GLYCOCALYX OF EPIDERMAL CELLS IN VITRO: DEMONSTRATION AND ENZYMATIC REMOVAL

P. FRITSCH, M.D., K. WOLFF, M.D., AND H. HÖNIGSMANN, M.D.

Division of Experimental Dermatology, Department of Dermatology I., University of Vienna, Vienna, Austria

Guinea-pig epidermal cells in culture possess a glycocalyx coat similar to that in vivo, as revealed by the ruthenium red staining technique. Trypsin, phospholipase C, and lysozyme do not produce any changes of the glycocalyx, while hyaluronidase and neuraminidase lead to partial and subcomplete removal, respectively. Cells stripped of their glycocalyx coat by neuraminidase do not detach from the support and do not show any signs of toxicity. There is complete reconstitution of the glycocalyx within 24 hr.

The surfaces of all or most cell types are coated by an acid mucopolysaccharide material to which a variety of functions has been ascribed: the maintenance of negative surface charge [1–3], masking of surface antigenicity [4,5], cellular recognition [3], pinocytosis [6], organ differentiation [7], and cellular adhesion [3,8]. The visualization of this coat, for which Bennett [9] has coined the term glycocalyx, requires histochemical techniques of which the most commonly used for electron microscopy are the lanthanum [10] and ruthenium red methods [11]; however, more than six other techniques have been described [12,13].

In skin, a periodic acid-Schiff (PAS)-positive substance was originally described within the intercellular spaces of the epidermis [14] and studied in detail at the light microscope level [15,16]. It was later demonstrated at the ultrastructural level by means of phosphotungstic acid [17], but it was not until the ruthenium red [18] and lanthanum [18,19] techniques were utilized that this substance was found to consist of the fused surface coats of adjacent epidermal cells. These results established that keratinocytes are also coated by a glycocalyx [18].

In order to gain a better understanding of the glycocalyx of epidermal cells, we have chosen a cell culture system as an investigational model to answer the following questions:

1. Do epidermal cells in vitro exhibit a glycocalyx as they do in vivo?
2. Can the glycocalyx of epidermal cells be removed by means of enzymes and does this affect the viability of the cells?
3. Do epidermal cells actively synthesize glycocalyx in vitro and, if they do, at what rate does it occur?

Manuscript received June 17, 1974; in revised form August 5, 1974; accepted for publication August 6, 1974.

This study was supported in part by Fonds zur Förderung der wissenschaftlichen Forschung, Vienna, and Schering AG, Berlin.

Presented, in part, at the 3rd Annual Meeting of the European Society for Dermatological Research, April 25–26, 1973, Amsterdam.

Reprint requests to: Dr. K. Wolff, Division of Experimental Dermatology, Department of Dermatology I., University of Vienna, A-1090 Vienna, Austria.

MATERIALS AND METHODS

Epidermal cell cultures were prepared from the ears of wild-type, spotted guinea pigs according to the technique of Cruickshank et al [20]. The cultures were grown in Falcon plastic flasks (Gateway Lab.) using Eagle's Minimal Essential Medium supplemented with 20% horse serum, gentamicin, and Hepes buffer (20 mmole/100 ml) (Flow Laboratories). Both primary cultures and subcultures were used; the ages of the cultures ranged from 2 days to 3 months.

Cytochemical and electron microscopical techniques. Ruthenium red (RR) (Johnson, Matthey & Co., Inc., New York) was employed to demonstrate the glycocalyx of cultured cells using a modification of Luft's [11] technique. A RR stock solution was prepared according to the directions given by Luft [11] and added in 1:2 proportions to fixative, buffer, and osmium solutions. The final concentration of RR in these solutions was 0.1%.

Specimens were fixed for 3 hr at room temperature in a RR–paraformaldehyde–glutaraldehyde [21] solution and rinsed 3 times in RR–0.1 M cacodylate buffer, pH 7.2, at 4°C. Postfixation was performed with RR–1.3% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.3, for 3 hr at room temperature. After a brief rinsing in distilled water the specimens were rapidly dehydrated in a graded series of alcohols and embedded in Epon 812.

Monolayers. Monolayers were stained, fixed, and dehydrated as described above; a small quantity of Epon was poured into the flasks which resulted in layers of resin not exceeding 1 mm in thickness. After polymerization, the bottoms of the flasks were cut out with a jigsaw and immersed in liquid nitrogen for separation. The resulting thin sheets of Epon were examined in the phase microscope. Appropriate areas were marked, excised, remounted in Epon, and sectioned parallel to the plane of the monolayer facing the support.

Cell suspensions. Cell suspensions were prepared by trypsinization (0.25% trypsin) (Flow Laboratories) or mechanically by scraping the cells from the support by means of a rubber policeman. Suspended cells were transferred into 2-ml plastic centrifuge tubes and spun down at 1200 rpm for 10 min. The pellets were first fixed in the RR-aldehyde fixative for 1 hr, minced, and then further fixed for 2 additional hr; further procedures were as described above and the pellets were embedded in Ladd rubber molds.

Sections were cut with a Reichert OM U2 Ultramicrotome and an LKB Ultrotome III and viewed with a Zeiss EM 9S electron microscope.

Enzymatic treatment. Enzymatic treatment was performed both on cultures in situ and on suspended cells. Before exposure the cultures were washed thoroughly in phosphate-buffered saline (PBS). Cell suspensions were first washed in serum-containing medium and thereafter twice in PBS (800 rpm for 10 min). The following enzymes and incubation conditions were employed:

Trypsin (Flow Laboratories), Lot 70127, 0.25% in PBS, pH 7.2, 10–30 min, 37°C

Hyaluronidase (testicular; Calbiochem), B grade, Lot 901889, 78 and 150 U/ml phosphate buffer, pH 6.05, 30 min, 37°C

Lysozyme (egg white; Sigma), grade I, No. L-6876, 1 mg:ml phosphate buffer, pH 6.2, 30 min, 37°C

Neuraminidase (*Vibrio cholerae;* Calbiochem), B grade, Lot 190004, 100–200 U/ml Ringer's solution, 30–60 min, 37°C

Phospholipase C (*Clostridium welchii;* Calbiochem), B grade, Lot 901450, 0.01 mg/ml PBS, 10–30 min, 37°C

After exposure to the enzymes the cells were washed twice in PBS and subjected to the RR procedure.

Reconstitution of glycocalyx. For this experiment six subcultures derived from the same primary culture and comparable in density and morphology were used. One culture served as a control; the others were exposed as monolayers to neuraminidase (100 U/ml Ringer's solution, 30 min, 37°C). After rinsing with PBS, one culture was immediately trypsinized, spun down to a pellet, and subjected to the RR procedure; the others were incubated in serum containing medium for 2, 4, 8, 18, and 22 hr, respectively, before being trypsinized, pelleted, and processed further.

Controls. Monolayers and cell suspensions were processed as above in the absence of RR. Controls of enzymatic treatment included incubation in buffer under identical conditions but in the absence of the respective enzyme both with and without RR treatment.

RESULTS

In Situ Preparations

In all specimens examined, the epidermal cells were lined by a prominent RR-positive surface coat which represented the glycocalyx. Controls not treated with RR were devoid of this material. The surface coat presented as an electron-opaque layer, approximately 100 to 200 Å in width, which rested on the outer leaflet of the cytomembranes and strictly followed their course to villi and invaginations (Fig. 1). It was fuzzy in appearance particularly on the free surfaces of the cells where it appeared as if it were composed of multiple, densely set and loosely interwoven threads aligned perpendicularly to the cell surface. Villous cell surfaces exhibited a more luxuriant surface coat than smooth ones. When the cells were in contact with each other their surface coats fused and filled the intercellular gap (Fig. 2) as they do in vivo (Fig. 3). RR-positive material was also found in micropinocytotic vesicles and invaginations of the membranes (Fig. 4) which, in tangential sections,

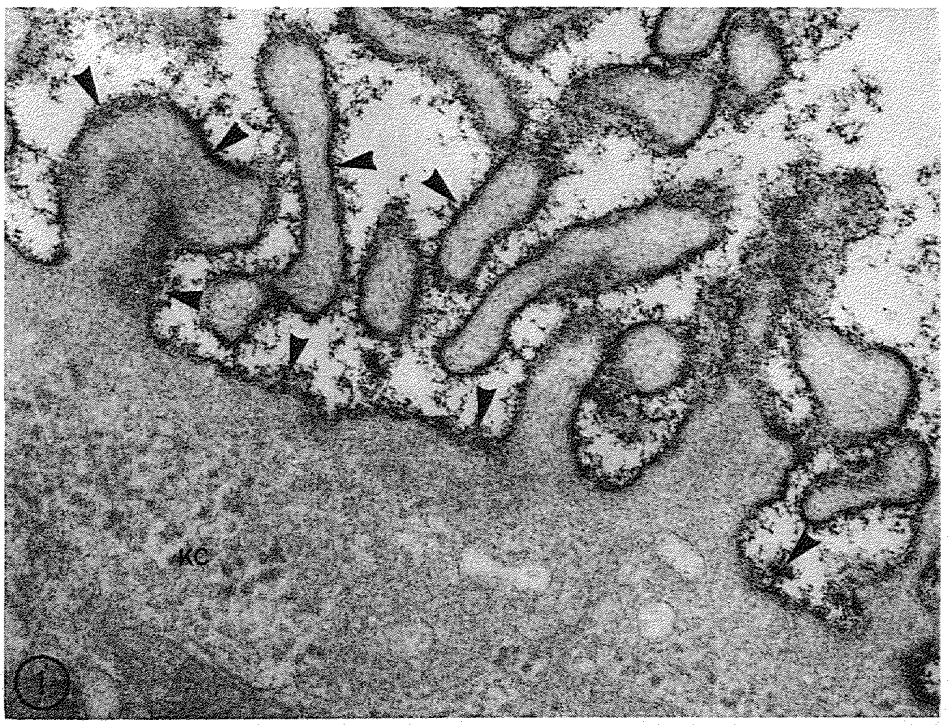

FIG. 1. Glycocalyx of epidermal cell (*KC*) in monolayer culture. RR staining. Note the fuzzy appearance of the RR-positive material (*arrow tips*) on the free surface of the cell (× 81,200).

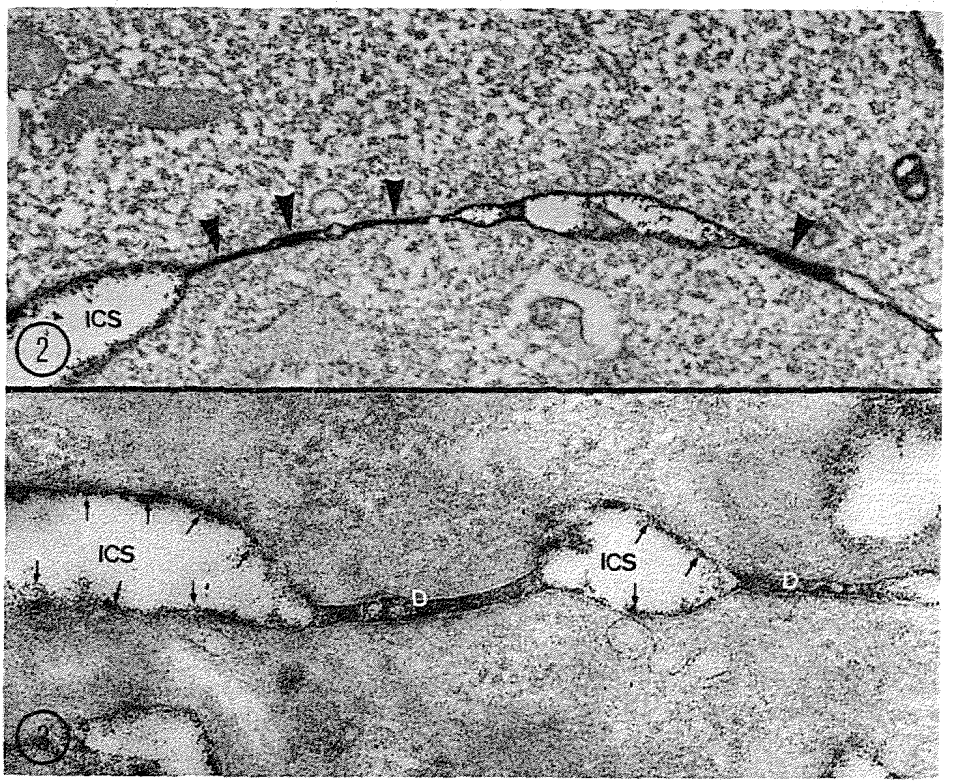

FIG. 2. Monolayer culture, RR staining. Partially fused glycocalyx (*arrow tips*) of two adjacent epidermal cells. *ICS*, intercellular space (× 23,000).

FIG. 3. Glycocalyx of guinea-pig epidermis in vivo (*arrows*); RR staining obscures inner structure of desmosome (*D*). *ISC*, intercellular space (× 81,200).

sometimes appeared as if they were intracellular. However, in intact cells, the RR-positive material never extended beyond the membrane into the cytoplasm, and only degenerated or lysed cells exhibited diffuse cytoplasmic staining.

There was some variation in the thickness of the glycocalyx among the different cultures investigated (Fig. 5) but it always appeared to be more luxuriant than in vivo. There was also some variation in glycocalyx thickness among individual cells of a given culture and even between different sites of a given cell. No unequivocal gross difference was found between cells of primary cultures that still exhibited markers of differentiation such as tonofilaments and desmosomes and older subcultures that were devoid of these. In general, however, the glycocalyx was thicker and less thready in old cultures.

Cell Suspensions

Trypsinization resulted in good yields of viable cells whereas mechanical suspension led to considerable cellular damage and often failed to detach cells from each other so that cellular sheets and aggregates were obtained. Since, under the conditions chosen, trypsin neither affected the stainability nor morphologic appearance of the glycocalyx of the suspended cells, trypsinized cells were employed in most of the experiments on cell suspensions.

Effect of Enzymatic Treatment

In monolayer cultures, trypsin induced detachment of cells from the support whereas all the other enzymes failed to do so. Since, with regard to the behavior of the glycocalyx, the results obtained with monolayers and cell suspensions were identical, they are discussed together.

Trypsin. As described above, trypsin did not affect the glycocalyx of keratinocytes to any significant degree. The glycocalyx remained unaltered even after prolonged trypsinization.

Phospholipase C. The glycocalyx was found unaltered in most cells. In some cells, however, there was partial destruction and removal of the entire cytomembrane which led to penetration of RR into and the staining of the cytoplasm.

Lysozyme. Lysozyme had no effect on the glycocalyx.

Testicular hyaluronidase. Testicular hyaluronidase exhibited a partial effect on the glycocalyx. In some areas of the cells the glycocalyx was thinned,

whereas it appeared irregularly clumped and partly detached from the surface in others (Fig. 6). Entire removal, however, did not occur, and in a portion of the cells the glycocalyx was not altered at all.

Neuraminidase. This enzyme induced considerable changes in the glycocalyx. The majority of cells were completely stripped of their glycocalyx and were delimited only by their triple-layered membrane (Figs. 7, 8). These cells appeared morphologically viable and perfectly healthy. In a small portion of the cells glycocalyx was not as completely removed and this was even the case after prolonged incubation (up to 2 hr) and with higher concentrations of the enzyme (up to 200 U/ml). Cells that had been stripped only partially of their glycocalyx were still lined by a discrete film of RR-positive material and, in places, irregular clumps and aggregates of this material were found to adhere to the cytomembranes (Fig. 10). These clumps often displayed a globular shape. A small number of cells even showed a seemingly unaltered surface coat, whereas others possessed an intact glycocalyx in some areas and were completely stripped of it in others. RR-positive material trapped in invaginations of the cytomembrane and micropinocytotic vesicles proved to be much more resistant to enzymatic removal and was not significantly diminished even after most vigorous treatment (Fig. 11).

Controls. Controls treated by buffer alone revealed an intact RR-positive surface coat (Fig. 9).

Reconstitution of Glycocalyx

Cells stripped of their surface coat by neuraminidase and subsequently incubated in serum containing medium for various time periods exhibited a reconstitution of their glycocalyx that followed a

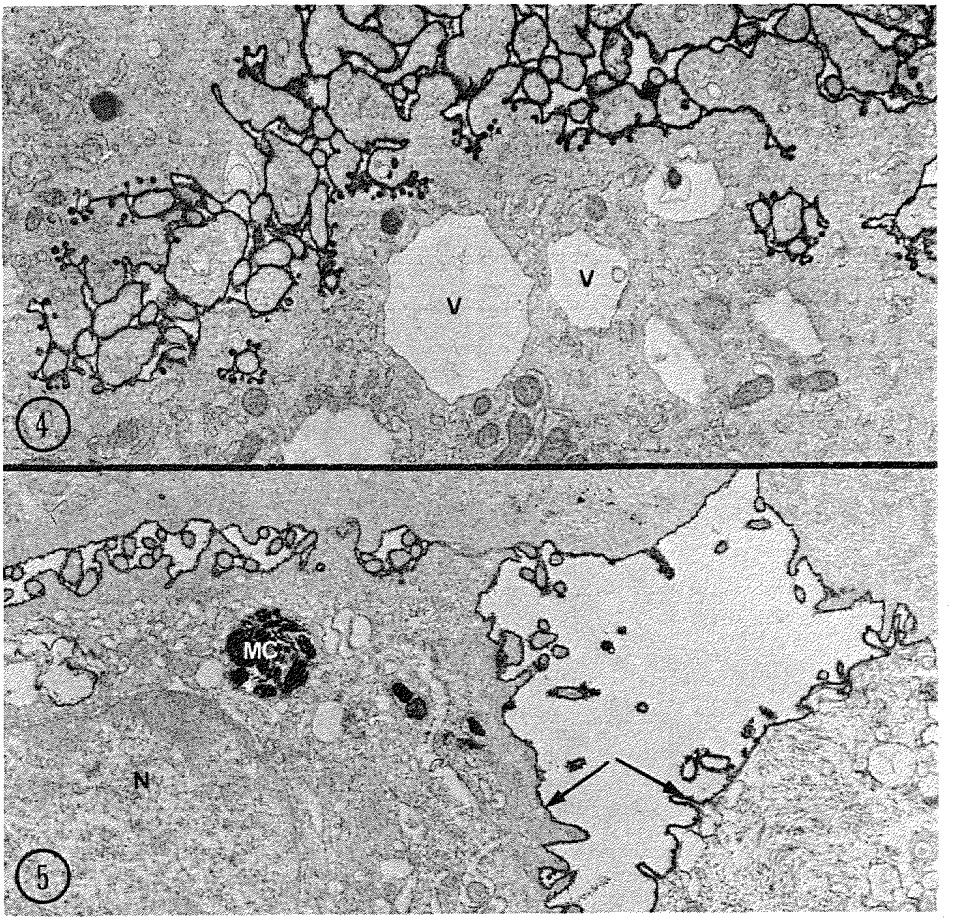

FIG. 4. Monolayer culture, RR staining. Micropinocytotic vesicles and invaginations of the cell membrane containing RR-positive material. Vacuoles (*V*) that do not communicate with the intercellular space are devoid of staining (× 10,800).

FIG. 5. Monolayer culture, RR staining. *Arrows* indicate the variability of the thickness of the glycocalyx. Compare also with Figure 1. *N*, nucleus; *MC*, melanosome complex (× 18,800).

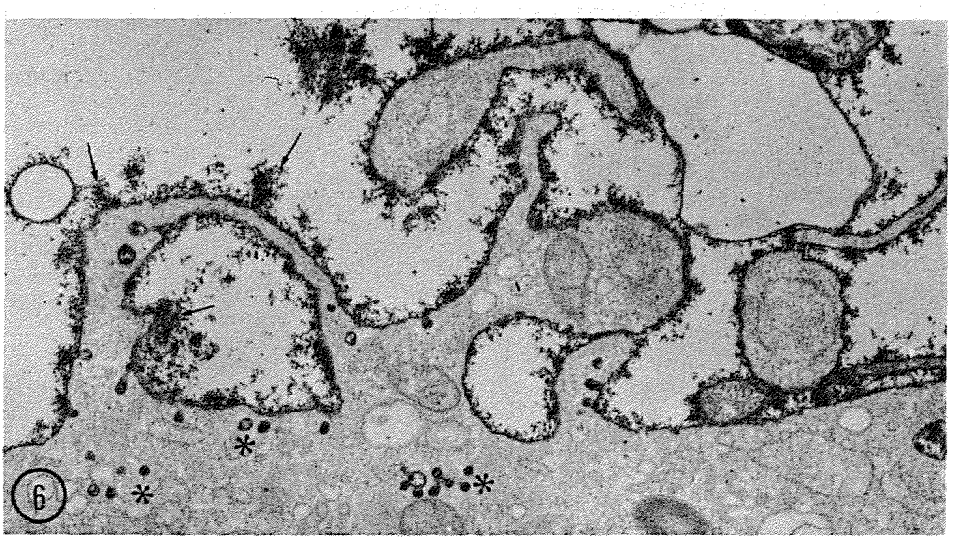

FIG. 6. Cell suspension treated with hyaluronidase and stained with RR. The glycocalyx appears partially clumped (*arrows*) but there is no removal of the surface coat. The *asterisks* indicate multiple micropinocytotic vesicles (× 23,000).

definite time course. Glycocalyx became discernible after 4 hr and was almost complete at 8 hr. At 18 and 22 hr the glycocalyx was fully reestablished.

DISCUSSION

This study shows that the glycocalyx of the epidermal cells in vitro is comparable to that in vivo. Cell islets with an epithelioid growth pattern exhibit a fusion of the surface coats of neighboring cells just as they do in vivo. However, on the free surfaces of cultured cells, the glycocalyx appears thicker and displays a fuzzy filamentous morphology that is not observed in the epidermis. A glycocalyx of similar appearance can be found, in vivo, on free surfaces of intestinal epithelial [22] or kidney endothelial [23] cells.

There was a considerable degree of variation in the thickness of glycocalyx among the cells of a given culture and also between young and old cultures. Since primary cultures contain cells from the different epidermal layers, cellular heterogeneity may be one reason for this finding. The age of a culture may also play a role as there was a tendency of glycocalyx to be more hyperplastic in older cultures. The glycocalyx was more luxuriant in villous, "ruffled" portions of the cell surface and its thickness could also reflect different functional states of the cell surface. Lastly, since there exists some evidence that changes in configuration and composition of cell surface coats take place during or prior to mitosis [24,25], there is also the possibility that some of the variations of glycocalyx thickness reflected different stages of the cell cycle.

Trypsin failed to produce any recognizable change in the glycocalyx. This finding stands slightly at variance with reports dealing with other cell types and raises some interesting questions. It is surprising that trypsin seemingly leaves the glycocalyx unimpaired while it is able to separate cells from the support; neuraminidase, on the other hand, strips the glycocalyx off the cell surfaces but fails to produce detachment even after prolonged exposure. This might be taken to suggest that the receptor sites are different, but it is in this respect that an important point needs clarification. For reasons of convenience the term "removal" of glycocalyx is used throughout this paper, but this is not meant to imply that the entire surface coat is removed or stripped from the cell surface. Rather, the term stands for the removal of those moieties of the glycocalyx that are visualized by the RR method, and this should be kept in mind in order to view our findings and those of others with the proper perspective.

Carbohydrates have been identified in the trypsinates of cells of various origins, using radioisotope [26] and chromatographic techniques [27], and it has been shown that trypsinized cells exhibit a decrease of dry weight [28] and electrophoretic mobility due to a loss of negative surface charge [26]. Also, cartilage cells have been reported to be stripped of their matrix by trypsin [29], and a thinning of glycocalyx has been observed in embryonic hamster cells after treatment with this enzyme as well as with neuraminidase [30]. On the other hand, the glycocalyx of chick embryonic cells is susceptible to phospholipase C whereas it is resistant to neuraminidase [31]; removal of glycocalyx by neuraminidase has been reported for ascitic tumor cells [32]. To add to the complexity, our own observations indicate that the glycocalyx of epidermal cells is resistant to trypsin and phospholipase C but sensitive to neuraminidase.

At present we have no other explanation for

these discrepancies than the fact that other cell types and different staining techniques were used by the different investigators. These apparent differences may reflect the true differences between the surface coats of different cell types or may simply result from the staining of different moieties by the methods employed by these authors and the RR technique employed by us. This could be in agreement with the observation that in the epidermis in vivo the staining patterns of the glycocalyx are not entirely identical with these two techniques.

RR is considered to stain carbohydrate moieties and since neuraminidase was the only enzyme in our experiments to exert a strong action on the glycocalyx, it may be concluded that sialic acid is present in the glycocalyx of keratinocytes. However, complete removal was not achieved in all cells and since this was also true for cells with free surfaces, this did not reflect an inability of the enzyme to gain access to the cell surfaces. The subtotal enzymatic removal was neither rendered complete by increasing the concentration of the enzyme nor by prolonging the exposure times and this could be explained by an observation that has been made on calf erythrocytes where only up to 65% of the sialic acids present on the surface are released by neuraminidase whereas the rest are bound by nonaccessible chemical linkages [33].

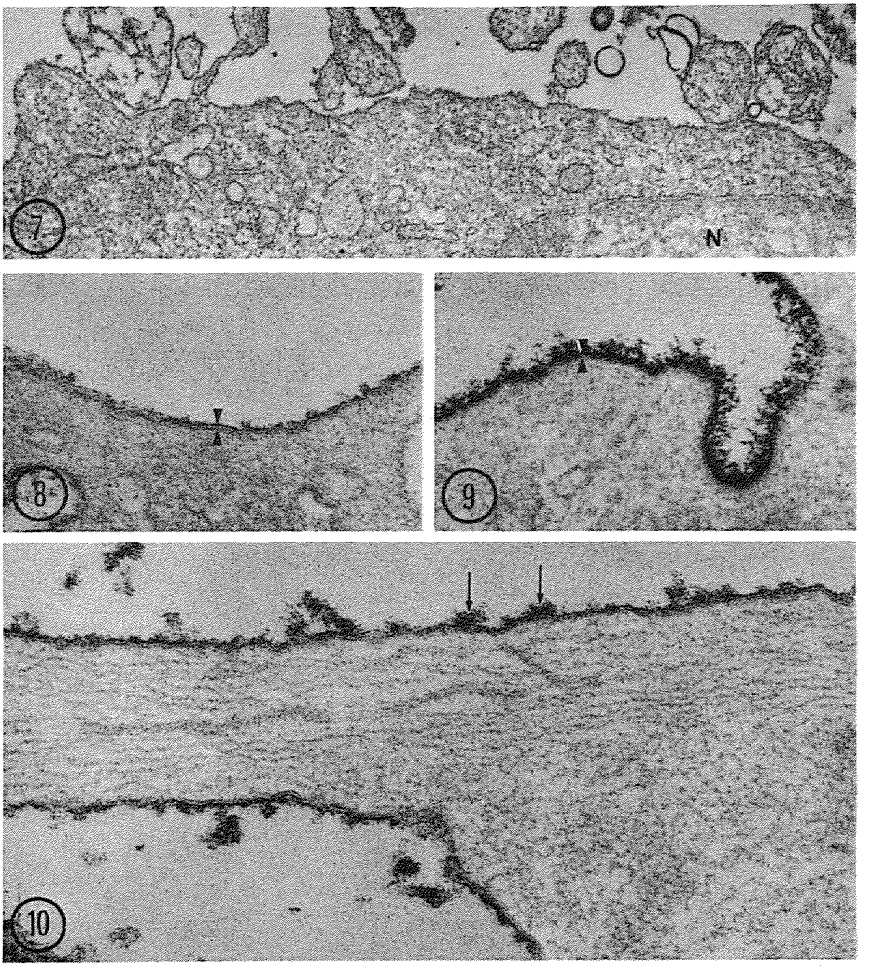

FIG. 7. Complete removal of RR-positive material from the cell surface with neuraminidase. *N*, nucleus (× 23,000). Figures 7–10 are all from cell suspensions.

FIG. 8. The higher magnification of Figure 7 shows an intact triple-layered membrane (*arrow tips*) devoid of RR-positive material (× 81,200).

FIG. 9. Control specimen not treated with neuraminidase. RR-positive coat is present on the cell membrane. Compare with Figure 8. *Arrow tips* indicate triple-layered cell membrane (× 81,200).

FIG. 10. Incomplete removal of glycocalyx by neuraminidase. A discrete film of RR-positive material forming clumps and globular aggregates (*arrows*) is present on the membrane of this keratinocyte (× 81,200).

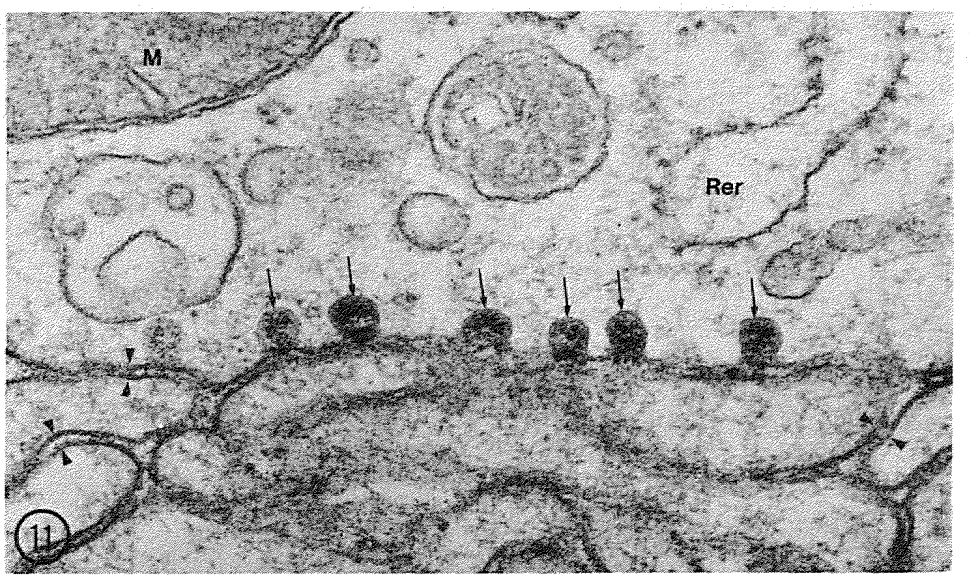

FIG. 11. Cell suspension treated with neuraminidase, RR staining. RR-positive material is still present within micropinocytotic vesicles (*arrows*) whereas no such material is seen on cellular membranes (*arrow tips*). *M*, mitochondrion; *Rer*, rough endoplasmic reticulum (× 81,200).

Removal of glycocalyx by neuraminidase often varied considerably in different areas of the surface of a given cell. Closer observation revealed that micropinocytotic vesicles scarcely showed a reduction of their RR-positive contents even when they were still in broad continuity with the intercellular space. The same was true for the shallow depressions of the cytomembrane that represent the initial stages of vesicle formation, and it may be speculated that, coinciding with or preceding pinocytotic activity, there are changes in the surface coat that render it more resistant to neuraminidase. The described globular aggregates of glycocalyx material on the cell surfaces may constitute the first visible signs of impending vesiculation, and this could correspond to what has been described in placental endothelium where globular aggregates of glycocalyx have been linked to early vesicle formation [6].

The mere fact that the cells grown in culture for several months still possess a glycocalyx which, if anything, is even more luxuriant than in young cultures indicates that the glycocalyx is a product of the cell itself. Our reconstitution experiment shows that the synthesis of glycocalyx is carried out at a rather speedy rate so that the glycocalyx cannot be regarded as a mere deposit of preformed material brought along from the tissue of origin. These observations are closely paralleled by those of Rosenberg and Einstein [4] who have reported full replacement of sialic acids on the cell surface of lymphocytes 12 hr after removal with neuraminidase.

Neuraminidase is unable to pass through an intact cell membrane [4]. Accordingly, our neuraminidase-treated cultures showed no signs of degeneration, either by phase microscopy or at the electron microscope level. Neuraminidase-treated cells were able to settle down again and resume growth under cell culture conditions. The enzyme is thus innocuous to the cell itself despite its action on the glycocalyx and may prove a useful tool for further investigations on the surface of epidermal cells.

The technical assistance of Mrs. Lotte Polasek, Mrs. Susan Csegezi, and Mrs. Heidi-Melitta Duffek is gratefully acknowledged.

REFERENCES

1. Forrester JA, Ambrose EJ, Stoker MGP: Microelectrophoresis of normal and transformed clones of hamster kidney fibroblasts. Nature (Lond) 201:945–946, 1964
2. Hause LL, Pattillo RA, Sances A Jr, Mattingly RF: Cell surface coatings and membrane potentials of malignant and nonmalignant cells. Science 169:601–602, 1970
3. Rambourg A, Leblond CP: Electron microscopic observations on the carbohydrate rich coat present at the surface of cells in the rat. J Cell Biol 32:27–53, 1967
4. Rosenberg SA, Einstein AB Jr: Sialic acids on the plasma membranes of cultured lymphoid cells. Chemical aspects and biosynthesis. J Cell Biol 53:466–473, 1972
5. Grothaus EA, Flye MW, Yunis E, Amos DB: Human lymphocyte antigen reactivity modified by neuraminidase. Science 173:542–544, 1971
6. Jollie WP, Triche TJ: Ruthenium labelling of micropinocytotic activity in the rat visceral yolk-sac placenta. J Ultrastruct Res 35:541–553, 1971
7. Bernfeld MR, Banerjee SD: Acid mucopolysaccharide (glycosaminoglycan) at the epithelial mesenchymal interface of mouse embryo salivary glands. J Cell Biol 52:664–673, 1972

8. Mercer EH, Jahn RA, Maibach HI: Surface coats containing polysaccharides on human epidermal cells. J Invest Dermatol 51:204–214, 1968
9. Bennett HS: Morphological aspects of extracellular polysaccharides. J Histochem Cytochem 11:14–23, 1963
10. Revel JP, Karnovsky MJ: Hexagonal array of subunits in intercellular junctions of the mouse heart and liver. J Cell Biol 33:C7–C12, 1967
11. Luft JH: Ruthenium red and violet. I. Chemistry, purification, methods of use for electron microscopy and mechanisms of action. Anat Rec 171:347–368, 1971
12. Behnke O, Zelander T: Preservation of intercellular substances by the cationic dye alcian blue in preparative procedures for electron microscopy. J Ultrastruct Res 31:424–438, 1970
13. Elias PM, Park HD, Patterson AE, Lutzner MA, Wetzel BK: Osmium tetroxide-zinc iodide staining of Golgi elements and surface coats of hydras. J Ultrastruct Res 40:87–102, 1972
14. Wislocki GB, Fawcett DW, Dempsey EW: Staining of stratified squamous epithelium of mucous membranes and skin of man and monkey by the periodic acid-Schiff method. Anat Rec 110:359–375, 1951
15. Roe DA, Flesch P, Esoda ECJ: Present status of epidermal mucopolysaccharides. Arch Dermatol 84:213–218, 1961
16. Braun-Falco O, Weber G: Zur histo- und biochemie des epidermalen interzellularraumes unter normalen und pathologischen verhältnissen. Arch Klin Exp Dermatol 207:459–471, 1958
17. Komura J, Ofuji S: Phosphotungstic acid stain and epidermal cell surface. J Invest Dermatol 49: 391–395, 1967
18. Wolff K, Schreiner E: An electron microscopic study on the extraneous coat of keratinocytes and the intercellular space of the epidermis. J Invest Dermatol 51:418–430, 1968
19. Komura, J, Shimada Y, Ofuji S: Lanthanum staining of the epidermal intercellular spaces. An electron microscopic study. Arch Klin Exp Dermatol 235:221–227, 1969
20. Cruickshank CND, Cooper JR, Hopper C: The cultivation of cells from the adult epidermis. J Invest Dermatol 34:339–342, 1960
21. Karnovsky MJ: A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy (abstr). J Cell Biol 27:137A–138A, 1965
22. Ito S: The enteric surface coat on rat intestinal microvilli. J Cell Biol 27:475–491, 1965
23. Groniowski J, Biszczyskowa W, Walski M: Electron microscope studies on the surface coat of the nephron. J Cell Biol 40:585–601, 1969
24. Pasternak CA, Warmsley AMH, Thomas DB: Structural alterations in the surface membrane during the cell cycle. J Cell Biol 50:562–564, 1964
25. Kraemer PM: Configuration change of surface sialic acid during mitosis. J Cell Biol 33:197–200, 1967
26. Kapeller M, Gal-Oz R, Grover NB, Doljanski F: Natural shedding of carbohydrate-containing macromolecules from cell surfaces. Exp Cell Res 79:152–158, 1973
27. Cook GMW: Linkage of sialic acid in the human erythrocyte ultrastructure. Nature (Lond) 195:159–161, 1962
28. Weiss L: The effects of trypsin on the size, viability and dry mass of sarcoma 37 cells. Exp Cell Res 14:80–83, 1958
29. Khan TA, Overton J: Lanthanum staining of developing chick cartilage and reaggregating cartilage cells. J Cell Biol 44:433–437, 1970
30. Huet CH, Herzberg M: Effects of enzymes and EDTA on ruthenium red and concanavalin A labeling of the cell surface. J Ultrastruct Res 42:186–199, 1973
31. Lesseps RJ: The removal by phospholipase C of a layer of lanthanum staining material external to the cell membrane in embryonic chick cells. J Cell Biol 34:173–183, 1967
32. Gasic G, Berwick L: Hale stain for sialic acid-containing mucins. Adaptation to electron microscopy. J Cell Biol 19:223–228, 1963
33. Mohr E, Schramm G: Reinigung und charakterisierung der neuraminidase aus *Vibrio cholerae*. Z Naturforsch [B] 15:568–575, 1960