

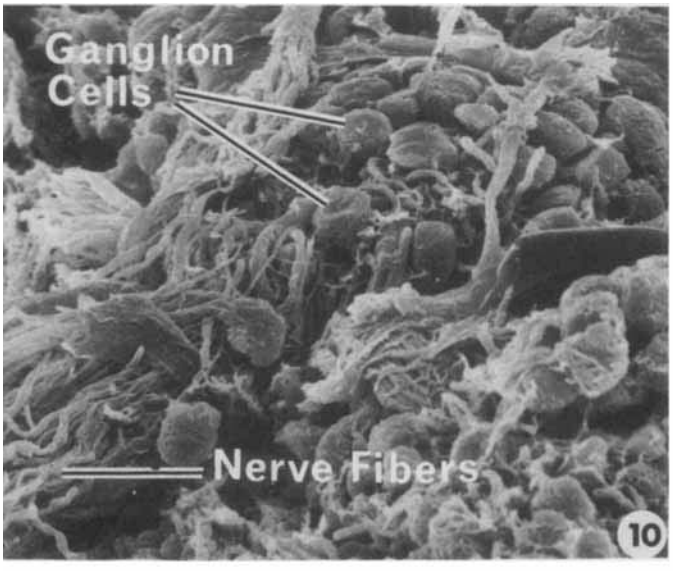
11 Higher magnification of ganglion cells, one of which has a long process (P). Note the irregular surface of the ganglion cell. The abundant excellular space can be attributed to digestion of excellular material and teasing. NR, Node of Ranvier. × 3,500.

12 Scanning electron micrograph of the soma of ganglion cells with their investment of neurites (N) and irregular-shaped satellite cells (arrows). Note the different shapes of ganglion cells due to crowding. × 3,600.

13 One micron section of HCl and collagenase-treated ganglion seen in figure 10. Autonomic ganglion cells, some of which have processes (P), are surrounded by nerve fibers and satellite cells (arrows). Note the gold-palladium coat (asterisk). Ganglion cells retain their large, pale nucleus and prominent nucleolus, although some holes may be seen in the cytoplasm. × 1,000.

14 HCl and collagenase-treated nerve bundle from pelvic autonomic ganglion. Note the perineural sheath (PS), Nodes of Ranvier (NR) and pits (arrows) along the nerve fiber. × 2,300.

15 Transmission electron micrograph of a cross section of a nerve fiber after treatment with HCl and collagenase. A pit (asterisk) is seen where myelin has been extracted. Areas where myelin has been removed are thought to correspond to the pits seen in figure 14 (arrows). × 27,000.


Ganglion Cells
Nerve Fibers
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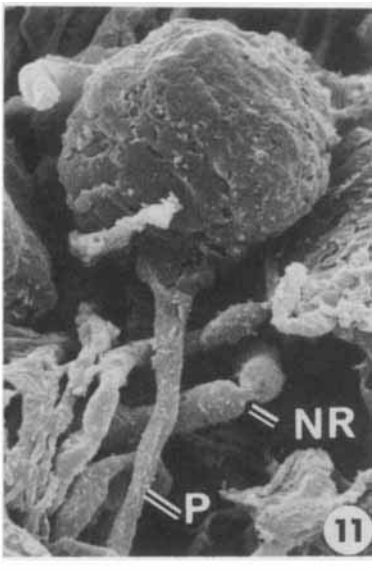
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P
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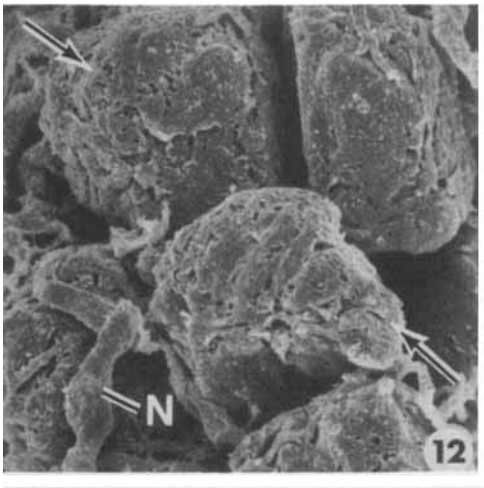
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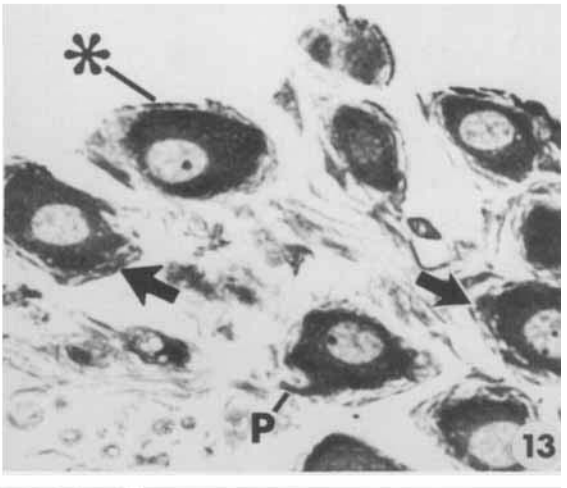
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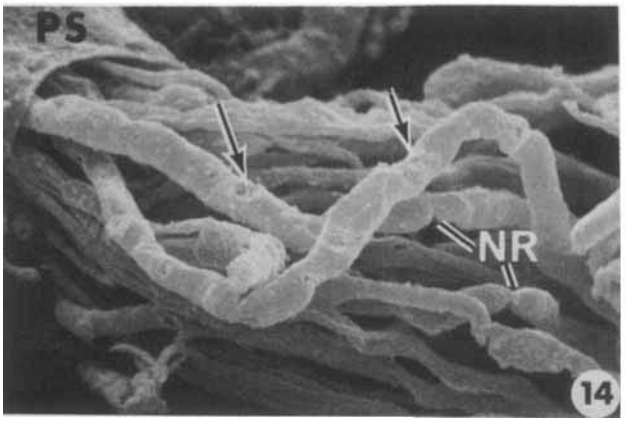
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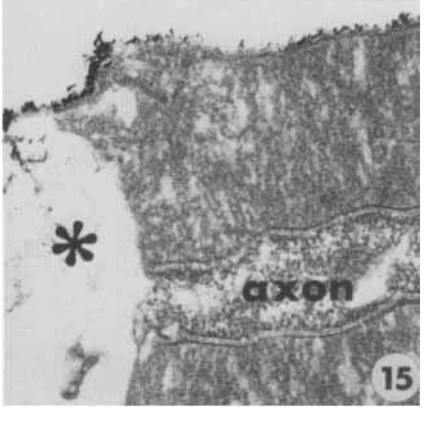
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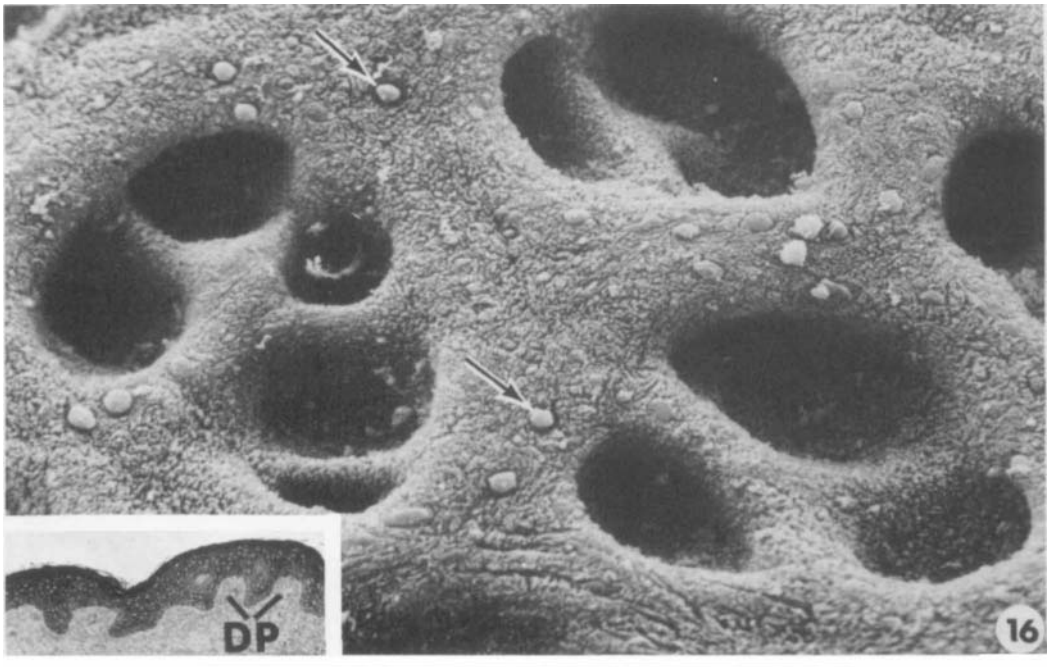
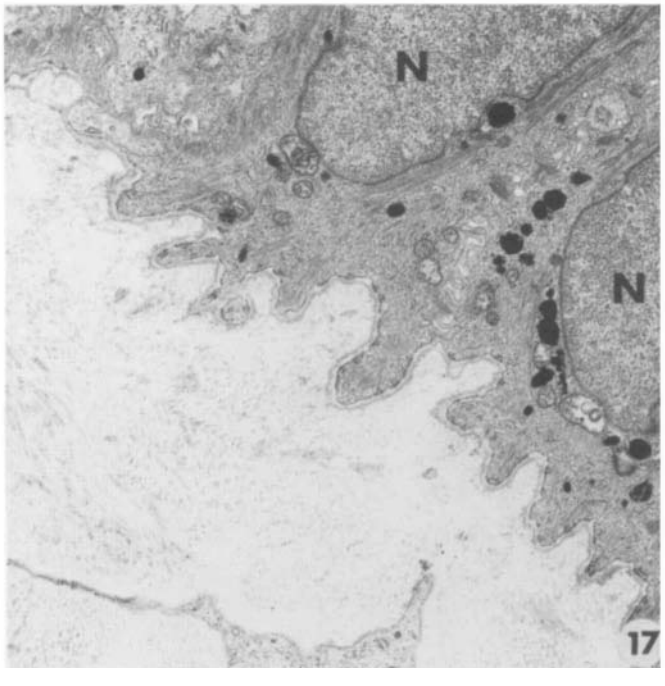
PLATE 4

EXPLANATION OF FIGURES

16 Basal surface of human epidermis. Removal of the dermal pegs (see inset, DP; dermal pegs) by HCl and collagenase has left numerous and branched pits. Processes of cells of the stratum germinativum (see figure 17) give a villous appearance to the basal surface of the epidermis. Rounded elevations (arrows) may represent melanocytes. × 800; Inset 450.

17 Transmission electron micrograph of the base of stratum germinativum cells. Note their irregular outline. N; nucleus. × 27,000.


DP
16


N
N
17