MEMBRANE-COATING GRANULES OF KERATINIZING EPITHELIA

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ABSTRACT

The purpose of this study has been to obtain information on the development of the envelop of horny cells that resists the action of keratinolytic agents. Toward this end the epidermis, oral mucosa, and tongue epithelium of various vertebrates, as well as the isolated envelopes of horny cells, were examined by electron microscopy. It was found that small cytoplasmic granules (1,000 to 5,000 A) that develop within differentiating epithelial cells move toward the cell periphery, and after fusion with the plasma membrane, empty their contents into the intercellular spaces. The content of the granules spreads over the cell surfaces, and subsequently a thickened and coated cell envelope is formed that resists the action of keratinolytic agent. The membrane-coating granule is regarded as a specific differentiation product of the keratinizing epithelium. It contains numerous inner membranes and is assumed to engage in synthetic activities such as, perhaps, the formation of polysaccharides.

INTRODUCTION

While considerable information about keratin is available, little is known about the membrane that envelops it. The envelope of the horny cell has been neglected for a long time because it was thought that the great imperviousness of the horny cell results from intense keratinization at the periphery of the cell (1). Attention has been focused on the cell envelope only since it was noted that this structure can be isolated by keratinolytic agents. The envelopes of horny cells have been isolated from human skin, loosened from sunburned backs, by the use of 1 per cent sodium sulfide (2) and from powdered callus with 0.1 N sodium hydroxide (3). Under the light microscope the isolated envelopes appear as wrinkled membranes or ghosts of horny cells (3), in the electron microscope as sheets about 100 A thick (2). Chemical studies have shown that the protein component of these isolated envelopes consists of a variety of common amino acids (4).

Szodoray (5) was the first to obtain significant information about the site of formation of these resistant cell envelopes. He treated skin sections with trypsin and observed that while the lower Malpighian cells were digested, the contours of the upper Malpighian cells remained recognizable, an indication that the cell periphery becomes increasingly resistant as the epidermal cells move toward the surface. Szodoray's observations were confirmed and extended by Matoltsy and Matoltsy (6) in light and electron microscope studies. They found that both trypsin and concentrated urea solutions dissolve the Malpighian cells as well as the lower granular cells of the epidermis. These solutions also attack the upper granular cells and disperse all components except the keratohyalin granules and the cell envelopes; the horny cells seem not to be affected. It appears,

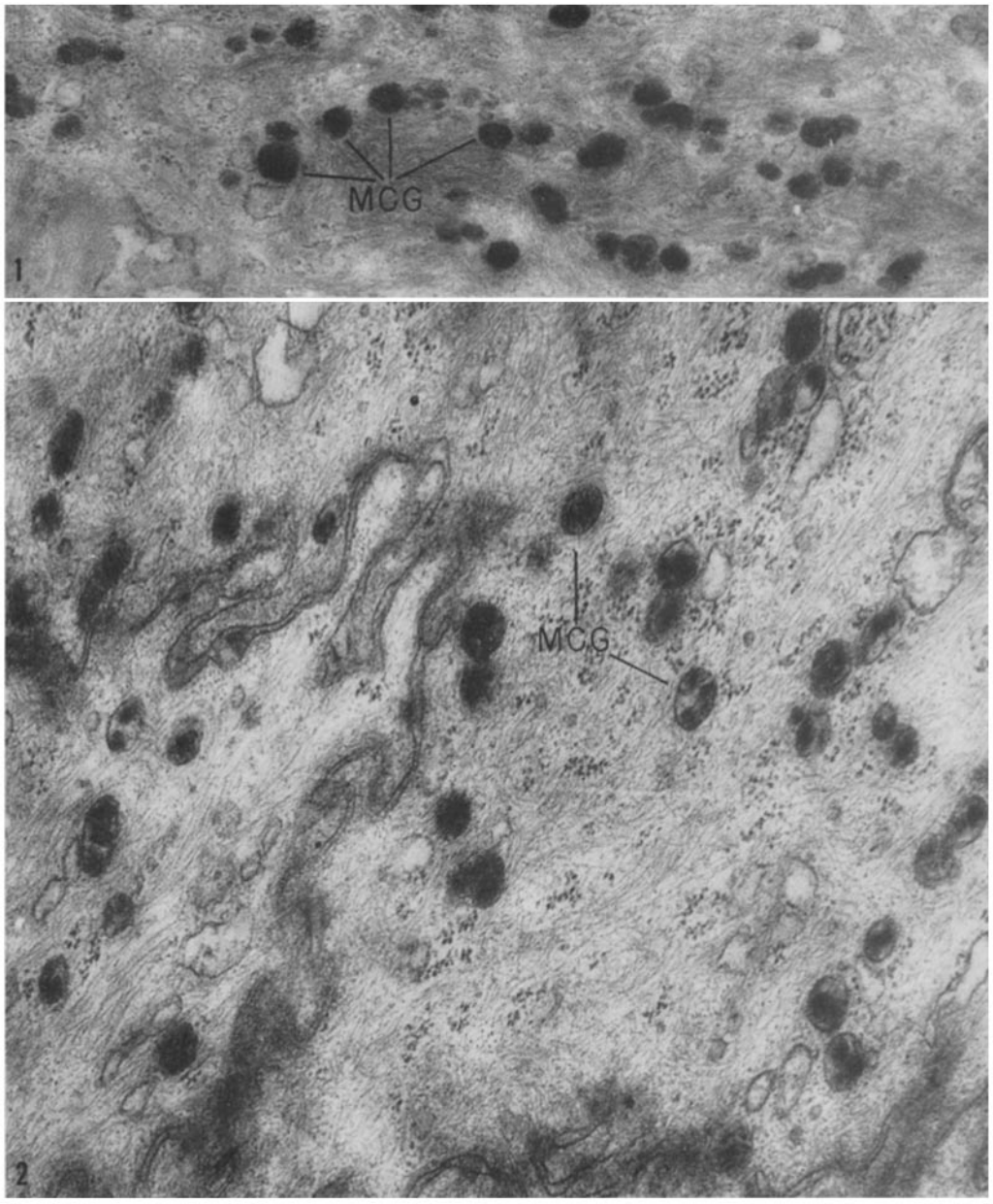


FIGURE 1 Membrane-coating granules (*MCG*) in the cytoplasm of differentiating human epidermal cells. Note that the granules are poorly preserved and their inner structure lacks detail. × 17,000.

FIGURE 2 Membrane-coating granules (*MCG*) of differentiating cells of the oral epithelium of the mouse. Note the well preserved inner structure and the great variation in the appearance of the granules. × 33,000.

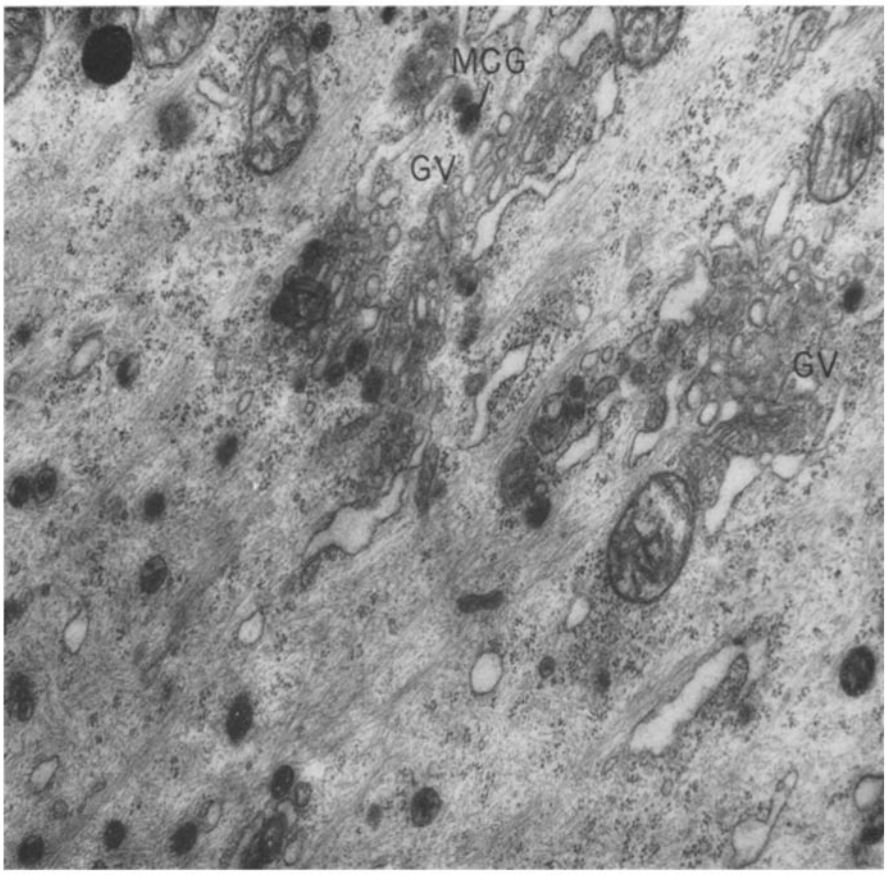


FIGURE 3 Membrane-coating granules (*MCG*) of the oral epithelium of the mouse distributed among Golgi vesicles (*GV*) of a cell that is in an early stage of differentiation. × 16,000.

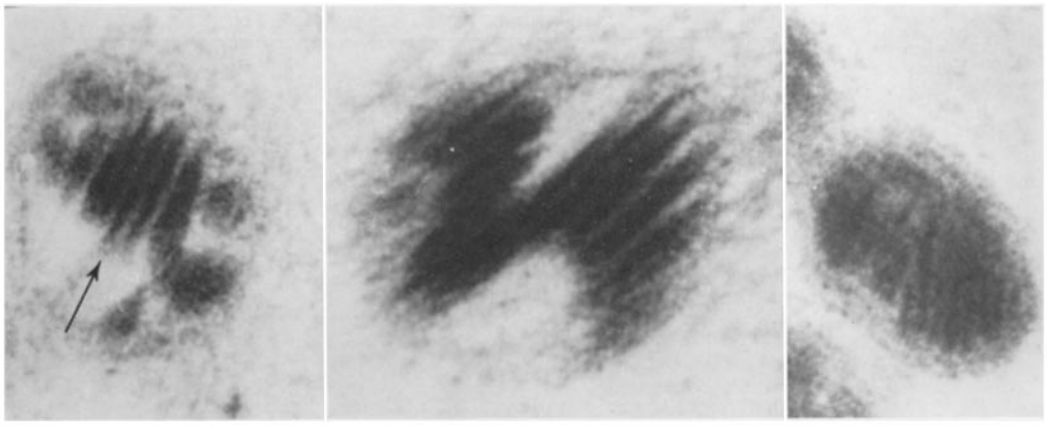


FIGURE 4 Membrane-coating granules of the oral mucosa and tongue epithelium highly magnified to demonstrate parallel orientation of inner membranes which occasionally occur in pairs (arrow). × 165,000.

therefore, that the plasma membrane of the epithelial cells acquires resistance during a late stage of differentiation, at the time when the cells are about to change into horny cells.

In order to learn more about the resistant cell envelopes, the authors have studied the keratinizing epithelium of man and other vertebrates by electron microscopy. In the cells beneath the horny layer, they have found numerous small granules fusing with the plasma membrane and pouring their contents over the outer surface of the cells. These observations suggested that the small granules may participate in development of the resistant envelope of the horny cells and have led to exploration of the structure, formation, and fate of these granules. In this report, a description is given of the membrane-coating granules (MCG) of various keratinizing epithelia and of findings in the plasma membranes at different levels of the tissue and in the isolated envelopes of horny cells.

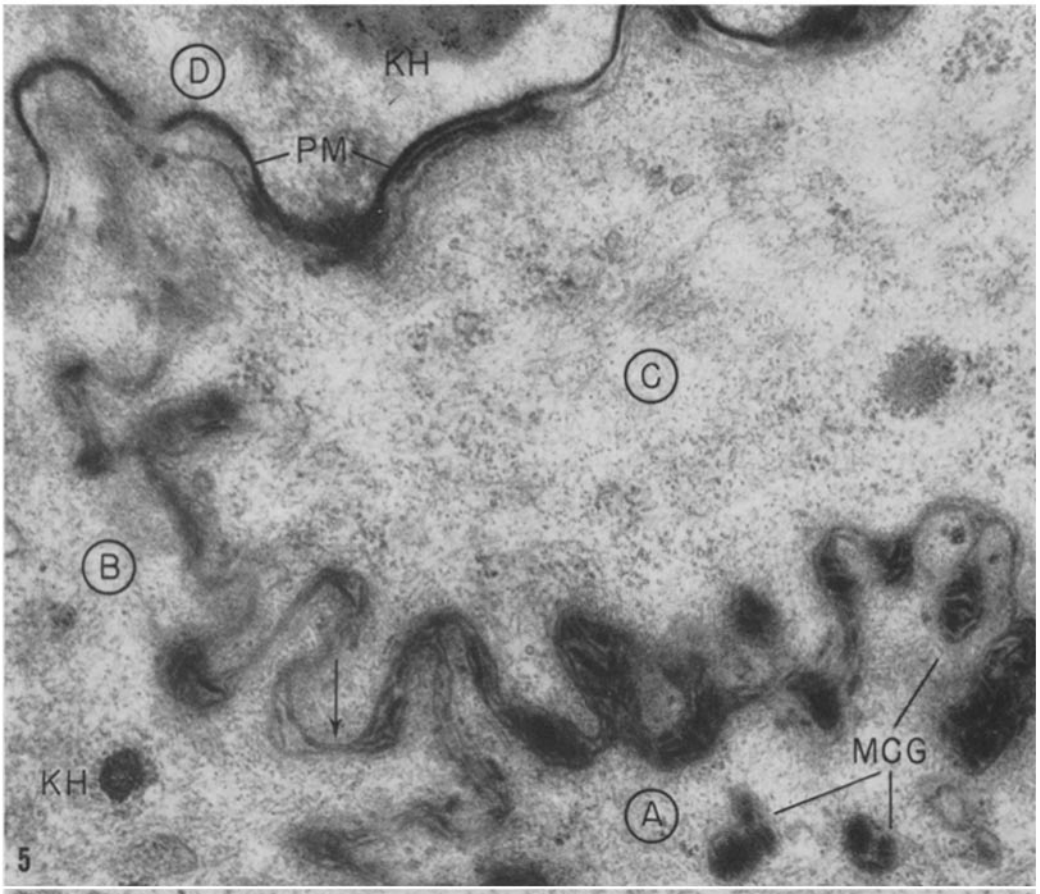
MATERIALS AND METHODS

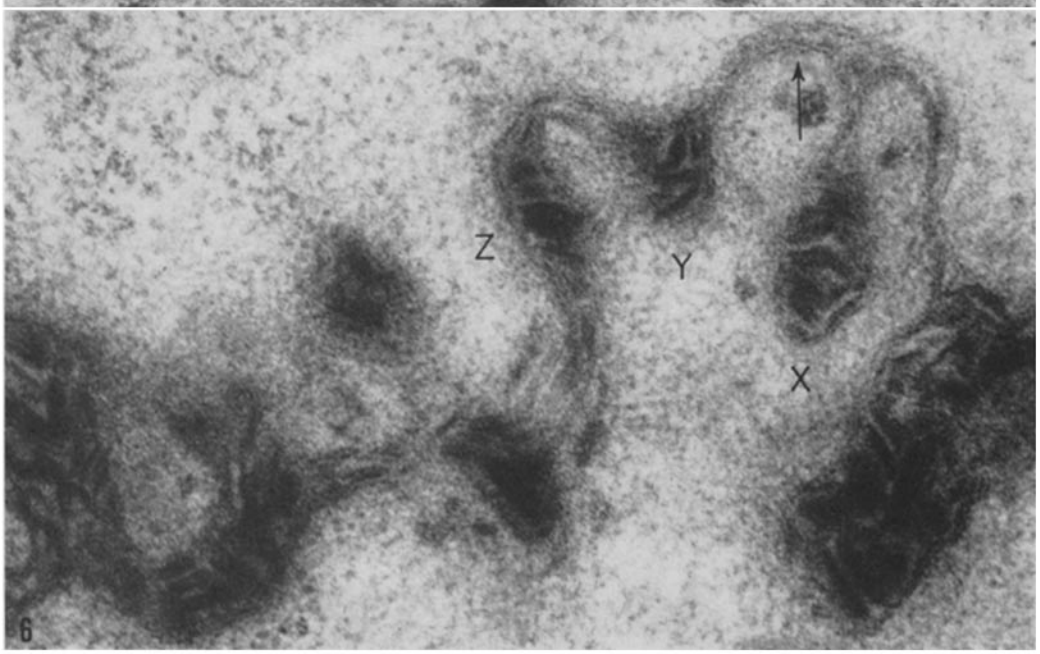
The tissues used for electron microscopy were: epidermis from the back of the frog, chicken, and mouse; the neck of the turtle; the nose of the cow; the thigh of man; and the oral mucosa and tongue of the mouse. For investigation of tissue sections, specimens were fixed immediately after excision in chilled 1 per cent osmium tetroxide buffered with Veronal-acetate to pH 7.5. After 2 hours of fixation, the tissue was dehydrated and embedded in epoxy resins according to the method of Luft (7). Thin sections were cut with the Porter-Blum microtome and stained with lead hydroxide. An RCA EMU-3F electron microscope was used for tissue examination.

Resistant membranes were isolated from the horny layer of the epidermis of the human foot, the epidermis of the cow's nose, and bovine oral epithelium by means of centrifugation after extraction in 0.1 N sodium hydroxide. Thickened stratum corneum from the sole of healthy men was powdered in a Wiley mill (No. 60 mesh screen), suspended in 0.1 N sodium hydroxide (500 mg powder in 100 ml), shaken with glass beads in a stoppered glass tube for 48 hours at about 5°C, and centrifuged at 3,500 RPM for 10 minutes. The sediment thus obtained was then resuspended in fresh sodium hydroxide and shaken for a second 48 hours. At the end of this period, the undissolved larger particles present in the suspension were removed by centrifugation at 600 RPM for 5 minutes. The cell membranes were removed from the resulting supernatant by centrifugation at 3,500 RPM for 10 minutes and washed three times in distilled water by repeated centrifugation and resuspension. The fresh, chilled bovine tissues studied were prepared for extraction immediately after being received from the slaughter house. Sheets of the epidermis of the nose and the oral epithelium (about 20 gm wet weight) were removed with the Storz-keratotome (set to cut sheets 0.2 mm thick), minced with scissors, suspended in 100 ml. 0.1 N sodium hydroxide, and homogenized in a VIR-TIS apparatus for 4 minutes. The homogenate was then shaken in 0.1 N sodium hydroxide solution (changed four times at intervals of 12 hours) and finally shaken in 0.1 N sodium hydroxide solution for 48 hours. The ensuing centrifugation procedures were the same as those used for powdered horny layer of the epidermis from the foot. Prior to fixation the isolated cell membranes were suspended in distilled water and centrifuged for 1 hour at 20,000 RPM until the sediment was thickly packed. The sediment was then immersed in agar-agar and

FIGURE 5 Four adjacent cells of the oral epithelium of the mouse showing the relationship between membrane-coating granules (*MCG*) and thickening of the plasma membrane (*PM*). At the lower right corner MCG can be seen migrating toward the plasma membrane of cell *A*. Above them, MCG appear attached to the plasma membrane or are dispersed in the intercellular space. Note fragments of the inner membranes of the MCG spreading along the cell surfaces and the double layers of the plasma membrane (at arrow). Between cells *C* and *D*, fragments of inner membranes are less numerous; the plasma membrane (*PM*) of cell *D* is thickened and smoothed out. Cells *A* and *B* are in an early stage of differentiation and contain small keratohyalin granules (*KH*) whereas cell *D* is in an advanced stage of differentiation and contains fully evolved keratohyalin granules (*KH*). × 18,000.

FIGURE 6 The lower right quadrant of Fig. 5 at a higher magnification showing the mode of attachment (*X*) of the membrane-coating granules to the double-layered plasma membranes (arrow) and later dissociation of the membrane-coating granules (*Y*) and the spreading of their contents (*Z*) in intercellular space. × 36,000.





processed for electron microscopy by the technics used for specimens of tissue..

RESULTS

Site of Formation and Structure of MCG

MCG first appear in the cytoplasm of epithelial cells located midway between the basal and the horny layers of keratinizing epithelium (Figs. 1 and 2). They are not present in the cells of the basal or the horny layers. They are first seen in the vicinity of the Golgi apparatus (Fig. 3). As their numbers increase, these granules disperse and scatter throughout the cytoplasm. In this study, they could be identified in the cytoplasm of cells located in the midportion of the epidermis of the turtle, chicken, mouse, cow, and man as well as in the middle layers of the oral and tongue epithelium of the mouse. MCG were not found in the epidermis of the frog. Those in the epidermis of the turtle, cow, and man (Fig. 1) were usually less well preserved than those in the epidermis of the chicken. The shape and inner structure of these granules could be most clearly seen in the oral (Fig. 2) and tongue epithelium of the mouse.

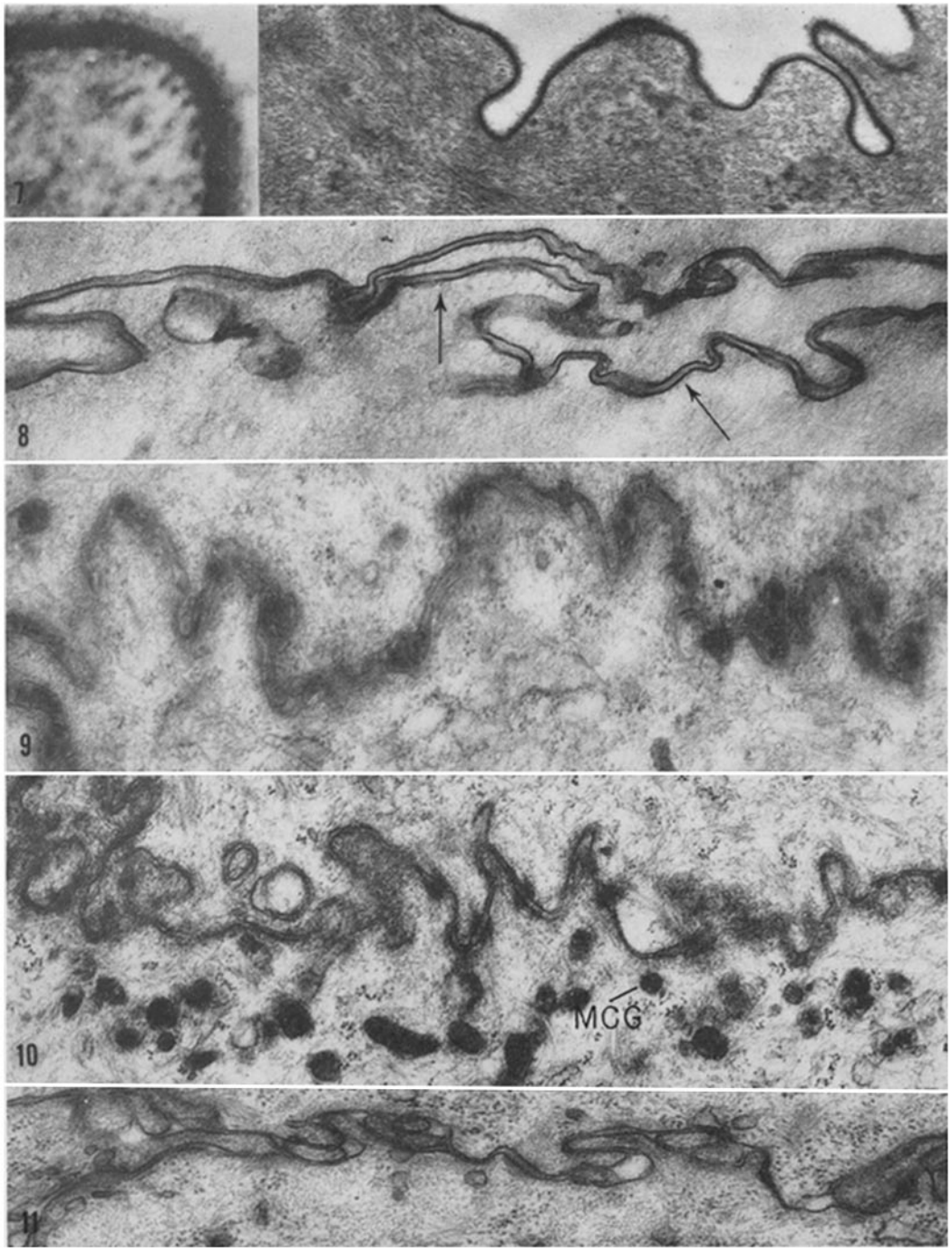
At low magnification, MCG appear as round or oval structures ranging in size from 1,000 to 5,000 A. Examination of many micrographs has made it possible to reconstruct them as ovoid bodies encased in smooth-walled membranes. As seen in the electron microscope, their internal structure consists of parallel membranes 60 to 70 A thick (Fig. 4), embedded in a lightly stained homogeneous medium. The inner membranes often appear in pairs and lie parallel to the short axis of the ovoid granules. The many variations of shape and inner structure observed seem to be related to the actual plane of sectioning of the MCG (Fig. 2).

Fate of the MCG

As the differentiating cells move to higher levels in the epithelium, the number of MCG within them greatly increases. As small keratohyalin granules are formed, the MCG migrate toward the periphery of the cell and become aligned in multiple rows along the plasma membrane (Fig. 10). After becoming attached to the membrane they empty their contents into the intercellular space (Figs. 6 and 9). At a later stage, when the epithelial cells change into flattened horny cells, no remnants of the MCG are apparent in the intercellular spaces, but the horny cells are enveloped by a thickened plasma membrane (Figs. 7 and 8).

The fate of MCG and the development of the thickened plasma membrane are demonstrated in detail in Figs. 5 and 6. Fig. 5 shows portions of four adjacent epithelial cells located in the midregion of the oral epithelium of the mouse. Cells *A* and *B* are in process of differentiation and contain small keratohyalin granules; cell *D* is in an advanced stage of differentiation and is filled with large keratohyalin granules and many cytoplasmic filaments. Cell *C* is in an intermediary stage. In cell *A* the MCG are migrating toward the cell periphery; a few of these granules can be seen in the vicinity of the plasma membrane in the lower right corner of the picture. A little higher up MCG can be seen attached to the plasma membrane and lying in the space between cells *A* and *C*. The mode of attachment of the MCG and the

FIGURES 7 to 11 Plasma membranes at different levels of the oral epithelium of the mouse. Magnification identical in all micrographs. × 11,000. Fig. 7 shows the free surface of an uppermost horny cell. Note that the thickened plasma membrane is coated with a thin layer of amorphous substance. Inset on the left shows the thickened plasma membrane and its outer coat at high magnification. × 56,000. Fig. 8 reveals smoothed out and thickened plasma membranes of keratinized cells coated with lightly stained amorphous material. Between the coated plasma membranes a narrow and densely stained material can be seen (arrows). Fig. 9 shows plasma membranes after the discharge of membrane-coating granules. Note the homogeneous dense substance and fragments of inner membranes of the MCG in the intercellular space. Fig. 10 demonstrates plasma membranes of differentiating epithelial cells while the membrane-coating granules (*MCG*) are migrating toward the cell periphery and lining up in multiple rows near the plasma membrane. Note that there are no intercellular gaps and that plasma membranes appear somewhat less convoluted than in Fig. 11. Fig. 11 shows highly convoluted plasma membranes of basal cells, both close together and separated by gaps.



way their content is dispersed are demonstrated in Fig. 6 which shows portions of cells *A* and *C* at high magnification. The MCG marked *X* is in an early stage of attachment; the double-layered plasma membrane is invaginating and has fused with the apical pole of the MCG. The MCG at *Y* is in a later stage; the inner membranes are disorganized and the granule itself seems to have been almost entirely extruded from the cell. An advanced stage can be seen at *Z* where the inner membranes of the granules lie outside the plasma membranes. Subsequent events can be followed more closely in Fig. 5. The cytoplasm of cell *C* contains no MCG, but there are many remnants of MCG in the intercellular spaces. The plasma membrane of cell *C* is not yet thickened. Cell *D*, which is in an advanced stage of differentiation, however, is enveloped by a thickened plasma membrane; there are very few remnants of MCG in the space surrounding this cell.

The Plasma Membranes

In order to study in more detail the sequence of events that lead to the development of resistant cell envelopes, an examination was made of the plasma membranes at different levels of the oral epithelium of the mouse. The basal cells were found to be enveloped by a relatively thin plasma membrane (80 to 90 A) which is highly convoluted (Fig. 11). The plasma membranes of adjacent basal cells lie close together except where the intercellular space is dilated. As the cells move to higher levels, dilatations of the intercellular space become less prominent and the plasma membranes appear somewhat less convoluted. When the MCG migrate toward the cell periphery, the plasma membranes lie close together and dilatations of the intercellular space are not apparent (Fig. 10). After MCG have been discharged, the intercellular spaces appear wider and are filled with fragments of inner membranes of MCG embedded in a homogeneous substance (Fig. 9). At this stage it was possible satisfactorily to resolve the double layers of the plasma membranes (Figs. 5 and 6). As the cells ascend to higher levels in the epithelium, their surface convolutions are characteristically smoothed out; the plasma membrane becomes thicker by approximately 60 to 80 A and an amorphous coat is deposited on its outer surface (Figs. 7 and 8). Between the coated plasma membranes a narrow and densely stained material may be seen under favorable conditions (Figs. 8 and 12). The complex membrane structure of the horny cells can be best seen in the uppermost layer where the cells have free surfaces. Cross-sections clearly reveal that the envelope of the horny cells consists of two layers: (1) a thickened plasma membrane and (2) an outer amorphous coat (Fig. 7, inset).

Isolated Envelopes of Horny Cells

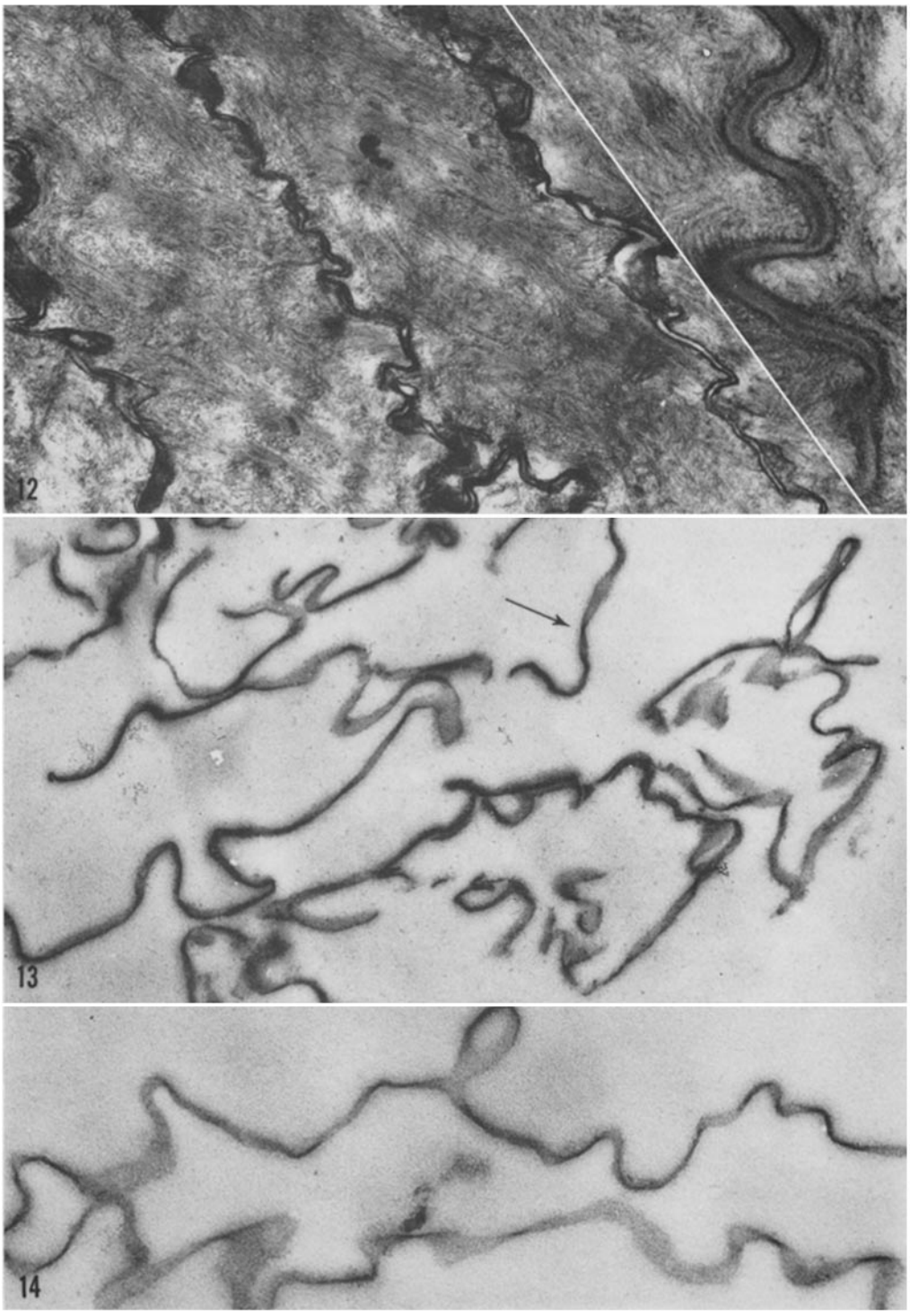
The preparations derived from horny layers of the sole of human foot, and the epidermis and oral epithelium of the cow, were found to consist of membrane fragments (Figs. 13 and 14). Since contaminants were not seen, it was concluded that the technics used permitted the isolation of pure fractions of cell membrane.

The membrane fragments appeared as twisted ribbons in the electron microscope (Figs. 13 and 14); in many respects they resemble the thickened plasma membrane of horny cells seen in thin sections (Fig. 12). The outer coat of the thickened plasma membrane, seen *in vivo*, is absent (Figs. 7 and 12). These findings confirm previous observation that the horny cells of the epidermis are protected by a highly resistant membrane (2, 3). Furthermore, they indicate that resistant properties of the envelope of the horny cells of the oral

FIGURE 12 Thin section of horny layer from the sole of the human foot. Note thick cell envelopes. × 10,000. Inset on the right shows cell envelopes and intercellular material at higher magnification. × 15,000.

FIGURE 13 Isolated envelopes of horny cells derived from the material shown in Fig. 12. The membrane fragments appear as twisted ribbons and at cross-sectional regions (arrow) reveal that the outer coat, seen *in vivo* as an intercellular material, is absent. × 12,500.

FIGURE 14 Isolated envelopes of horny cells derived from bovine oral epithelium. Compare with Fig. 13. × 13,000.



epithelium (Fig. 14) are similar to those of the horny cells of the epidermis (Fig. 13).

DISCUSSION

The protective layer of the keratinizing epithelium is formed by cells which pass through a specific form of differentiation. While the basal cells ascend to higher levels they cease to undergo mitosis and their main activity is directed toward the development of specific products needed for protection. The ascending cells form abundant cytoplasmic filaments which ultimately constitute a large portion of the terminal horny substance; these cells also produce many keratohyalin granules which are dispersed during the terminal stage of differentiation and become a part of the interfilamentous substance. While the differentiation products increase in quantity, cell constituents such as the endoplasmic reticulum, Golgi vesicles, ribosomes, and mitochondria decrease in quantity and finally the nucleus disintegrates and is partially or fully eliminated from the cell (8).

The results of this study demonstrate that in addition to the described events other important changes also take place during the formation of the protective layer. Electron microscope studies show that the plasma membrane of the basal cells is relatively thin. There is no appreciable change in the thickness of the plasma membrane of the cells located above the basal cells. MCG are formed in these cells, and as these cells ascend to a higher level, the MCG are discharged and the intercellular spaces become filled with a dense amorphous substance as well as with fragments of inner membranes of MCG. Following this event the plasma membranes become thickened and coated by an amorphous substance (Figs. 7 to 11). These sequential changes strongly suggest that the MCG are actively involved in the thickening and coating of the plasma membranes of differentiated epithelial cells and play a role in the development of the protective layer.

The MCG, as seen in this study, appear as specific differentiation products of keratinizing epithelia of the "higher" vertebrates. They are formed by ascending cells undergoing differentiation, but they are absent in basal cells. As far as the sequential events are concerned, it is interesting to note that the formation of the MCG begins earlier than that of the keratohyalin granules and that MCG are usually not present in the cytoplasm by the time the keratohyalin granules are fully evolved. The MCG migrate toward the cell periphery at a time when the other differentiation products are being formed and their discharge through the plasma membrane and spreading of their contents over the cell surface precede formation of the horny layer. The sequence of these events suggests that the content of MCG may progressively seal the intercellular spaces thus isolating differentiated cells from their cellular environment. This change may gradually lead to the reduction of their metabolic activities and in turn promote their transformation into inactive horny cells. Subsequent thickening of the plasma membrane appears as the final stage in the formation of the external protective system of the body.

The membranous inner structure of MCG suggests that these cell organelles are engaged in synthetic activities. In this regard, they appear comparable to melanosomes which actively synthesize melanin on a protein skeleton and to mitochondria which form enzymes on the surface of their inner membranes. The nature of the product of MCG is not yet known. On the basis of histochemical studies, however, one could assume that MCG synthesize a polysaccharide; Wislocki, Fawcett, and Dempsey (9) found in light microscopic studies a periodic acid–Schiff-positive substance in the intercellular spaces of the upper cells of stratified squamous epithelia. They thought that it was a local product and was either discharged from the cells or was formed in or on their surfaces.

Small granules, such as the MCG, have been previously noted by several investigators in various keratinizing epithelia and have been called by different names and assigned various functions. Selby (10) was the first to observe small granules in differentiating epidermal cells; she assumed them to be small keratohyalin granules. Three years later, Odland (11) found small granular components in the upper spinous and lower granular cells of the human epidermis. He noted numerous tubules and membranes within them and presumed that these cell constituents correspond to attenuated mitochondria. Frei and Sheldon (12) later observed small granules (corpuscula) in the normal and hyperplastic epidermis of the mouse which appeared homogeneously dense and structureless or revealed a clear center or some internal structure. Such granules were seen exclusively in the deeper part of the stratum granulosum. In some sections, the granules lie close to the intercellular space within invagina-

tions of the "cell-surface membrane." These authors believe that corpuscula are not related to mitochondria, as Odland (11) suggests, but postulate that they are either an unidentified virus or secretory granules of some kind. Rhodin and Reith (13) and Albright and Listgarten (14), in studying cells from the intermediate layer of the epithelium of the esophagus, tongue and skin of the mouse and cheek pouch of the hamster, have seen small spherical granules about 0.1 μ in diameter that are composed of both light- and dark-staining material. Most of these granules lie close to the plasma membrane, but some were scattered throughout the cytoplasm. Their origin and function were not known. Zelickson and Hartmann (15) studied human oral epithelium and described smooth-walled "vesicles" frequently present near the plasma membrane of differentiating cells. Since these cells contained abnormally few mitochondria, the vesicles were considered to be degenerated mitochondria. Horstmann and Knoop (16) found small granules that they assumed to be viruses in epidermis from the footpad of rat. Small granules identical in appearance with MCG have also been seen in psoriatic epidermis (17); Wettstein and his colleagues (18) concluded that these granules are either casual agents (viruses) or secondary products of the disease.

The chemical studies show that the thickened plasma membrane of the horny cells of keratinizing epithelia possesses a higher resistance than their keratin content. The primary function of the thickened plasma membrane therefore appears to be the protection of the content of the horny cell. Hence, keratin and the resistant plasma membrane together seem to constitute a very efficient means of protection against physical and chemical agents which may impinge upon the surface of the various keratinized epithelia (3). The amorphous coat appearing upon the surface of the thickened plasma membrane does not resist the action of keratinolytic agent; this material may affect the flow of solutes between the horny cells.

Frei and Sheldon (12) noted that a dense material is visible in the space between the superficial granular cells of the mouse epidermis, and Rhodin and Reith (13) observed in epithelium from the esophagus of the mouse that the membrane of the horny cells is thickened and surrounded by intercellular material, but they offered no explanation for these phenomena. The investigation described here provides data related to the development and function of the thickening of the membrane of horny cells and to the origin of the intercellular material.

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