A POLYSACCHARIDE-CONTAINING CELL COAT ON KERATINIZING CELLS OF THE ROMNEY WOOL FOLLICLE

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Summary

The periodic acid–silver methenamine test revealed the presence of a polysaccharide-containing cell coat on the surfaces of keratinizing cells of the Romney wool follicle. This coat was present throughout the changes involving cellular adhesion, cell growth, and cell shape which occur before keratinization is complete. Once these changes were complete, the cell membrane, or regions close to it, and the cell coat of the cortex and the cortex cuticle cells became modified. Modifications of the cell membrane, or regions close to it, in the inner root sheath occurred on or just prior to hardening of these cells.

I. Introduction

Recent reports (Rambourg 1967; Rambourg and Leblond 1967; Mercer, Jahn, and Maibach 1968) have shown that the periodic acid–silver methenamine (PA–silver) test is a reasonably reliable and fairly specific indicator of the presence of polysaccharides, mainly glycoproteins, at the ultrastructural level (see Rambourg and Leblond 1967 for a review of literature and discussion). In particular, the test revealed polysaccharide-containing cell coats on many cell types in mammalian tissues. While in most tissues this coating formed a continuous cover, except possibly at tight junctions (Rambourg and Leblond 1967), Mercer, Jahn, and Maibach (1968) found a differential distribution of cell-coating material in the human epidermis. The epidermal basal layer showed little or no reaction to the PA–silver test, while the cell layers above reacted with increasing intensity until the cell keratinized. At this stage the reaction became non-specific. It was concluded that the cell coat had considerable significance in the development of intercellular adhesion, with resultant importance in the overall growth and differentiation of the epidermis.

The wool follicle, a derivative of the epidermis, follows broadly similar growth and differentiation stages to produce a keratinized fibre. However, the process is more complex because, amongst other differences, the mitotic cells in the follicle bulb give rise to more than one type of keratinized cell. Thus, the keratinized cell may form part of the following follicular structures: (1) the cortex; (2) the cortex cuticle which surrounds it; (3) the inner root sheath (IRS) which encompasses the cortex and its cuticle, and comprises the IRS cuticle, Huxley's layer, and Henle's layer. These layers

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are enclosed in the non-keratinizing outer root sheath (ORS) which is continuous with the epidermis.

The membranes of these follicle cell types have been found to undergo characteristic changes during keratinization to form a cell membrane complex. These changes consist of an increase in material between cell membranes and altered staining properties of the membranes (Birbeck and Mercer 1957; Rogers 1964).

As in the epidermis, increasing cell adhesion is also a feature of keratinization in the follicle (Birbeck and Mercer 1957; Roth and Helwig 1964*a*, 1964*b*; Forslind and Swanbeck 1966).

This ultrastructural study was undertaken to determine whether there is a similar cell coat on keratinizing wool follicle cells and, if so, its occurrence in relation to keratinization and the described membrane changes.

II. Materials and Methods

Wool follicles were dissected from biopsies of prefixed skin of 9-month-old Romney sheep (castrated males) and fixed for a further 4–5 hr in 4% paraformaldehyde in 0·1M phosphate buffer at pH 7·3, or in 2·5% glutaraldehyde in the same buffer. After rapid dehydration through a graded series of ethanols the follicles were embedded in Epon. "Silver" sections of the keratinizing region of the follicles were cut on a LKB ultramicrotome and transferred to the staining solutions with a platinum wire loop. The treated sections were picked up on Formvar–carbon coated grids for examination in a Philips EM300 electron microscope.

Histochemical Procedure

All solutions were made up using deionized distilled water.

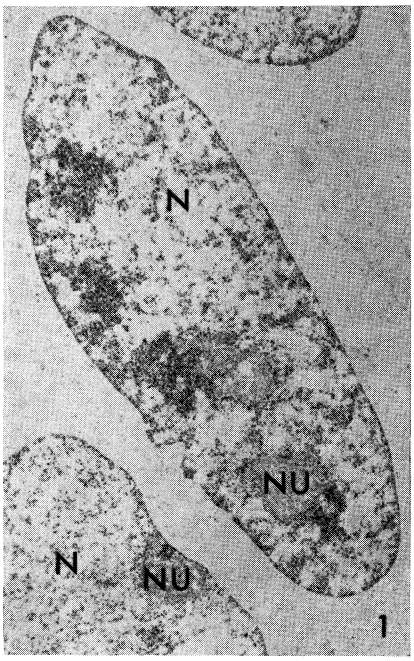
The preparation of the staining solution was adapted from the methods of De Martino and Zamboni (1967) and Rambourg (1967). 2 ml of 5% silver nitrate were added to 18 ml of 3% hexamethylenetetramine. The precipitate formed was redissolved by agitation and 20 ml of distilled water added. Finally, 0·8 ml of 5% sodium borate was added.

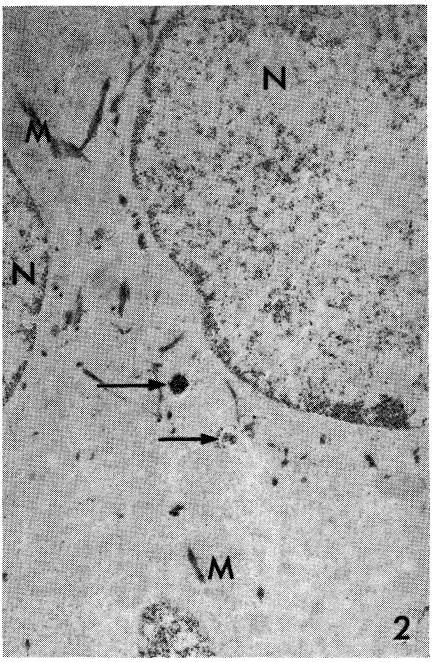
Oxidation was carried out with 1·5% periodic acid. A range of oxidation times was tried and 10–15 min proved adequate. The sections were then washed for from 15 min to overnight in several changes of distilled water before transfer to the freshly made up staining solution. Control sections for comparative purposes were not treated with periodic acid but were placed directly onto the silver methenamine solution for similar time periods as the treated sections.

Sections on the silver methenamine solution were incubated at 55–60°C in a dark oven. Periodic examinations were made to ensure that the sections were removed immediately they became tinged a yellow-brown colour. Failure to do this resulted in an increasingly dense non-specific background deposit of silver grains, which obscured the definition of the periodic acid-positive material. The time required for suitable staining varied but was generally about 20–30 min. The sections were then rinsed in distilled water, placed in a 2·5% sodium thiosulphate solution for 5 min, and finally rinsed again.

Fig. 2.—Control. Cortex cells in upper bulb. Dense deposits of silver are found over the chromatin of the nuclei (*N*) and the forming macrofibrils (*M*). Arrows point to one heavily and one lightly stained cytoplasmic granule. Ribosomes are lightly stained. Glutaraldehyde. × 10,000.

Fig. 3.—PA–silver. Lower bulb. Cells are outlined by silver deposits wherever membranes are in apposition (arrows). Intercellular spaces (*I*) are common especially near the dermal papilla (*DP*). Ribosomes, chromatin in nuclei (*N*), and dense bodies (*G*) are heavily stained. The basement membrane (*BM*) of the dermal papilla shows a moderate deposition of silver while collagen in the dermal papilla is stained black. One cell is dividing (*DC*). *D*, desmosome. Glutaraldehyde. × 7,500.





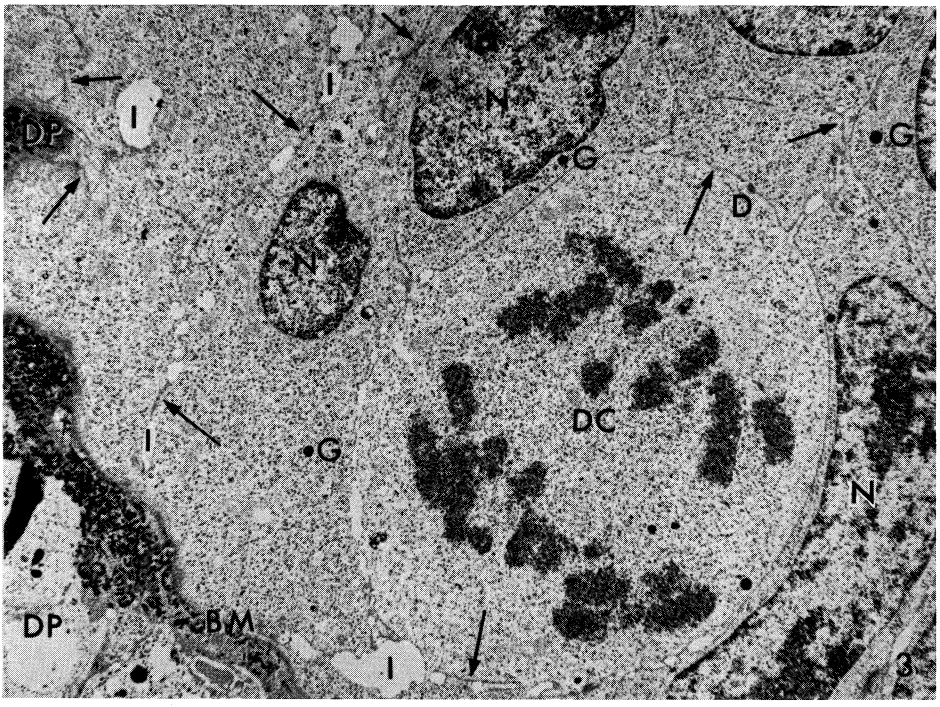


Fig. 1.—Control. Lower bulb region. Chromatin of the nucleus (*N*) and the nucleolus (*NU*) is stained. Ribosomes are lightly stained. Glutaraldehyde. ×12,000.

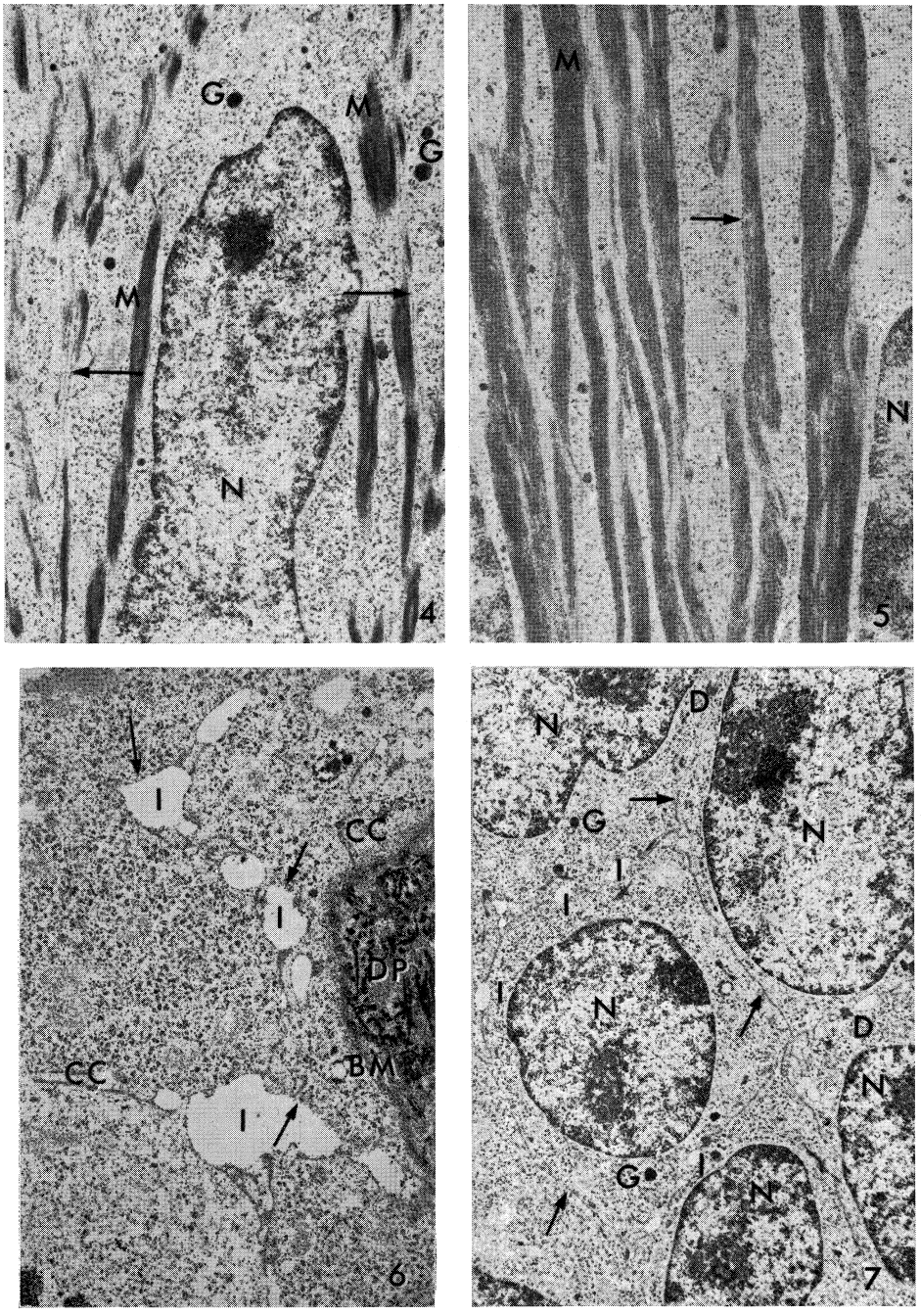


Fig. 4.—PA–silver. Early keratogenous zone. In some areas, cell outlines are still marked by silver deposits (arrows). Some forming macrofibrils are closely associated with the cell coat. *N*, nucleus; *G*, dense bodies. Glutaraldehyde. ×10,000.

III. Results

(a) *Cell Components Showing Non-specific Staining*

Observation of control sections revealed that the chromatin and nucleoli of nuclei stained, together with ribosomes (Figs. 1 and 2). Forming keratin macrofibrils in cortex cells reacted strongly (Figs. 2 and 11) as did cortex cuticle keratin (Figs. 11 and 12). Although trichohyalin droplets stained lightly or not at all, the filamentous form extending from the droplets stained positively until the IRS cell was completely hardened. At this stage staining became light or undetectable (Figs. 11 and 12).

Unidentified cytoplasmic granules (dense bodies), ranging in diameter from about 150 to 250 nm, were observed in cortex cells and were found to stain non-specifically (Fig. 2). However, it is unlikely that all dense bodies visible in periodic acid-treated sections were stained non-specifically. Silver sections taken before and after the one shown in Figure 1 and treated with PA–silver revealed 10 positively staining dense bodies in areas where no dense bodies were seen in the control section.

Collagen was argentophilic in both the dermal papilla and the connective tissue sheath. Non-specific staining was also noted for the cell membranes and dense bodies of one cell type in the dermal papilla. Periodic acid treatment increased the intensity of staining of nuclear chromatin, ribosomes, collagen, and trichohyalin (Figs. 3 and 9).

Membranes of the mitotic zone, cortical, and presumptive IRS cells were unreactive although very faint staining of tight junction-like regions was occasionally observed. Membranes of hardened IRS cells and parts of the cortex cuticle cell membranes showed staining. However, this type of membrane staining will be detailed in the context of specific staining.

(b) *Specific Staining of Cell Components*

Positive reactions after periodic acid oxidation were shown by the basement membranes surrounding the follicle and the dermal papilla (Fig. 3). However, the most significant result was the appearance of dense silver deposits outlining cells. These were considered to indicate a polysaccharide-containing cell coat. As the appearance of this coat altered during the keratinization process of each cell type, the changes found in each cell line will be described separately.

(i) *Mitotic Zone (Lower Bulb)*

In general, all cells in the mitotic zone, including regions of apposition near the dermal papilla, were outlined by silver deposits wherever cell membranes were

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Fig. 5.—PA–silver. Middle keratogenous zone. Faintly stained line (arrow) may indicate presence of cell coat. *M*, forming macrofibril; *N*, nucleus. Glutaraldehyde. ×11,000.

Fig. 6.—PA–silver. Lower bulb. Intercellular spaces (*I*) appear to have silver deposits on some surfaces but not on others (arrows). *CC*, silver deposits on cell coat; *DP*, dermal papilla; *BM*, basement membrane. Black deposits in the dermal papilla indicate collagen. Glutaraldehyde. ×10,500.

Fig. 7.—PA–silver. Mid-bulb. These undifferentiated cells are outlined by silver deposits (arrows). Intercellular spaces (*I*) are small and relatively few. *G*, dense bodies; *N*, nuclei; *D*, desmosomes. Glutaraldehyde. ×7,900.

apposed (Figs. 3, 6, and 7). The average width of these deposits was about 16 nm. Silver deposits were not found on the surfaces of basal layer cells in contact with the basement membrane of the dermal papilla (Fig. 3).

The deposition of silver on the surface of intercellular spaces seemed to be variable. These spaces, which are frequent in the bulb of the follicle and especially between the basal cells surrounding the dermal papilla, often showed regions which lacked the dense deposits associated with the cell coat material (Fig. 6).

Desmosomes were indicated by deposits of silver thicker than those found in other regions of cell contact (Figs. 3 and 7). In favourably oriented sections, the deposits could be resolved as two lines (Fig. 15). Dense bodies were readily apparent in the cytoplasm and were frequently located close to cell membranes (Figs. 3 and 7).

(ii) *Cortex Cells*

Cells passing from the mitotic zone to the growth zone (upper bulb) were characterized by marked convolutions of the cell coat. In the elongation zone and the early keratogenous zone, where the final form of the cell is assumed, the cell was still outlined by the reaction product. However, regions occurred where it was difficult to determine the position of the cell coat (Fig. 4). A close association of cell coat and some forming macrofibrils was noted.

About one-third to half-way up the keratogenous zone, the staining properties of the cell coat changed and it became either undetectable or very faintly stained, with apparent loss of its former granularity (Fig. 5). This transition occurred at the approximate level where the cortex cuticle assumes its final form.

(iii) *Cortex Cuticle Cells*

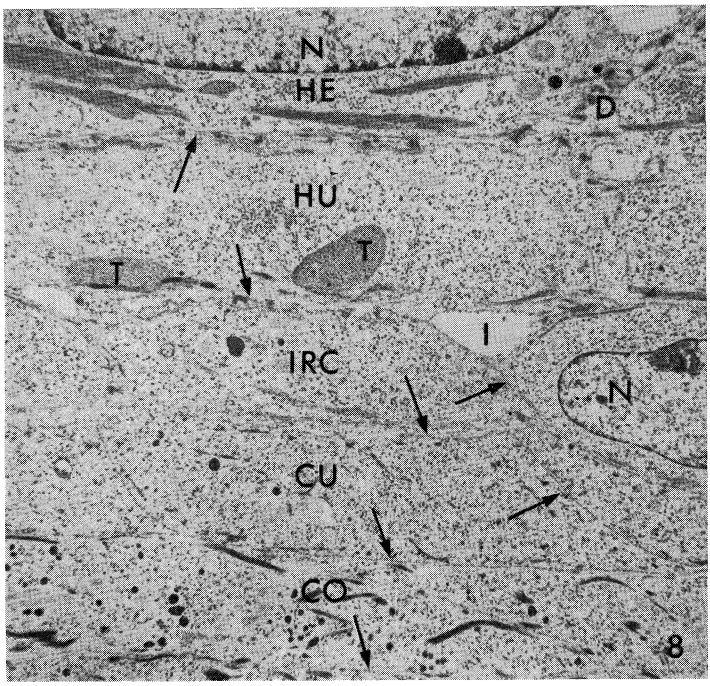
The coat of the cortex cuticle was discernible up to where the cuticle assumed its approximate final shape but before it was completely keratinized (Fig. 8). At this point, the granular nature of the usual reaction product seemed to be lost and, instead, a non-specific and often discontinuous stained line became visible around many cuticle cells (Fig. 10). Measurements of the gap between the two lines found when two cuticle cells were in apposition ranged from about 20 to 29 nm. The double line in regions of apposition and the agranular nature of the staining suggest that this staining was not due to cell coat material.

(iv) *Inner Root Sheath Cells*

In general, IRS cells were outlined by a single layer of silver deposits during differentiation (Figs. 7, 8, and 9). On hardening, staining became non-specific although intensified after periodic acid oxidation (Figs. 12 and 13).

Henle's layer is the first to harden. Prior to hardening, changes in cell shape, loss of intercellular spaces, and increasing numbers of desmosomes (especially between Henle's cells) were clearly depicted by the cell coat in sections treated with PA–silver (Fig. 8). In control sections, the hardened cells were outlined by a single discontinuous

Fig. 9.—PA–silver. Differentiating IRS cuticle and Huxley's layers. Cell outlines marked by silver deposits (arrows). The junction of IRS cuticle (*IRC*) and cortex cuticle (*CU*) is lightly stained (crossed arrow). Henle's layer (*HE*) has hardened and cell margin has no gaps in it. *CO*, cortex; *T*, trichohyalin; *D*, desmosome. Glutaraldehyde. ×8,800.



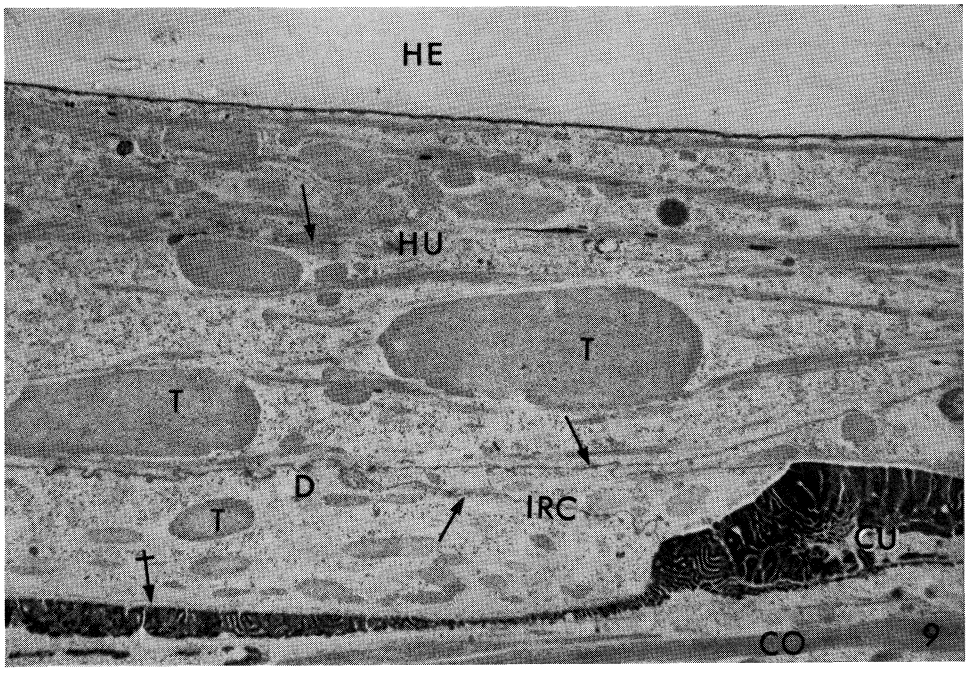


Fig. 8.—PA–silver. Differentiating IRS. Silver deposits (arrows) outline cells in Henle's (*HE*), Huxley's (*HU*), IRS cuticle (*IRC*), cortex cuticle (*CU*), and cortex (*CO*) layers. Thickened regions in cell coat represent desmosomes (*D*). *T*, trichohyalin; *N*, nuclei; *I*, intercellular space. Glutaraldehyde. ×6,800.

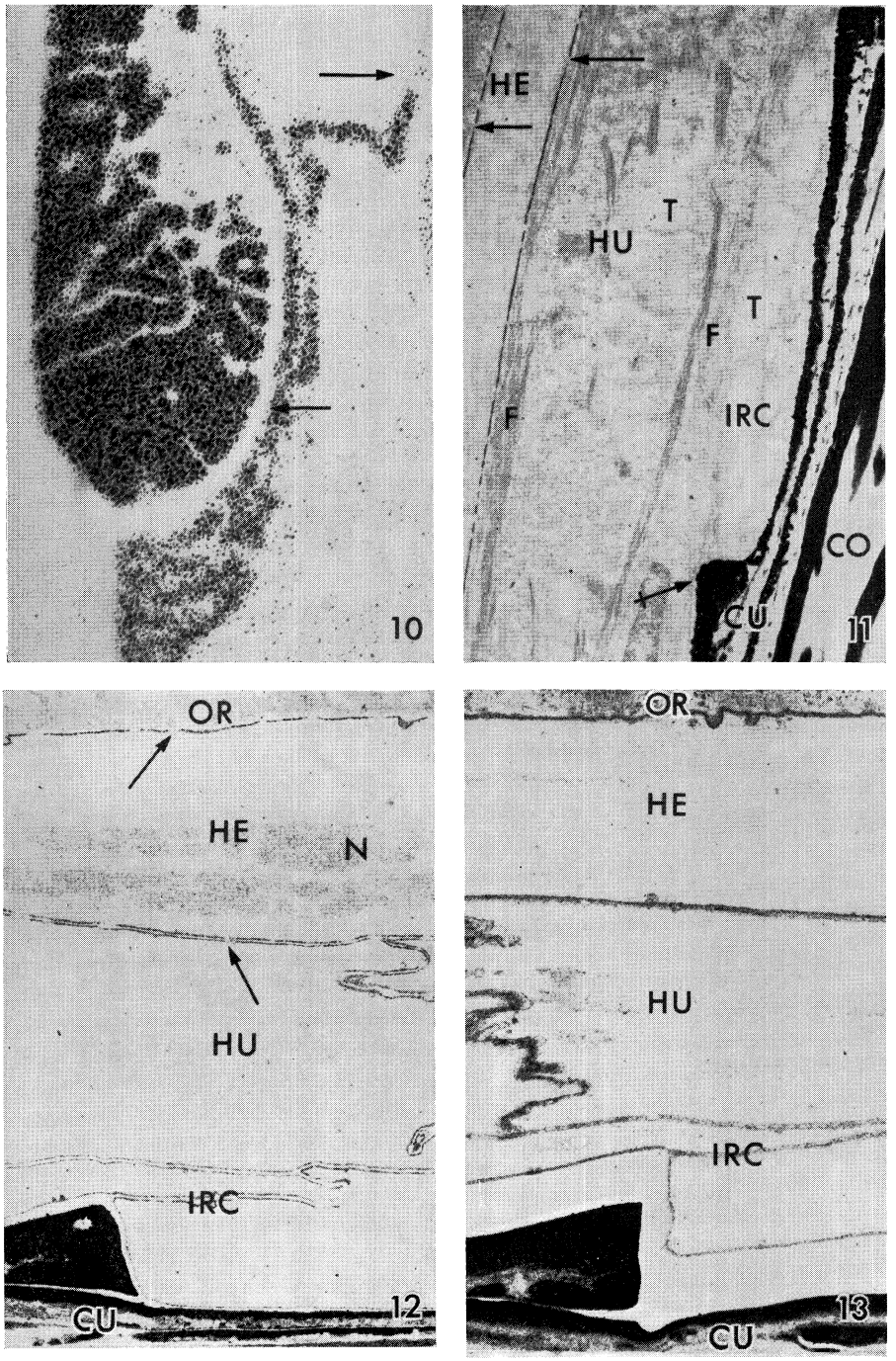


Fig. 10.—Control. Keratinizing cortex cuticle. Non-specific staining of two layers at apposition of two cortex cuticle cells (arrows). Stained line is not continuous over whole cell surface. Formaldehyde. ×46,000.

line of silver deposits, indicating non-specific staining (Figs. 11 and 12). The density of these deposits was increased after periodic acid oxidation and the gaps disappeared (Figs. 13, 14, and 16). The increased intensity of staining was probably the result of the juxtaposition of the cell coats of ORS and Huxley's cells with Henle's cells (Fig. 16).

Huxley's layer and the IRS cuticle both harden at about the same level, with similar changes in the cell coat material. In differentiating cells of these layers, PA–silver-treated sections showed the usual silver deposits outlining the cells. However, at the junction of the more differentiated IRS cuticle and the cortex cuticle cells, lighter-staining material was usually apparent which in parts stained non-specifically (Figs. 9 and 11). Control sections again showed non-specific staining where hardened Henle's cells adjoined Huxley's cells.

Further changes were apparent in the fully hardened IRS. In control sections, cell outlines were non-specifically stained. However, where the cells were in apposition, the silver deposits did not fuse into one line but remained separated by a gap of about 20–30 nm except at the junction of Henle's and ORS cells (Fig. 12). This staining was interpreted as resulting from the alteration of properties of membranes or regions immediately adjacent to them, not from changes in cell coat material. As in control sections of hardened Henle's cells, the non-specific staining was discontinuous over the cell surface in IRS cuticle and Huxley's layers. These changes were sometimes seen in IRS cells immediately prior to hardening. After periodic acid treatment, an increased granularity was observed in the spaces between the non-specifically stained lines (Fig. 13), which in favourably oriented sections could be resolved as an extra line of discontinuous silver deposits (Fig. 14). The gaps seen in non-specifically stained cell outlines disappeared after periodic acid oxidation. The cell outline at the junction of ORS and Henle's cells could be resolved, in places, into two short lines (Fig. 16) which were similar to the structures interpreted as desmosomes in non-hardened cells (Fig. 15).

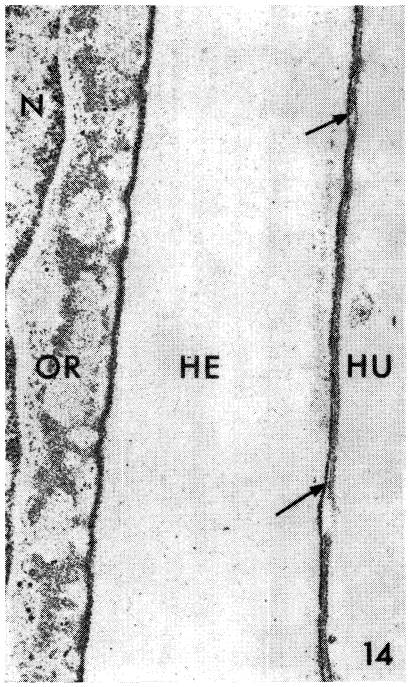
(v) *Outer Root Sheath Cells*

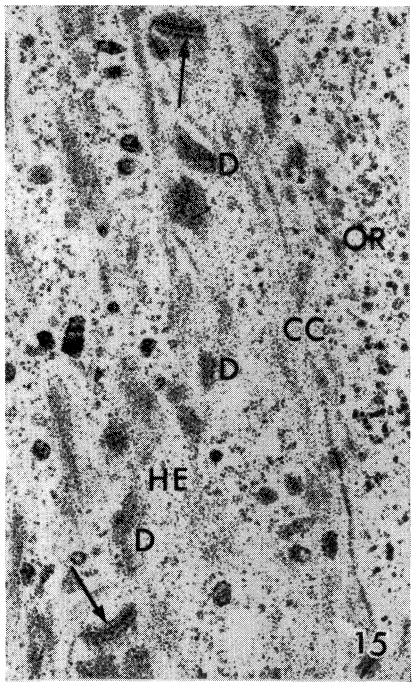
In the regions of the follicle studied, cells of the ORS were outlined by a single line of silver deposits.

Fig. 11.—Control. Early keratogenous zone. Forming macrofibrils in cortex (*CO*) and cuticle (*CU*) keratin are heavily stained. In Huxley's (*HU*) and IRS cuticle (*IRC*) cells, trichohyalin (*T*) and ribosomes are lightly stained. The filamentous form (*F*) of transforming trichohyalin stains while the hardened Henle's layer (*HE*) does not. Henle's layer is outlined by heavily staining material which is discontinuous (arrows). Faintly staining material is visible over part of the surface of the IRS cuticle cell where it is juxtaposed with the cortex cuticle (crossed arrow). Glutaraldehyde. ×7,000.

Fig. 12.—Control. Hardened IRS. Non-specific staining is found in nuclear remnants (*N*) and cell outlines of Henle's (*HE*), Huxley's (*HU*), and IRS cuticle (*IRC*) cells. Gaps occur in these cell outlines (arrows). Cortex cuticle (*CU*) keratin and ribosomes in the ORS (*OR*) stain. Glutaraldehyde. ×10,000.

Fig. 13.—PA–silver. Hardened IRS. Granularity of cell outlines has increased. Gaps do not occur in cell outlines. The IRS has separated slightly from the cortex cuticle (*CU*) during processing. *OR*, outer root sheath. *HE*, Henle's layer; *HU*, Huxley's layer; *IRC*, IRS cuticle. Glutaraldehyde. ×11,300.





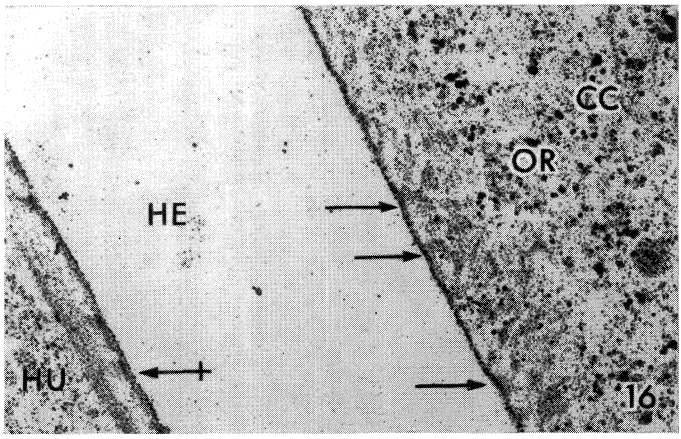


Fig. 14.—PA–silver. Hardened IRS. At junction between Huxley's (*HU*) and Henle's (*HE*) layer, a discontinuous line of silver deposits (arrows) can be seen between two continuous lines. *OR*, outer root sheath; *N*, nucleus. Glutaraldehyde. ×17,000.

Fig. 15.—PA–silver. Differentiating Henle's layer. Desmosomes (*D*) are numerous at the junction of two Henle's cells (*HE*). Two desmosomes (arrows) are favourably oriented so that the separation of the cell coat (*CC*) into two lines can be seen. *OR*, outer root sheath. Glutaraldehyde. ×21,000.

Fig. 16.—PA–silver. Hardened Henle's cell. At the junction of Henle's (*HE*) cell and the ORS (*OR*), the cell outline can be resolved as two lines in some regions (arrows). The silver deposits at the junction of Henle's and Huxley's (*HU*) layer can also be resolved into two to three lines in places (crossed arrow). *CC*, cell coat. Glutaraldehyde. ×19,000.

IV. Discussion

The results from the wool follicle support the contention that polysaccharide-containing material indicated by the PA–silver technique is present at or near the surface of various types of mammalian cells [Rambourg and Leblond (1967) and Rambourg (1967) in the rat; Mercer, Jahn, and Maibach (1968) in human epidermis]. In this study, evidence for the presence of a cell coat was derived from three observations. First, the average width (about 16 nm) of the silver deposits found between cells was consistent with an intercellular spacing of about 15 nm described between osmium-fixed cells in the bulb of human and guinea pig hair follicles (Birbeck and Mercer 1957; Rogers 1964). Second, if this material were present within or beneath cell membranes, the silver deposits would resolve as a double line where cells were in apposition. However, the only double lines seen were those resolved in the desmosomes (see also Mercer, Jahn, and Maibach 1968). Third, in osmium-fixed follicles (Birbeck and Mercer 1957; Rogers 1964) the space between the cell membranes of hardened IRS cells was about 25–30 nm, which is similar to that found between the non-specifically stained cell outlines in this study. Following periodic acid treatment, the silver deposits indicating the cell coat clearly lay between these non-specifically stained cell outlines.

The marked differential occurrence of cell coat material observed in the epidermis (Mercer, Jahn, and Maibach 1968) was not found during the differentiation of lower bulb cells in the wool follicle. Cell coat material was found in all regions where the cell membranes were in apposition, including the basal layer, except where basal cells were attached to the basement membrane of the dermal papilla (see also Rambourg and Leblond 1967). The only differential deposition of cell coat material seen occurred around intercellular spaces, which often appeared partially coated and decreased in size and frequency during keratinization.

In osmium-fixed follicles, the decrease in intercellular spaces was associated with the appearance of an "intercellular cement" between membranes of apposing cells (Birbeck and Mercer 1957; Roth and Helwig 1964*a*, 1964*b*; Forslind and Swanbeck 1966). This was considered an essential part of the differentiation of the keratinizing cell (Birbeck and Mercer 1957). The present study indicates that the "intercellular cement" consists, in part at least, of a polysaccharide-containing material. Polysaccharide-containing material (usually glycoproteins) has been found between apposed cells in many other cell types (Emmelot *et al.* 1964; Langley and Ambrose 1964; Pease 1966; Rambourg and Leblond 1967). The fact that intercellular spaces appear partially coated supports the idea that this polysaccharide material may be necessary for cell adhesion.

In osmium-fixed follicles, an increase in the spacing between cell membranes from about 15 nm to 25–30 nm was observed about the middle of the keratogenous zone in cortex cells, where the cortex cuticle cells reach their imbricate form, and where the IRS cells harden (Birbeck and Mercer 1957; Rogers 1959, 1964). This increase is due to the appearance of a central dense component in the space between membranes. These findings coincide closely with the changes in staining properties of the cell coat in PA–silver treated sections in this study. The results indicate that in the cortex and the cortex cuticle, the polysaccharide nature of the cell coat is

apparently modified markedly or lost entirely about the level where the spacing between cell membranes increases. Cell coat material was retained after separation of membranes in hardened IRS cells, although it appeared discontinuous. The retention of cell coat material may have significance in the breakdown of the IRS higher in the follicle.

The non-specific staining at or near the membranes of the IRS cells and cortex cuticle reported in this study may represent the material observed in osmium-fixed follicles, adjacent to the membranes of these cells just prior to their increased separation (Birbeck and Mercer 1957). In cortex cells, this material was not reported as occurring before the spacing between membranes increased.

Overall, the results presented in this paper indicate that a polysaccharide-containing cell coat is present during the changes associated with cellular adhesion, cell growth, and cell shape during keratinization. Cells then seem to be stabilized in their appropriate layers by modifications of the cell coat and the cell membranes, or regions close to them. The site of synthesis and the mechanism of transport of cell coat material were not resolved. However, the possible presence of specifically stained cytoplasmic granules may be significant. It is possible that a mechanism involving the Golgi apparatus, similar to that thought to operate in the epidermis (Matoltsy and Parakkal 1965) and intestinal columnar cells (Rambourg, Hernandez, and Leblond 1969), is present in the wool follicle.

V. Acknowledgments

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