

Ultrastructure of the Surfaces of Cells Infected with Avian Leukosis-Sarcoma Viruses

HERBERT R. MORGAN¹

Institut de Recherches sur le Cancer, Villejuif 94 France, and the Louis A. Wehle Virus Research Laboratory, Department of Microbiology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14620

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When stained with ruthenium red (RR), chick embryo cells infected with various strains of Rous sarcoma virus (RSV) and with avian leukosis viruses RAV-1 and RAV-3 showed an increase in the layer of acid mucopolysaccharides (AMPS) at their surfaces as compared with uninfected cells. This increase was most prominent in cells infected with the Fujinami strain of RSV. The layer was resistant to digestion with neuraminidase or trypsin but was readily removed by exposure to hyaluronidase. The thickness of this AMPS layer was not correlated with the varying degree of loss of contact inhibition exhibited by cells infected with the different strains of virus. The staining of the cell envelope with a solution of phosphotungstic and chromic acids (PTA-CR) suggested the presence of glycoproteins. The outer surface of the virions showed the same staining as the cell surface with RR and PTA-CR, and the budding virus particle was seen to incorporate the RR layer of the cell into its structure. The RR layers of cells and virions appeared to fuse, as did those between virus particles, suggesting that these layers play a role in the aggregation of virus particles and in their adherence to the surface of the cell.

Previous studies (3) demonstrated that colonies of transformed cells developing in cultures of chick embryo fibroblasts infected with Bryan Rous sarcoma virus (Br-RSV) accumulate acid mucopolysaccharides (AMPS), as indicated by intense staining with the Hale colloidal iron technique. Other investigators (5, 12) showed that infection of chick embryo fibroblasts in vitro with Rous sarcoma viruses resulted in a significant increase in cellular levels of hyaluronic acid synthetase and in the production of hyaluronic acid. Since morphological transformation of cells occurs after infection with these viruses and is associated in several with a marked loss of contact inhibition of the cells, it was of interest to examine the surface of infected cells for the presence of mucopolysaccharides. To detect these materials, we used the ultrastructural, cytochemical method with ruthenium red (RR) staining (8). In addition, the intercellular relationships were studied by means of in situ embedding of the cultivated cells (9).

Several strains of the avian leukosis-sarcoma virus group were selected for study since these strains produce different types of morphological

transformation of cells, which in turn differ in the degree of loss of contact inhibition exhibited; thus, it was possible to relate the layer of mucopolysaccharides at the surfaces of the cells to the behavior of the cells in vitro.

MATERIALS AND METHODS

Viruses. All viruses were prepared by removing the supernatant medium from chick embryo fibroblast cell cultures infected with the various viruses. The fluids were then stored at -70°C until used. The Bryan strain (Br-RSV) was derived from virus CT-845 supplied by W. R. Bryan (National Cancer Institute, Bethesda, Md.), and the morph-r and morph-f strains were received from H. Temin (University of Wisconsin, Madison) who derived them from the Bryan standard Rous sarcoma virus. The Fujinami strain (Fu-RSV) was obtained from E. Reich (Rockefeller University, New York, N.Y.), and an RAV virus containing a mixture of RAV-1 and RAV-3 was obtained from J. Bader (National Cancer Institute, Bethesda, Md.).

Cells and cell culture. Chick embryo fibroblasts were derived from susceptible embryonated eggs obtained from a flock of chickens maintained by R. E. Luginbuhl (University of Connecticut, Storrs) and were supplied by the research resource program of the National Cancer Institute. All cells were tested for uniform susceptibility to infection with Br-RSV as used in each experiment. After mincing of the embryo

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tissues and treatment with trypsin, the cells were cultured in plastic petri dishes according to techniques previously described (10).

To infect cells derived from a single chick embryo with the various strains of viruses, we used between 2×10^4 and 3×10^4 focus-forming units per 100-mm plastic petri dish and a dose of RAV which produced a solid resistance of infected cells to superinfection by Br-RSV 7 days after infection. After 1 week, all cells were subcultured in prescription bottles, Leighton tubes, and 100-mm plastic petri dishes containing cover slips. The cultures were observed daily until morphological transformation and formation of foci were clearly visible in those strains producing these changes; then (about 2 weeks after infection) appropriate cultures were selected for histological and ultrastructural examination. Uninfected cells were handled in the same manner. Some infected cultures were examined up to 33 days after infection, at which time almost all cells had developed the characteristic morphology of viral infected cells.

Histological examination. Cover slips were removed, fixed with neutral buffered Formalin, and stained with May-Grunwald Giemsa dye.

Electron microscopy. The method described by Martinez-Palomo et al. (9) for preparation and examination of cells was employed. Cells in prescription bottles and Leighton tubes were fixed in situ with 2.5% glutaraldehyde-cacodylate buffer solution (pH 7.3) for 1 hr at 4 C. Postfixation was carried out with a solution of 2% osmium tetroxide in cacodylate buffer for 3 hr at room temperature. The RR dye was added to both fixatives at a concentration of 50 mg/100 ml. The cells were then dehydrated in increasing concentrations of acetone and were embedded in situ in tubes or bottles with a layer of Epon 2 to 3 mm thick. After polymerization at 60 C, the glass containers were broken and the layer of Epon containing the cells was detached after a brief immersion in boiling water. The cells were located with the aid of a microscope, and suitable colonies were selected; these colonies were then cut out in blocks, approximately 2 by 3 by 1 mm, and were oriented to obtain vertical sections through the colonies. The sections were stained with lead citrate (Reynolds) for 10 min.

In other instances, cells were scraped from the glass surface into a 1.6% solution of glutaraldehyde in Sorenson's buffer kept for 15 min at 4 C; then the cells were embedded in glycol methacrylate according to the method of Leduc and Bernhard (7) and were subsequently polymerized with ultraviolet irradiation.

Sections were prepared from the cell button and were stained with an aqueous solution containing 1% phosphotungstic and 10% chromic acids according to the method described by Rambourg (11).

Sections were examined with a Siemens Elmiskop I electron microscope.

Enzyme digestions. Cell cultures were rinsed with 0.85% saline and were treated with (i) a solution containing 300 National Formulary units of hyaluronidase (Wyeth) per ml in 0.85% saline or (ii) a solution comprised of 500 units (number of micrograms of *N*-acetyl neuraminic acid liberated at 37 C from acid α -1-glycoprotein) of neuraminidase (Behringwerke) in

a sodium acetate-acetic acid buffer (pH 5.5) containing 0.9 g of NaCl and 0.1 g of CaCl₂ per 100 ml for 3 hr at 37 C. In both instances, digestion was continued until the cells began to detach from the glass surface.

For trypsin digestion, cells were fixed for 1 hr with 2.5% glutaraldehyde-cacodylate buffer solution, rinsed repeatedly with cacodylate buffer, and then exposed to a 0.25% solution of trypsin (Difco) in phosphate-buffered saline (pH 7.3) for 2 hr at 37 C.

In all instances, the cells were then carefully rinsed with cacodylate buffer and were processed as described for RR staining.

RESULTS

Giemsa and RR stains. Normal chick embryo fibroblasts grew in typical patterns (Fig. 1) with occasional overlapping of cells (Fig. 1 and 2) and possessed a layer of dark material at the cell surface when stained with RR; this material varied in thickness and density over the cell surface (Fig. 2, 10a).

Foci of fibroblasts infected with Br-RSV showed characteristic, rounded, dark cells (Fig. 3) piled up in multiple layers (Fig. 3-5) with many long cell processes (Fig. 5). The RR layer at the cell surface was heaviest at the free surfaces in the foci, as the dye did not penetrate well into the mass of cells. The RR layer was slightly more prominent in infected (Fig. 10b) than in control cells (Fig. 10a). Intercellular contacts of the type known as close junctions were seen; in these junctions, the RR staining layer between the cells was continuous (Fig. 5). Foci of cells infected for 2 weeks showed the same type of RR layer as those from cultures infected for as long as 33 days. Since only cells in foci of transformation were selected for study, the possibility of examining uninfected cells in the culture was eliminated and cells at the upper surface of a colony were used to permit maximal interaction of the RR stain with the free cell surface.

Cells infected with morph-r-RSV developed sharp, round foci of piled up cells (Fig. 7) with many processes (Fig. 6) at the cell surface, which was covered with an RR layer (Fig. 6, 10c) somewhat more prominent than that seen in control cells; cells infected with morph-f-RSV showed typical, fusiform cells in foci, with overlapping of cells (Fig. 8) but without the formation of multiple layers. Contacts between cells infected with morph-r-RSV were of the type characterized as close junctions (Fig. 6). The RR layer of morph-f-RSV-infected cells (Fig. 10d) was as prominent as that seen with the other two strains of virus; however, few cell processes were seen and they were not as long.

The Fu-RSV-infected cells became rounded and produced multilayered, dense foci (Fig. 9) in

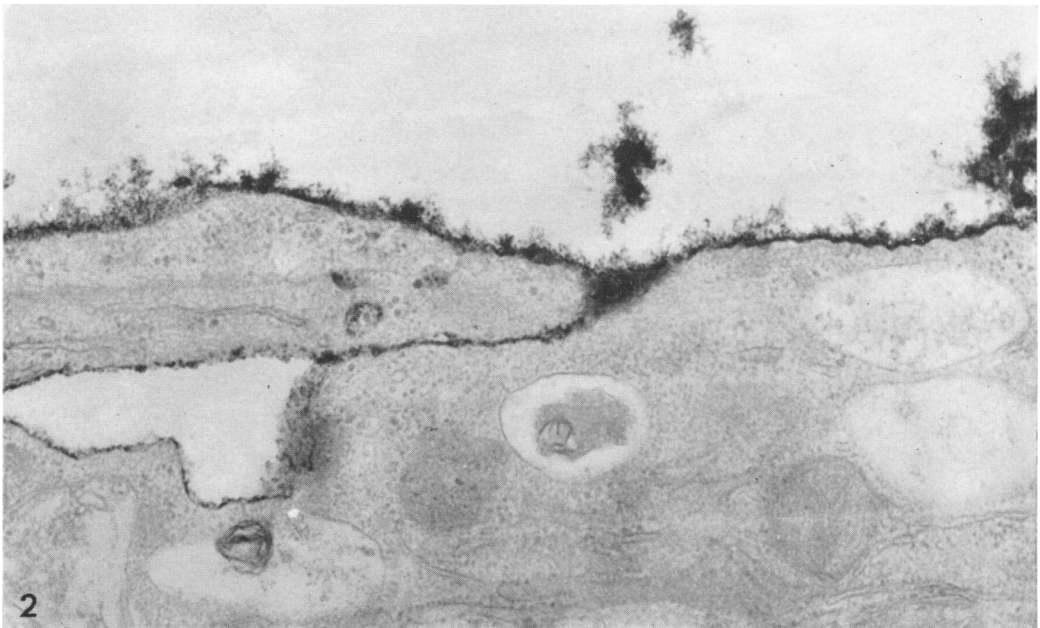
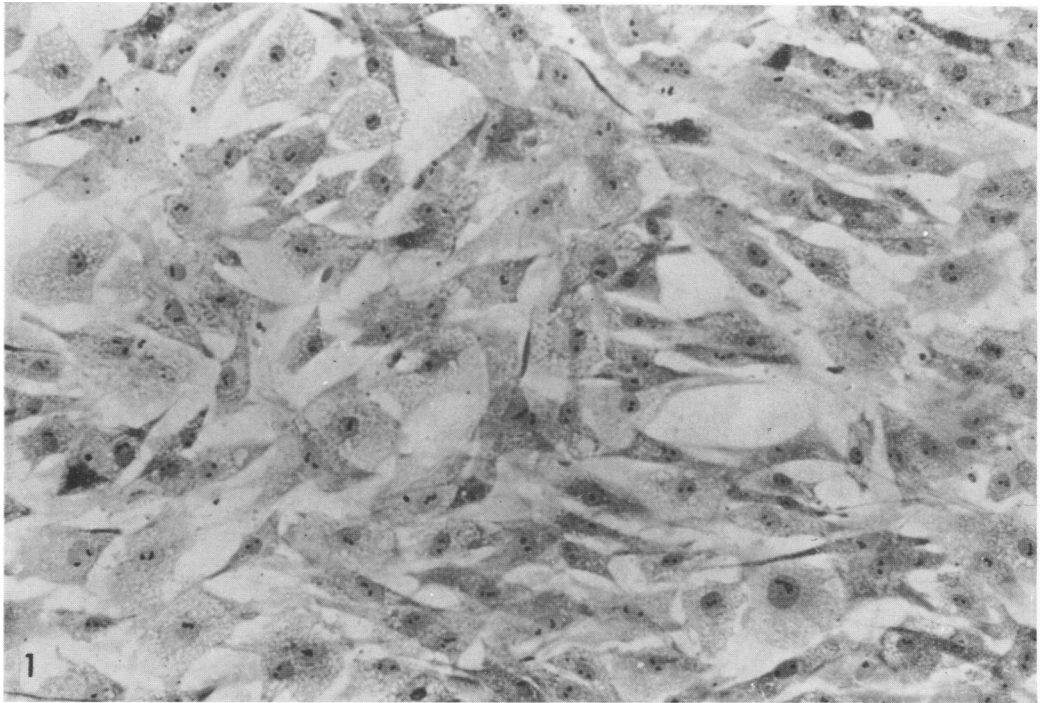


FIG. 1. Monolayer culture of normal chick embryo fibroblasts with occasional overlapping of cells. Giemsa stain. $\times 320$.

FIG. 2. Normal chick embryo fibroblasts showing overlapping of cells and ruthenium red stained layers which are more prominent at the free cell surface. Ruthenium red stain. $\times 30,000$.

which the cells showed many processes which interdigitated; this was also seen in cells infected with Br-RSV and morph-r-RSV. In Fu-RSV-infected cells, the RR layer was denser and much thicker than in uninfected cells or in cells infected with the other strains of virus (Fig. 10e).

The infection of cells with a mixture of RAV-1

and RAV-3 produced no obvious changes in cell morphology (Fig. 11); the cells remained as a monolayer with occasional overlapping. The RR layer of the cells was similar to that found on cells infected with various strains of RSV except Fu-RSV (Fig. 12).

Virus particles were seen in all infected cul-

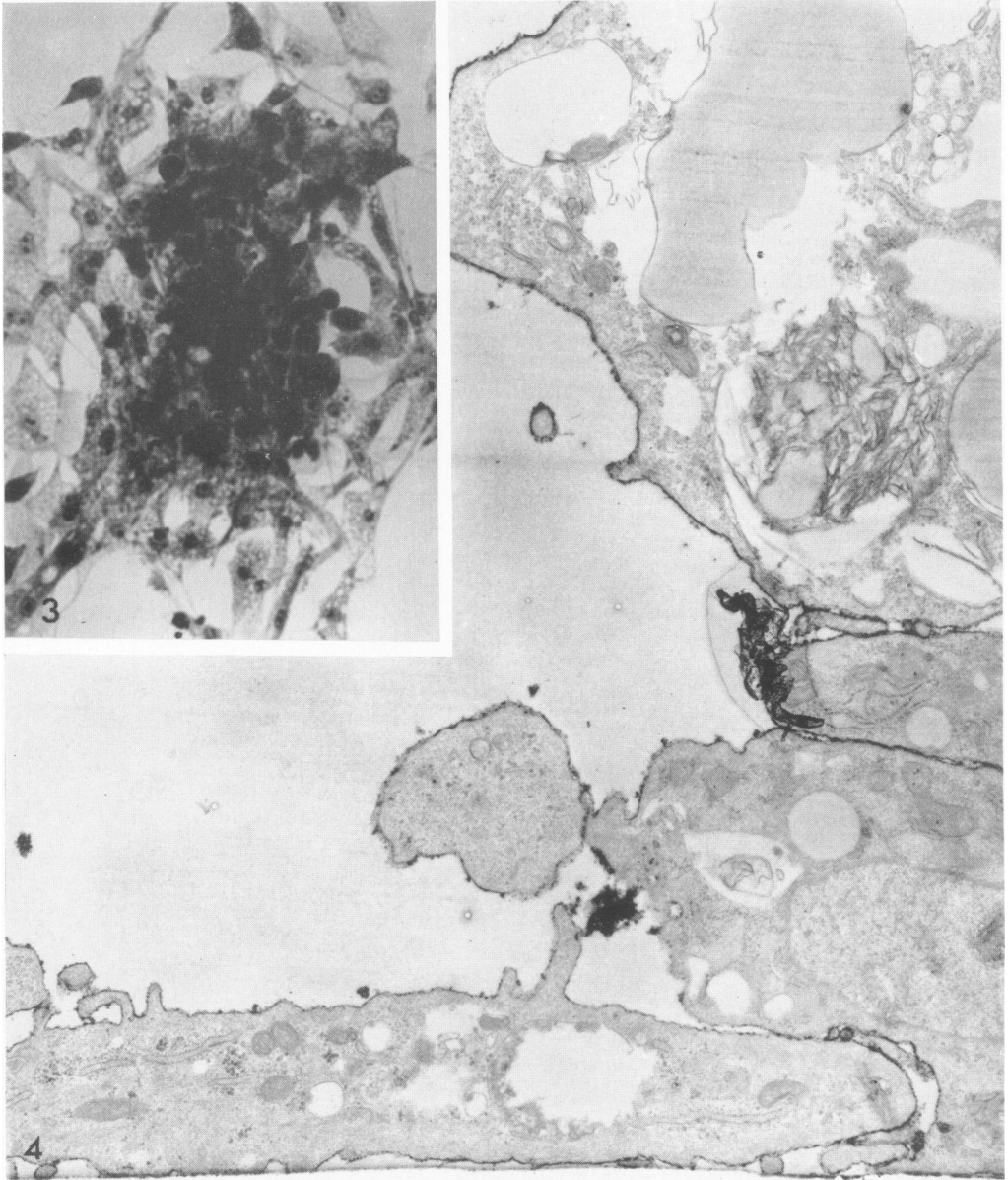


FIG. 3. Colony of chick embryo fibroblasts infected with Br-RSV showing piled up cells. Giemsa stain. $\times 320$.

FIG. 4. Vertical section through a colony of cells infected with Br-RSV demonstrating loss of contact inhibition and the layer of ruthenium red staining material at the cell surfaces. Ruthenium red stain. $\times 12,000$.

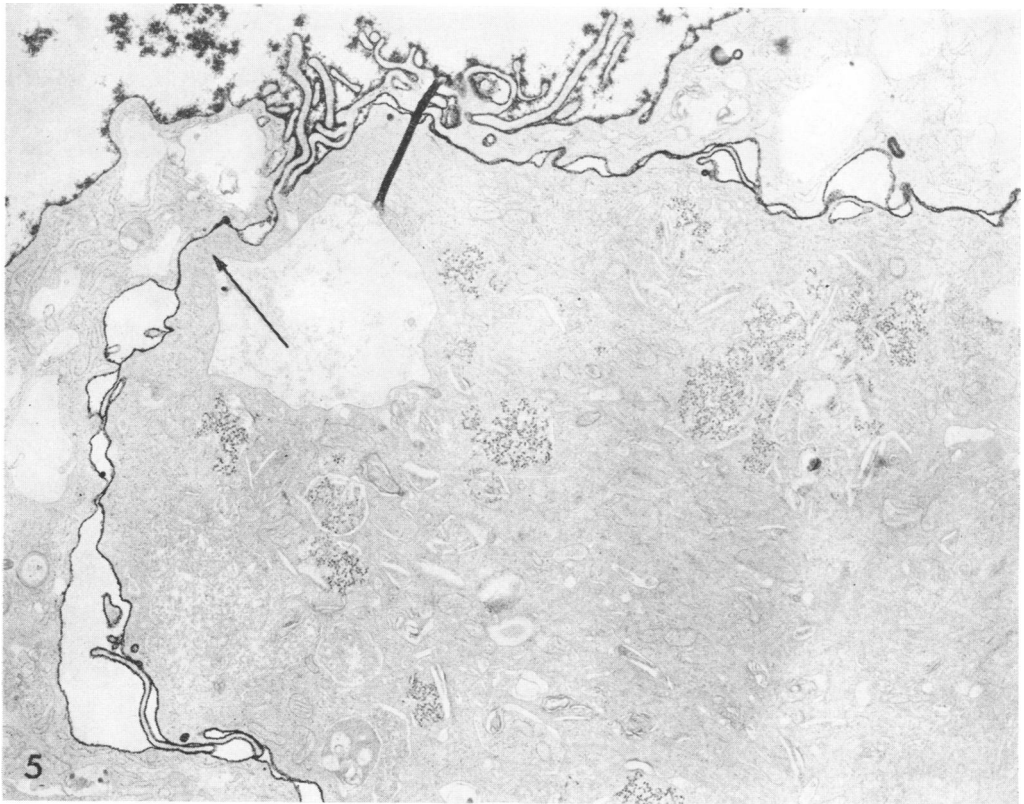


FIG. 5. Vertical section through a colony of cells infected with Br-RSV showing characteristic long processes extending from the cells and intercellular contacts characterized as close junctions (arrow). Ruthenium red stain. $\times 6,000$.

FIG. 6. Cellular processes of chick fibroblasts infected with morph-r-RSV having many intercellular contacts of the type identified as close junctions (arrows). Ruthenium red stain. $\times 60,000$.

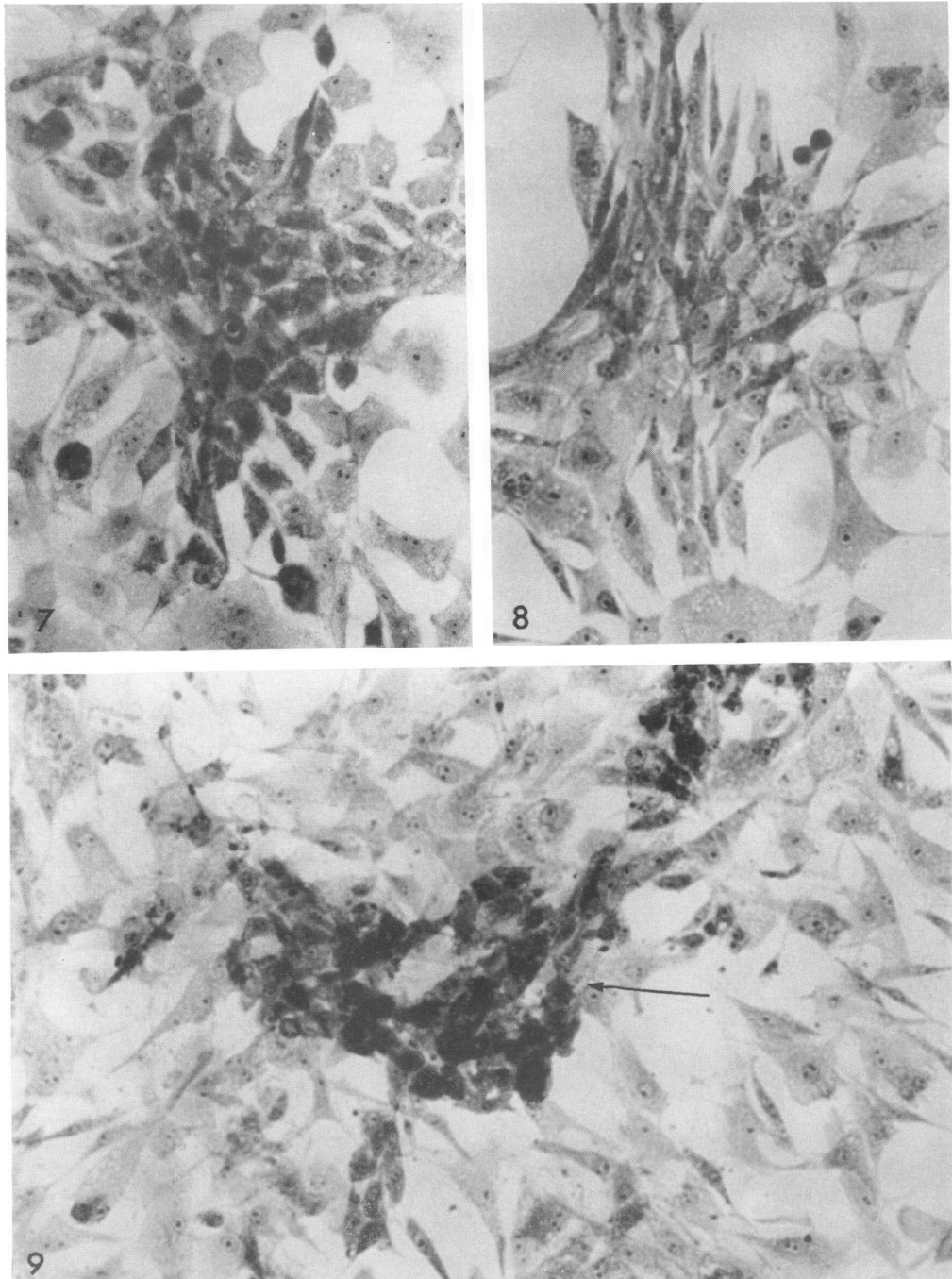


FIG. 7. Cells infected with morph-r-RSV forming a clump of rounded cells. Giemsa stain. $\times 320$.

FIG. 8. Cells infected with morph-f-RSV. The characteristic fusiform cells show some overlapping. Giemsa stain. $\times 320$.

FIG. 9. Colony of rounded cells infected with Fu-RSV (arrow) in a culture of chick embryo fibroblasts. Giemsa stain. $\times 320$.

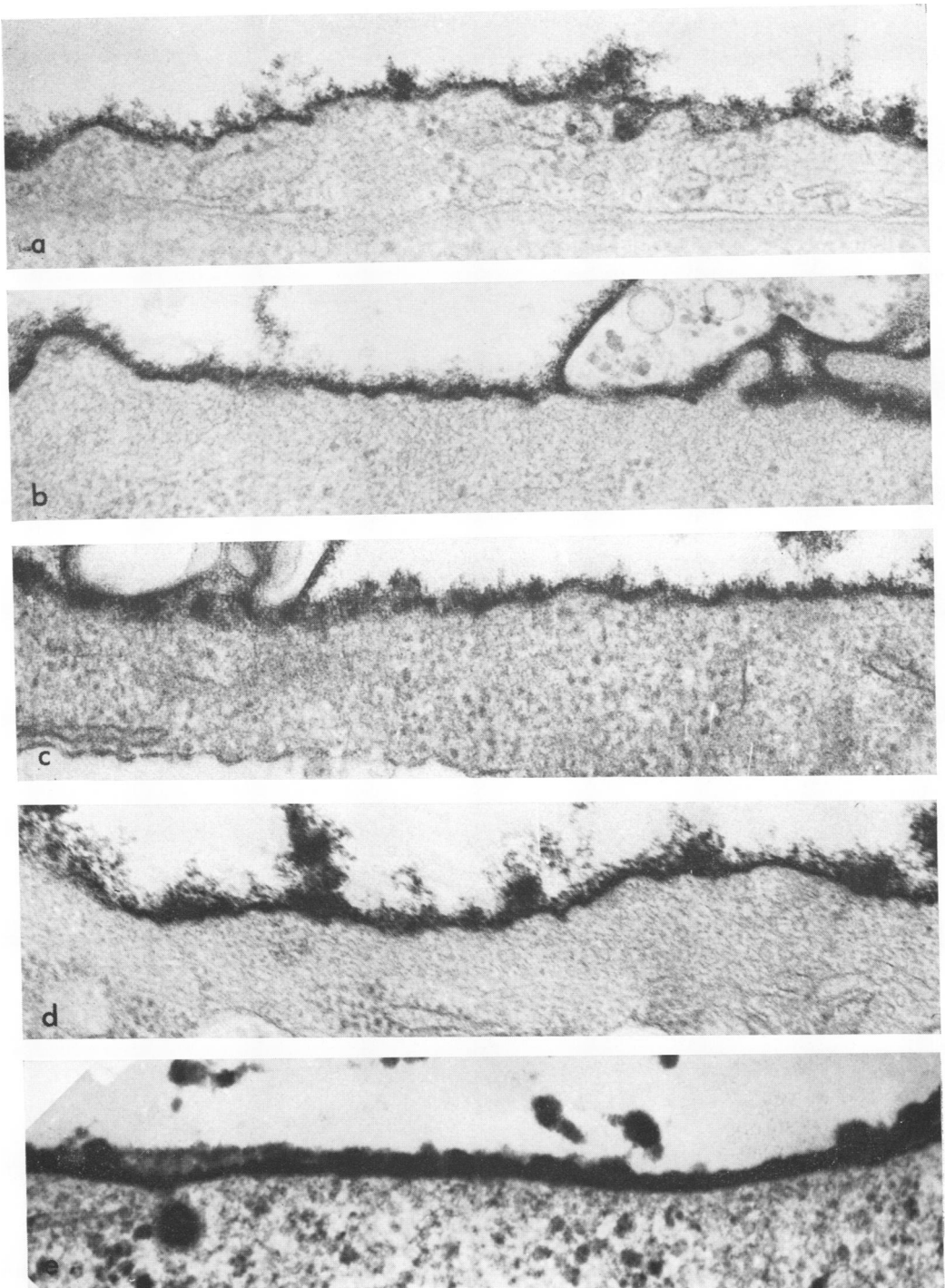


FIG. 10. Electron micrographs illustrating the ruthenium red layer at the upper free surface of (a) normal cells and of cells infected with (b) *Br-RSV*, (c) *morph-r-RSV*, (d) *morph-f-RSV*, and (e) *Fu-RSV*. Ruthenium red stain. $\times 60,000$.

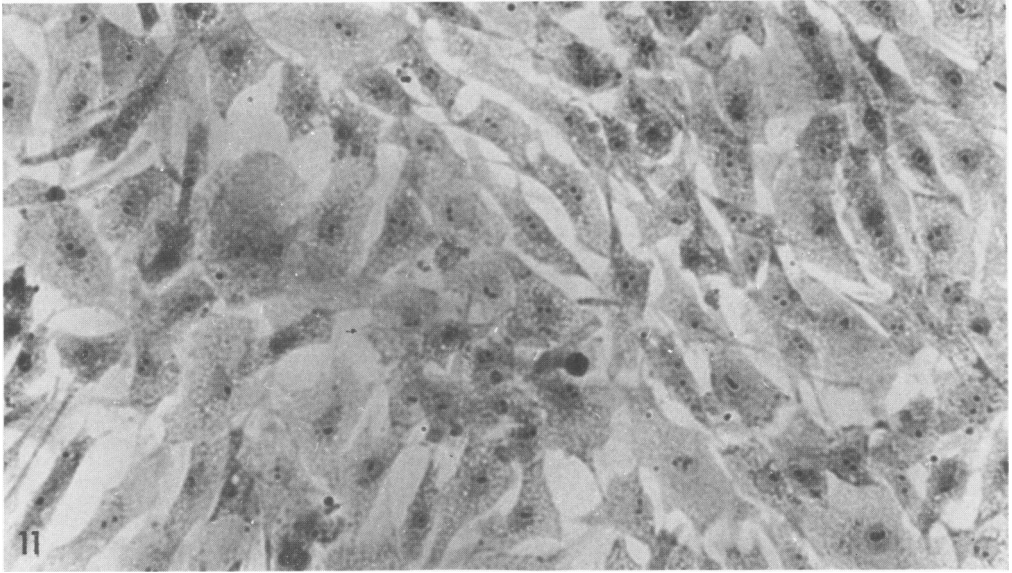


FIG. 11. Cells infected with RAV-1 and RAV-3. Giemsa stain. $\times 320$.

FIG. 12. Cells infected with RAV-1 and RAV-3. Ruthenium red stain. $\times 60,000$.

tures, but not in normal cell cultures, and their morphology is described below.

Phosphotungstic-chromic acid (PTA-CR) stain. Examination of the buttons of cells embedded in

GMA and stained with PTA-CR showed a discontinuous layer of material at the surface of normal (Fig. 13) and infected (Fig. 14) cells; this discontinuous layer probably resulted from

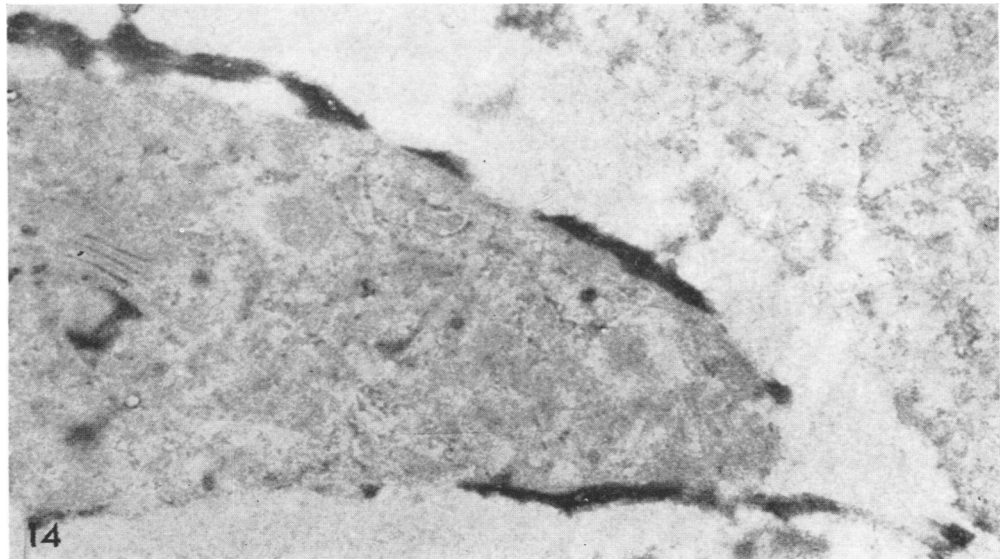
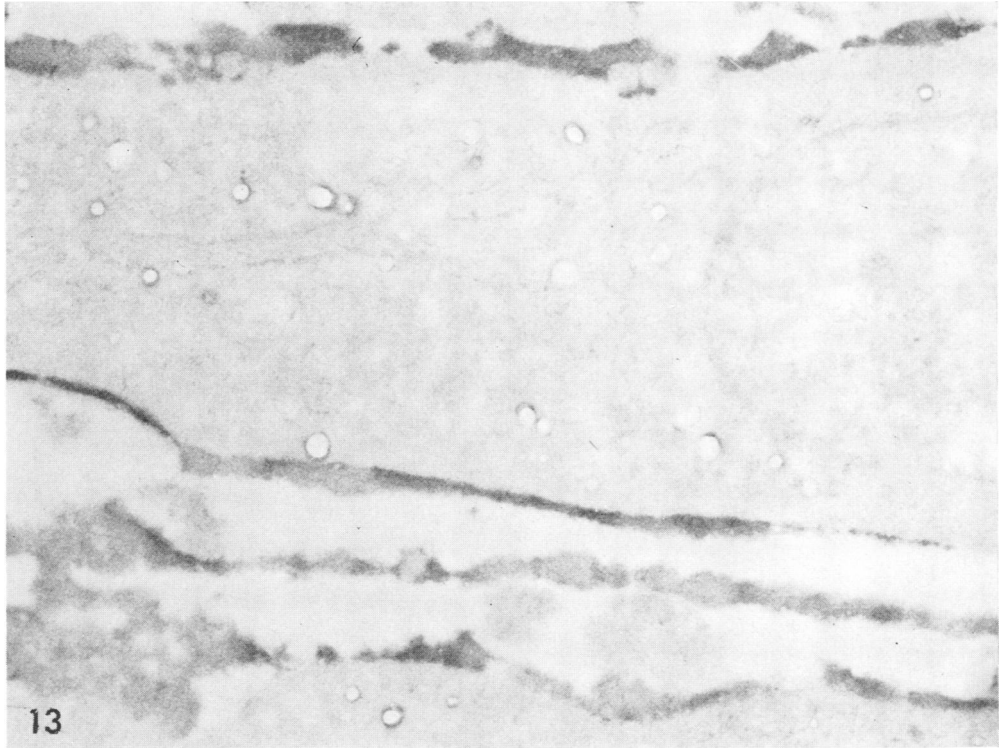


FIG. 13. Normal cells. Phosphotungstic-chromic acid stain. $\times 30,000$.

FIG. 14. Cells infected with Fu-RSV. Phosphotungstic-chromic acid stain. $\times 30,000$.

trauma to the cell surface when the cells were removed from the glass substrate in the preparation of the cell buttons for examination.

Enzyme treatment. Neuraminidase reduced the thickness and density of the RR-staining layer of

normal cells somewhat (Fig. 15a, b) but had no effect on the RR layer of cells infected with Fu-RSV (Fig. 15d, e) and other strains of RSV. Hyaluronidase markedly reduced the RR layer of normal fibroblasts (Fig. 15c) and almost en-

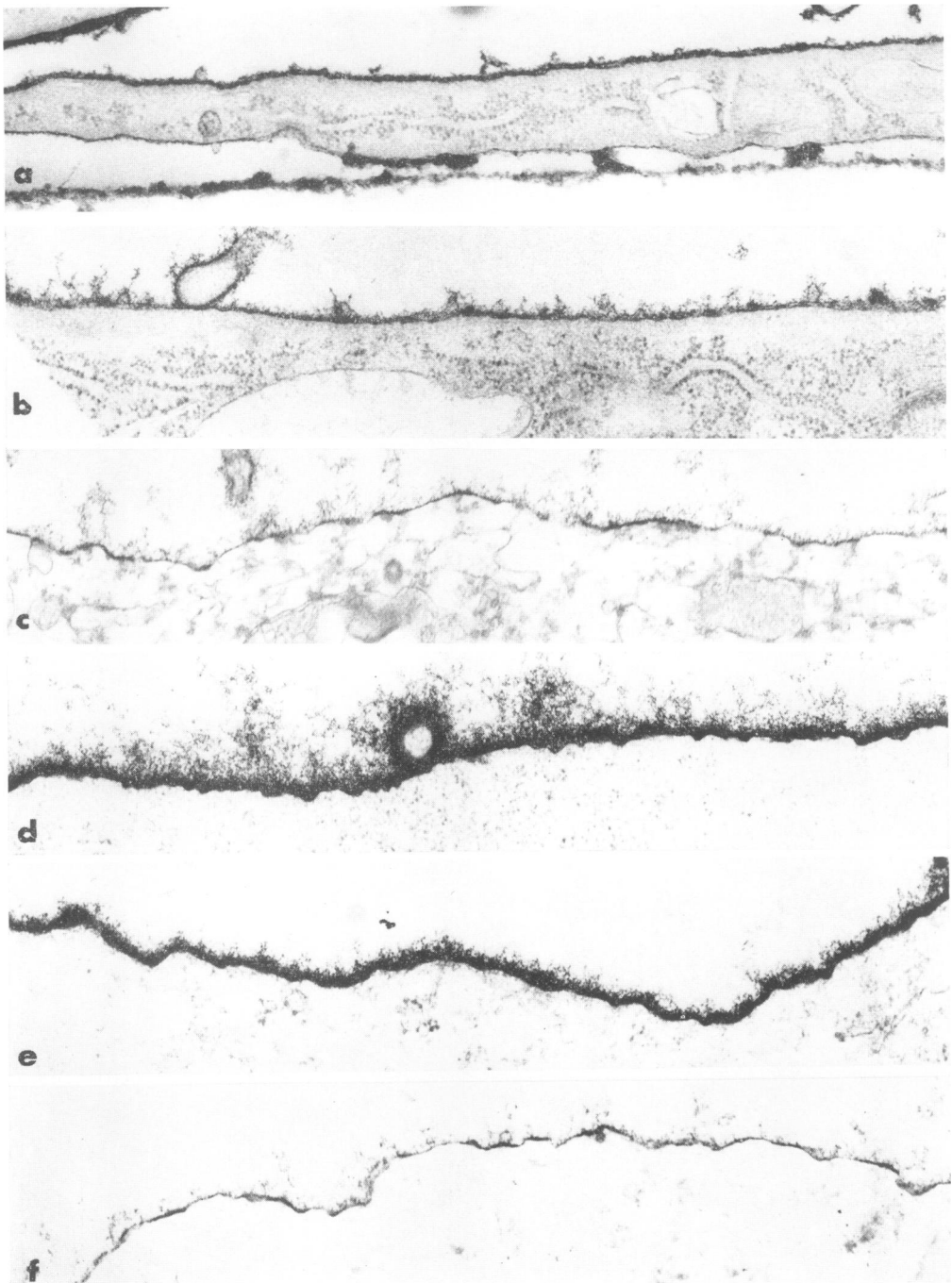


FIG. 15. *Electron micrographs illustrating the action of various enzymes on normal cells and on cells infected with Fu-RSV. Normal cells: (a) control, (b) neuraminidase, and (c) hyaluronidase. Cells infected with Fu-RSV: (d) control, (e) neuraminidase, and (f) hyaluronidase. Ruthenium red stain. $\times 60,000$.*

tirely removed the thick RR layer from cells infected with Fu-RSV (Fig. 15f). Trypsin did not affect the RR layer of glutaraldehyde-fixed normal

cells or the RR layer of cells infected with morph-r-RSV, although the enzyme penetrated the cells and disrupted intracellular structures.

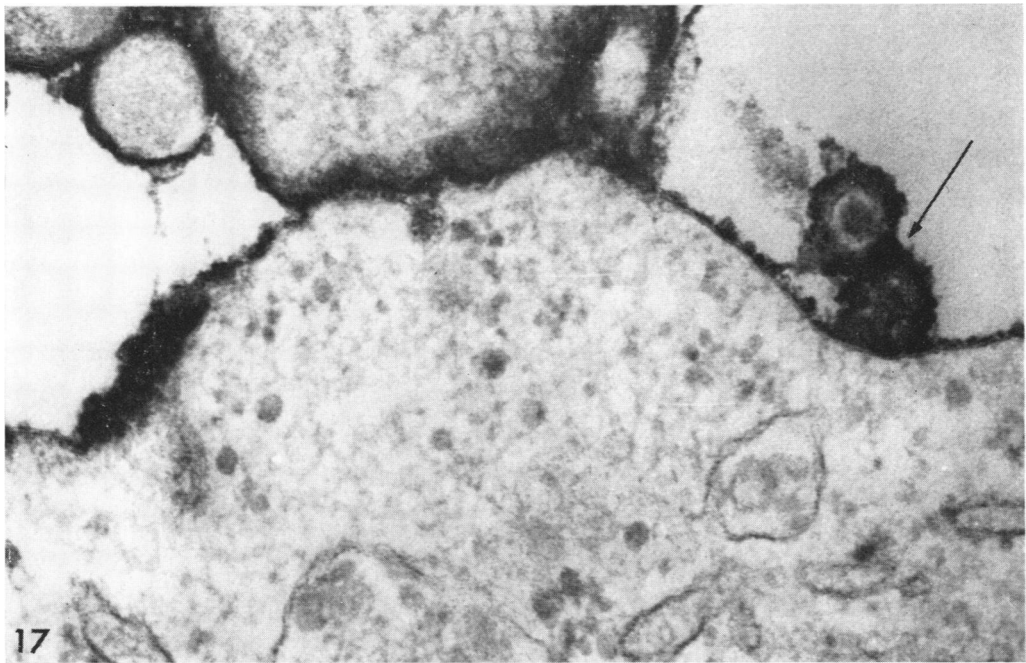
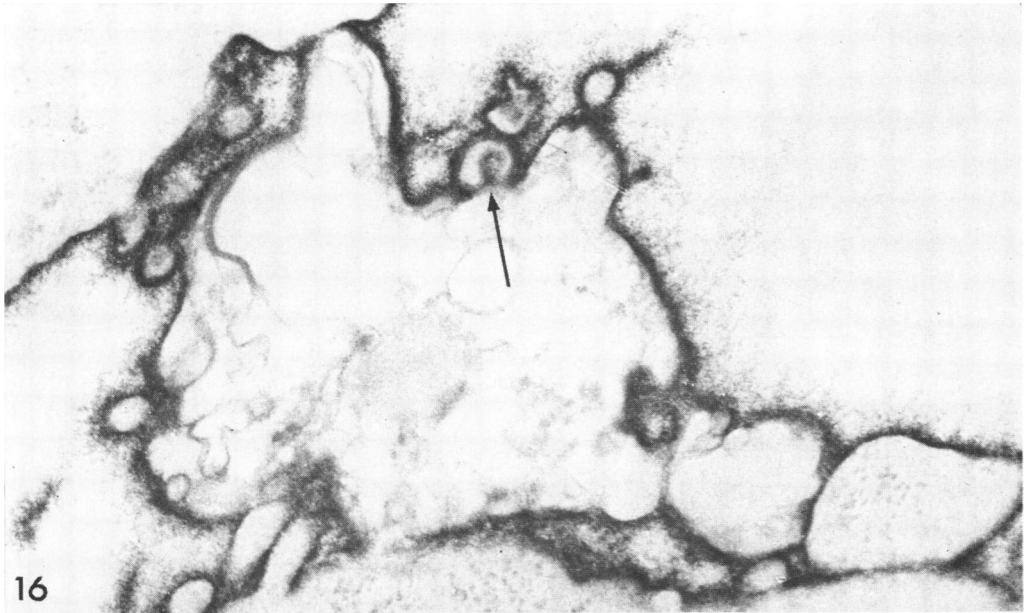


FIG. 16. Virus particle (arrow) budding from surface of cell infected with Fu-RSV. Ruthenium red stain. $\times 90,000$.

FIG. 17. Two virus particles (arrow) at the surface of a cell infected with Fu-RSV. Ruthenium red stain. $\times 90,000$.

Virus. With all strains of RSV studied and with RAV-1 and RAV-3, the virus particles were surrounded with a thick layer of RR staining material (Fig. 17 and 18). As the virus was seen to bud from the cell surface (Fig. 16), the RR layer was

incorporated as part of the viral envelope. Large clumps of virus particles were frequently seen (Fig. 18), with the RR layers of the virions closely packed together. These clumps and single virus particles were often attached to the cell surfaces,

with the RR layer of the virus and the cell forming a continuous structure (Fig. 17 and 18). The viral RR layer was not affected by treatment with neuraminidase and the virions remained attached to the cells; however, in cultures treated with hyaluronidase the abundant virus particles disappeared, suggesting that the enzyme had released them from the cells and that they had dispersed and were washed from the cultures when the enzyme solution was removed. Virus particles sedimented from the enzyme solution by centrifugation and stained with RR possessed no dark outer layer.

The virus particles in the GMA sections stained with PTA-CR were embedded in a stained matrix in clumps which usually adhered closely to the surfaces of cells, with continuity of the viral and cellular envelope material (Fig. 19).

DISCUSSION

When used in combination with osmium tetroxide, the RR stain which has a particular affinity for highly polymerized acidic polysaccharides produces a heavy black layer at the surface of cells containing such substances (8). The dye is known to combine readily with polymerized hyaluronic acid and chondroitin sulfate but not with sialic acid (J. H. Luft, *personal communication*). Thus, it is a good stain for the study of the surface layer of cells infected with Rous sarcoma viruses, since this layer has been shown to contain AMPS belonging to the hyaluronic acid-chondroitin sulfuric acid group (3). The easy removal of this layer by hyaluronidase and its resistance to dissolution by neuraminidase and trypsin indicate that hyaluronic acid-containing AMPS are responsible to a large extent for the RR staining. Since hyaluronidase appears to have a slightly greater effect on RSV-infected cells than on normal cells and the AMPS layer of infected cells is totally resistant to treatment with neuraminidase, differences in the AMPS layer of normal and RSV-infected cells may exist. Chemical analyses (6) have shown that the hyaluronic acid isolated from Rous sarcoma tissues has a smaller particle size than the hyaluronic acid isolated from normal tissues. Furthermore, the hyaluronic acid isolated from Rous sarcoma tissues produced increased spreading of dye in tissues and increased capillary permeability when injected locally, whereas the hyaluronic acid from normal tissues did not (6). Therefore, it is possible that the AMPS layer on the tumor cells may be different from that on normal cells, even though Ishimoto et al. (5) were unable to detect differences between the hyaluronic synthetase derived from RSV-infected or uninfected cells cultured in vitro.

This surface layer is probably the same as the amorphous material, which was thought to contain AMPS, previously described at the surface of Rous sarcoma tumor cells in electron microscopic studies (4). The staining of the layer with PTA-CR suggests that it also contains glycoproteins (11).

In these investigations, no correlation was observed between the thickness of the RR layer and the characteristic social behavior of cells infected in vitro with the various strains of RSV or leukemia viruses. RSV strains such as Br-RSV and morph-r-RSV, which induce the formation of dense foci of cells exhibiting a prominent loss of contact inhibition, had an RR layer of the same thickness as cells infected with morph-f-RSV or RAV-1 and RAV-3, which showed little or no loss of contact inhibition. It is of interest to note, however, that the Fu-RSV infected cells which produced the most significant increase in AMPS after infection in vitro (12) also had the densest and thickest layer of AMPS at the cell surface.

These findings with the RSV strains are at variance with the results obtained by Defendi and Gasic (2) and Martinez-Palomo et al. (9); these investigators found a correlation between an increase of AMPS at the surface of cells transformed by infection with polyoma, SV40, or adeno-12 viruses and their loss of contact inhibition. The situation between these DNA viruses and the ribonucleic acid leukemia-sarcoma viruses may be different and the increase in surface AMPS is not necessarily related to the loss of contact inhibition in all cases.

The development of long processes at the surfaces of RSV-infected cells was most common in those cells found in the multilayered foci and is probably correlated with the increased surface activity of these cells as they migrated over one another to form the foci.

The intercellular contacts between both normal and virus-infected cells involved the formation of close junctions (intermediate junctions and tight junctions were not observed) and no essential differences in the numbers or nature of these cell junctions was seen. The differences in cell contacts observed by Martinez-Palomo et al. (*unpublished data*) in hamster cells transformed with SV40 virus or adenovirus 12, where the tight junctions seen in controls had practically disappeared in the transformed cultures, could not be studied in this investigation since no tight junctions were observed in either control or infected cells.

The outer envelope of the virus particles showed the same RR staining and reaction to PTA-CR as did the cell surface. This was expected since the budding virus particle (Fig. 16) was seen to incorporate the RR layer of the cell into its struc-

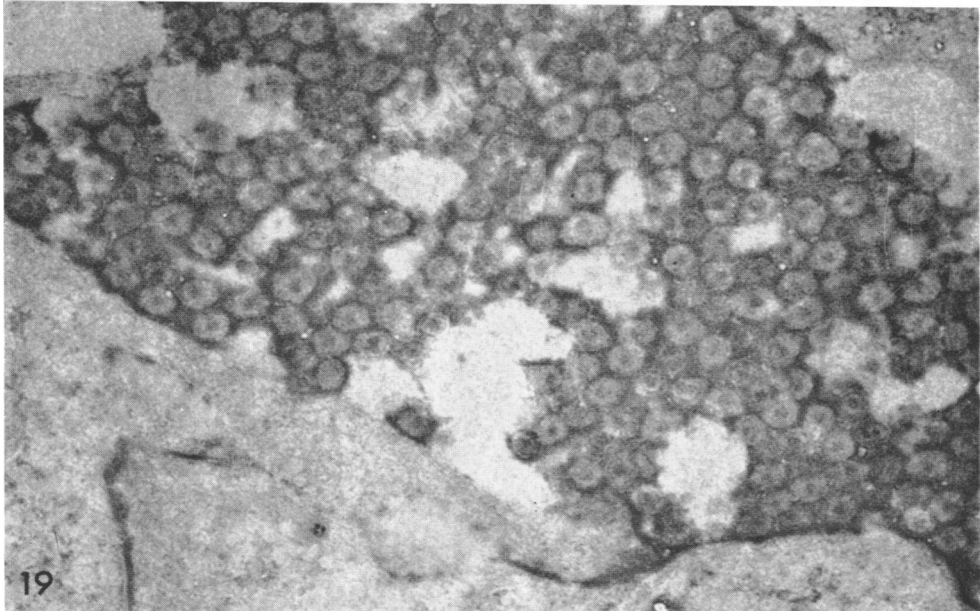
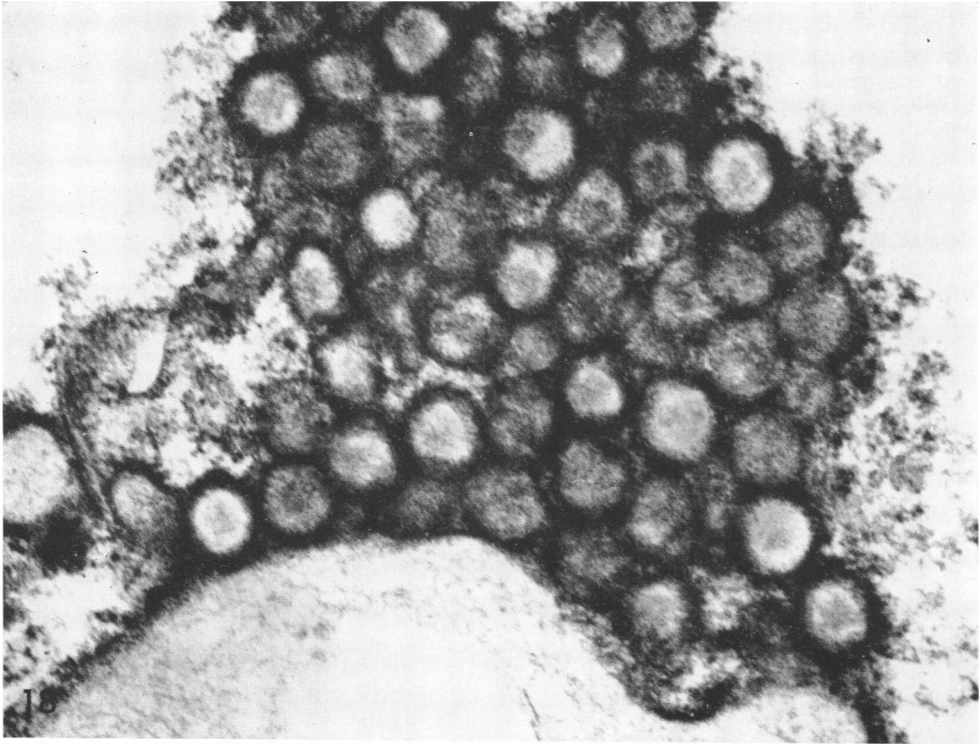


FIG. 18. Aggregation of virus particles at the surface of a cell infected with Br-RSV. Ruthenium red stain. $\times 90,000$.

FIG. 19. Virus particles in clumps at the surface of a cell infected with Fu-RSV. Phosphotungstic-chromic acid stain. $\times 30,000$.

ture, which thus contained AMPS and glycoproteins. The viral envelope appeared to merge with the cellular envelope to which the virions were frequently attached, suggesting that these envelopes play a role in this attachment as well as in the formation of large clumps of virus particles which were frequently seen in the infected cultures. The relationship between the viral and cellular envelopes was the same when stained with RR or PTA-CR. Since most of the virions in the cultures were present in aggregates, the importance of releasing the virus particles from the cells and dispersing them before attempting to assay the virus content of fluids or attempting to prepare stock virus from infected tissue cultures is obvious.

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