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Inhibition of Sindbis Virus Release by Media of Low Ionic Strength: an Electron Microscope Study

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Release of Sindbis virus from infected cells is inhibited by lowering the ionic strength of the medium. To determine the nature of the inhibited step, we examined, by electron microscopy, both freeze-etched and thin-sectioned preparations which had been fixed with either glutaraldehyde or formaldehyde. Inhibitory medium had two different effects on Sindbis virus release: virus budding was partially inhibited, and those virions which did mature were precipitated on the surface of the cell. Freeze-etched, inhibited cells showed very few viral buds. After shift to normal medium, the number of budding virions increased dramatically, far exceeding the quantity found in normal controls. Thus, low ionic strength medium clearly inhibited an early stage of virus maturation. The results were the same regardless of the fixative. Thin sections of glutaraldehyde-fixed, inhibited cells contained large extracellular aggregates of mature virus which were not present in similar, formaldehyde-fixed preparations. Fixation of radioactively-labeled, inhibited cultures revealed that approximately half of the virus that could be released from inhibited cells by raising the ionic strength of the medium could also be released by formaldehyde, but not by glutaraldehyde. This fraction probably represents mature virus attached to the cell surface by the ionic conditions.

Sindbis, a group A arbovirus, acquires its membranous outer coat as the nucleocapsid buds through the modified membranes of infected cells (1, 5, 9, 10). Waite and Pfefferkorn (14, 15) showed that reducing the NaCl concentration of the medium prevented the appearance of Sindbis virus without inhibiting viral RNA or protein synthesis, nucleocapsid formation, or the appearance of viral proteins in the cell membrane. This inhibition could be readily reversed by restoring the ionic strength of the medium to normal. Greatly accelerated virus release was detected within 20 sec. Within 15 to 20 min after shift, the normal medium contained as much virus as was produced by control cultures incubated in normal medium throughout infection. Neither cycloheximide nor inhibitors of energy metabolism prevented reversal of the inhibition. Virus could be released from sonic extracts of inhibited cells only under the same conditions of temperature and ionic strength that were required for release from intact cells. Disruption of inhibited cells in low ionic strength medium, or in normal medium in the cold, did not result in virus production. These observations suggested that the inhibition caused by low ionic strength medium occurred at a very late step in virus maturation.

Two models were consistent with these observations (14, 15): (i) the virus was fully mature and precipitated on the surface of the cells, or (ii) maturation of the virus was inhibited at a terminal stage of the budding process but after a firm association between nucleocapsid and cell membrane had taken place, so that maturation could occur after cellular disruption. Since our biochemical studies failed to distinguish between these models, we turned to electron microscopy. We reasoned that examination of thin sections might fail to differentiate nearly mature virions from mature virions precipitated onto the surface of the cell. Therefore, we examined cells using the freeze-etching technique (8, 13). This method is well suited for examining membranes with their associated structures because it allows observations of large contiguous areas of their surfaces. These results were then compared with those obtained by conventional ultrathin-sectioning techniques.

MATERIALS AND METHODS

Cells, media, and viruses. Methods for the growth and titration of Sindbis virus in primary cultures of chicken embryo fibroblasts have been described (12). Cultures were infected by allowing 20 to 100 plaque-forming units (PFU) per cell to adsorb to the mono-

layers for 1 hr at room temperature from Hanks balanced salt solution (BSS). Low ionic strength medium (sometimes termed inhibitory medium) and low ionic strength BSS had ionic strengths of 0.105. They were prepared by reducing the amount of NaCl as described previously (15).

Fixation. At 6.5 hr after infection, cells were washed in low or normal ionic strength BSS and fixed for electron microscopy with 2 to 3% formaldehyde or glutaraldehyde in phosphate-buffered fixatives (*p*H 7) of the same ionic strength (i.e. 0.045 M or 0.068 M) as the medium in which the cells were incubated just before fixation. The low ionic strength fixative was brought to the same osmotic pressure as the normal by the addition of sucrose.

Formaldehyde was used as a fixative to avoid the marked cross-linking effect of glutaraldehyde (6). We felt that the dialydehyde might bind newly released virions to the cell surface, especially in cultures that were shifted from low to normal ionic strength. These cultures are known to release virus at about 3,000 PFU/cell in the first few minutes after shift (15), and we wished to avoid attaching these virions to the cells during fixation.

Electron microscopy. Cells were prepared for freeze etching as described in the accompanying paper (3). For thin sectioning, cultures were grown in 250-ml plastic tissue culture flasks (Falcon), and were flat-embedded by the method of Brinkley, et al. (2). Thin-sectioned and freeze-etched preparations were observed in a Siemens Elmiskop 101.

Detection of virus released during fixation. After infection, cultures were incubated for 1 hr in low ionic strength medium containing actinomycin D (1 μg/ml). This medium was replaced with similar medium containing ^{3}H-uridine (2.5 to 50 μCi/ml, 20 Ci/mmole, Schwartz BioResearch). Incubation was continued for an additional 5.5 hr. One set of cultures was then shifted to normal medium that was harvested 30 min later. Other cultures were fixed with glutaraldehyde or formaldehyde in low ionic strength solutions. After 1 hr of fixation, the solutions were decanted, Millonig's buffer (reference 7; ionic strength 0.37) was added, and the cultures were incubated at 37 C for an additional hour. Virus in the medium from the shifted cultures, the two fixatives, and the Millonig's buffer were purified by gradient ultracentrifugation as described by Scheele and Pfefferkorn (12). Fixation apparently did not significantly alter the density of the virus because a single symmetrical peak of radioactivity was found at approximately the same position on all gradients.

RESULTS

All cultures in these experiments were processed for electron microscopy 6.5 hr after infection. This relatively early time, about 3 hr into the 7-hr linear phase of virus production (15), was chosen to avoid any artifacts accompanying cellular disorganization late in infection.

Freeze etching. Figure 1 shows the surface of a Sindbis virus-infected cell, incubated in normal medium and fixed with glutaraldehyde. Prior to

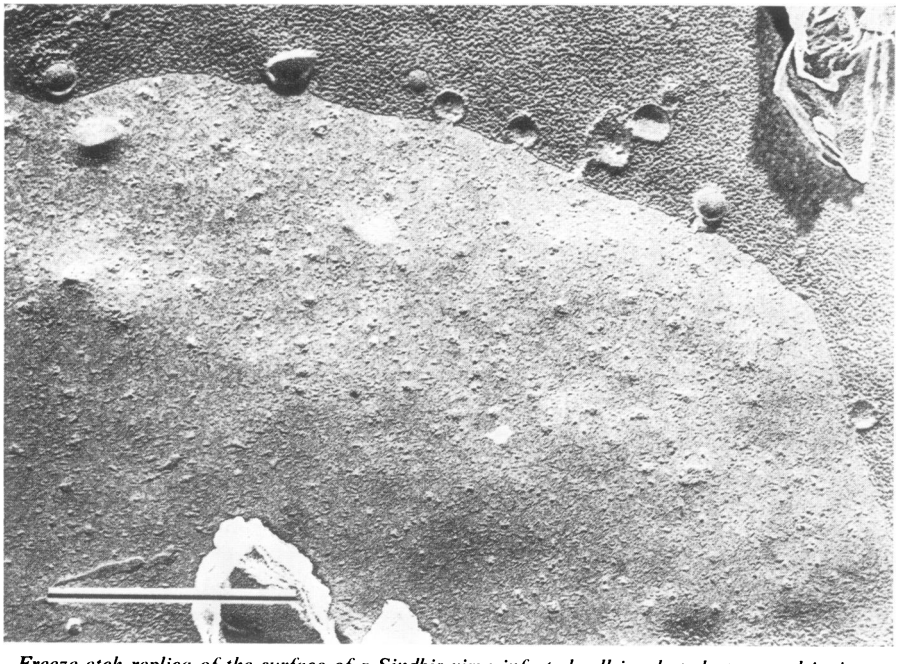

FIG. 1. *Freeze-etch replica of the surface of a Sindbis virus-infected cell incubated at normal ionic strength for 6.5 hr and fixed in glutaraldehyde. Some nearly mature virions are seen at the level of the ice. Magnification bar is 0.5 μm.*

fixation, the culture had released 2,000 PFU/cell into the medium, or about 11 PFU per cell per min. The surface of the cell membrane shows one small bump that probably represents the beginning of a viral bud (3). Along the periphery at ice level are two intact virions. Several others were split by the cleavage plane, thereby revealing an inner surface of the viral envelope which is continuous with the membrane of the cell. This relatively small number of budding virions is characteristic of a normal, infected cell. Since this culture released about 11 PFU per cell per min, and the particle per PFU ratio of Sindbis virus is about 5 (9), only 55 particles/cell are released each minute. Since only a small fraction of the cell surface is visible, only a few budding virions would be expected.

Cells incubated in low ionic strength medium were fixed with glutaraldehyde or formaldehyde in phosphate-buffered solution of the same ionic strength. The membrane surfaces of inhibited cells typically showed no signs of virus budding. It was possible to find occasional areas with immature buds resembling that on the surface of the cell in Fig. 1. These were rare, however, and very few mature or nearly mature virions of the type seen at ice level in Fig. 1 were found.

The above results suggested that viral budding was inhibited by the low ionic strength medium. Restoring the ionic strength of the medium to normal results in the rapid release of large numbers of virions (15). We decided, therefore, to examine inhibited cells that were fixed 1.5 min after exposure to normal medium. A typical view of such a culture, which had released 500 PFU/cell between reversal of the inhibition and fixation, is shown in Fig. 2. All the small bumps on the surface of the cell represent *budding* virus. This enhancement of the number of buds by the normal ionic strength medium confirms the idea that maturation had been inhibited by the low ionic strength medium.

Identical results were obtained in the above experiments regardless of whether the cells were fixed with glutaraldehyde or formaldehyde.

Thin sections. To amplify the results obtained with freeze etching, we examined ultrathin sections of similar cultures. In confirmation of the freeze-etching data, a typical section of a control cell at 6.5 hr after infection showed few budding virions and few nucleocapsids underlying the membrane. However, in some areas, large numbers of nucleocapsids were visible under the membrane (Fig. 3a) or budding from the surface of the cell (Fig. 3b). Few mature virions were apparent, probably because they were washed

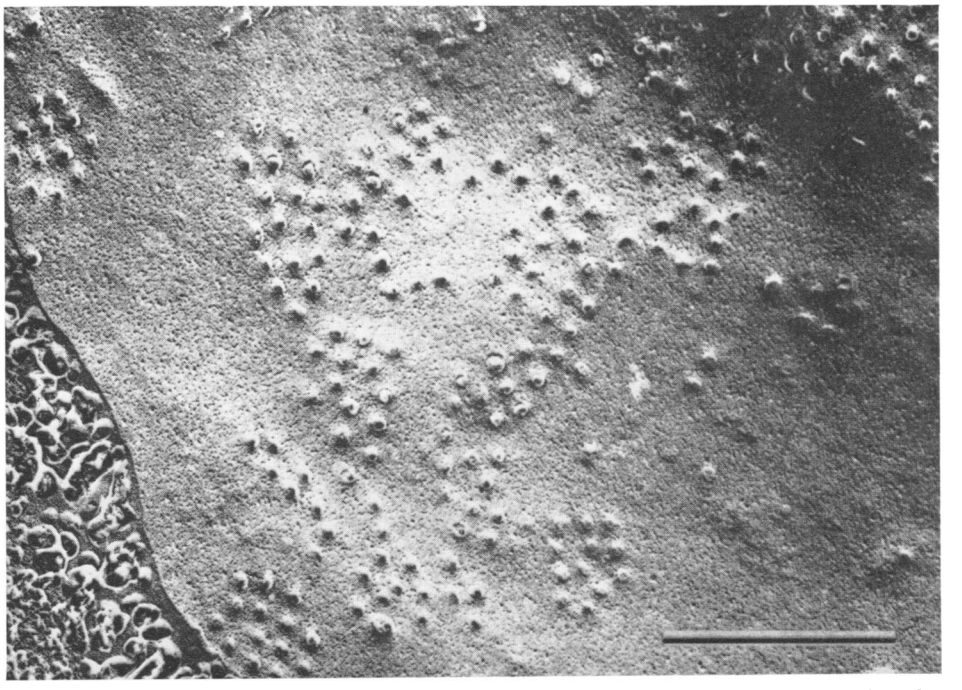

FIG. 2. *Freeze-etch replica of a Sindbis virus-infected cell shortly after reversal of inhibition. The culture was incubated for 6.5 hr in medium of low ionic strength, shifted to medium of normal ionic strength for 1.5 min, and fixed with formaldehyde. Magnification bar is 0.5 μm.*

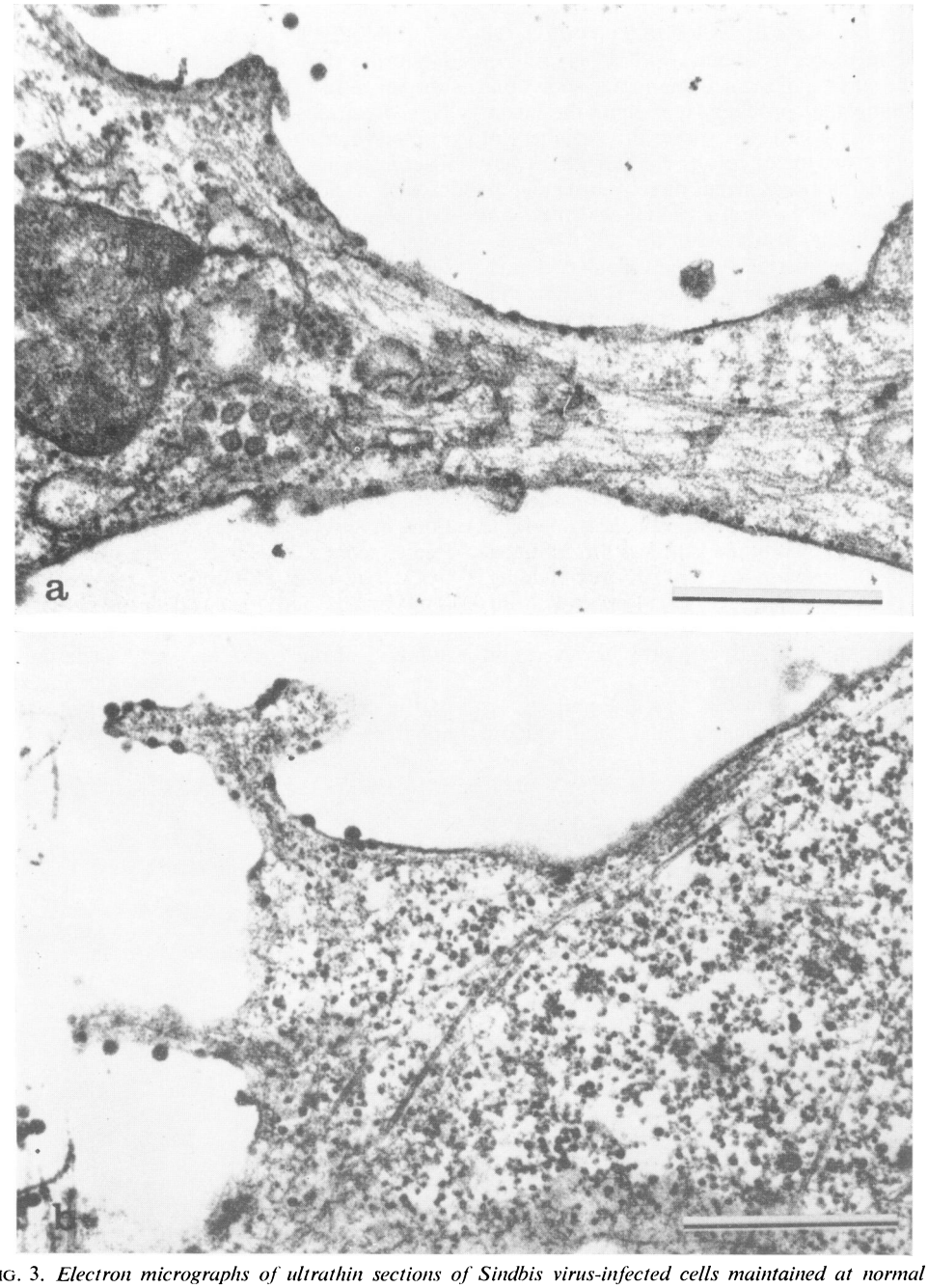

FIG. 3. *Electron micrographs of ultrathin sections of Sindbis virus-infected cells maintained at normal ionic strength for 6.5 hr. The preparation shown in 3a was fixed in glutaraldehyde, whereas that in 3b was fixed in formaldehyde. Magnification bars are both 0.5 μm.*

away when the culture was rinsed before fixation. Control cultures fixed with glutaraldehyde (3a) or formaldehyde (3b) were similar, except that the cytoplasmic morphology was better preserved by the glutaraldehyde.

The differences between control cultures and those incubated in low ionic strength medium and fixed with formaldehyde were mainly quantitative. More areas resembled the views shown in Fig. 3 with accumulations of capsids under the

membrane and virions starting to bud. In addition, there were also areas of the inhibited cells which were unique. Cross-sections of two such cells are presented in Fig. 4. Here the nucleocapsids are very closely arrayed under the membrane.

Inhibited cultures fixed with formaldehyde appeared to have more mature virions in the medium

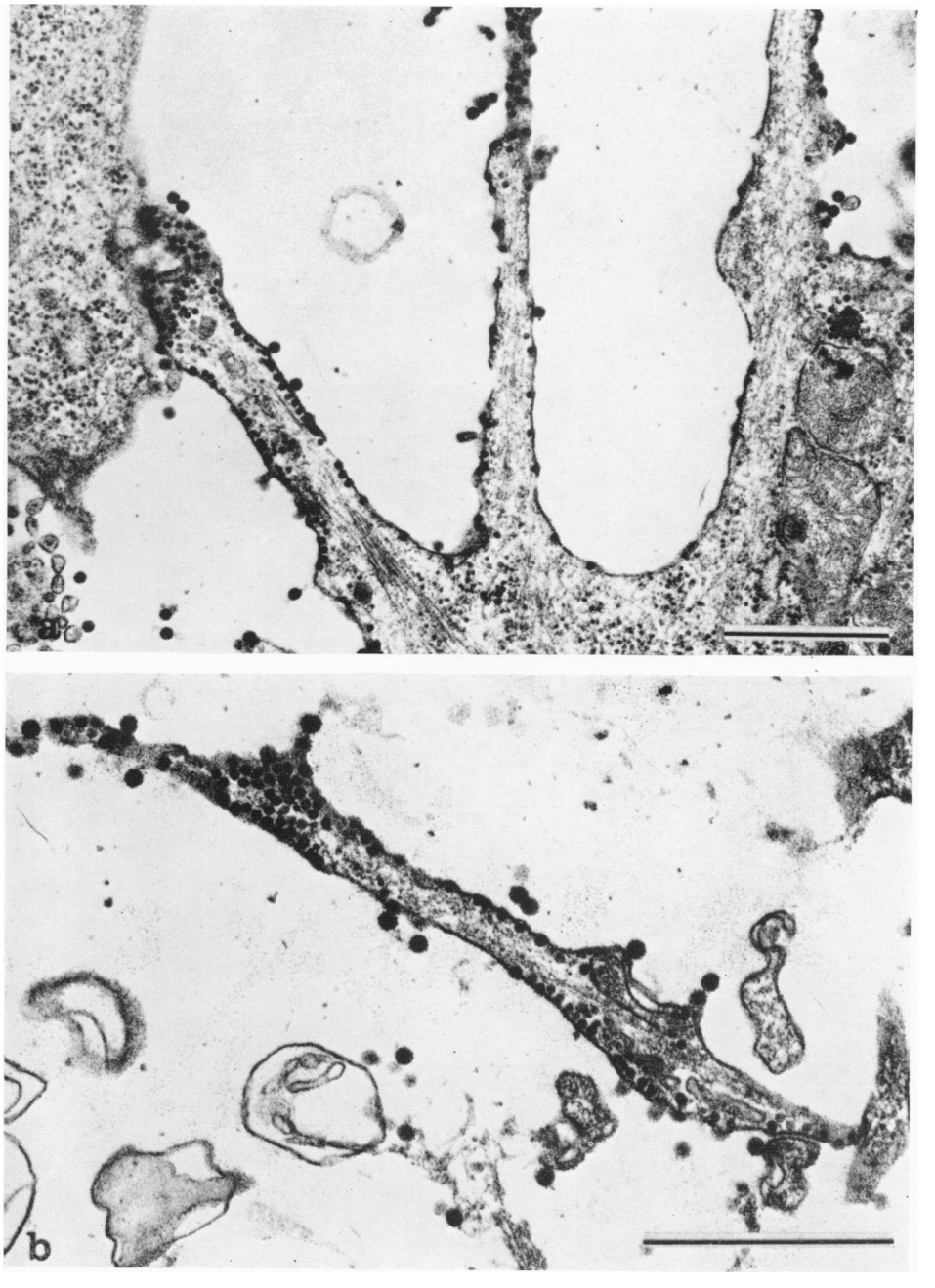

FIG. 4. *Ultrathin sections of Sindbis virus-infected cells incubated at low ionic strength for 6.5 hr and fixed with formaldehyde. Magnification bars are for 4a, 1.0 μm; for 4b, 0.5 μm.*

surrounding the cells than normal control cultures. This was surprising, because we had found less than 1 PFU/cell in the inhibitory medium prior to fixation. The use of glutaraldehyde fixation provided an explanation, for it revealed that inhibited cultures contained many mature virions attached to the cells, often in paracrystalline arrays (Fig. 5). The results with glutaraldehyde fixation suggested that much mature virus was present on the surface of the inhibited cells.

We thought that the failure to find many extracellular virions in formaldehyde-fixed, thin-sectioned preparations might be explained by a difference in the cross-linking properties of the two fixatives, since glutaraldehyde, a dialdehyde, cross-links more efficiently than formaldehyde (6). Thus, the virions not found in association with the cells after formaldehyde fixation should be found in the fixative.

To determine whether this was the case and to quantitate the amount of virus released during fixation, we treated inhibited cultures with actinomycin D and labeled them with ^{3}H-uridine. At 6.5 hr after infection, one set of cultures was shifted from low to normal ionic strength medium; this normal medium was harvested after 30 min. Other sets of inhibited cultures were fixed in low ionic strength formaldehyde or glutaraldehyde fixatives. The quantity of radioactive virus released into each preparation was determined as described in the Materials and Methods section, and the results are presented in Table 1. The total amount of radioactive virus that could be released by inhibited cultures was measured by the shift to normal medium. As anticipated, the glutaraldehyde fixative contained only a small amount of radioactive virus, i.e. 6% of that released by the shifted cultures. The formaldehyde fixative also did not contain as much radioactive virus as the shifted cultures produced. In three experiments, the formaldehyde contained only 30 to 55% of the potential viral crop, and negligible amounts were found in the subsequent washes with Millonig's buffer.

The majority of the residual radioactive virus that was not released in the formaldehyde was not mature at the time of fixation for it was not seen in thin sections of formaldehyde-fixed cells. The synchronous maturation of this residual virus, when inhibited cells were shifted to medium of normal strength, presumably results in the striking increase in budding seen in freeze-etched preparations.

The failure to detect large paracrystalline arrays on the surface of inhibited, glutaraldehyde-fixed cultures examined by freeze etching can be explained in two ways, either separately or in

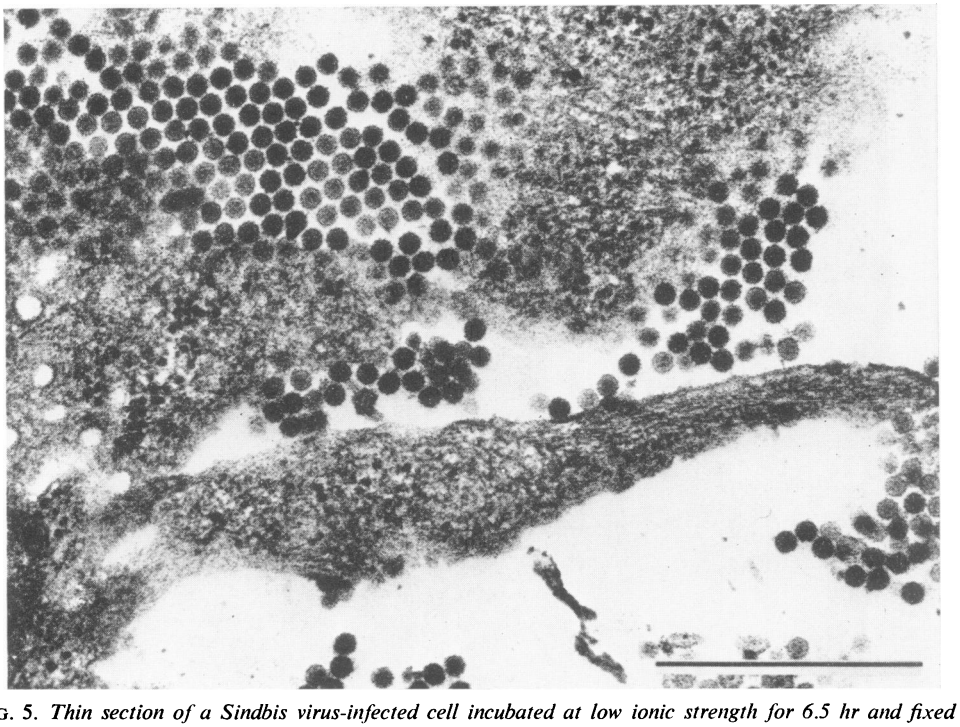

FIG. 5. *Thin section of a Sindbis virus-infected cell incubated at low ionic strength for 6.5 hr and fixed with glutaraldehyde. Magnification bar is 0.5 μm.*

TABLE 1. *Radioactive virus released by fixation of inhibited cultures*[a]

Fixative	Virus released into fixative (%)[c]	Virus subsequently released into Millonig's buffer (%)[c]
2% Formaldehyde. . . .	47[d]	9[e]
2% Glutaraldehyde. . .	6[e]	

[a] Infected cultures were incubated in low ionic strength medium containing actinomycin D (1 μg/ml) for 1 hr and then exposed for 5.5 hr to similar medium containing from 2.5 to 50 μCi of ^{3}H-uridine per ml. Some cultures were then shifted to normal medium at 37 C for 30 min (controls) and the remainder were fixed with the indicated fixative at room temperature for 1 hr. After fixation, they were exposed to Millonig's phosphate buffer (7) for 1 hr at 37 C. The solutions were analyzed by ultracentrifugation (12), and the radioactivity in the virus bands was compared. In three independent experiments, control cultures released between 10,000 and 550,000 counts/min of labeled virus.

[b] In low ionic strength phosphate buffer.

[c] Counts per minute per monolayer as percentage of shifted controls.

[d] Triplicate experiments.

[e] Duplicate experiments.

combination. (i) The cross-linking effect of glutaraldehyde may not be sufficient to hold the mature virions on the surface of the cell during high vacuum etching. Clark and Branton have shown that free particles such as latex beads are lost from the fracture face under similar conditions (4). (ii) The gentle handling of the virus-cell complexes in the flat-embedding procedure employed here may have retained, in thin-sectioned preparations, the loosely bound virions which were removed by the scraping and centrifugation involved in preparing cells for freeze etching.

DISCUSSION

A relatively small number of buds are seen on the surface of fixed, inhibited cells when they are examined by freeze-etching. A large number of buds, far exceeding anything in control cells, are seen if the cultures are fixed shortly after reversal of the inhibition. Thus, low ionic strength medium appears to inhibit the budding of Sindbis virus from infected cells.

Initially we thought that low ionic strength medium simply inhibited a very early stage in budding, after the attachment of the nucleocapsid to the membrane and before the membrane had begun to roll around the capsid. Precipitation of extracellular virus seemed unlikely because Sindbis virus is soluble in solutions with the ionic composition of the inhibitory medium (14, 15). Unfortunately, examination of thin sections of inhibited cells reveals that this inhibition is not as simple as we had hoped.

Much mature extracellular virus was attached to the surface of inhibited cells fixed with glutaraldehyde at low ionic strength. Formaldehyde fixation released almost half of the "inhibited" virus from the monolayers. Comparison of the results obtained from thin sectioning and freeze etching suggests that about half of the potential virus crop was presumably immature and was represented by intracellular nucleocapsids that could bud rapidly upon exposure to medium of normal ionic strength; the other half was apparently mature and could either be released by formaldehyde or bound to the surface of the cell by glutaraldehyde. Why are some virions released in a mature form whereas others remain immature within the inhibited cell?

We assume that medium of low ionic strength partially inhibits the nucleocapsid-membrane interaction that follows attachment and initiates the budding process (see the transition of stage a to b in Fig. 1 of the accompanying paper; reference 3). This inhibition results in the accumulation of membrane-associated nucleocapsids that are capable of rapid maturation upon exposure to medium of normal ionic strength. Those nucleocapsids that circumvent this inhibited step rapidly progress through the remaining morphogenetic stages and are released as mature virions. However, the low ionic strength of the extracellular fluid precipitates these virions onto the cell surface. Thus, although the inhibition is not complete, the use of low ionic strength medium provides the only way of reversibly inhibiting the maturation of a membrane-bound virus.

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