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SURFACE COATS CONTAINING POLYSACCHARIDES ON HUMAN EPIDERMAL CELLS*

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The development of reliable histochemical methods for demonstrating polysaccharides at a fine structural level by means of electron microscopy has made it possible to reconsider problems relating to the existence and function of polysaccharide containing coats or layers on the surfaces of closely packed and adhering cells in tissues. Rambourg, Neutra and Leblond (13) have reviewed this question recently and marshalled strong evidence from light microscopical histochemistry that substances containing carbohydrates (positive PAS substances) occur universally at or near the surfaces of cells in epithelial type tissues. They later applied to numerous tissues (Rambourg and Leblond (12)) the modified PAS test (PA-Ag) in which the aldehyde groups produced by the oxidation of sugars containing adjacent OH groups or ONH_2 groups are caused to reduce silver preparations and to deposit metallic silver. See (12) for previous references. The silver grains are visible electron microscopically and the seat of the reaction appeared to be immediately adjacent and external to the plasma membrane.

An entirely new method developed by Luft (6), which depends on the combination of the substance "ruthenium red" with the surface coat, has also been introduced to electron microscopy and promises a more accurate localization of the acidic surface polymers (5). Its chemical specificity is not yet as well established as that of the older PAS and the modified PA-Ag tests.

With these methods at hand an investigation of the occurrence and functional role of polysaccharide-rich coats in the stratified, squamous keratinizing epithelia becomes possible. These tissues are constantly proliferating and differentiating. There are problems concerning the specialized intercellular adhesion (8) followed by desquamation of their keratinized layers, which are imperfectly understood.

The experiments described below were carried out using human skin and the periodic acid-silver method, which was favored on the grounds of its acknowledged specificity. See discussion in Pease (11) and Rambourg and Leblond (12). It is proposed to repeat the work using Luft's ruthenium red technique with the object of achieving finer resolution.

METHODS

Materials

Thin slivers of human skin, about 1–2 mm square and a little thicker than the epidermal layer, were cut by slicing with a fragment of razor blade held in a special holder and processed at once. The inner forearm or back of minimally pigmented human volunteers were the sites selected for study. Prior to sampling, the skin was anesthetized by an injection of 1% Lidocaine solution.

Fixation and Embedding

The fixative was 2.5% glutaraldehyde in a phosphate buffer at pH 7.2. Specimens were fixed for 2-4 hours at room temperature and then washed for two hours in the buffer with at least three changes to remove the aldehyde.

After washing, the specimens were dehydrated in an alcohol series of increasing strength, transferred to dry acetone and then to propylene oxide before being embedded in an Epon mixture made according to Luft (6). Sections were cut using a diamond knife and a Reichert microtome and were examined after the required processing (see below) in a Siemens Elmiskop IA.

Histochemical Details

The procedure followed that outlined by Rambourg and Leblond (12) following numerous other authors. Appropriate times of oxidation and of silver reaction were found by trial and error and differ somewhat from those found best for other tissues.

Periodic acid: 1% in water.

Silver methenamine: Drops of 5% silver ni-

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trate solution (2 ml) were added to 18 ml of 3% hexamethylene tetramine followed by 2 ml of 2% sodium borate in water.

Silver to gold sections were cut and allowed to float on the collecting bath until needed. Some sections were stained with uranyl acetate and examined in the microscope to check the quality of the preparation and to facilitate the later identification of reactive structures.

Sections in small groups for the PA-Ag test were transferred from the bath by means of a platinum wire ring to the surface of the periodic acid and left there for 20, 40, 60 or 80 minutes to provide a range of degrees of oxidation of the embedded tissues. After oxidation sections were thoroughly washed by transferring them to the surface of a succession of three water baths, being left on each for 30 minutes.

After washing they were transferred to the surface of a freshly made up solution of silver methenamine at 60° in a darkened oven. It is a common experience that some uncontrollable factor intervenes when silver preparations of this nature are used; our experience confirms this. Some solutions deteriorated rapidly and became covered with silver, which contaminated the sections; others seemed more stable, but slow to react with the sections. We followed Rambourg and Leblond's suggestion and examined the preparations from time to time for evidence of darkening. Times of 20, 30, 40, 50 and 60 minutes of treatment were tried. The results were variable, but 20–30 minutes treatment most frequently gave the desired degree of reaction. The deposition of silver probably takes place first on sites of reducing material in the embedded specimen, which may or may not be specific for polysaccharide. As exposure continues, deposits form more generally over the entire section producing a "background" as may be noted in some of the micrographs. This undesirable development is signalled by a darkening of the section.

Treated sections were picked up on Formvar coated grids.

Controls

For this test a reactive site is taken as positive f it reacts with silver methenamine after oxidaion, and not without prior oxidation.

Control sections were therefore placed on the ilver solution without prior oxidation with eriodic acid and treated for similar times. They vere compared with oxidized sections exposed for he same times on the same solutions under he same conditions.

It is not possible to quantitate this method trictly since the factors controlling the rate of rowth of silver grains are not known. However, it may be assumed that the number of grains initiated and the size to which they grow both increase with the concentration of reducing substances present. A semi-quantitative measure follows them from noting numbers of grains and their size found per unit area.

RESULTS

Components Reacting Non-specifically

Dermis: As noted by others the dermal collagen reacts strongly with silver reagents. The fibrils (Fig. 6) appear banded suggesting some periodic localization of reactive sites. Other fine, ill-defined clusters of unbanded filaments (Fig. 6 at R) located between the collagen bundles, react equally strongly. This may be reticulin, which is partly defined histologically by its ready reaction with silver preparations (11).

The basal lamella (BM Fig. 1) reacts weakly and the tapering, banded connecting filaments, described by Farquhar and Palade from frog skin, also react (3). All these components react more strongly after oxidation. This may mean that they also have a carbohydrate associated with them as is suggested by independent evidence.

Epidermis: The most conspicuous non-specific reaction is given by pigment granules found both in melanocytes and keratinocytes. Melanin contains a silver reducing substance as has been often noted. In the nucleus certain areas, which also counterstain with uranyl uranyl acetate and correspond with the distriacetate and correspond with the distribution of nucleic acid, react strongly (Fig. 1). The nuclear membrane is defined by these deposits but is itself unreactive.

The membranes of the flattened, keratinized cells of the stratum corneum (Figs. 4 and 7) react non-specifically before oxidation and more strongly after PA treatment. The special problem of these membranes will be referred to below. Various unidentified inclusions in these cells also stain.

Specific components: (*i.e.* components reacting only after PA oxidation).

Epidermal cell membranes: The cell surfaces throughout the epidermis with the exception of those of the cells in the basal layer react positively with the reagent.

The silver grains are variable in size. The smallest are less than 100Å in diameter and

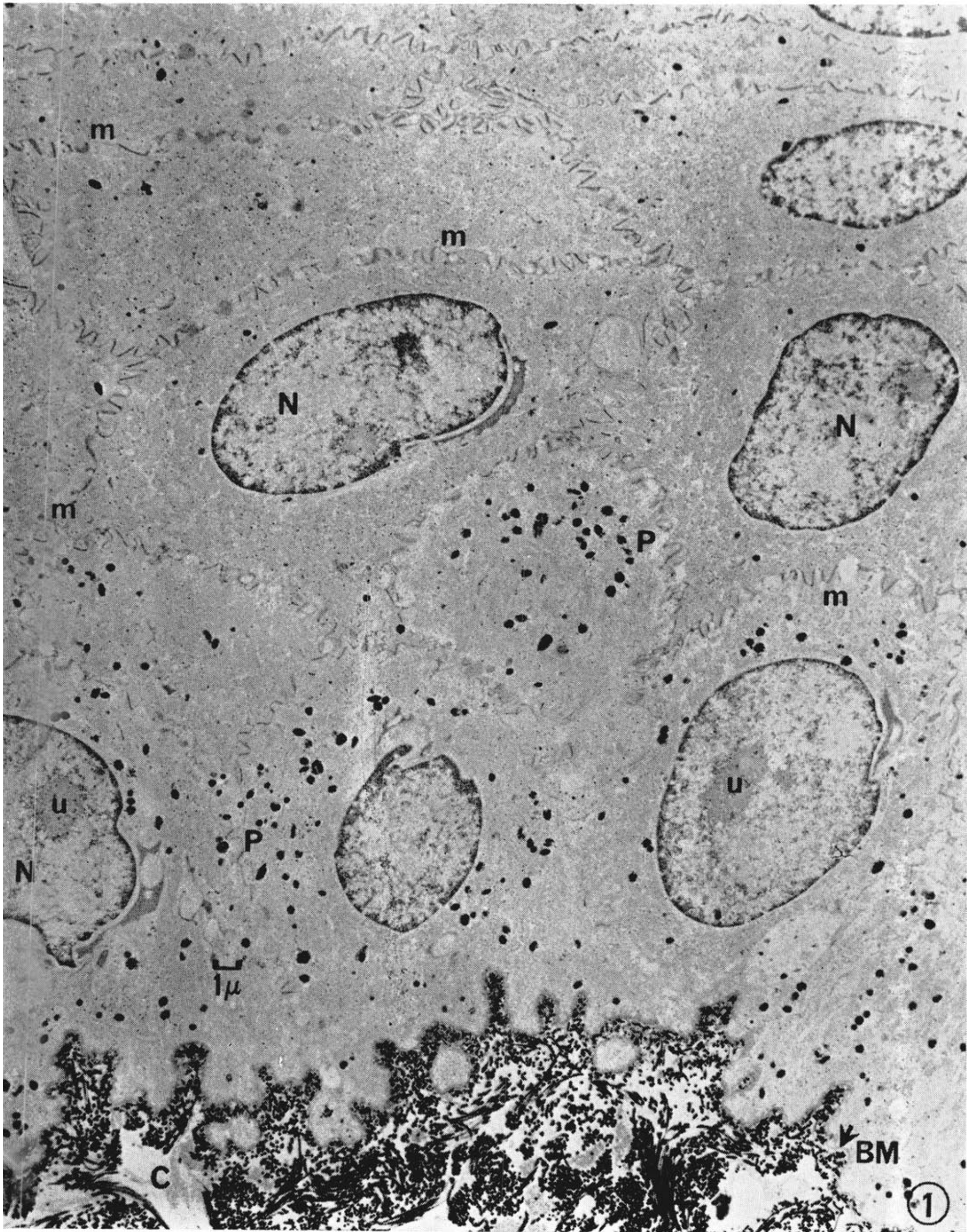


PLATE 1

FIG. 1. Low magnification survey micrograph extending from to basal lamella almost to the stratum granulosum to show major sites of silver deposition.

Non-specific sites are: collagen, nuclei pigment granules.

Specific deposition on membranes as at m. Note absence of silver on membranes near basal lamella and generally weak reaction of membranes of basal layer cells compared with higher cells.

some confluent clumps may be as large as 0.1μ. The commonest size is between 100 and 200Å. The reaction as judged by density of silver grains increases towards the stratum corneum (Fig. 1). Desmosomes, common between cells above the basal layer react strongly. At higher power (Fig. 5) it is apparent that the reactive layers immediately outside of the plasma membranes of the participating cells continue without break through each desmosome and that these two layers do not fuse to form a single reacting layer. This relationship is more obvious in sections counterstained with uranyl acetate to indicate the position of the other components of the desmosome. In Figure 5A for example, the several desmosomal components: plasma membranes P (unstained), cytoplasmic deposits A, fibrils of keratin and the central dividing line C are recognizable. The silver grains are found between the plasma membrane and the central line (Text Fig. 1).

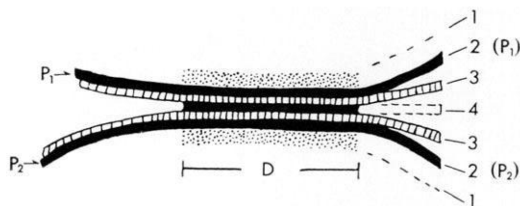


FIG. 1. Structural elements of desmosome. P1 and P2 the two plasma membranes; 1–4 the several components of the desmosome D. (1) intracellular protein deposit. (2) phospholipid—protein plasma membrane. (3) polysaccharide surface coat. (4) intermediate or central disc uniting surface coats (nature not known).

Magnification of each micrograph is indicated by the index line (——) 1 μ long.

Preparation: Normal human skin fixed in glutaraldehyde. Periodic acid oxidation followed by treatment with silver methenamine.

BM—basal lamella
C—dermal collagen fibers
N—nuclei
U—nucleoli
P—pigment granules
D—desmosomes
d—hemidesmosomes facing basement membrane
k—stratum corneum
g—stratum granulosum
f—keratin fibrils
m—membranes shown by silver deposits.

In locations of close contact other than desmosomes only a single line of silver grains defines the 150Å intercellular gap. Where the membranes dilate more widely, the grains appear on each surface except in the wider openings between cells in the basal layer, where, as stated above, the reactive coat seems wanting.

Half or Hemidesmosomes of Basal Layer Cells

The membranes of basal layer cells rarely yielded a positive reaction except at the sites of the hemidesmosomes which attach the basal layer cells to the basal lamella and thus to the dermis. Here a patch of cell membrane reacts positively (d, Fig. 6). The basal lamella itself reacts weakly in a non-specific fashion (Fig. 6) and more strongly after PA oxidation; it may therefore contain some polysaccharide. The peculiar tapered abnormally banded collagen segments, which bridge the massed dermal collagen and the basal lamella, stain non-specifically like collagen itself (Fig. 6 at T). It was difficult to decide whether the thin threads running extracellularly between the lamella and the plasma membrane at sites of the hemidesmosomes were positive. The plasma membrane in these areas reacts positively and thus it is impossible to decide whether the silver deposits arise entirely from the reaction with a surface coat in these areas or from the associated extracellular threads.

The Membranes of the Keratinized Cells of the Stratum Corneum

The membranes of fully keratinized cells are thicker (300Å) than those of the unkeratinized living cells beneath the stratum corneum, which are about 70–80Å. The triple layered structure is also obscured in these membranes and this is a further structural sign of modification (1, 8) occurring during keratinization. These altered membranes reacted with the silver reagent in a non-specific manner before PA oxidation and more strongly after oxidation (Figs. 4 and 1). When the contact between the cell membrane of the last of the cells of the stratum granulosum and the first of the keratinized cells is examined (Fig. 7 at g), this difference in reactivity is very obvious.

Desmosomes between keratinized cells of the stratum corneum (as at D in Fig. 7) react strongly, but their fine strucutre is less visible.

A further point of interest here concerns the small granules referred to as keratinosomes (diameter about 1000 Å) which Matoltsy and

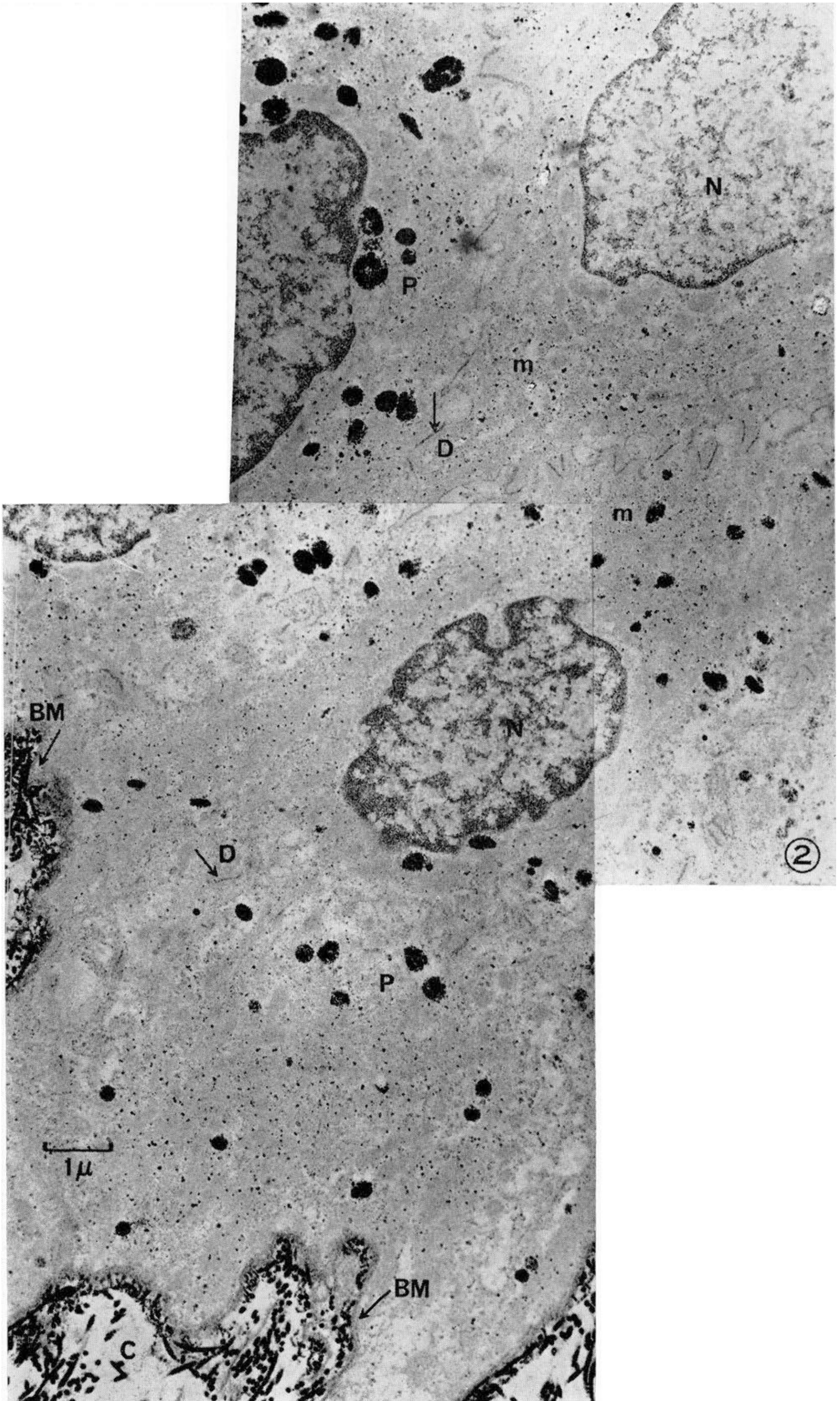


PLATE 2

Parakkal (7) have shown are secreted into the intercellular space just prior to the transformation of a granular cell into a keratinized cell. The small pockets between the cells, where the granules have entered, may be seen at points g in Figure 7. No obvious deposits of silver grains were noted in these pockets nor in the dilated intercellular gaps occupied by secreted granules at higher levels.

Intracellular keratinosomes, small rounded granules of diameter about 0.1μ also called Odland bodies or "membrane coating granules" (7), are usually noted in considerable numbers beneath the plasma membrane in cells of the stratum granulosum where they gather prior to secretion. No localized silver deposits, which could be associated with these granules, were noted in these regions. Other components not stained under any conditions include: keratin fibrils, keratohyalin granules in the stratum granulosum (See Figs. 4 and 7), intracellular membranes (mitochondrial membranes, nuclear membranes and the small amounts of ER).

Discussion

Plasma Membranes of Epidermal Cells

The positive reaction given by most of the cell membranes of the epidermis is evidence that these membranes have a polysaccharide component associated with them. Since in situations where the two membranes are in close contact only one line of silver grains is observed, it is concluded that this deposit lies between the two membranes (*i.e.*, in the 150Å extracellular gap). The reactive sites cannot lie on the cytoplasmic side of membranes, or actually on the membranes themselves, for this would give rise to two lines of grains separated by about 150Å.

The other intracellular membranes: nuclear, mitochondrial, vesicular apparatus, etc., do not react. An occasional small, elongated vesicle with silver grains within it, may be part of the vesicular apparatus synthesizing polysaccharide.

The greatly convoluted and often well-separated membranes of the basal or germinal layer of cells react either weakly and irregularly or not at all, (except at sites of hemidesmosomes) (d, Fig. 6). Since the reaction occurs principally where cell membranes are closely opposed, it appears to be correlated with the adhesion of these membranes to form the familiar image of closely parallel membranes spaced about 150Å apart. The dimensions of the intercellular space suggest that a surface coat of about 70–80Å thick acting as a "spacer" covers the external surfaces of the cells and is the site where silver grains appear.

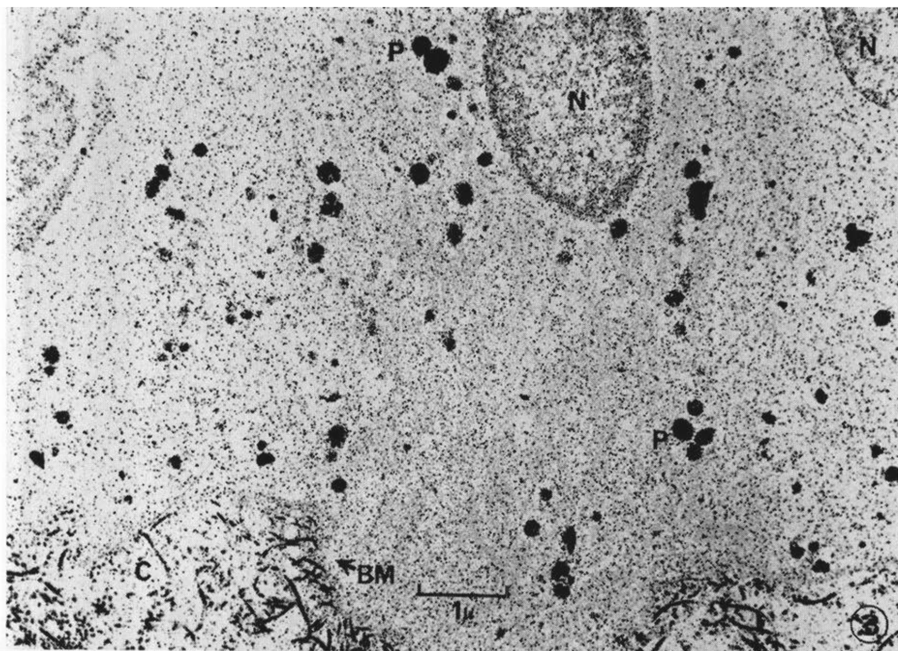
At desmosomes the intercellular space is increased and here two separate silver reacting surfaces become apparent. Since the space between the layers of silver grains appears distinct and not filled with other grains, it may be concluded that either the two coats are separate, one associated with each cell surface, or that some other factor prevents reaction in the central zone. This clear central zone appears to correspond to the central dense line recognized in the desmosomal complex by Odland (10) and by Farquhar and Palade (3).

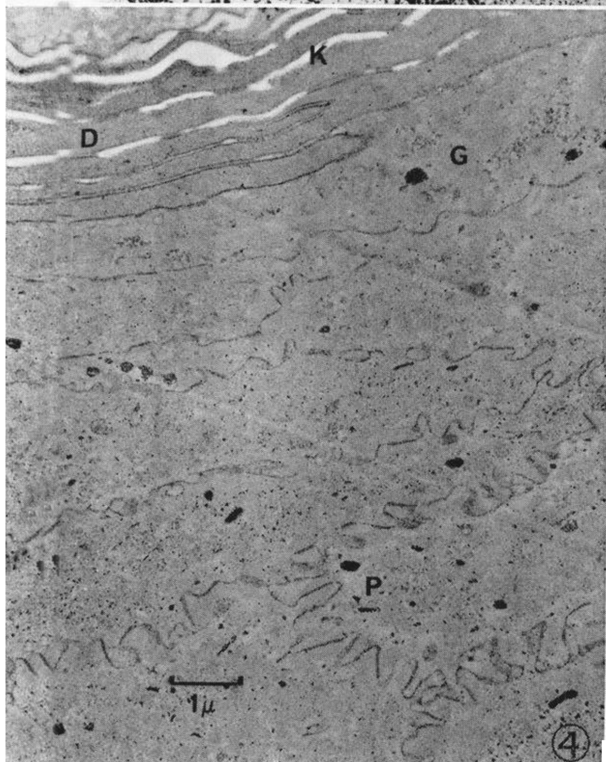
The Surface Coat and Differentiation

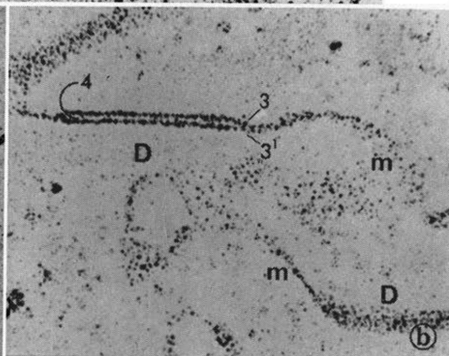
Rambourg and Leblond (12) reported that in the intestinal epithelium the cell surfaces in the germinal layer were lacking a surface coat. We note the same phenomenon in the epidermis; the surfaces of the basal layer cells are largely non-reactive in the PA-Ag test. A reaction occurs only at the sites of hemidesmosomes facing the basement membrane and occasionally on the surfaces most distal from the basal lamella. Further, although the test is not quantitative, an increase in the amount of silver deposited between cells is evident as the cells move towards the stratum corneum. This suggests an increase in the amount of polysaccharide accompanying cellular differentiation. The strongest reaction occurs on the membranes of the keratinized cells of the stratum corneum but here the reaction is partly non-specific and its interpretation is obscure.

The surfaces of the basal layer cells are very irregularly convoluted with numerous projecting processes and invaginations; wide in-

FIG. 2. Higher magnification of cells in lower layers. Note non-reactive membranes near BM, early reaction on desmosomes D, stronger reaction on membranes at m at higher level (mosaic of two micrographs).







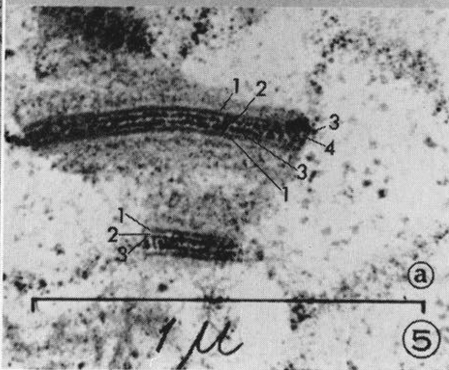


PLATE 3

tercellular gaps and channels are common. These localized gaps diminish in size immediately above the basal layer but do not entirely disappear, although the membranes of the cells at these levels are largely in close contact with an intercellular spacing reduced to about 150Å and desmosomes are very common. Two considerations suggest that diminished intercellular openings form persistent or transient channels leading from the open dermis into the upper epidermal layers. Firstly, the most active site of protein synthesis is the stratum granulosum, the layer of cells immediately preceding the fully keratinized layer, as is shown by the massive accumulation of keratohyalin in these cells and by the rapid labelling that occurs here when certain tritiated amino acids (e.g. histidine) are injected in the dermis (4). Secondly, following injection of ferritin into the dermis, molecules can be found intercellularly and in cellular vesicles in the stratum granulosum after 60 minutes. Nevertheless, while small local channels between cells seem to persist, the greater part of their surfaces are in close contact and these same surfaces react positively in the PA-Ag test. It seems a probable conclusion then that the close contact of the cell surfaces with the effacing of the larger intercellular gaps is due to adhesion and that this adhesion is in fact effected by the polysaccharide coats which the cells have acquired on leaving the basal layer.

The notion that cells in tissues are held together by intercellular components (cements) has long been held, although the evidence for the cement substance was not convincing to all histologists. Rambourg and Leblond have argued convincingly for its presence as demonstrated by the PAS and the PA-Ag test used electron microscopically. The present work supports their contentions. It seems to us now fairly well established for these two proliferating tissues, intestinal epithelium (12) and epidermis, that an increase in surface coat accompanies differentiation and the development of close intercellular contacts between the cells of the tissue. While the full details of intercellular adhesion remain unclear, a polysaccharide containing layer closely associated with the outside surface of the plasma membrane (Text Fig. 1) is clearly involved. Other components (Ca^{++} ions and proteins) are probable also, as shown by experiments aimed at separating cells from tissues (see reviews in (2) and (14)).

The fine structure of the desmosome is of particular interest in this connection. The cell membranes separated to about 400Å at a desmosome and the intercellular layer is more densely stained by heavy metal stains (UO_2 and Pb). The PA-Ag positive layer on each participating plasma membrane continues without break through the desmosome (Fig. 5) and does not seem to fuse to yield a continuous intercellular layer since the silver grains form two distinct lines in micrographs without grains between them. The dense intermediate line described by several authors after staining can be distinguished as a clear zone. It may represent a surface of contact between the two coats where a further material non-reactive in the PA-Ag treatment links them, or may be a layer too dense to allow the reagents to penetrate as might be implied by Kelly's model (5).

Cell Membranes in the Stratum Corneum

The cell membranes in the stratum corneum are known to be peculiar in several respects. They are thicker and stain more densely in routine preparations for electron microscopy; chemically they are insoluble in keratinolytic reagents, such as caustic soda, and strong solu-

FIG. 3. Control test silver deposits on section not oxidized with periodic acid as control. Note: non-specific reactivity of collagen fibers, pigment granules, chromatin areas in nuclei. The background of silver grains indicates some degree of over treatment.

FIG. 4. Upper levels of epidermis to show strong reaction with membranes and desmosomes. Note increased reactivity of first membranes of stratum corneum (stratum compactum).

FIG. 5. Enlarged areas to display details of desmosomal structure and PA-Ag reactivity. 5B shows silver deposits only. 5A has been counterstained with uranyl acetate to stain protein components. In 5B m indicates membranes in adhesive contact with normal intercellular spacing of about 150A. Only one line of silver grains is evident. D are desmosomes and where favourably oriented, the silver deposits separate into two well separated lines (3 and 3′) with a gap (4). In 5A other components are apparent. At (1) the intracellular "backing plate". Between (1) and (3) the site of the cellular coat is a clear area (2) which represents the unstained plasma membrane.

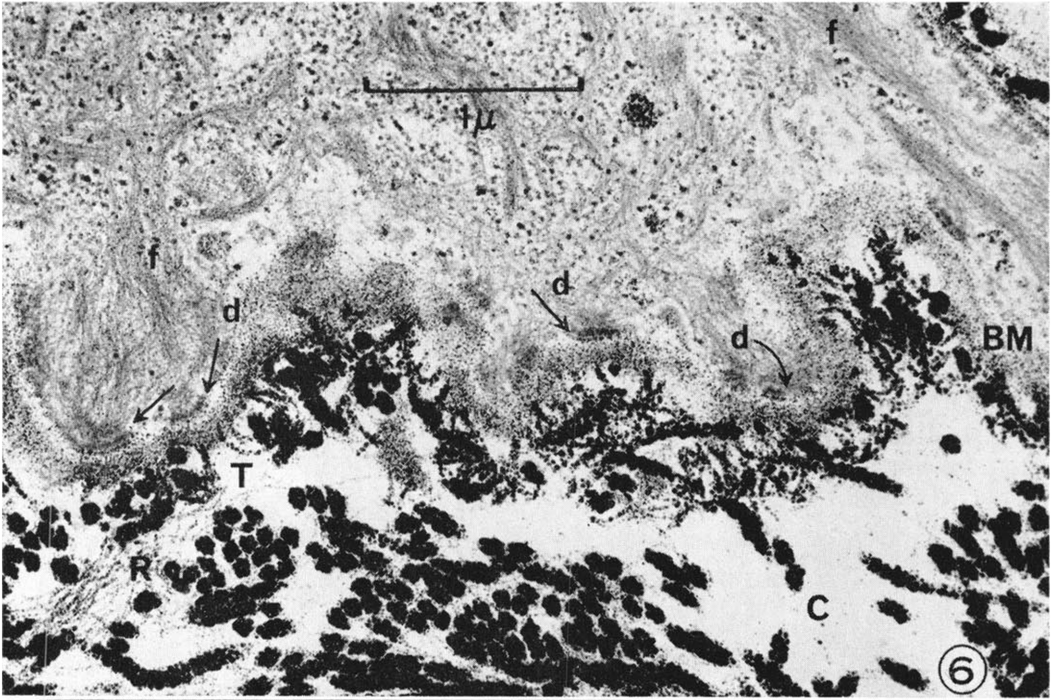


PLATE 4

tions of urea containing reducing compounds. Similarly modified membranes are found in all keratinized tissues (8).

The membranes of the stratum corneum react with silver methenamine without prior oxidation with periodic acid and react more strongly after PA treatment. These findings suggest that these membranes have been chemically modified and also that they probably still contain polysaccharides. The change occurs suddenly in cells passing from the stratum granulosum into the keratinized layer probably in the transitional T-cell identified by Brody (1). Where a granular cell and a keratinized cell are in contact the differences in the reactivity of their membranes is obvious. At such zones of contact the sites where keratinosomes have entered the intercellular gap are visible but no marked deposition of silver was noted. This may mean that these granules are not PA-Ag positive and do not contain polysaccharide. However, it is also possible that the greater part of the granules has been leached out during processing. We have independent evidence that glutaraldehyde does not stabilize the granules completely.

Desmosomes continue to react strongly in the stratum corneum and appear to be the main device holding the formation together. Elsewhere the cell surfaces, although positive on the PA-Ag test, are separated to a variable degree. This separation may indicate a lessened adhesion or perhaps is an indication of unsatisfactory fixation. We have encountered considerable difficulty in obtaining a reproducible morphology of the stratum corneum.

SUMMARY

The presence and distribution of surface coats containing sugars on the cells of the human epidermis have been established using a periodic acid-silver reaction and the electron microscope. The basal (germinal) layer cells have little reactive surface coat; the reaction increases in the layers above the basal layer and is associated with the development of close, adhesive contact between cells. The surface coats are continuous through desmosomes but do not fuse. The keratinized cell surfaces react non-specifically with the silver reagent but, since the reaction increases after periodic acid oxidation, a sugar component is probably present. The development of the surface coat causing intercellular adhesion is probably an important factor in controlling epidermal growth and differentiation.

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FIG. 6. Detail of structures adjacent to basal lamella BM. Counterstained with uranyl acetate. The BM has reacted but the adjacent plasma membrane of the basal layer cell has only clearly reacted at the sites of half desmosomes d. f indicates "keratin" fibrils entering the half desmosomes. The dermal collagen stains (partly specifically) and the finer fibrils linking them to the BM can also be seen (T).

FIG. 7. Details of upper layers of epidermis. Counterstained with uranyl acetate. Compare with Figure 4, Plate 3. Note increased staining of membranes of keratinized cells (k) and associated desmosomes D. g are intercellular pockets unreactive indicating sites of keratinosomes (membrane coating granules) entering the intercellular space.

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