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Electron Microscopic Observations of the Surface of L-Cells in Culture

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Summary. Techniques are described for the preparation of preshadowed replicas of both the upper and lower surfaces of L-cells in culture, and of cross sections of L-cells growing on a cellophane substrate. These revealed long slender microvilli, 800 to 1,100 A in diameter, projecting from both upper and lower surfaces of the cells. These microvilli were frequently observed to contact other cells and substrate, and to leave material behind on the substrate. The plasma membrane of the lower surface was separated from the substrate by an electron-lucent gap 200 to 300 A wide. The surface coat of the L-cell was visualized by staining with colloidal iron and ruthenium. Staining with colloidal iron was most intense on the surface of the microvilli. The gap between cell and substrate was intensely stained with ruthenium red. Enzymatic digestion of living cells revealed that both trypsin and neuraminidase reduced the staining of the cell coat by colloidal iron, whereas only trypsin altered its staining with ruthenium red. After trypsin treatment, fragments of an amorphous material with the staining characteristics of the cell coat were observed between the denuded cells. Treatment with ribonuclease, chymotrypsin or hyaluronidase did not affect the staining of the cell coat.

The cell surface plays an important role in the process of cellular adhesion. However, in spite of a great deal of productive investigation, the nature of the cell surface still remains in large part a mystery. In recent years, various electron microscopic techniques have been used in several studies of the ultrastructural configuration of the cell surface. Sectioned material, shadowed wholemounts and replicas have all revealed the presence of fine microvilli projecting from the free surfaces of cells [6, 7, 19]. These microvilli have been observed in phase-contrast studies as well [9, 24], where they were seen to be highly motile. Optical methods have been used to study that surface of cultured cells which is in contact with the substrate [1, 5] in an attempt to learn how cells adhere to their substrate. The presence

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of a cell surface coat, first suggested by Chambers [3], has been demonstrated in the electron microscope with various histochemical techniques [2, 14, 20].

The aims of this investigation were to characterize the ultrastructural configuration of the cultured L-cell, to demonstrate the surface coat of this cell, and to determine what effects various enzymes would have on these two parameters.

Materials and Methods

The L-cells were grown either as monolayers or as suspension cultures. Monolayer cultures were grown in 100-ml glass medicine bottles at 37 °C with a gas phase of 5 % CO_2-95 % air. Cells were harvested from the glass by 20-min incubation in 0.25 % trypsin solution (Gibco). The suspension cultures were maintained in 50-ml Bellco spinner flasks, at 37 °C with the same gas phase. Routine growth medium was a mixture of Medium 199 (Gibco) and McCoy's Medium 5A (Gibco), with a fetal calf serum concentration of 10 or 15 %.

Specimen Preparation

To prepare shadowed replicas of the upper cell surface, monolayer cultures on glass cover slips were fixed in 2 % phosphate-buffered glutaraldehyde (made up from 25 % commercial stock solutions), dehydrated in a graded series of ethanol solutions and then in an acetone series, and dried from benzene in a dry atmosphere of nitrogen gas. They were then shadowed with carbon/platinum and replicated with carbon in an Edwards vacuum evaporator. The shadowed replicas were floated off the glass in 10 N NaOH and cleaned in a mixture of H_2SO_4, $KMnO_4$ and $K_2Cr_2O_7$ before they were mounted on clean copper grids.

To prepare shadowed replicas of the bottom cell surface, the cells, grown as monolayers on Formvar-covered glass cover slips, were fixed in 2 % phosphate-buffered glutaraldehyde and then dehydrated in an ethanol series only. They were then dried from absolute ethanol in dry nitrogen gas. A heavy layer of aluminium (about 0.0025 gm, pure aluminium wire) was evaporated onto the dried cover slip from a distance of about 10 cm in the vacuum evaporator. During this procedure, the cover slip was rotated to ensure a complete covering layer in all irregularities of the specimen surface. The aluminium film with its attached cells was floated off the cover slip by dissolving the Formvar substrate in chloroform, and dried down with the cells uppermost to a glass slide in a dry atmosphere. This inverted film was then shadowed and replicated in the usual manner. The aluminium was removed from the replica in 50 % HCl, and the replica was cleaned of cellular material with the acid mixture described above.

For cross sections of monolayer cultures, the cells, grown on strips of washed and sterilized cellophane, were fixed in 2 % phosphate-buffered glutaraldehyde and post-fixed in 2 % osmium tetroxide. They were dehydrated in a graded ethanol series and then in an acetone series, and embedded in Durcupan ACM (Fluka AG) as described in the manufacturer's instructions. At the 70 % ethanol stage, the cellophane strips were cut into small pieces of a size to fit lengthwise into a gelatin embedding capsule. This orientation of the strips permitted ready cutting of cross sections of the cellophane with attached cells. Sections were mounted on Formvar-covered grids and stained for 2 min with lead citrate [21].

To prepare sections of cells from suspension culture, the cells were spun into pellets, and the pellets fixed in 2 % phosphate-buffered glutaraldehyde and in 2 % osmium tetroxide as described above. After dehydration in a graded ethanol series and an acetone series, the pellets were passed through styrene and embedded in Vestopal W (Martin Jaeger) as described in the manufacturer's intructions. Sections were mounted on Formvar-covered copper grids and stained for 2 min with lead citrate.

All specimens were observed in an RCA EMU 3D electron microscope modified to operate at 65 KV.

Staining of the Cell Coat

Staining with colloidal iron hydroxide was by the method of Bennedetti and Emmelot [2], in which the specimen is exposed to the stain at low pH (1.8) between fixation with glutaraldehyde and post-fixation with osmium tetroxide. Sections of these preparations were not stained with lead citrate.

Staining with ruthenium red was according to Luft [14], using the stain in both fixative stages. Fixation was for 3 hr at 4 °C in 1.4 % glutaraldehyde buffered to pH 7.4 with cacodylate, and containing 500 ppm ruthenium red. Post-fixation was for 3 hr at room temperature in 1.1 % osmium tetroxide buffered to pH 7.4 with cacodylate and containing 500 ppm ruthenium red. Sections of these preparations were not stained with lead citrate.

Enzyme Solutions

The enzymes used were prepared as follows: trypsin (Gibco), a 0.25 % solution in balanced salt solution, used directly from the bottle; chymotrypsin from bovine pancreas (Sigma), type IV, made up to 0.1 mg/ml with Hanks' solution; hyaluronidase from bovine testis (Sigma), type IV, supplied at 75 RF units/mg, and made up to 0.1 mg/ml with Hanks' solution; neuraminidase from *Vibrio cholerae* (Calbiochem), B grade, supplied at 500 units/ml, and made up to 50 units/ml with Hanks' solution; and ribonuclease (Worthington), crystalline, made up to 0.1 mg/ml with Hanks' solution.

All enzymatic digestion was of living cells in suspension culture, in sealed glass containers at 37 °C with a gas phase of 5 % CO_2-95 % air. Trypsin digestion was for 20 min; all other enzymes were incubated with the cells for 2 hr.

Results

Morphology

Replicas of L-cells growing in a protein-containing medium revealed a quite variable cell morphology (Fig. 1). The cells were usually well spread on the substrate, and the raised nuclear area could be easily distinguished.

Fig. 1. Replica of L-cells growing on glass in protein-containing medium. Spreading and cell shape are quite variable. The cells possess several pseudopodia and many microvilli

Fig. 2. Replica of an L-cell growing on glass in protein-containing medium. The cell edge is applied quite closely to the substrate. Many microvilli of variable length but fairly constant diameter arise from the cell surface

1
10μ
2
1μ

Many large pseudopodia extended outwards from each cell, and varied considerably in size and in number per cell. These were applied closely to the substrate, appearing towards their edges as quite flat with no evidence of large underlying organelles. All cells possessed many fine surface microvilli which were located in large numbers toward the center of the cells where they were short and curving (Fig. 2). Many microvilli also extended from the cell edges, sometimes for great distances over the substrate, making contact with the substrate and with other cells. These microvilli were of fairly constant diameter (800 to 1,000 A), and their length varied from 0.1 μ to more than 10 μ.

The fine structure of the cell surface in these replicas had a rough and granular appearance. The approximate size of these irregularities was 200 to 300 A.

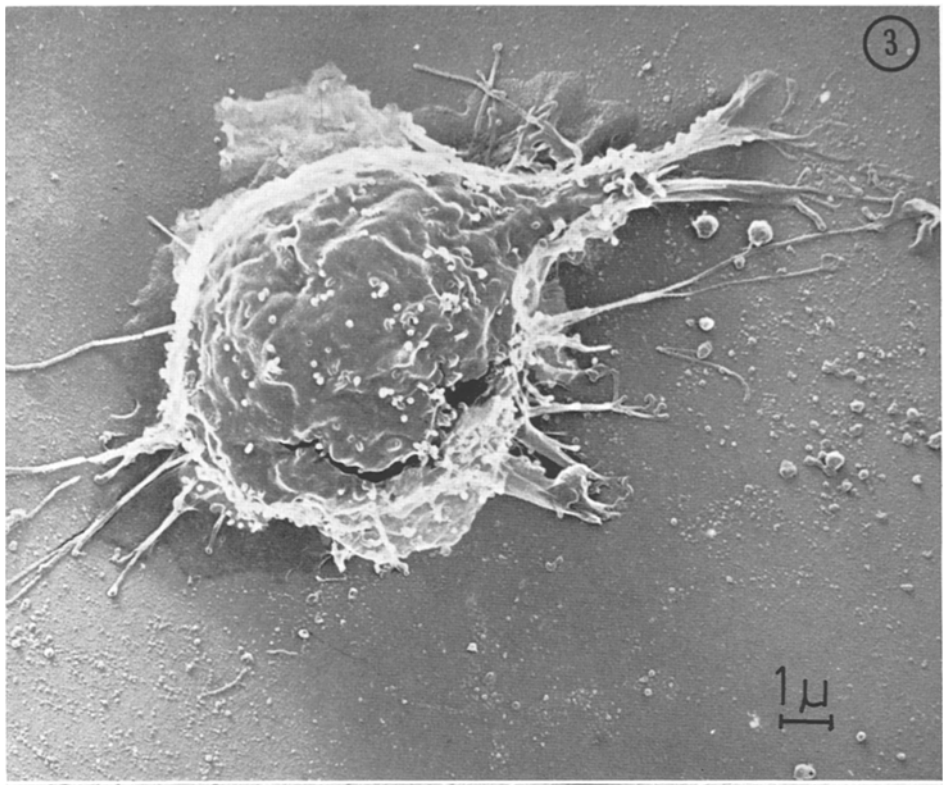
When the cells were cultured in a protein-free medium (Medium 199), certain differences were observed (Fig. 3). The cells did not spread as well, but remained high and hemispherical, and pseudopodia were fewer. Microvilli were as plentiful and as variable, but there were often peculiar elevations on the substrate seen about clusters of microvilli which were in contact with the substrate. These elevations were small, and seemed from their size and distribution to be cellular material left behind when microvilli had become detached from the substrate surface.

No differences were observed in the fine texture of the cell surface in specimens grown in protein-free and in protein-containing media.

Control replicas indicated that the various steps of the lower-surface replication procedure did not introduce any detectable artifact beyond that inherent in the standard replication procedure, provided that all of the drying steps were carried out in the absence of water. In these lower-surface replicas (Figs. 4 & 5), the cells spread well on the substrate, and their bottom surface appeared quite flat, apparently closely applied to the substrate. Some indentations were visible in the surface (Fig. 4), and often a few flattened microvilli were seen arising from this surface (Fig. 5). The edges of the cell indicated that this part of the cell was at a slight distance from the substrate surface (Fig. 4). The scarcity of microvilli on this surface was

Fig. 3. Replica of an L-cell on glass in a protein-free medium. The cell is not well spread, and several long microvilli reach to the substrate surface. Many small bits of material are seen on the substrate near the microvilli

Fig. 4. Replica of the lower surface of an L-cell. This cell surface appears to be flat and relatively featureless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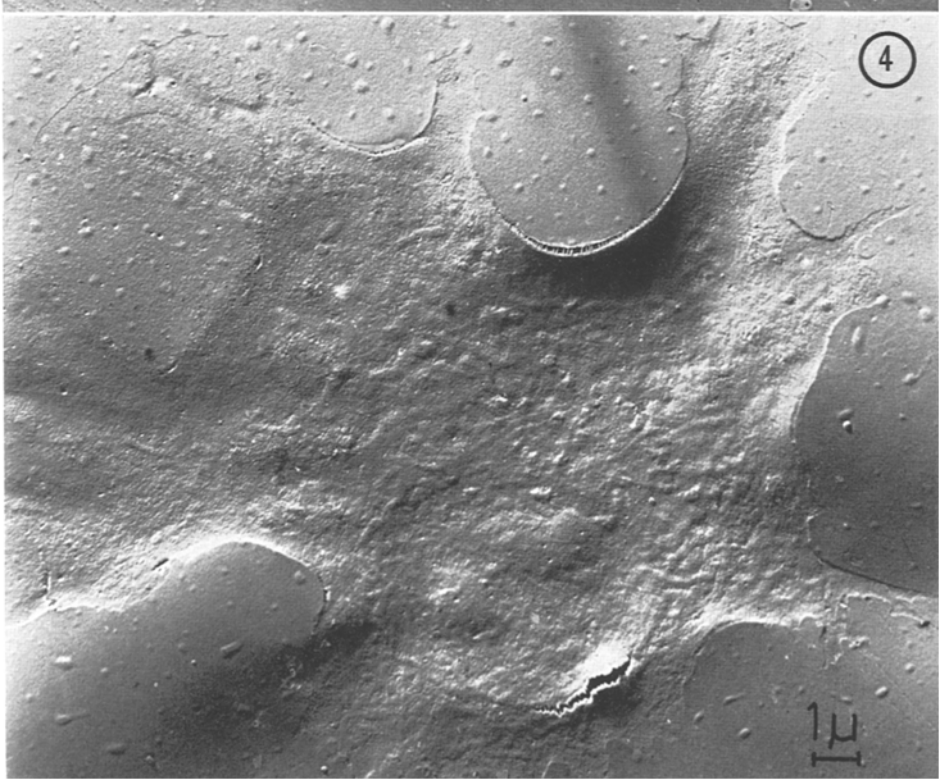
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probably not due to surface disruption of the cells during their removal from the substrate because the Formvar substrate was dissolved in chloroform, making examination of it for cell remnants impossible.

Sections of cells grown on a cellophane substrate (Fig. 7) showed cells that were well spread, with many microvilli on their upper surfaces. The internal organization of the cells was not remarkable. The plasma membrane of the cell's lower surface was applied quite closely to the cellophane, although there were occasional folds in the membrane, a few pinocytotic vesicles, and occasional microvilli. A homogeneous, electron-lucent gap was observed between the cell membrane and the cellophane, which was quite constant in width (200 to 300 A).

Cells in suspension culture, pelleted and sectioned, were generally spherical in outline (Fig. 6). The internal organization of the cells was not remarkable. Many microvilli were observed projecting from the cell surface. These were straight or curved, varied greatly in length, but had a fairly constant diameter of 800 to 1,100 A.

Staining of the Cell Coat

With ruthenium red, a dense reaction product was seen on the free surfaces of cells in monolayer culture (Fig. 8) and, to a lesser degree, in the intercellular gap and in the gap between cell and substrate. This reaction was also demonstrable in cells from suspension culture (Fig. 9). The intensity of staining was found to vary somewhat from preparation to preparation in a random manner. Microvilli stained with the same intensity as did the rest of the cell surface. The stain did not appear to penetrate to the interior of the cell.

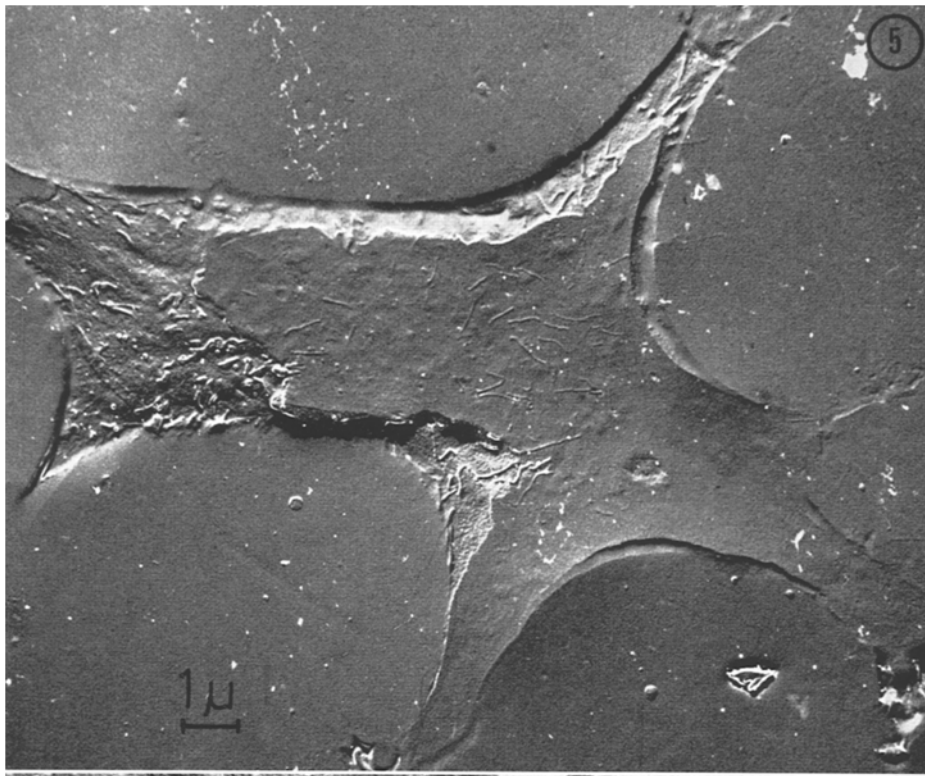
A surface reaction was also demonstrated with colloidal iron (Fig. 10) in which fine dense iron particles were located at the cell surface. With this stain, the density of iron particles was greater on the surface. With both staining reactions, the stained layer appeared to be just outside the plasma membrane.

Enzyme Effects

In the replica preparations, trypsin was the only enzyme used which affected cell morphology, producing a general rounding up of the cell to a

Fig. 5. Replica of the lower surface of an L-cell. Several short microvilli are seen arising from this lower cell surface

Fig. 6. Section of an L-cell from suspension culture, stained with lead. Several microvilli are seen arising from the cell surface, with a diameter similar to that seen in replicas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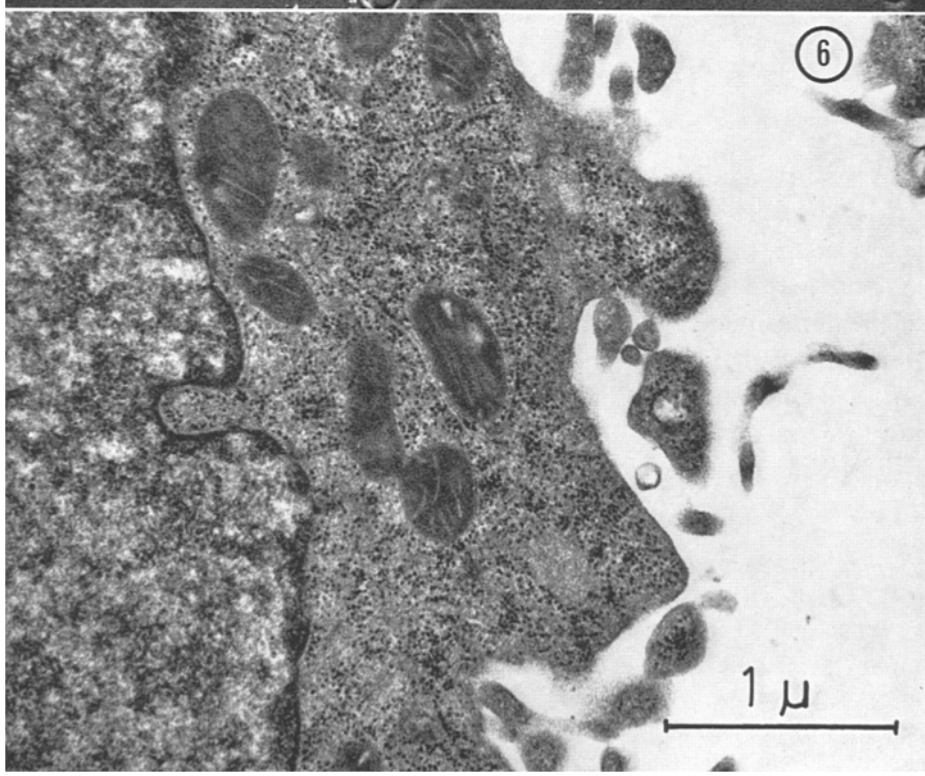
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more hemispherical form. No changes were noted in the character or in the distribution of the microvilli, or in the nature of the fine surface granularity.

In the lead-stained sections of cells from suspension culture, no significant morphological changes were observed with any of the enzymes used, although with trypsin an amorphous material was seen between the cells (Fig. 11). In the cellophane sections, no changes were noted except for the general rounding up of the cells with trypsin. In particular, no changes in the cell-substrate gap were seen.

Trypsin and neuraminidase were observed to alter the character of the staining of the cell surface. In the ruthenium red preparations which reacted with trypsin (Fig. 12), there were patches of the cell surface where the reaction product was absent. In addition, the amorphous material seen between the cells stained with both ruthenium red and colloidal iron, and resembled in appearance the reaction product on the cell surface. Trypsin was also observed to produce a general decrease in the reaction of the cell surface with colloidal iron (Fig. 13).

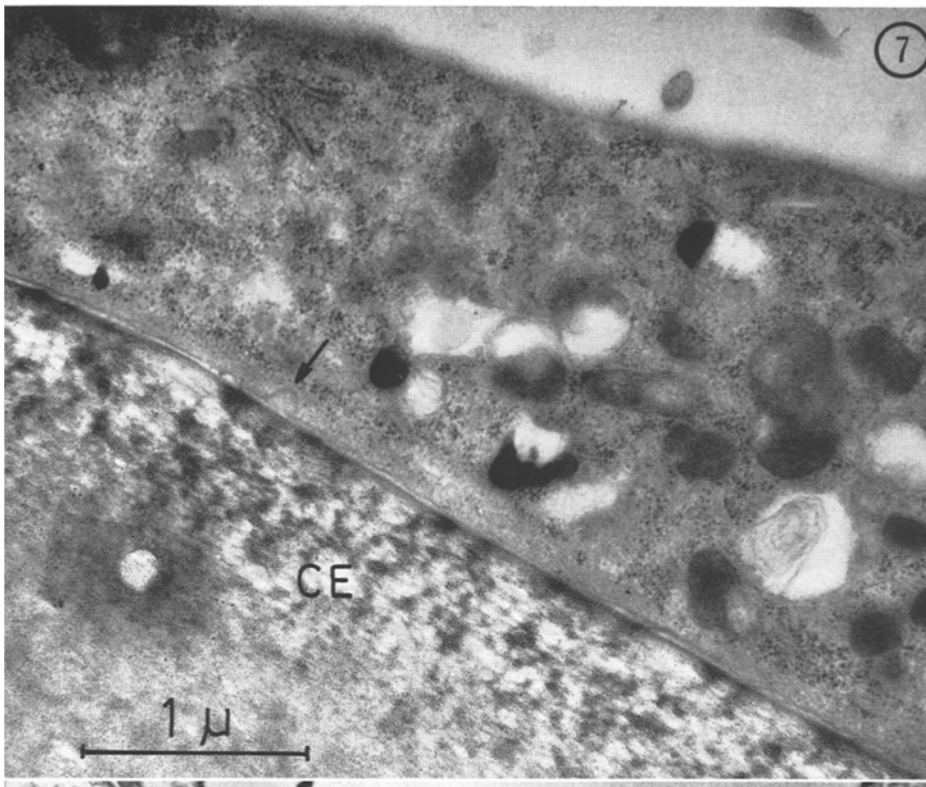
Neuraminidase had no effect on the ruthenium red staining reaction, but was observed to virtually eliminate the colloidal iron reaction (Fig. 14). Chymotrypsin, hyaluronidase, and ribonuclease were not effective in altering the morphology or the staining properties of these cells.

Discussion

Two methods for preparing specimens for replication have been reported: drying fixed cells from water [6, 24] and freeze-drying [7, 10]. The technique described in this report, that of drying fixed and dehydrated cells from benzene in a dry atmosphere, is simple and fast, requires no special equipment, and produces reproducible results. Cellular detail was revealed as well as with the other techniques: surface granularity was similar to that reported previously, and the dimensions of the cells and their microvilli were similar to those found with the freeze-dry technique [7]. It was found that the presence of water during the drying procedure tended to obliterate a

Fig. 7. Section of an L-cell growing on cellophane (*CE*). Some microvilli are seen arising from the upper surface. A pale gap, fairly regular in width, lies between the plasma membrane and the cellophane. One pinocytotic vesicle (arrow) is seen on this surface of the cell

Fig. 8. Section of L-cells on cellophane, stained with ruthenium red. The stain reacts densely with the cell surface, but penetrates less well into the intercellular junctions (arrows) and cell-cellophane gap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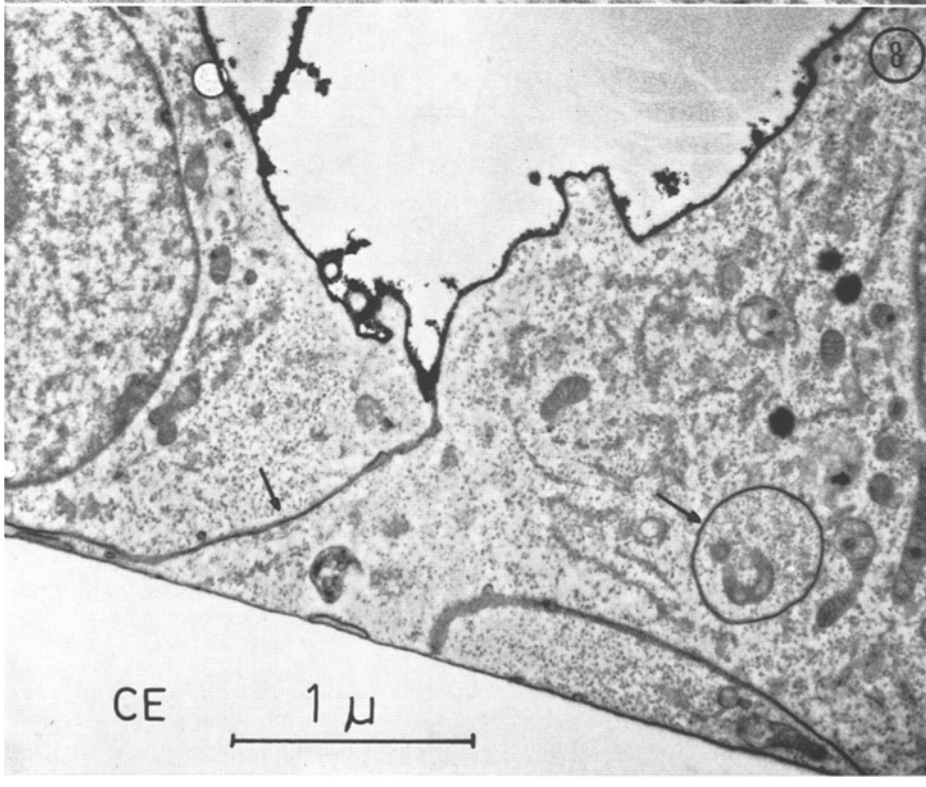
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great deal of cellular detail; therefore in this respect, this present method seems superior to methods in which the cells are dried from water.

Microvilli similar to those seen with this technique have been observed in shadowed wholemounts [13, 18, 19, 24], in shadowed replicas [7], and in sections [7, 12, 23]. In all of these studies, the microvilli have shown a similar structure: they varied greatly in length, but had a fairly constant diameter of 800 to 1,200 A; they were covered with plasma membrane continuous with that of the rest of the cell surface; they were usually straight, but were occasionally curved or bent; and they often had a fibrillary core which was described by Taylor [23] as consisting of microtubules. The microvilli observed on L-cell surfaces in this study were similar in all respects to those seen by other authors. Also, the elevations of cellular-like material on the substrate in the protein-free cultures suggested that the microvilli were motile and were actively investigating their environment, as suggested by Taylor and Robbins [24] and Fisher and Cooper [7].

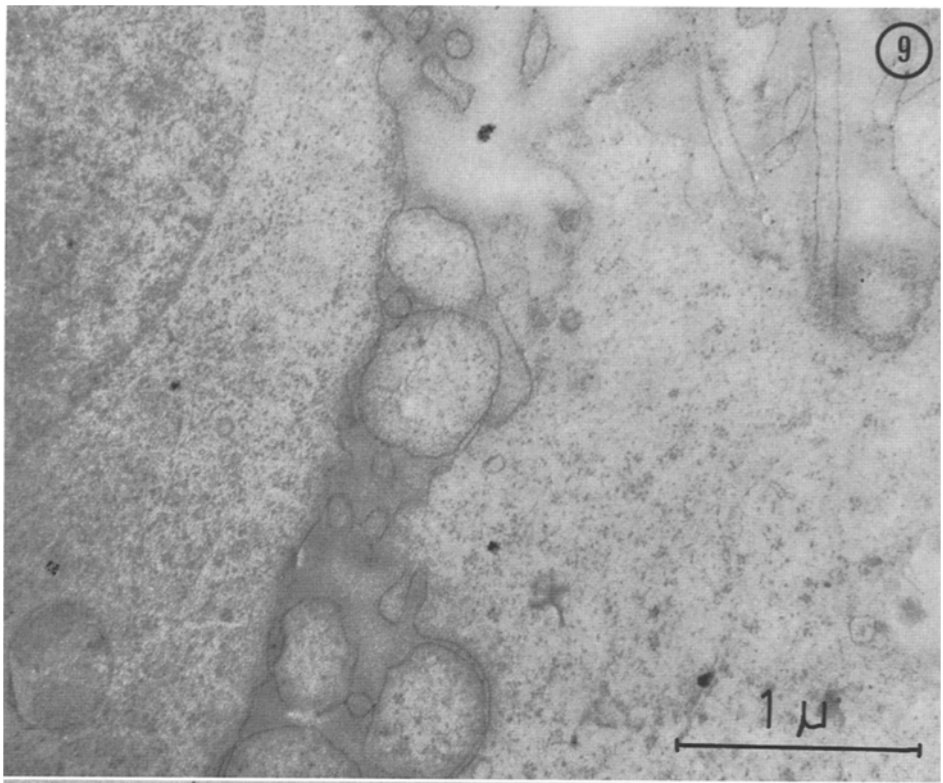
These elevations were repeatedly seen surrounding groups of microvilli which were attached to the substrate, and seemed from their character and location to be bits of cellular material left behind by the detachment of microvilli from the substrate. If so, this observation would support the suggestion of Weiss and his colleagues [27, 29] that cells in moving about or detaching in cultures leave cellular material behind on the substrate.

The other two techniques described in this report permit observation at a fine structural level of that surface of a cultured cell in contact with its substrate. Both methods seemed to be free of special artifact, and were capable of presenting a reproducible and consistent picture of this aspect of cellular morphology.

Both techniques indicated that the bottom surface of the L-cell in monolayer culture is quite flat and relatively featureless. No evidence was found to support the suggestion of Curtis [5] and of Nordling [17] that a well-spread cell has a small area of actual contact with its substrate. The lower plasma membrane was, in these preparations, separated from the cellophane substrate by a fairly constant electron-lucent gap of 200 to 300 A. This separation is considerably larger than that observed by Curtis [5], but

Fig. 9. Section of L-cells from suspension culture, stained with ruthenium red. A dense line outlines the cell surface, including the surfaces of the microvilli

Fig. 10. Section of L-cells from suspension culture, stained with colloidal iron. Fine dense particles of iron are seen lying on the cell and microvilli surfaces. There is an increased density of particles on the microvilli surfaces. At one point (arrow), the iron particles are seen to lie just outside the plasma membrane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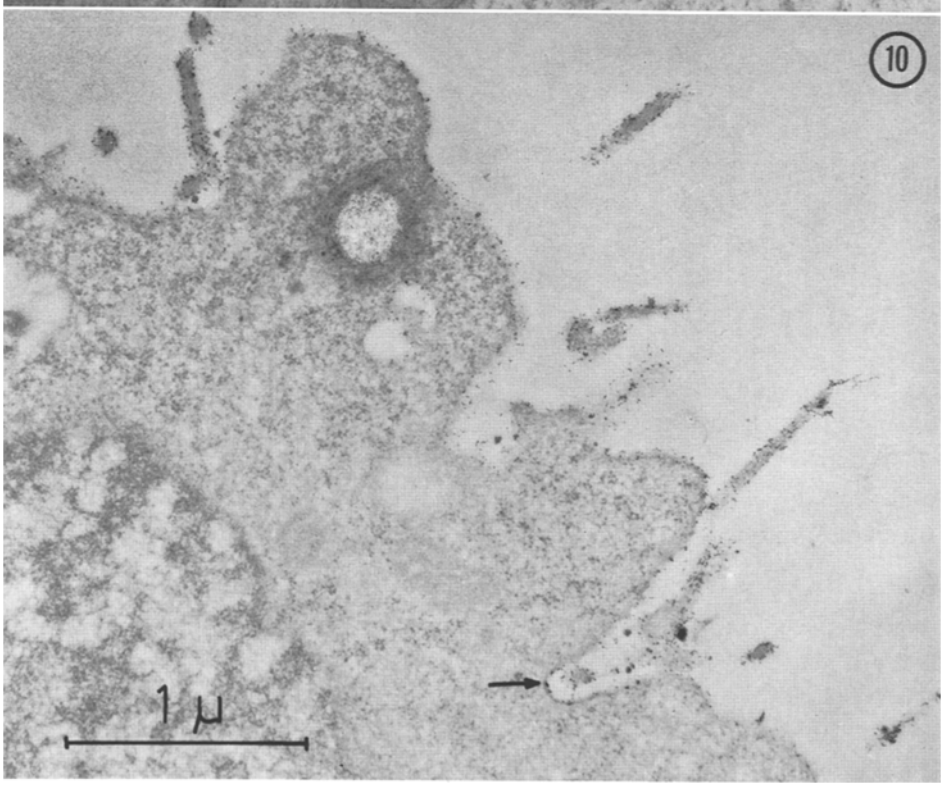
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comparisons are difficult because the present observations were of fixed and sectioned cells and the previous measurements were of living cells. The space may indeed be artifactual. Ruthenium red did react with this space (Fig. 8), but it also reacted with the surface of untreated cellophane which had not been in contact with cells or with culture medium.

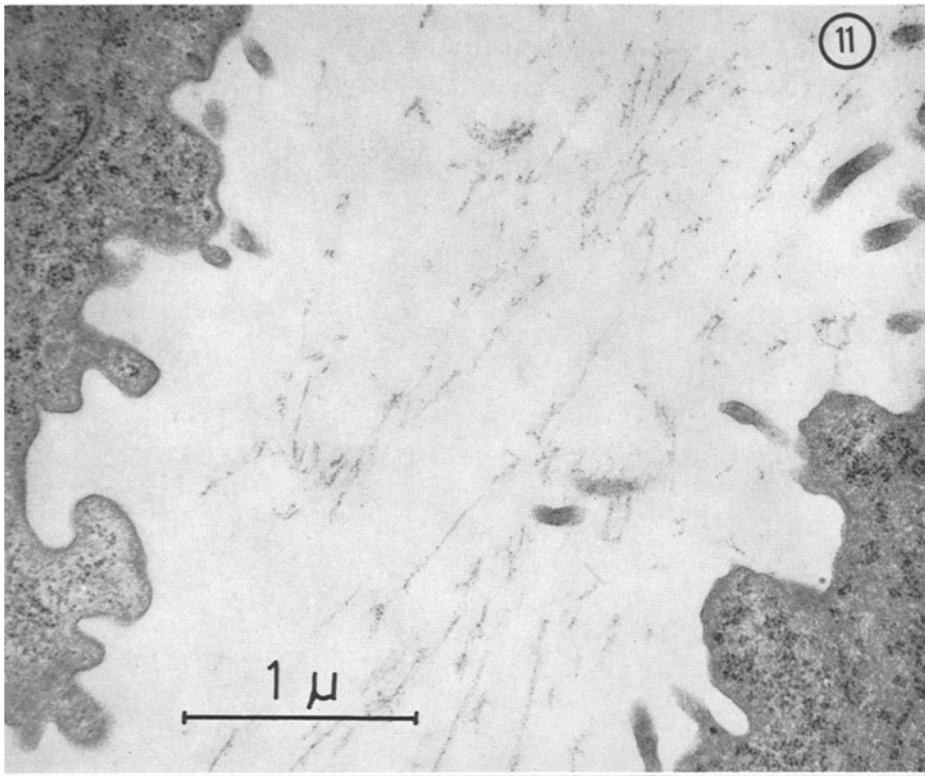
Ruthenium red has been shown by Luft [14] to stain an endocapillary layer in blood vessels, and he has suggested that it is a specific stain for acid mucopolysaccharides. Colloidal iron has long been used as a specific histological stain for acid mucopolysaccharides [4], and recently has been shown in electron microscopic preparations to be specific for the sialic acid component of these substances [2]. Both of these stains, then, can be used to demonstrate a cell coat, and in this study the stain reaction product in both procedures was observed at the surface of the L-cell, apparently just outside the plasma membrane. The greater density of iron particles seen on the microvilli surfaces would indicate a greater concentration of sialic acid in these locations, and, according to suggestions of both Gottschalk [11] and Weiss [26], a more rigid surface for the microvilli compared to the rest of the cell surface. This conclusion is consistent with the observations of Taylor and Robbins [24] of the rod-like movements of microvilli in cultured cells.

The enzymes used in this study have all been shown to affect either cell surfaces or closely related substances. Trypsin, an endopeptidase, has long been used to separate cells from a substrate or from their parent tissue [16]. Chymotrypsin, another endopeptidase, has been used on cell surface proteins [28]. Neuraminidase has a well-documented and specific action on cell surface sialic acid [8]. Ribonuclease has been shown to reduce cellular electrophoretic mobility by its action on surface ribonucleic acid phosphate groups [30, 31]. Hyaluronidase is known to act on the polysaccharide hyaluronic acid [15] and therefore might be expected to react with cell surface mucopolysaccharides.

Of these enzymes, trypsin was the only one which altered cell morphology in replicas or in sections. It would appear that any chemical changes produced by the other enzymes were at a level beyond that detectable by these routine techniques.

Fig. 11. Section of L-cells in suspension, treated with trypsin and stained with lead. An amorphous material is seen in the space between the cells

Fig. 12. Section of L-cells in suspension, treated with trypsin, and stained with ruthenium red. The amorphous material stains well, but the cell surface stains well only in patches (arrows)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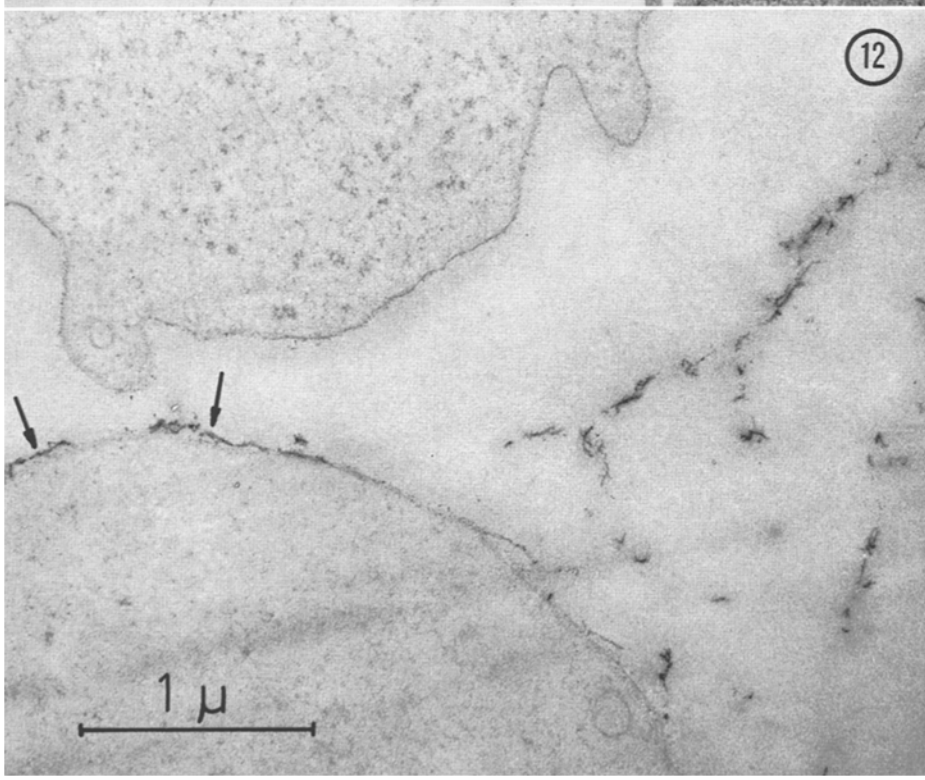
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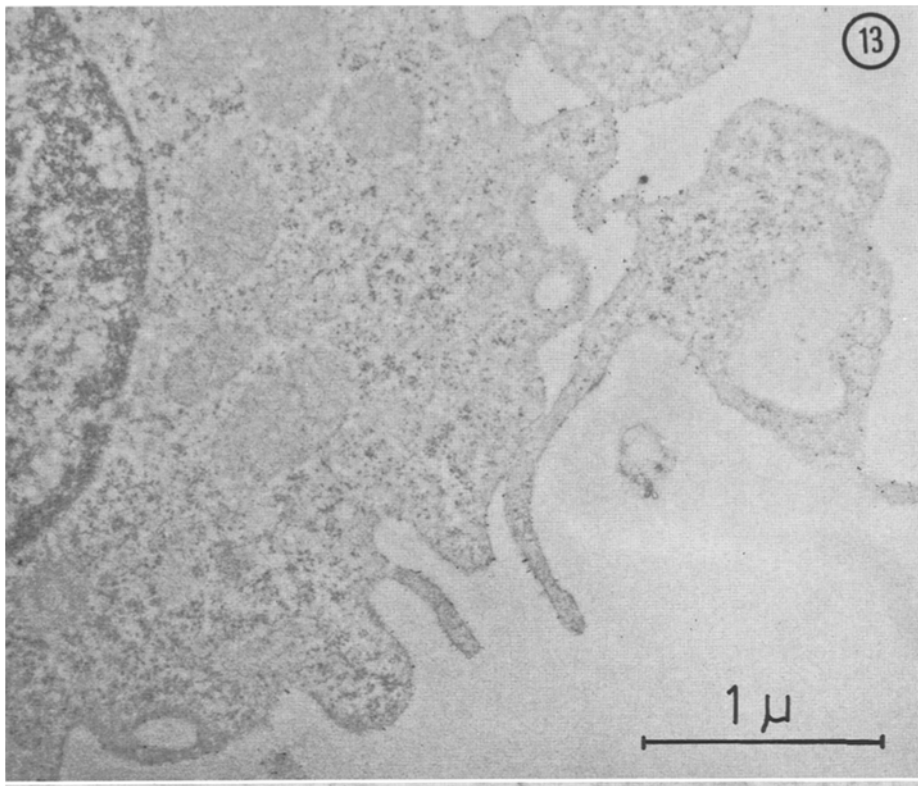
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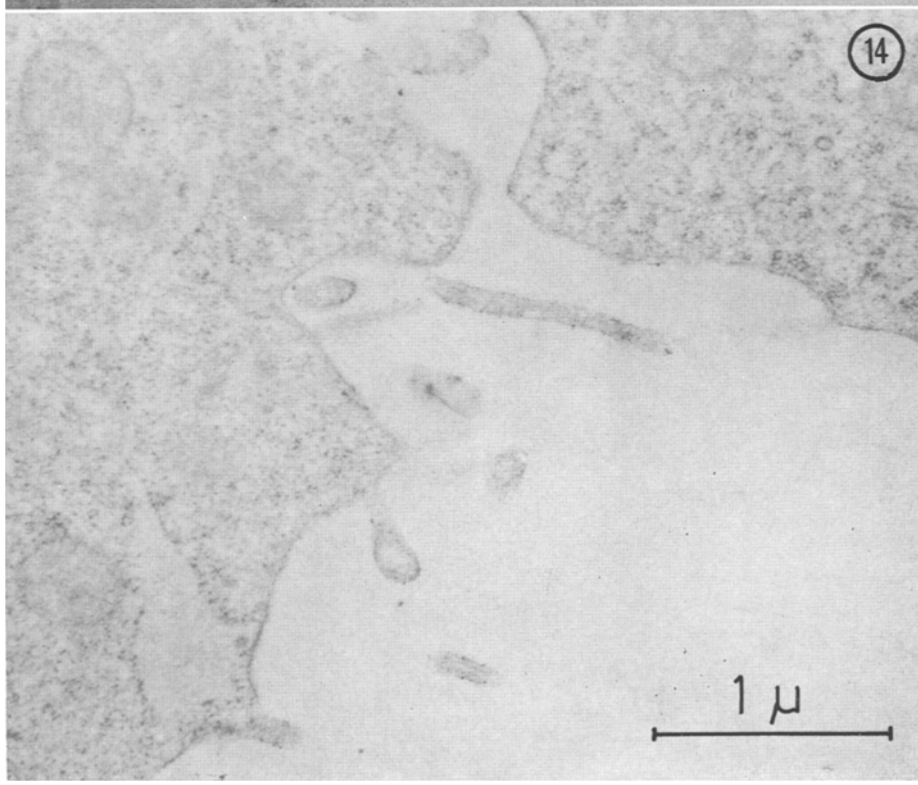
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1 μ

Fig. 13. Section of L-cell in suspension, treated with trypsin, and stained with colloidal iron. There are fewer iron particles on the cell surface

Fig. 14. Section of L-cells in suspension, treated with neuraminidase, and stained with collodial iron. The treatment has virtually eliminated the staining of the cell surface by the iron particles

With the two staining reactions, no changes were noted with chymotrypsin, ribonuclease, or hyaluronidase, so any effects of these enzymes on the cell surface do not affect the reaction sites demonstrated by these stains. However, staining of the cell surface by colloidal iron was reduced by trypsin and virtually eliminated by neuraminidase, and staining by ruthenium red was patchily remolved by trypsin but was not affected by neuraminidase. Thus, both sialic acid and the ruthenium red reaction sites are susceptible to a proteolytic enzyme, but only the sialic acid sites are susceptible to neuraminidase. This would suggest that the sialic acid is more peripherally located in the cell surface coat.

The amorphous material seen between cells in trypsinized preparations stained well with both colloidal iron and ruthenium red. This supports the suggestion of Weiss [25] that trypsinized cells suffer a loss of dry mass. It has been suggested [22] that this mucoid material is cellular debris, as it is digested by desoxyribonuclease, but the similarity of its staining by ruthenium red with the stained patches of the cell surface coat would support a cell surface origin for this material.

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