

## Replication of Sindbis Virus

### IV. Electron Microscope Study of the Insertion of Viral Glycoproteins into the Surface of Infected Chick Cells

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Received for publication 15 April 1974

The appearance of Sindbis virus-envelope glycoproteins in the surfaces of chicken embryo fibroblasts was studied by an indirect labeling technique. This technique involved treating infected cells sequentially with rabbit immunoglobulin G (IgG) specific for Sindbis virus followed by hemocyanin-conjugated goat (anti-rabbit IgG) IgG; surface replicas of these cells were then prepared and examined in the electron microscope. As early as 2 h after infection (and at least 1 h before mature virions were released), newly synthesized virus-envelope glycoproteins were detected at the cell surface. By 3 h after infection, cell surface membranes were extensively modified by the insertion of the Sindbis glycoproteins. When infected cells were prefixed with glutaraldehyde before labeling, the glycoproteins were distributed fairly evenly over the cell surface, although a slight clustering was observed on cells labeled early in infection. However, no evidence for large-scale clustering of virus glycoproteins corresponding to patches of budding virus was observed. Similar results were found with unfixed cells labeled at 4 C. However, when unfixed cells were labeled at 37 C, the glycoproteins were shown to be in discrete clusters, demonstrating that these glycoprotein antigens can diffuse laterally through the cell membrane at this temperature.

Sindbis virus, a group A arbovirus, consists of a lipoprotein envelope surrounding a nucleocapsid or "core" (1, 22, 25). The membrane contains two glycoproteins (19, 24, 25) and the lipid of the virus; the core contains the viral RNA complexed with a nucleocapsid protein (24, 25). Since the membrane of the virus is acquired as the nucleocapsid buds through the cell surface (1, 12), the current model for virus maturation involves insertion of the two envelope glycoproteins into the plasma membrane and budding of virus through these regions. Some of the other groups of enveloped viruses have a similar mechanism of release. Host membrane glycoproteins must be rearranged during this process since the only proteins found in the virus are synthesized de novo after infection (14, 24), being encoded in the viral RNA (D. T. Simmons and J. H. Strauss, *J. Mol. Biol.*, in press). On the other hand, the majority of the phospholipids of the virus preexist in the uninfected cell (15). Little is known about how the envelope glycoproteins are incorporated into the cell surface, but because Sindbis is a relatively

simple virus, it offers a good system for studying the mechanisms of virus-specific changes in cell surfaces.

One approach to studying the alteration of the plasma membrane after virus infection is to label the infected cell surface with markers specific for viral antigens and then to examine these cells under the electron microscope. We have recently examined Sindbis virus-infected cells using the surface replica technique, with which we can examine large areas of the cell surface (4). We used this technique to study the insertion of Sindbis envelope glycoproteins into the cell surface. Infected cells were labeled with rabbit immunoglobulin G (IgG) specific for Sindbis virus followed by labeling with a conjugate goat (anti-rabbit IgG) IgG coupled to hemocyanin. Hemocyanin can be easily identified in replicas, and the location of the viral glycoproteins is indicated by the position of hemocyanin on the cell surface.

#### MATERIALS AND METHODS

**Cells and virus.** Secondary cultures of chicken embryo fibroblasts were seeded in 35-mm petri plates containing a 12-mm cover glass, using Eagle minimal

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essential medium supplemented with 10% fetal calf serum. After 18 to 24 h at 37 C, when the cells were still subconfluent, they were infected with approximately 50 PFU per cell of Sindbis virus (HR strain) at 37 C, as previously described (5) except without actinomycin D.

**Antisera.** Sindbis virus was purified by polyethylene glycol precipitation followed by sucrose gradient velocity and isopycnic centrifugations, as previously described (16, 24). The virus was dialyzed against phosphate-buffered saline, pH 7.4 (PBS of Dulbecco and Vogt [10] but lacking Ca and Mg) at 4 C before injection. Antiserum against purified Sindbis virus was prepared in rabbits by a toe pad injection of 250  $\mu$ g of Sindbis mixed with an equal volume of complete Freund adjuvant. Three weeks later the same amount of virus with adjuvant was injected intraperitoneally. Three days after the second injection, 250  $\mu$ g of Sindbis was injected intravenously without adjuvant. Rabbits were bled by cardiac puncture on the 8th and 9th days after the final injection. The serum was collected and adsorbed against uninfected chick cells by incubating serially 10 ml of serum for 30 min at 37 C in roller bottles with confluent monolayers of chicken embryo fibroblasts (about  $10^8$  cells per bottle). Anti-Sindbis activity in the serum was monitored by hemagglutination inhibition, using pigeon erythrocytes (9); the hemagglutination inhibition titer was found to be  $10^4$ . The IgG fraction was prepared by ammonium sulfate precipitation followed by DEAE-cellulose chromatography (8). This fraction gave one precipitation band when tested by immunodiffusion against goat (anti-rabbit IgG) serum.

Goat (anti-rabbit IgG) IgG was prepared by the method of Avrameas and Ternynck (3). Purified rabbit IgG (Miles Laboratories) was cross-linked with glutaraldehyde (TAAB, England) and used to pour an immunoadsorbent column. Goat (anti-rabbit IgG) serum (Miles Laboratories) was then passed through the column; the adsorbed goat IgG was subsequently eluted with a low-pH buffer.

**Hemocyanin conjugation.** Hemolymph was collected from snails (*Busyon canaliculatum*). Particulate material was removed by centrifugation at  $12,000 \times g$  for 10 min, and the hemocyanin was then collected by centrifugation at  $150,000 \times g$  for 30 min at 4 C. The hemocyanin pellet was dissolved in PBS overnight at 4 C, and large aggregates were removed by centrifugation at  $12,000 \times g$  for 10 min at 4 C. The supernatant was applied to a column (2.5  $\times$  100 cm) of agarose (Bio-Gel A-1.5 m; Bio-Rad Laboratories) in PBS. The void volume fractions, which contain the hemocyanin, were pooled, filtered through a 0.22- $\mu$ m membrane filter (Millipore Corp.), and stored at 4 C.

The conjugation procedure is essentially that of Avrameas (2), with all reagents prepared in PBS. Goat (anti-rabbit IgG) IgG and hemocyanin were concentrated on Amicon PM-30 membranes to 20 and 70 mg/ml, respectively, and 1 ml of each was mixed together. While the mixture was vortexed, 0.5% glutaraldehyde was added slowly to a final concentration of 0.05% glutaraldehyde. The conjugation was allowed to proceed for 45 min at room temperature, after which glycine was added to a final concentration of 0.1 M. After 15 min, the mixture was subjected to

centrifugation at  $12,000 \times g$  for 10 min at 4 C and the supernatant was dialyzed exhaustively against PBS at 4 C. The dialyzed conjugate was then applied to an A-1.5 m column, and the void volume fractions were pooled, filtered, and stored at 4 C. The purified goat (anti-rabbit IgG) IgG-hemocyanin conjugate (G-Hcy) gave one precipitation band against rabbit IgG in immunodiffusion agar gels.

**Cell labeling.** All reagents were prepared in PBS. At various times after infection, cover glasses with cells on them were washed several times in PBS and treated in one of the following ways.

(i) Prefixation. Cells were prefixed on ice in 1% glutaraldehyde for 10 min, rinsed with PBS, treated with 0.15 M glycine at 37 C for 10 min to remove any unreacted glutaraldehyde, and rinsed again. Cells were then treated with anti-Sindbis IgG (500  $\mu$ g/ml) at 37 C for 10 min, rinsed with PBS, treated with G-Hcy (1 mg/ml) at 37 C for 10 min, and finally rinsed again with PBS. Control (uninfected) cells were treated in the same way.

(ii) No prefixation. Cells were treated as above, except that the prefixation and glycine treatments were omitted. For labeling at 4 C, cells were rinsed with cold PBS and then treated with anti-Sindbis IgG for 10 min at 4 C followed by G-Hcy for 10 min at 4 C. For labeling at 37 C, all operations were performed at 37 C.

**Electron microscopy.** Fixation of cells and preparation of surface replicas for electron microscopy has been previously described (4). Replicas were examined on a Philips model 300.1 electron microscope. All electron micrographs are presented in reverse contrast.

## RESULTS

**Control experiments.** It was found necessary to adsorb the rabbit anti-Sindbis antiserum against uninfected chick cells to remove interfering components from the antibody preparation, thus producing an IgG preparation specific for infected cells. When uninfected cells were treated with anti-Sindbis IgG and G-Hcy, very little hemocyanin could be detected on the cell surface (Fig. 1 and 2). These figures illustrate, however, that the binding of hemocyanin to the background (i.e., the protein layer deposited from the serum on the glass substrate; ref. 17) was quite variable from experiment to experiment. This background label appeared to be nonspecific, although its origins were unclear. A similar nonspecific binding to the background was found when hemocyanin was used to study the distribution of concanavalin A binding sites on the surface of cells (18, 23). Thus, although this background labeling is poorly understood, it does not interfere with the interpretation of results of binding to the cell surface and was ignored in these experiments.

**Time of appearance of Sindbis virus glycoproteins at the cell surface.** All of the labeling experiments discussed in this section were

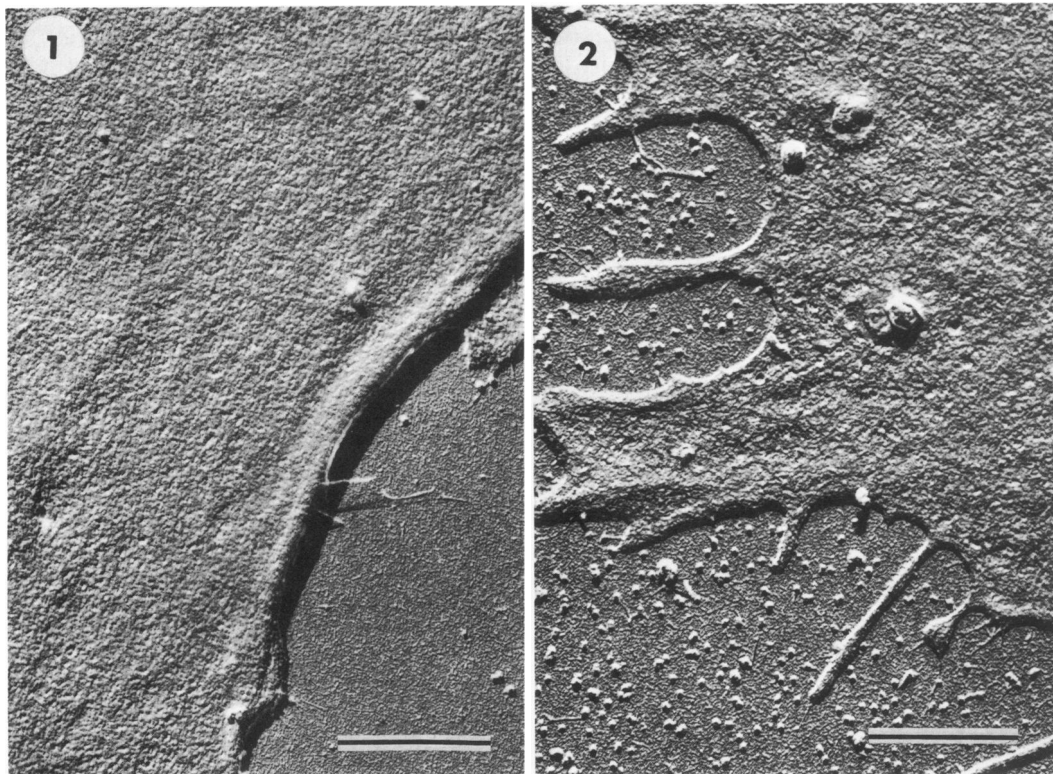


FIG. 1 AND 2. Surface replicas of prefixed, uninfected cells treated with anti-Sindbis IgG and G-Hcy at 37 C, showing light and heavy background labeling with hemocyanin, respectively.  $\times 20,000$ . Scale bars are 1  $\mu\text{m}$ .

done on prefixed cells to prevent clustering artifacts caused by the use of multivalent reagents.

At 1 h after infection (Fig. 3), there was little or no detectable hemocyanin on the cell surface, illustrating that under our conditions the antigens contributed by the inoculum did not interfere with the assay. However, by 2 h after infection, significant amounts of hemocyanin were seen (Fig. 4 and 5). The cell in Fig. 4 was lightly labeled and represents a very early stage in the insertion of the viral glycoproteins. The hemocyanin label was fairly evenly distributed over the surface of the cell, although close inspection reveals that the distribution was not random. For example, there is an area in the right center of the picture (arrow) where 15 to 20 hemocyanin molecules are found, whereas areas of comparable size to the right (at the edge of the picture) and to the left are almost devoid of hemocyanin. The cell in Fig. 5 was more heavily labeled. Again the label was fairly evenly distributed over the surface, but there was some clustering of the hemocyanin. This is illustrated by two adjacent circles of equal size on the picture. One encircles about 10 hemocyanin

molecules, the other none. This slight clustering may represent a real deviation from a random distribution. However, it might be an artefact of the labeling procedure or a consequence of statistical fluctuations. In any event, when large areas of the cell surface are considered the hemocyanin is randomly distributed; the virus glycoproteins do not preferentially accumulate in particular regions as might be expected from previous observations that the virus matures in patches (4, 6). This uniformity of label also implies that the viral glycoproteins are either inserted randomly or else diffuse rapidly from the site of insertion to take up a uniform configuration.

The cell in Fig. 6 was infected for 2.5 h, and illustrates that at this time a few of the cells were heavily labeled. By 3 h after infection most cells were heavily labeled (Fig. 7). The label is fairly evenly distributed, with no detectable tendency to form patches, at least on a large scale.

The cell in Fig. 8 was infected for 5.5 h. Virus-specific processes where most virus release occurs are evident (arrow) and are solidly packed with hemocyanin, as expected, since

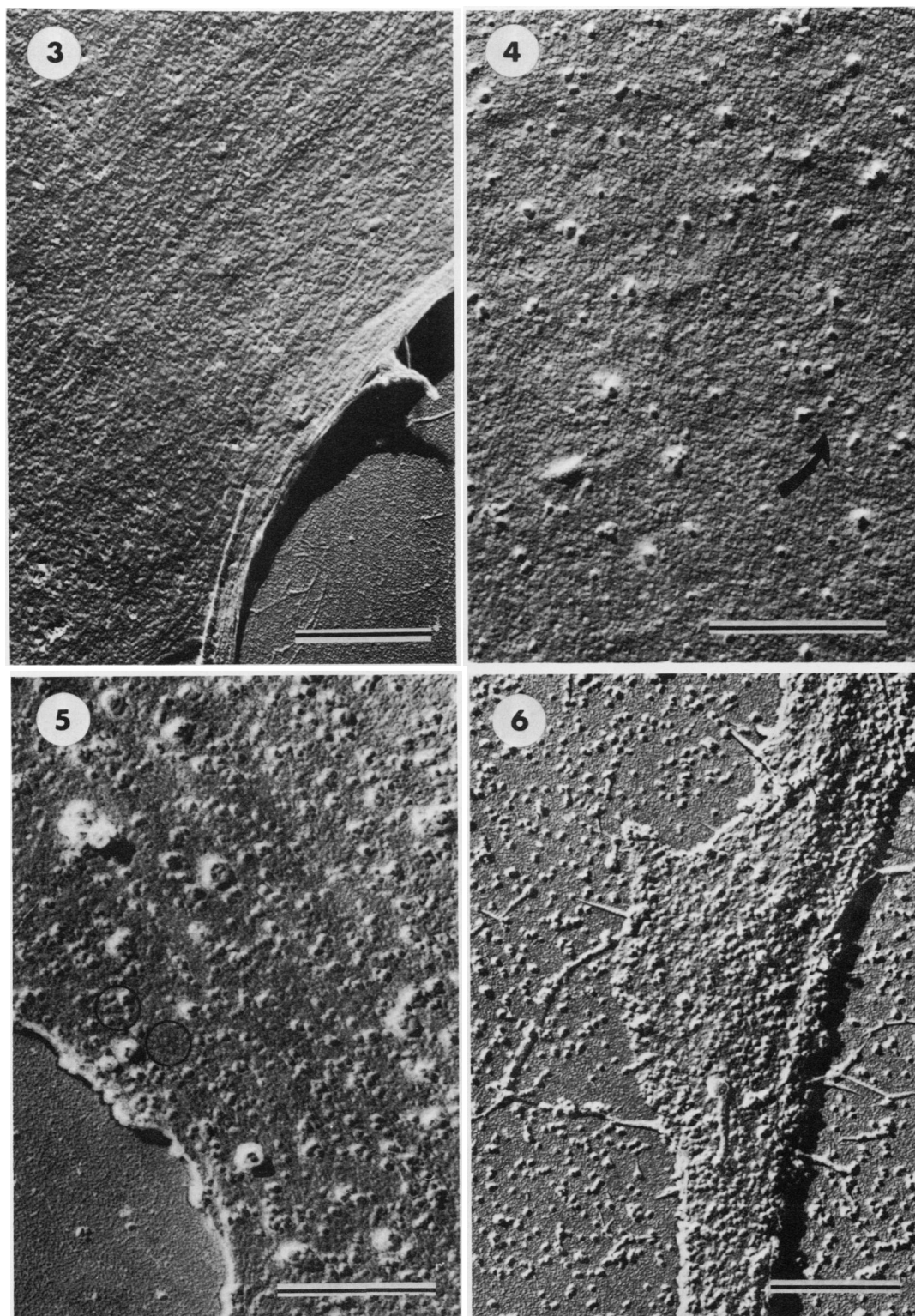


FIG. 3-6. Surface replicas of prefixed cells treated at 37 C with anti-Sindbis IgG and G-Hcy at 1 (Fig. 3), 2 (Fig. 4 and 5), and 2.5 h (Fig. 6) after infection with Sindbis virus. The arrow in Fig. 4 indicates a slight clustering of hemocyanin; the circles in Fig. 5 show a cluster of hemocyanin adjacent to an area with no hemocyanin. Fig. 3,  $\times 20,000$ ; Fig. 4,  $\times 26,000$ ; Fig. 5,  $\times 24,000$ ; Fig. 6,  $\times 20,000$ . Scale bars are 1  $\mu\text{m}$ .



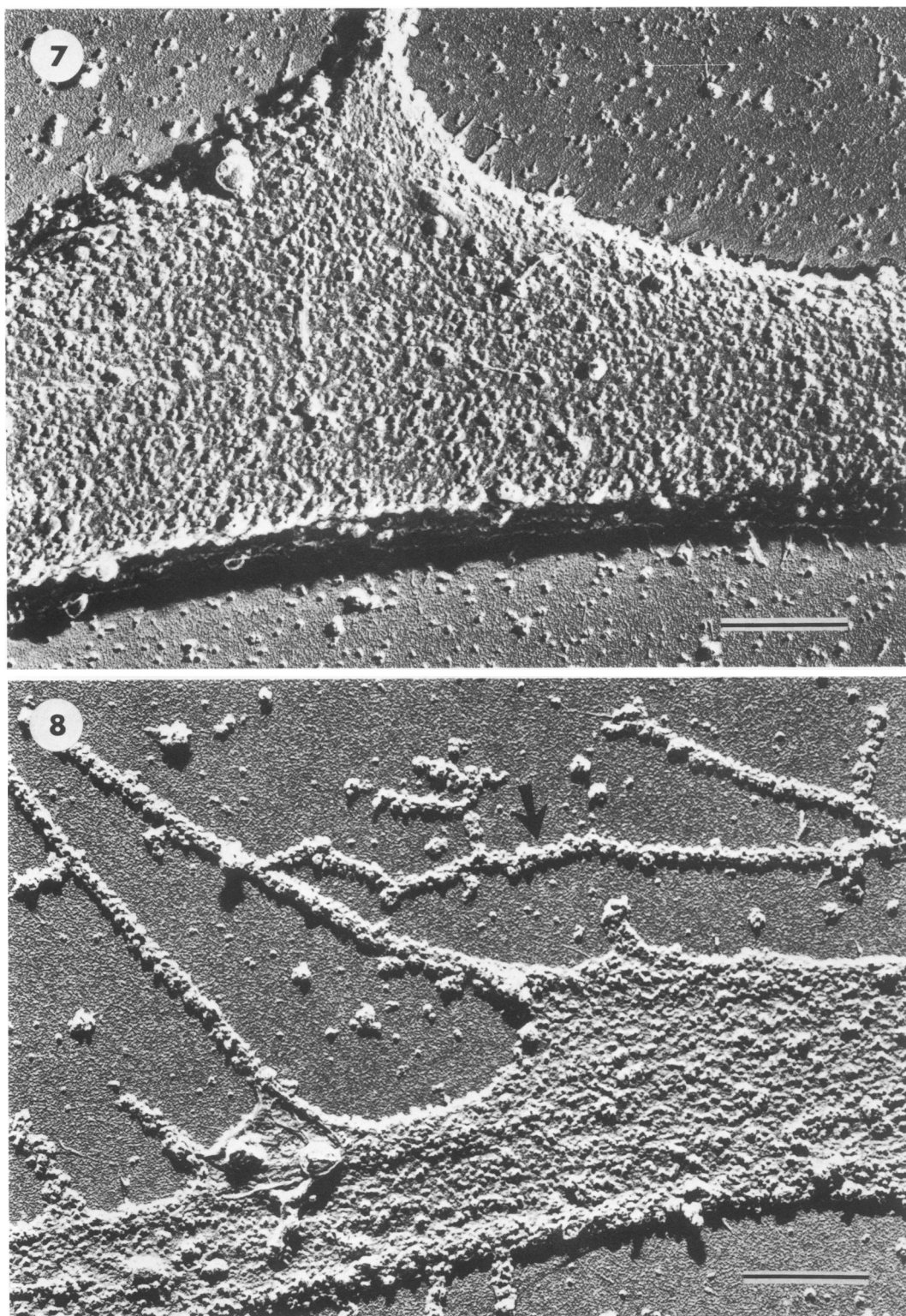


FIG. 7 AND 8. Surface replicas of prefixed cells treated at 37 C with anti-Sindbis IgG and G-Hcy at 3 and 5.5 h after infection with Sindbis virus, respectively. The arrow in Fig. 8 indicates a virus-specific process.  $\times 20,000$ . Scale bars are 1  $\mu\text{m}$ .

these processes contain a linear array of maturing virions (4). The remainder of the cell was also heavily labeled, however, although somewhat less so than the processes. In Fig. 9 is shown a cell at 6.5 h after infection. This cell was very heavily labeled with hemocyanin over its entire surface. Several virus particles are seen lying on the background, coated with hemocyanin; one such particle is circled. Numerous clumps of hemocyanin of similar size are seen over the surface of the cell, probably representing budding virus; one of these clusters is also circled.

To compare the insertion of virus glycoproteins to the release of virus at various times after infection, surface replicas were prepared of infected cells that had not been treated with rabbit anti-Sindbis IgG and G-Hcy; these experiments were done simultaneously with the labeling experiments. At 3 h after infection, the few cells that were releasing virus had only a few budding figures on the cell surface. Substantial numbers of budding virions were not detected until 3.5 to 4 h after infection, in agreement

with our earlier studies (4). Even by 5.5 h after infection, many more cells were labeled with hemocyanin than were releasing virus.

Thus the virus glycoproteins are found fairly evenly distributed over the surface of the cell, and insertion of these proteins precedes virus maturation by 1 to 2 h.

**Labeling of infected cells at 4 C and 37 C without prefixation.** When infected cells were treated with anti-Sindbis IgG and G-Hcy at 4 C before fixation, the hemocyanin label was evenly dispersed over the cell surface, as was true for prefixed cells labeled at 37 C. However, when unfixed cells were treated at 37 C, the hemocyanin label was clustered. Figure 10 shows a cell with clustered labeling at 2 h after infection. This cell was lightly labeled, so the clustering is quite obvious. At 2.5 h after infection, when more glycoprotein has been inserted into the cell surface, many more clusters of hemocyanin were found on the cell than at 2 h after infection. Later in infection, treating unfixed cells at 37 C did not produce discrete clusters but rather large patches of hemocyanin

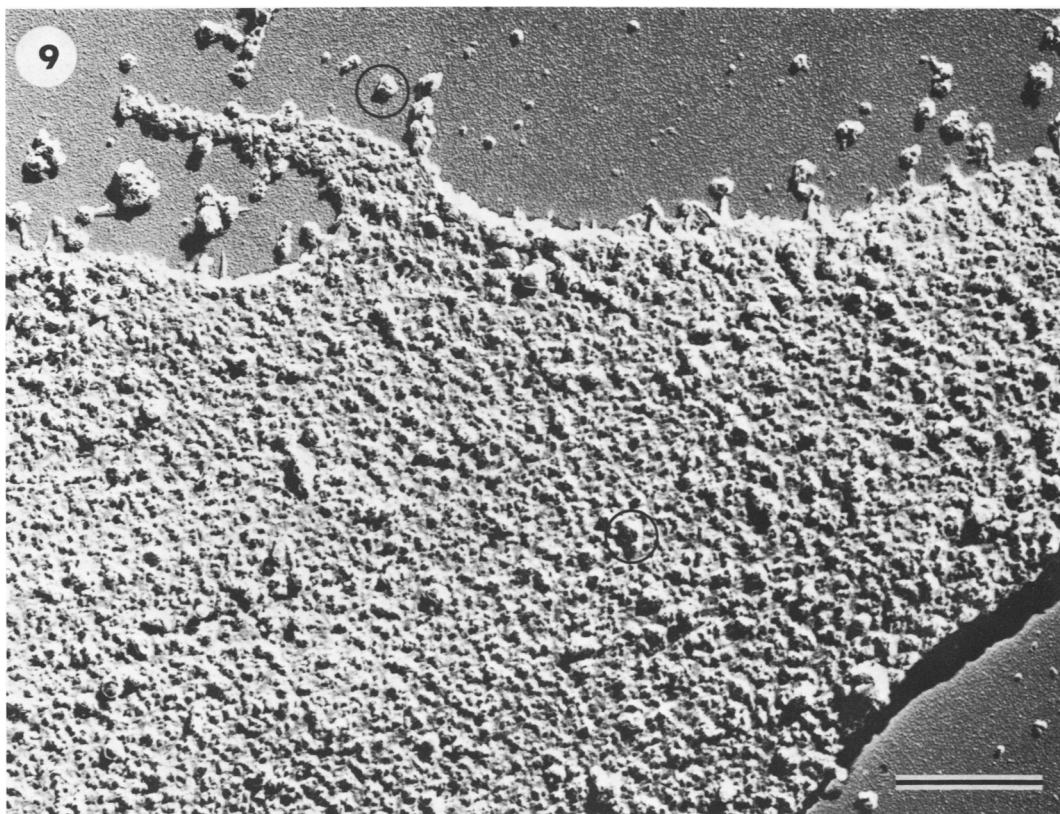


FIG. 9. Surface replicas of a prefixed cell treated at 37 C with anti-Sindbis IgG and G-Hcy at 6.5 h after infection with Sindbis virus. Circles indicate aggregates of hemocyanin molecules which are probably attached to released virions.  $\times 19,000$ . Scale bar is 1  $\mu$ m.

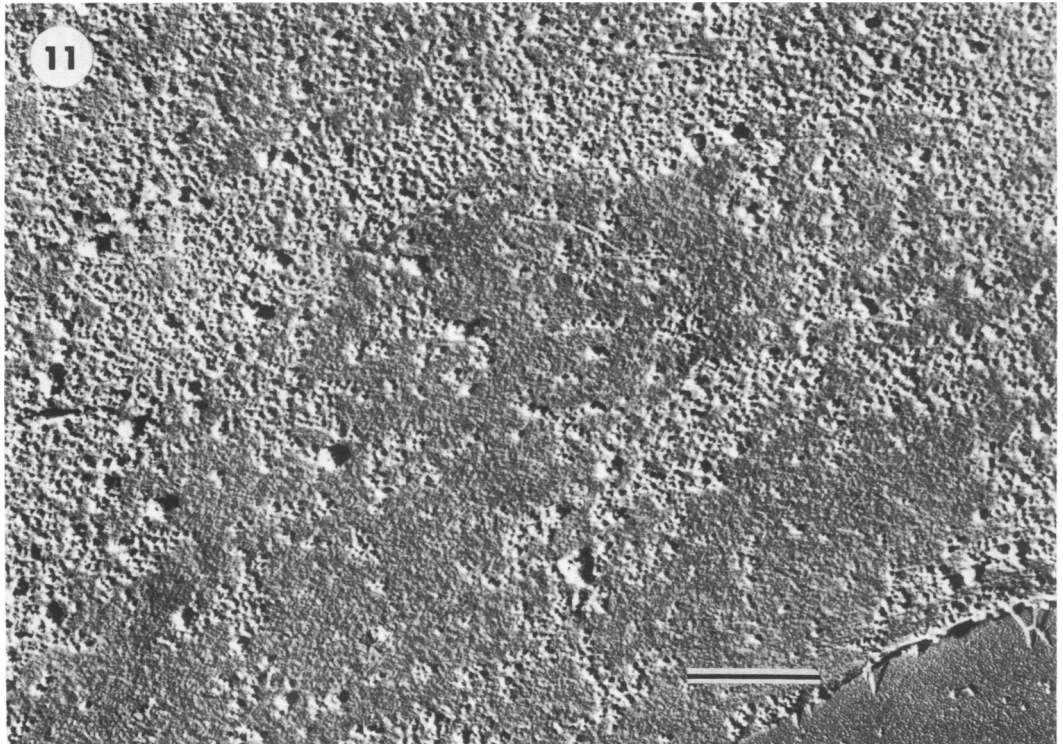
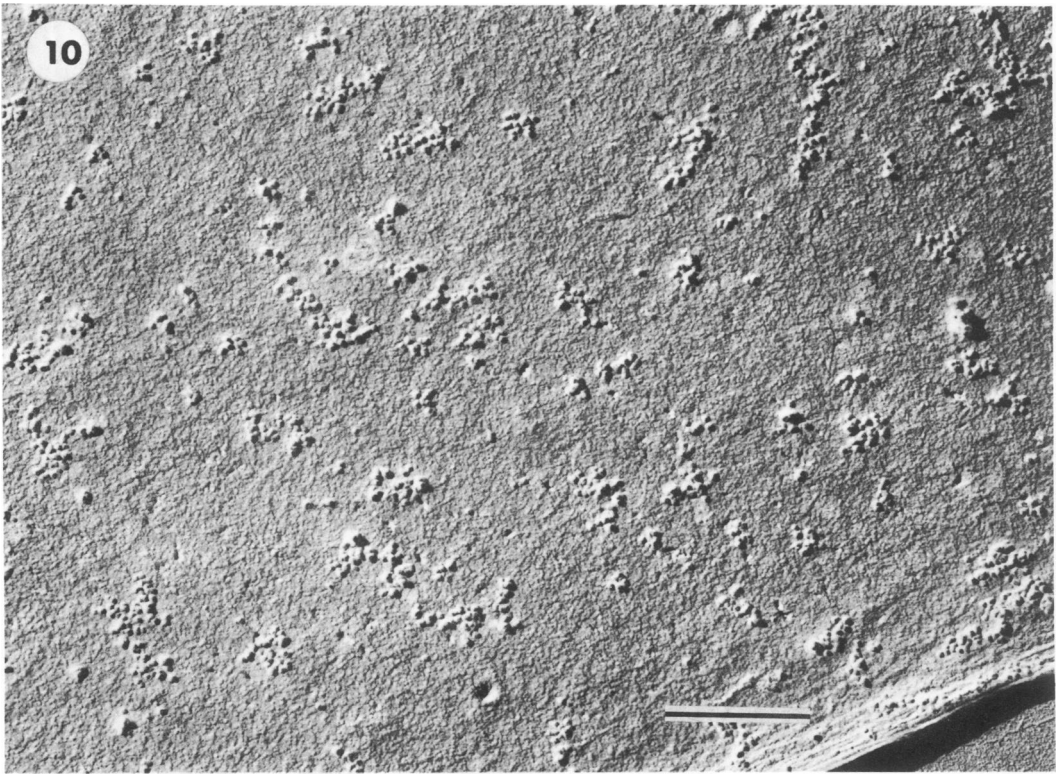


FIG. 10 AND 11. Surface replicas of unfixed cells treated at 37 C with anti-Sindbis IgG and G-Hcy at 2 and 3 h after infection with Sindbis virus, respectively, showing clustering of hemocyanin on the cell surface. Fig. 10,  $\times 20,000$ ; Fig. 11,  $\times 18,000$ . Scale bars are 1  $\mu\text{m}$ .

adjacent to large areas of the cell surface devoid of hemocyanin (Fig. 11). Clustering was also produced when cells were treated with anti-Sindbis IgG and G-Hcy at 4 C and subsequently allowed to warm to 37 C. Therefore, it would appear that under conditions in which membrane components are free to diffuse laterally, i.e., at 37 C and without prefixation, multivalent Sindbis antibody can cross-link the Sindbis virus glycoproteins which have been inserted into the cell surface and cause them to become clustered.

## DISCUSSION

Our results show that the insertion of Sindbis virus envelope glycoproteins into the plasma membrane of an infected cell begins as early as 2 h after infection and appears to occur fairly evenly over the entire cell surface (Fig. 4 and 5). By 3 h after infection the surface has been modified extensively (Fig. 7), although few virions have been released at this time. Moreover, once the glycoproteins are inserted into the cell surface, they appear to be able to move laterally through the membrane (Fig. 10 and 11).

The very early insertion of the glycoproteins into the plasma membrane as evidenced by the reaction with hemocyanin-conjugated antibody molecules agrees with the previous results of Burge and Pfefferkorn (7), who found that infected cells became capable of hemadsorption at 2 to 2.5 h after infection, at least 1 h before any mature virions were produced. From our data it appears that insertion of relatively few glycoprotein molecules is sufficient to cause this change.

Two other surface properties of Sindbis virus-infected cells occur somewhat later in the replication cycle, about 3 to 3.5 h after infection, when surface modification is much more extensive. One of these is the change in agglutinability of infected cells with concanavalin A (5); the other is the phenomenon of superinfection exclusion, where the amount of superinfecting Sindbis which can bind tightly to an infected cell declines rapidly at 3 h after infection, until by 7 h after infection the amount of tightly bound virus is only 10 to 20% of that to uninfected cells (16).

All of these effects begin before the production of mature virions. Although a few virions are produced at 3.5 h after infection, maximal linear release of infectious particles begins at about 4 to 4.5 h after infection and continues for many hours. There are several possibilities to explain the lag between initial insertion of the

glycoproteins and the release of virus from the cell surface. This may represent the time required to accumulate sufficient quantities of viral components, either glycoproteins or fully developed core particles. Another explanation is suggested by recent experiments on the biosynthesis of Sindbis glycoproteins. Pulse-chase experiments have shown that both envelope glycoproteins  $E_1$  and  $E_2$  appear concomitantly and in equal amounts in mature virions (20). However, the appearance of  $E_1$  in the cell surface membrane precedes  $E_2$ . It has been suggested that  $PE_2$  (a precursor of  $E_2$ ) and  $E_1$  exist as a complex, and virus release can only occur after the cleavage of  $PE_2$  to  $E_2$  (20). The proposed complex may be present in the surface membrane, although  $PE_2$  is not accessible to iodination by lactoperoxidase (21). The early indications of the presence of glycoproteins on the cell surface (either the antibody-labeling studies in this paper or the previous hemadsorption results) may be due to the insertion of  $E_1$  alone, or the  $PE_2$ - $E_1$  complex; the delay between these events and the eventual release of virus could be the time required for conversion of  $PE_2$  to  $E_2$ . Similarly, both  $E_2$  and  $E_1$  in the membrane may be necessary to produce changes in agglutinability and superinfection exclusion; it is also quite possible that concentration effects play the deciding role in one or both of these cases.

A virus particle contains several hundred glycoprotein molecules, and a budding virus particle represents a small patch of glycoprotein. On a large scale, previous studies have shown that the virus matures in patches (4, 6), with many virions budding from some regions of the cell and few or none from other regions. In addition, we have shown that the virus preferentially buds from the edge of the cell, either singly or in long processes. This existence of preferred budding sites is somewhat surprising in light of the fairly uniform distribution of virus glycoprotein over the surface of the cell. It is possible that even though the glycoproteins are inserted evenly (or diffuse rapidly to take up this configuration), they are later organized by nucleocapsids at the preferred regions into patches of one or more virions. It is also possible that during infection different regions of the cell surface release virus at different times, so that eventually the whole cell surface is used for virus maturation.

A random distribution of certain cell surface antigens and the lateral movement of glycoproteins through the cell surface has been reported in other systems (11, 13, 18, 23). Using either ferritin or hemocyanin as a label, the



distribution of concanavalin A binding sites on the surfaces of normal and transformed cells has been studied (13, 18, 23). In these studies, the concanavalin A binding sites on the surfaces of normal cells are randomly dispersed under all labeling conditions. Transformed cells, however, show a clustering of concanavalin A binding sites when the cells are labeled at 37 C without prefixation, suggesting that the transformed cell surface is more fluid than the normal cell surface and allows a rapid lateral diffusion of concanavalin A binding sites. We have also shown that the receptor sites for Sindbis virus on chick cells are randomly distributed over the cell surface when cells are prefixed, but are capable of clustering, even at 4 C, upon adsorption of virus (Birdwell and Strauss, *J. Virol.*, submitted for publication). Thus, as a cell surface marker the Sindbis glycoproteins show similarities to other surface markers that have been studied.

#### ACKNOWLEDGMENTS

Jean-Paul Revel played an important role in the conduct of these experiments, pointing out the advantages of the surface replica technique and providing instruction in how to apply it. Ellen G. Strauss provided invaluable help in the preparation of the manuscript. Edith Lenches and Sharman Christoph provided competent technical assistance. This work was supported by grant GM 06965 from the U. S. Public Health Service and by grant GB 31763X from the National Science Foundation. These results are taken from the Ph.D. thesis of CRB, who was supported by U. S. Public Health Service Training grant GM 00086.

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