STUDIES ON THE INFECTION OF ANIMAL CELLS WITH SINDBIS VIRUS: ADSORPTION, CELL SURFACE MODIFICATION, AND MATURATION

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To Glynn and Jason

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ABSTRACT

The surface replica technique was used to study the distribution of Sindbis virus receptors on chick embryo fibroblasts. Using the position of adsorbed Sindbis virions on the cell surface to indicate the position of Sindbis virus receptors, an even distribution of Sindbis virus receptors over the cell surface was observed if the cells had been previously fixed with glutaraldehyde before adsorption of virus. The number of receptors observed varied from cell to cell, being on the order of 10^5 per cell. Occasionally areas of the cell surface with different concentrations of virus receptors were seen on the same cell. The virus particle-receptor complexes were found to move laterally within the plasma membrane, and the virus particles appeared clustered on the cell surface, when unfixed cells were adsorbed with Sindbis virus at 4 C.

The insertion of Sindbis virus glycoproteins into the cell surface was studied by an indirect electron microscopic labeling technique, which involved treating infected cells sequentially with rabbit anti-Sindbis IgG followed by hemocyanin-conjugated goat (anti-rabbit IgG) IgG, and examining the cells in the electron microscope by the surface replica technique; the position of hemocyanin on the cell surface was used to indicate the position of viral glycoproteins. Sindbis virus glycoproteins were detected at the surface of chick embryo fibroblasts by 2 hours after infection. When infected cells were prefixed with glutaraldehyde before labeling at 37 C, the distribution of virus glycoproteins in the cell surface was fairly even,

although a slight clustering was noticed early in infection. However, when infected cells were labeled at 37 C without prefixation, the hemocyanin was clustered, suggesting that the viral glycoproteins moved laterally within the plasma membrane after treatment with the antibody.

The modification of the cell surface by Sindbis virus was also studied by examining the agglutination of infected cells by two of the plant lectins, concanavalin A and Ricinus communis agglutinin. By 3-3.5 hours after infection, Sindbis virus-infected chick embryo fibroblasts were more agglutinable by these lectins than uninfected cells. Evidence is presented that this increase in agglutination was not due to an increase in the number of lectin binding sites on the cell surface.

Using the surface replica technique, the maturation of Sindbis virus in baby hamster kidney cells and chick embryo fibroblasts was studied. Sindbis virus was found to bud through certain areas of the cell surface in patches, and through virus-specific processes extending only from the cell periphery; budding virions were also seen on the undersides of infected cells. The virus-specific processes were also seen in cells infected with vesicular stomatitis virus, but budding through processes was not a major mode of virus release in these cells. Electron microscopic examination of thin-sections of Sindbis virus-infected cells revealed that these processes contained viral nucleocapsids in the process of budding. The

maturation of Sindbis virus may be somewhat dependent on the structural integrity of cellular microtubules and microfilaments, as both colchicine and cytochalasin B were found to inhibit Sindbis virus release.

A temperature-sensitive mutant of Sindbis virus, ts-103, was found to bud much differently than wild type Sindbis virus. Cells infected with ts-103 contained abnormal virus-specific processes extending from the cell edge; these mutant processes often contained large virus particles containing many viral nucleocapsids.

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General Introduction

Despite the tremendous amount of research done on animal cell surfaces, or plasma membranes, in the last several years, many of the interesting questions about cell surfaces remain unanswered. For example, although it has been recognized for a long time that proteins are important in determining the specificity of the cell surface, little is known about the insertion of proteins into the cell surface. There are many enveloped viruses which modify cell surfaces by inserting newly synthesized structural proteins into the plasma membrane, followed by budding of the viral nucleocapsid through the modified regions. By studying such systems, one should be able to learn a great deal about the biosynthesis and organization of the plasma membrane. This reasoning has been a primary motivation for the work presented in this thesis, which is concerned with the interaction of Sindbis virus, a group A arbovirus, with the surface of animal cells. The following introduction discusses some of the more important findings in recent years in cell surface research, and the techniques which have been used in these studies.

For many years the accepted model for membrane structure was the Danielli-Davson model (Danielli and Davson, 1943), which stated that the lipid of the membrane was arranged in a static bi-layer and was covered by pleated sheets of proteins. On the basis of such techniques as calorimetry and X-ray diffraction, the arrangement of membrane lipids in a bilayer has been established (Steim et al., 1969; Caspar and Kirschner, 1971). However, objections have been raised recently which indicate that the Danielli-Davson model is incomplete.

First, the arrangement of lipids and proteins in this model is energetically unfavorable (Singer, 1971). Second, many of the glycoproteins in cell surfaces appear to be globular (Branton, 1969). Third, both lipids and glycoproteins can move laterally in the plane of the cell surface (Kornberg and McConnell, 1971a; Frye and Edidin, 1970). These and other findings have been incorporated into the recent Fluid Mosaic Model of membrane structure (Singer and Nicolson, 1972), which proposes that most of the membrane proteins are globular and that they can migrate laterally through a fluid lipid bilayer.

In this model membrane proteins which can be removed from the membrane by relatively mild treatment, such as dissociation by chelating agents, are called peripheral membrane proteins. These proteins are isolated free of lipids and are relatively soluble in aqueous buffers. However, most of the membrane proteins are integral proteins, which can only be removed from the membrane by harsh treatment, such as solubilization with detergents. The integral proteins are globular and amphipathic, or structurally asymmetric -- that is, they contain both polar and nonpolar regions. The polar regions contain all the carbohydrate of the protein and are exposed at the cell surface to the aqueous environment around the cell; the nonpolar regions are found in the lipid bilayer. If large enough, an integral protein could penetrate the entire lipid bilayer, but the polar region containing the carbohydrate would remain exposed at the cell surface. The nonpolar region of an integral protein interacts with the lipid bilayer and probably secures the protein in the membrane, but because the lipid bilayer is fluid, integral proteins can

diffuse laterally within the membrane. It appears that many of the recent observations on membranes are compatible with this membrane model, although another model has been proposed which places more emphasis on long-range protein-protein interaction in the membrane (Capaldi and Green, 1972).

The isolation of plasma membranes is often a tricky procedure, being hindered by contamination from intracellular membranes (see review by dePierre and Karnovsky, 1973). Because erythrocyte membranes can be isolated relatively free of contamination, the most convincing data on the organization of structural proteins come from studies on these membranes. When the proteins of human erythrocyte membranes are examined on SDS-polyacrylamide gels, more than 10 polypeptides are found with molecular weights ranging from about 15,000 to 250,000 (Fairbanks et al., 1971). Two of these polypeptides with molecular weights of approximately 220,000 and 250,000 have been called spectrin collectively (Marchesi et al., 1970). Spectrin is probably located on the inner side of the plasma membrane because it is extractable from ghosts (which are hemolysed erythrocytes) but not from intact cells; also, when ghosts are labeled with ferritin-conjugated antibodies against spectrin, the label is found on the inner side of the membrane (Nicolson et al., 1971).

Studies on the organization of the erythrocyte membrane proteins have been aided by the development of various labeling reagents which react with proteins on the surface but do not penetrate the membrane. The most important of these are the diazonium salt of

^{35}S-sulfanilic acid (Berg, 1969), ^{35}S-formylmethionyl (sulfone) methylphosphate (Bretscher, 1971a), and ^{125}I labeling by lactoperoxidase (Phillips and Morrison, 1970). All of the erythrocyte membrane proteins are labeled when erythrocyte ghosts are treated with the reagents, but only two proteins with molecular weights of 100,000 and of 90,000 can be labeled when intact cells are treated with the reagents (Bretscher, 1971a; Phillips and Morrison, 1970, 1971); several different values have been reported for the molecular weight of the smaller protein, which is extensively glycosylated. Comparison of tryptic digests of these two proteins from both labeled intact erythrocytes and from erythrocyte ghosts suggests that these two proteins penetrate the membrane (Bretscher, 1971b,c). More convincing results have been obtained by comparing the SDS-polyacrylamide gel patterns of right-side-out and inside-out vesicles (prepared from erythrocyte membranes) after treatment with proteases (Steck, 1972); these experiments strongly suggest that the two proteins labeled in intact erythrocytes penetrate the lipid bilayer. Thus, all of these results collectively demonstrate that there is an asymmetry in the distribution of proteins in the human erythrocyte membrane, and that two of these proteins may penetrate the membrane.

The protein with a molecular weight of 90,000 has been studied in great detail, possibly because reagents are available which can extract it from the membrane in a water-soluble form (Kathan et al., 1961; Blumenfeld, 1968; Marchesi and Andrews, 1971). This protein, recently called glycophorin, is almost 60% carbohydrate and

contains blood group determinants, lectin receptors and a receptor for influenza virus (Marchesi et al., 1972; Segrest et al., 1973). Amino acid sequence data on cyanogen bromide and tryptic peptides indicate that the N-terminal end and C-terminal end of glycophorin are hydrophilic, whereas the region between is hydrophobic (Segrest et al., 1972; Jackson et al., 1973). From these results it has been proposed that the N-terminal end, which contains all of the carbohydrate, is exposed on the outside of the membrane and the C-terminal end extends into the cytoplasm, while the hydrophobic middle region interacts with the lipids of the membrane. Similar models for the orientation of glycophorin in the plasma membrane have been proposed (Morawiecki, 1964; Winzler, 1969), although these earlier models did not consider extension of the protein into the cytoplasm.

The amphipathic structure of glycophorin may be a general characteristic of all membrane proteins which penetrate the lipid bilayer to any extent, i.e., the integral proteins in the Fluid Mosaic Model. A survey of the amino acid compositions reported for many integral membrane proteins has shown that most of these proteins have a low polarity (Capaldi and Vanderkooi, 1972), which would be expected for proteins which are partially exposed to a hydrophobic environment. Cytochrome b_5, an integral membrane protein isolated from microsomal membranes of liver cells, does not have an excessively hydrophobic amino acid composition, but this protein nevertheless has a strongly hydrophobic region which penetrates the bilayer and secures the

protein in the membrane (Spatz and Strittmatter, 1971). Another interesting example of an amphipathic integral protein is cytochrome oxidase, which apparently can interact so strongly with membrane lipids that it can immobilize enough lipid to cover itself with a layer of "boundary lipid" (Jost et al., 1973).

Evidence that many integral proteins have a globular structure in the membrane comes from experiments using the freeze-fracture and freeze-etching techniques, which expose material embedded in the lipid bilayer (Branton, 1966; Pinto da Silva and Branton, 1970). Most cell membranes which have been examined by these techniques possess globular particles of various diameters in the lipid bilayer (Branton, 1969). There is a large body of evidence which suggests that these intramembranous particles contain glycoprotein, and therefore represent some of the integral proteins which penetrate the lipid bilayer. For example, the fracture faces of lamellar phases of lipids do not contain these particles (Deamer et al., 1970), but lipid phases that contain membrane proteins do show particles on their fracture faces (MacLennan et al., 1971). Also, red blood cell A-antigens and glycophorin are located in these particles (Pinto da Silva et al., 1971; Tillack et al., 1972).

An important part of the Fluid Mosaic Model for membranes is the fluidity of the cell surface, which has now been demonstrated in various cell types. Much of the evidence for the movement of lipids in the membrane comes from the use of electron spin-resonance

spectroscopy. In this technique a "spin-label" group, usually a nitroxide group with an unpaired electron, is coupled to a fatty acid molecule, which is then incorporated into the lipids of a particular membrane; the motion of the "spin-label" group is indicated by changes in its spectrum. Both lateral movement of lipids in the plane of the membrane, and flip-flop movement from one side of the lipid bilayer to the other, have been detected (Kornberg and McConnell, 1971a,b), but lateral movement occurs much more rapidly than flip-flop movement. Experiments with E. coli plasma membranes involving the induction of lactose transport in an unsaturated fatty acid auxotroph have shown that the membrane must be fluid for normal transport function (Fox, 1969; Wilson and Fox, 1971; Overath et al., 1971).

There are also many experiments which demonstrate the movement of proteins in the plane of the cell surface. Photodichroism studies on rhodopsin in frog retinal membranes indicate that rhodopsin, the only protein in these membranes, can rotate in the membrane (Brown, 1972; Core, 1972). Much of the data on movement of proteins in membranes has been shown by techniques which involve labeling of cell surface glycoproteins with various markers, such as fluorescein and ferritin, that can be identified by either light or electron microscopy. Two different experimental approaches are often used in such studies: 1. A monovalent or divalent antibody against the appropriate cell surface glycoprotein is prepared and is coupled directly to the marker; this conjugate is then used for labeling the

cell. 2. Cells are treated with a specific antibody, such as rabbit IgG, and then with a conjugate of the marker coupled to another antibody, such as goat (anti-rabbit IgG) IgG, directed against the first. This second approach is convenient when several different antigens are being tested. For high resolution work, thin-sections of appropriately labeled cells are then examined in the electron microscope; however, thin-sections are inadequate for studying the topography of the cell surface.

High resolution techniques which reveal large areas of the cell surface have recently been developed. One such technique involves lysing cells on an air-water interface and picking up the membranes on an electron microscope grid which is covered with a carbon-coated collodion film (Nicolson and Singer, 1971). The grids are then treated with the appropriate label and the membranes are air-dryed. An obvious criticism of this technique, however, is the possibility of redistribution of cell surface glycoproteins during lysis of the cell. Another technique is the surface replica technique, which involves fixation of cells <u>in situ</u> and preparation of a shadow-cast replica (Smith and Revel, 1972; Revel and Wolken, 1973). Lysis of the cell is not required in this technique, which makes it better suited for studying the cell surface; this technique is discussed in more detail in Part III of this thesis.

Examples of the translational movement of proteins within the cell surface have been found using all of the above techniques.

Frye and Edidin (1970) used fluorescent-labeled antibodies to show that mouse and human cell surface antigens mixed completely in 40 minutes when the two cell types were fused by Sendai virus. Several groups studying different cell surface antigens have shown that multivalent reagents can cross-link proteins and cluster them in the membrane, implying protein movement (Taylor et al., 1971; Davis, 1972; Unanue et al., 1972; de Petris and Raff, 1973; Rosenblith et al., 1973); monovalent reagents do not produce this clustering on the cell surface.

The distribution of many antigens on the cell surface appears to be random, but in some cases membrane proteins are concentrated into specific regions on the cell surface -- for example, the localization of acetylcholinesterase at one end of a nerve cell. Also, many membrane proteins are enzymes whose catalytic activity depends on proper association with lipid as a lipoprotein complex in the membrane. It is not known how the proper orientation of lipids and proteins is maintained in the cell surface, but there is evidence to indicate how membrane organization may be accomplished in some cells. In human erythrocyte membranes, aggregation of the protein spectrin, which is located on the inner side of the membrane, caused aggregation of glycoproteins on the cell surface (Nicolson and Painter, 1973), suggesting that spectrin is linked to integral proteins of the membrane, and therefore exerts some sort of control on the topography of the membrane components. It appears that microtubules may determine the organization of plasma membrane components

in polymorphonuclear leucocytes (Berlin and Ukena, 1972; Ukena and Berlin, 1972).

A very active area of cell surface research is the characterization of the surface components of cells transformed by both DNA and RNA tumor viruses. Much of the activity in this field stems from the general belief that cell surface glycoproteins, or enzymes involved in their synthesis, may play an active role in such processes as cell-cell recognition and contact inhibition. Since transformation by tumor viruses often involves the appearance of new antigens at the cell surface and loss of contact inhibition, many workers have tried to distinguish normal from transformed cells by studying their cell surface components. This approach has been only partially successful, probably because the complexity of the cell surface has been underestimated, but a few interesting observations have been made. A number of different cell types transformed by both DNA and RNA tumor viruses release a specific glycopeptide when treated with trypsin (Buck et al., 1970, 1971a,b); control cells contain much less of this material, so the difference is quantitative. There is also evidence that increased activity of a sialyltransferase is partly responsible for the appearance of this glycopeptide in transformed cells (Warren et al., 1972). The glycolipids of transformed cell surfaces have also been investigated in great detail, and in general there is an incomplete synthesis of the carbohydrate chain (Hakomori and Murakami, 1968). However, nothing is known definitely about how the appearance or disappearance of material at the cell surface contributes to the transformed state of the cell.

A different experimental approach to the study of transformed cell surfaces involves the use of the plant lectins. These are proteins (often glycoproteins) which bind to specific carbohydrates (see review by Sharon and Lis, 1972), and it was found that transformed cells are agglutinated by lower concentrations of the lectins than normal cells (Burger and Goldberg, 1967; Inbar and Sachs, 1969). This observation has now been extended to several types of transformed cells, and to cells infected with non-oncogenic viruses (Poste and Reeve, 1972; Becht et al., 1972; Birdwell and Strauss, 1973). The mechanism for this increased agglutination is a subject of much controversy. It was initially thought that transformation exposed lectin binding sites which were masked in normal cells, because trypsinization of normal cells increased their agglutination with lectins (Burger, 1969); thus, transformed cells should bind more lectin than normal cells. However, many groups have since reported that normal and transformed cells bind the same number of radioactive lectin molecules (Arndt-Jovin and Berg, 1971; Cline and Livingston, 1971; Ozanne and Sambrook, 1971), although there have been recent claims that transformed cells do bind more lectin than normal cells (Nicolson and Lacorbiere, 1973; Burger, 1973). Electron microscopic labeling techniques have also been applied to this problem, and here again the results are confusing. It had been shown that the binding sites for concanavalin A, a plant lectin, were clustered on transformed cells and randomly dispersed on normal cells (Nicolson, 1971).

The interpretation of these results was that a rearrangement of binding sites was responsible for increased agglutination. However, it now appears that concanavalin A binding sites are randomly dispersed on both normal and transformed cells, and that an increased fluidity of transformed cell surfaces may be responsible for increased agglutination (Nicolson, 1973; Rosenblith et al., 1973).

To summarize thus far, little is known conclusively about the organization of proteins in plasma membranes, except in the case of the human erythrocyte membrane. In this membrane, the protein spectrin on the inner side of the membrane appears to influence the organization of the integral proteins. For more complicated membranes, much less is known, but the organization of membrane components appears to vary from one cell type to another. Despite the immense amount of work which has been done on the transformed cell surface, little is known conclusively about how surface changes occur after transformation. However, there are simpler systems, such as the infection of cells with enveloped RNA viruses, which involve cell surface changes similar to those found in transformed cells.

Many of the enveloped RNA viruses appear to have basically the same mechanism of assembly (Compans and Choppin, 1971; Choppin et al., 1972). The viral nucleocapsids or cores align themselves under specific regions of the plasma membrane into which newly synthesized viral glycoproteins have been inserted. A mature virion is formed as the nucleocapsid buds through the modified region of the plasma membrane and surrounds itself with the lipid bilayer of the host cell

plasma membrane. Many electron microscopic studies have confirmed this sequence of events in the assembly process of several enveloped RNA viruses (Morgan et al., 1961a,b, 1962; Howatson and Whitmore, 1962; Compans et al., 1966; Acheson and Tamm, 1967; Howe et al., 1967; Compans and Dimmock, 1969). Although the maturing virion obtains its lipid bilayer from that of the host cell plasma membrane, the lipid composition of the viral membrane does not always resemble that of the host cell plasma membrane. For example, it has been reported that the lipid composition of simian virus 5, a paramyxovirus, resembles that of the host cell plasma membrane (Klenk and Choppin, 1969, 1970a,b), but two other paramyxoviruses, Newcastle disease virus and Sendai virus, have different lipid compositions when grown in the same host (Blough and Lawson, 1968). Also, when Sindbis virus, a group A arbovirus, was grown in two different hosts, the lipid compositions of the two virus preparations were very similar (David, 1971). Thus, for some of the enveloped RNA viruses, cellular lipids may be selectively incorporated into the viral envelope.

Another important feature of the modification of the cell surface by these viruses is that host cell membrane proteins are excluded from the altered regions (Howe et al., 1967; Holland and Kiehn, 1970); thus, only virus-specific proteins are found in virions released from the cell. All of the enveloped RNA viruses studied thus far contain glycoproteins which appear as spikes, or projections, on the viral envelope. Examples of these are group A arboviruses

(Strauss et al., 1970; Schlesinger et al., 1972), group B arboviruses (Stollar, 1969), rhabdoviruses (Wagner et al., 1969), myxoviruses (Schulze, 1970), paramyxoviruses (Evans and Kingsbury, 1969), and RNA tumor viruses (Duesberg et al., 1970). Various biological activities have been associated with these glycoprotein spikes; for example, it has been shown that the neuraminidase and hemagglutinating activities of influenza virus are located on separate glycoprotein spikes (Laver and Valentine, 1969). The envelope glycoproteins do not appear to exert any control over the organization of the lipid bilayer phase of the viral membrane, because the glycoprotein spikes can be removed by proteases without affecting the other viral structural proteins (Compans et al., 1970; Compans, 1971; Chen et al., 1971; McSharry et al., 1971; Rifkin and Compans, 1971), and because removal of the spikes does not affect the lipid phase of the viral membrane, as determined by electron spin resonance studies (Landsberger et al., 1971). Furthermore, other electron spin resonance studies have shown that the rigidity of the viral membrane is probably determined by its lipid composition (Landsberger et al., 1973).

Other viral membrane proteins have been isolated which may be more intimately involved in the organization of the viral membrane. Influenza, parainfluenza, and vesicular stomatitis virions have a non-glycosylated membrane protein which may be associated with the inner side of the viral envelope (Choppin et al., 1972); it has been postulated that these nonglycosylated membrane proteins could impart stability to the viral envelope.

I have been studying the infection of cells with Sindbis virus, a group A arbovirus. Sindbis virus has a membrane which consists of a lipid bilayer and two glycoproteins (Strauss et al., 1970; Schlesinger et al., 1972); this membrane surrounds a nucleocapsid composed of RNA and one nonglycosylated structural protein. The glycoproteins, which appear as spikes, or projections, on the viral envelope (Compans, 1971), do not appear to penetrate the lipid bilayer (Harrison et al., 1971). Thus, Sindbis is a relatively simple virus. The phospholipids of the virus are derived from the host cell (Pfefferkorn and Hunter, 1963), whereas the glycoproteins are synthesized de novo after infection (Pfefferkorn and Clifford, 1964). The carbohydrate composition of the glycoproteins is determined, at least in part, by the host cell (Strauss et al., 1970). As just discussed, the modification of the plasma membrane by Sindbis virus involves insertion of the viral envelope glycoproteins into the cell surface and budding of the nucleocapsids through these modified regions (Acheson and Tamm, 1967). For Sindbis virus, the initial insertion of viral glycoproteins occurs as early as 2-2.5 hours after infection. Temperature-sensitive mutants of Sindbis virus have been isolated which are defective in membrane components (Burge and Pfefferkorn, 1968); these mutants may be very useful for membrane studies.

Three different aspects of Sindbis virus-cell surface interactions are examined in this thesis: adsorption, cell surface modification by the insertion of viral glycoproteins, and maturation.

In Part I, the distribution of Sindbis virus receptors is studied by examining surface replicas of cells adsorbed with Sindbis virus; the position of an adsorbed virion on the cell surface is used to locate the Sindbis virus receptor. In Part II, cell surface modifications are studied in two different ways. First, the agglutination of Sindbis virus-infected cells by plant lectins is examined. Second, the insertion of viral glycoproteins into the cell surface is studied by examining surface replicas of infected cells treated sequentially with rabbit anti-Sindbis IgG followed by hemocyanin-conjugated goat (anti-rabbit IgG) IgG. In Part III, the maturation of wild type Sindbis virus and some of its temperature-sensitive mutants is studied by examining the top and underside of Sindbis virus-infected cells.

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Part I

The Distribution of the Receptor Sites for Sindbis Virus in the Surface of Chick Cells

The following material has been submitted to the Journal of Virology.

THE DISTRIBUTION OF THE RECEPTOR SITES FOR SINDBIS VIRUS ON THE SURFACE OF CHICK CELLS

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ABSTRACT

Sindbis virus was adsorbed to chick cells, and the distribution of virus over the surface of the cell was examined by electron microscopy of surface replicas. The distribution of virus particles on the cell was used to indicate the position of virus receptors at the cell surface. When purified Sindbis virus was adsorbed at 37 C to cells prefixed with glutaraldehyde, the virus particles were evenly distributed over the surface of most cells. There was a large variability from cell to cell, however, in the number of virus particles adsorbed, and regions with different concentrations of virus particles were sometimes observed on the same cell. The concentration of virus receptors observed varied from 20 to 160 per square micron of cell surface, and the total number of virus receptors per cell is thus on the order of 10^5. When virus was adsorbed to unfixed cells at 4 C, the virus particles were clustered into aggregates varying from a few particles to large crystalline arrays. These conditions are apparently conducive to virus aggregation and this, coupled with free lateral diffusion of the virus-receptor complex in the cell membrane, leads to the observed clustering.

INTRODUCTION

The early events in the infection of cells by animal viruses are the adsorption of the virion to a receptor on the cell surface, loss or "eclipse" of the virus particle infectivity, and penetration of the virus into the cell (1, 13, 15). Adsorption occurs at 0 C, but eclipse does not. At higher temperatures (20 C or above) eclipse and penetration occur. A large fraction of the eclipsed particles can be washed off the cell; these particles contain all of the macromolecules of the virus, but they will not readsorb to the cell (5, 8, 15). There seem to be two ways for the virion to penetrate the cell: pinocytosis (4, 14), followed by the breakdown of the virus inside the cell, or fusion of the membrane of the virus with the plasma membrane of the cell and release of the viral nucleic acid (20).

The virus receptors on the cell surface are quite specific. For example, the adsorption of influenza virus at 4 C to red blood cells is a specific reaction, on the basis of differences in hemagglutination with different species of red blood cells (3, 11). The adsorption of the picornaviruses also involves binding to a specific receptor, based on two lines of evidence. First, poliovirus infects only a few different cell types in vivo. However, some cells not susceptible to poliovirus in vivo become so after culturing in vitro (12); this susceptibility is accompanied by the appearance of virus receptors. Second, receptors specific for several picornaviruses have been distinguished from each other on the basis of sensitivity to

enzyme inactivation and time required for the regeneration of receptor activity (17, 22, 28). In the case of adenovirus, the specificity of the receptor has also been established (23). Although the specificity of virus-receptor interactions is quite certain, little is known about the structure of virus receptors. For the myxoviruses and paramyxoviruses, sialic acid appears to be part of the receptor (10); the receptor of influenza virus in erythrocyte membranes seems to be a glycoprotein (16, 19).

Studies thus far on the adsorption of viruses to cells have not examined the distribution of virus receptors on the cell surface, in part, because of the lack of a technique which can reveal large areas of the cell surface. We have successfully used the surface replica technique to study the topography of Sindbis virus-infected cells (2), and we have now used this technique to study the distribution of Sindbis virus receptors on chick embryo fibroblasts.

MATERIALS AND METHODS

Cells. The culturing of chick embryo fibroblasts and their growth on 12 mm coverglasses have been previously described (2).

Adsorption of virus to cells. Sindbis virus (HR strain) was purified by polyethylene glycol precipitation followed by velocity and isopycnic sucrose gradients, as previously described (24, 27), and dialyzed overnight at 4 C against phosphate-buffered saline, pH 7.4 (PBS of Dulbecco and Vogt, ref. 7). The virus solution before dialysis had a protein concentration of 1 mg/ml, as determined by Lowry's assay (18). After dialysis, fetal calf serum was added to a concentration of

1%, and the virus was further diluted to protein concentrations of 25, 50, 125 and 250 µg/ml with PBS containing 1% fetal calf serum.

For adsorption at 37 C, all manipulations were done in the 37 C room, and all cells were prefixed before adsorption to prevent movement of the virus receptors. Cells were washed in PBS, fixed in 1% glutaraldehyde in PBS for 10 min at 37 C, and then washed again in PBS. To remove unreacted glutaraldehyde, cells were treated with .15 M glycine in PBS for 10 min at 37 C. After washing again, cells on coverglasses were treated with .1 ml of a virus dilution for 30 min at 37 C, and then washed several times in PBS.

For adsorption at 4 C, all manipulations were done in the 4 C room. Before adsorption, cells were allowed to cool to 4 C in PBS with 1% fetal calf serum. Except for the temperature difference, the procedures for prefixation and adsorption at 4 C were the same as those at 37 C.

Electron microscopy. Fixation of cells and preparation of surface replicas have been previously described (2). All micrographs are presented in reverse contrast.

RESULTS

Since the adsorption of virus to cell surfaces involves attachment to a specific receptor, we have used the distribution of adsorbed Sindbis virus on the cell surface to determine the distribution of Sindbis virus receptors. Adsorbed virus particles will penetrate the cell at 37 C, so the adsorption of Sindbis virus was studied under

conditions which inhibit penetration into the cell, i.e., adsorption at low temperatures or prefixation with glutaraldehyde before adsorption.

In the following sections, density refers to the number of virus receptors or virus particles per μm^2 of cell surface.

Adsorption of virus at 37 C. For these experiments cells were prefixed at 37 C before adsorption. Figure 1 shows a surface replica of two cells, A and B, after adsorption of Sindbis virus at 37 C. The cells are partially overlapping; the solid and black-and-white arrows indicate the edges of cells A and B, respectively. These cells illustrate two points about the distribution of Sindbis virus receptors. First, receptors are evenly distributed over the entire cell surface; even pseudopodia extending from the cell periphery are uniformly covered with virus particles. Second, the density of receptors on the cell surface varies from cell to cell. In Fig. 1, cell A has a density of 96 virus particles per μm^2, whereas cell B has a density of 46 virus particles per μm^2. Virus particle densities on other cells were found to vary from about 20 per μm^2 to about 160 per μm^2. The virus receptors were saturated under the conditions used, since the same results were obtained with all the virus dilutions used. Assuming that chick embryo fibroblasts in tissue culture have an average surface area of 2×10^3 μm^2, the number of Sindbis virus receptors on these cells varies from 4×10^4 to 3×10^5. This probably represents only a minimum value, since virus particles may bind to or overlap more than one receptor molecule.

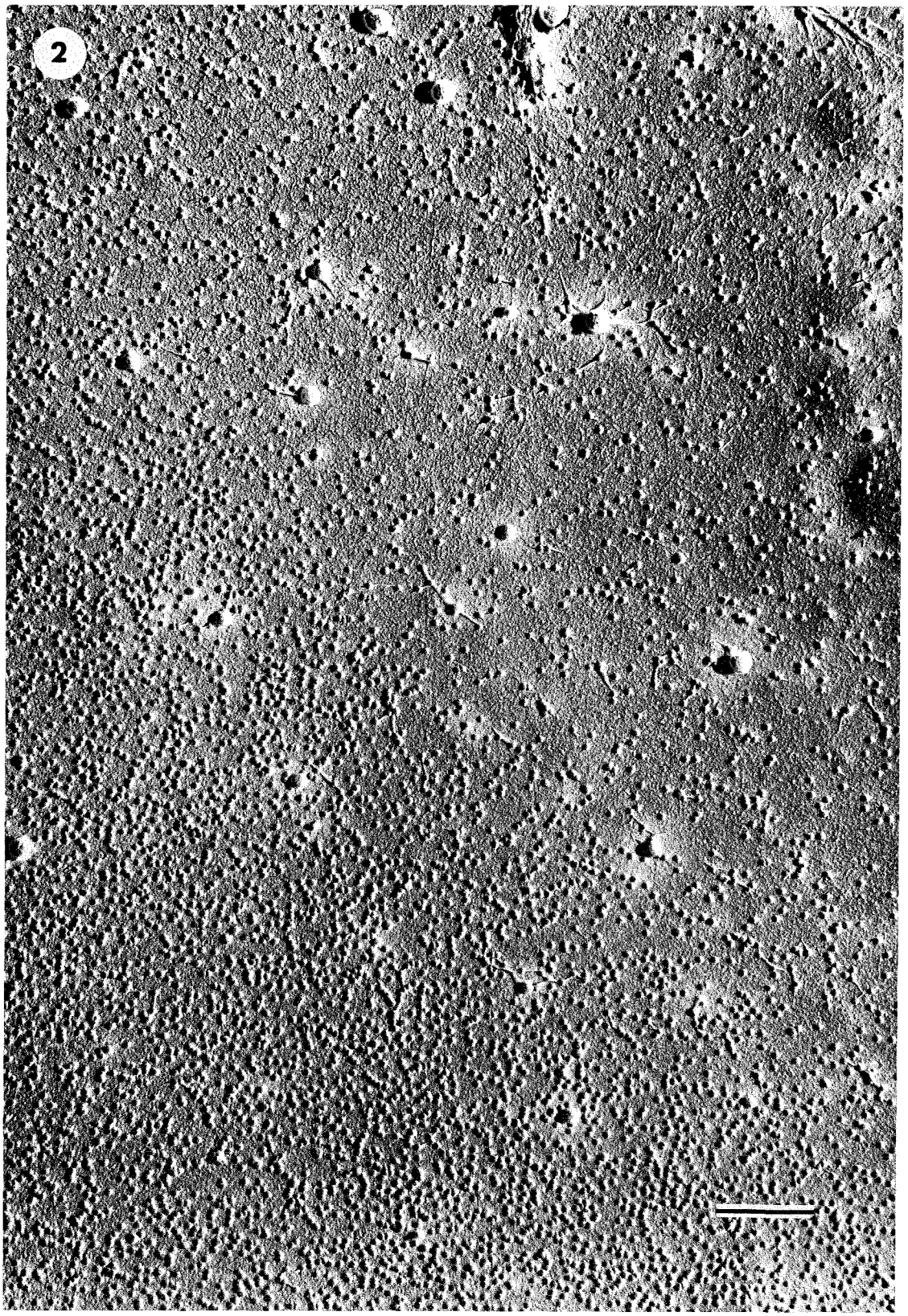
FIG. 1. Surface replica of Sindbis virus adsorbed to prefixed cells at 37 C. The solid and black-and-white arrows indicate the edges of cells A and B, respectively. X14,500. Scale bar is 1 μm.


1
B
A

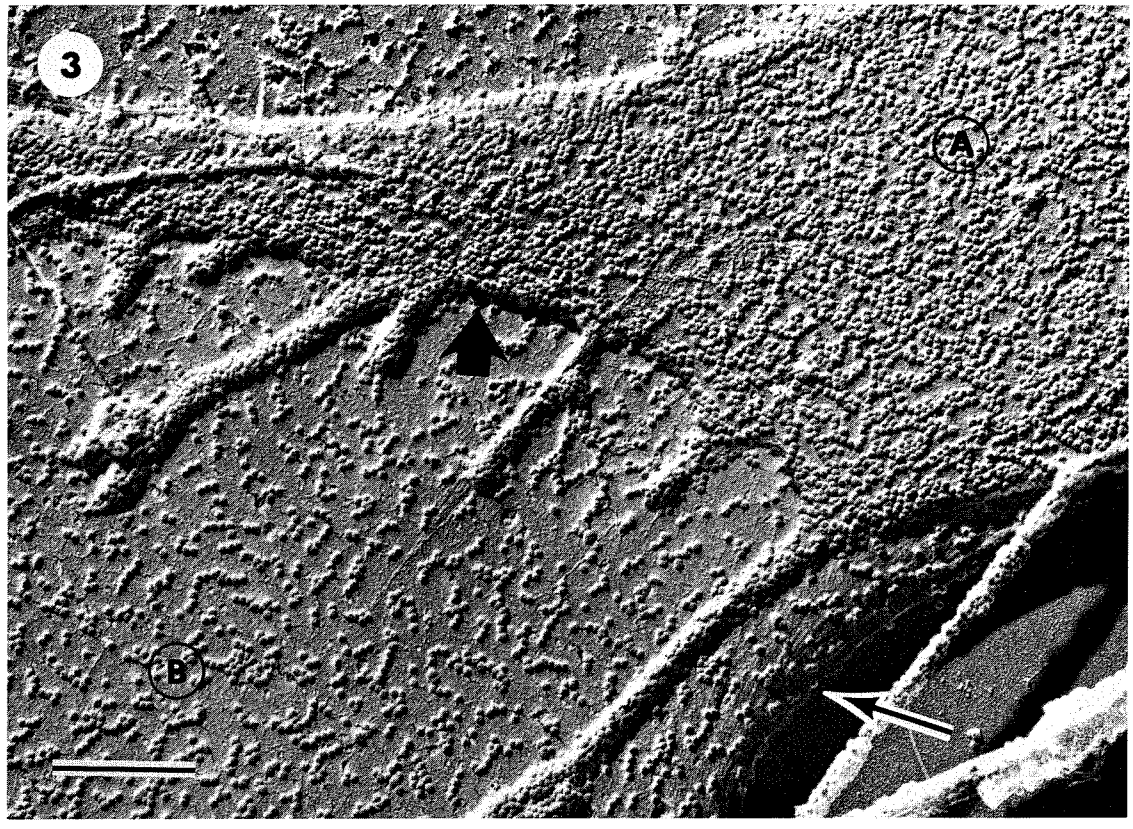
Although most cells had the same density of virus particles over their entire surface, areas with different particle densities were sometimes observed on the same cell, as shown in Fig. 2. The denser area of the cell has a density of 77 virus particles per μm^2, whereas the less dense area has a density of 20 virus particles per μm^2.

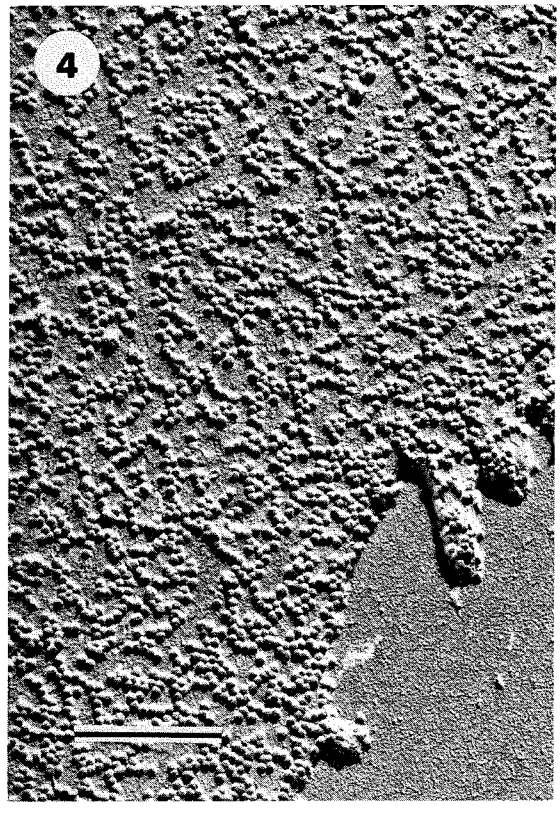
Adsorption of virus at 4 C. When virus was adsorbed to cells at 4 C, there was a large variability from cell to cell in the density of virus particles on the cell surface, as had been seen at 37 C. In contrast to the even distribution of virus particles seen on prefixed cells adsorbed at 37 C, however, unfixed cells adsorbed at 4 C showed a clustering of virus particles on the cell surface. In Fig. 3 is seen a surface replica of two unfixed cells adsorbed at 4 C; as in Fig. 1, these cells are also overlapping. The solid and black-and-white arrows indicate the edges of cells A and B, respectively. Cell A has a density of 200 virus particles per μm^2 on its surface, whereas cell B has a density of 50 virus particles per μm^2. The virus particles on cell A are aggregated into large clusters in which the virus is tightly packed, whereas the clusters on cell B are much smaller and there are many single particles. At an intermediate density the clustering is more apparent, as on the cell in Fig. 4, which has a density of 150 virus particles per μm^2 on its surface. On a few cells the clustering at 4 C was so extensive that the virus particles appeared to form a crystalline lattice on the cell surface, as shown in Fig. 5. The large cluster seen in Fig. 5 has a density of 450 virus

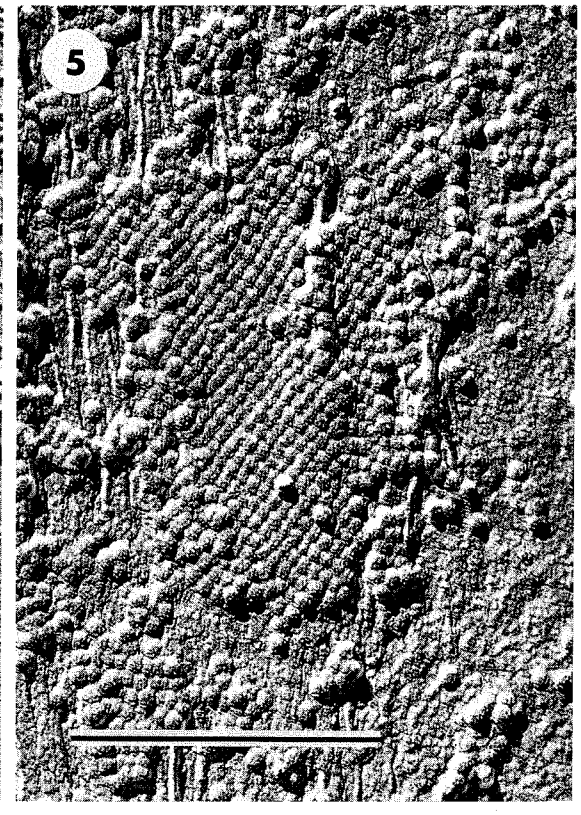
FIG. 2. Surface replica of Sindbis virus adsorbed to a prefixed cell at 37 C. X14,500. Scale bar is 1 μm.


2

FIGS. 3-5. Surface replicas of Sindbis virus adsorbed to unfixed cells at 4 C. Fig. 3, X17,000. The solid and black-and-white arrows indicate the edges of cells A and B, respectively. Fig. 4, X18,000; Fig. 5, X39,000. Scale bars are 1 μm.


3
A
B


4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particles per μm^2. At this density the virus particles have an effective diameter of only 60 nm, assuming hexagonal close packing, which indicates that in such clusters the particles are very tightly packed, since the measured diameter of a Sindbis virion is approximately 70 nm.

There seem to be three possible explanations for this clustering of virus particles at 4 C. First, Sindbis virus particles aggregate in solution at 4 C and are therefore adsorbed as aggregates at this temperature. Second, cooling cells to 4 C causes the virus receptors to aggregate or "freeze out" of the plasma membrane. Third, after virus particles bind to receptors on the cell surface, the virus receptor complexes migrate laterally along the cell surface to form aggregates. The following experiments were done to test these possibilities (Table 1). One set of cells was prefixed at 37 C, then cooled and adsorbed with Sindbis virus at 4 C. Two other sets of cells were cooled and prefixed at 4 C, then one set was warmed and adsorbed at 37 C while the other was adsorbed at 4 C. Adsorption at 37 C of cells prefixed at 37 C and adsorption of unfixed cells at 4 C were used as controls, since it was known that under these conditions the virus particles had even and clustered distributions, respectively. Adsorption at 37 C to unfixed cells was not attempted because of the complications in interpretation caused by virus penetration.

As shown in Table 1, all conditions of adsorption used produced an even distribution of virus particles, except adsorption at

TABLE 1. Adsorption of Sindbis virus to chick embryo fibroblasts

Preadsorption Treatment	Temperature of Adsorption	Virus Particles
Prefixed at 37 C	4 C	Even
Prefixed at 37 C	37 C	Even
Prefixed at 4 C	4 C	Even
Prefixed at 4 C	37 C	Even
None	4 C	Clustered

Procedures for prefixation and adsorption are explained in Materials and Methods.

4 C of unfixed cells. If the virus had aggregated in solution at 4 C, then adsorption at 4 C of cells prefixed at either 4 C or 37 C would have produced a clustered distribution of particles. If cooling cells to 4 C clustered the virus receptors, then adsorption at either 4 C or 37 C of cells cooled and prefixed at 4 C would have also produced a clustered distribution. Therefore, it appears that the virus receptors can diffuse laterally in the plasma membrane, even at 4 C.

DISCUSSION

Fixation of cells with glutaraldehyde inhibits movement of proteins through the plasma membrane and prevents the penetration of adsorbed virus into the cell, thus allowing one to examine the true distribution of virus receptors on the cell surface by adsorbing virus onto prefixed cells. Using this procedure, we have found that Sindbis virus receptors are randomly distributed over the surface of chick embryo fibroblasts. Random distributions have also been reported for other cell surface antigens, such as the concanavalin A-binding sites on normal and transformed cells (21, 26). The density of Sindbis virus receptors on the cell surface varies from cell to cell, ranging from 20 to 160 virus particles adsorbed per μm^2 on prefixed cells adsorbed at 37 C. Areas of different particle densities were sometimes found on the same cell. Variability in the number of influenza virus particles adsorbed to endodermal cells of the chorioallantoic membrane of chick embryos has been reported (20), but as

thin-sectioning techniques were used in this study, the observed variability may not apply to the whole cell surface. It is not clear why different cells have different numbers of Sindbis virus receptors on their surfaces, but this may be partly due to the asynchrony of the cell population.

The virion-receptor complex could diffuse laterally over the cell surface at 4 C in unfixed cells, leading to clustering of the virus particles on the cell surface. This clustering appears to be due to a tendency for the virions to crystallize or aggregate under these conditions, since the receptors do not clump in the absence of virus. It is known that low temperature inhibits the movement of certain antigens on the cell surface (9), but there are examples of such movement at low temperatures. Treating mouse lymphocytes at 0 C with divalent antibodies against mouse IgG produces a patchy distribution of IgG molecules on the cell surface, although capping (movement of cell surface IgG to one pole of the cell) is inhibited at this temperature (6). Also, the intramembranous particles of erythrocyte membranes can be aggregated at low temperatures (25). Thus, when studying the cell surface distribution of any antigen, one cannot assume that low temperatures prevent movement of the particular antigens under study.

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Part II

Modification of the Cell Surface by Sindbis Virus

The following material has been submitted for publication to the Journal of Virology.

REPLICATION OF SINDBIS VIRUS

IV. AN ELECTRON MICROSCOPIC STUDY OF THE INSERTION OF VIRAL GLYCOPROTEINS INTO THE SURFACE OF INFECTED CHICK CELLS

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ABSTRACT

The insertion of Sindbis virus-envelope glycoproteins into the surfaces of chick embryo fibroblasts has been studied by an indirect labeling technique. This technique involved treating infected cells sequentially with rabbit 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 hrs after infection (and at least 1 hr before mature virions were released), newly synthesized virus-envelope glycoproteins were detected at the cell surface. By 3 hrs 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.

INTRODUCTION

Sindbis virus, a group A arbovirus, consists of a lipoprotein envelope surrounding a nucleocapsid or "core" (1, 23, 26). The membrane contains two glycoproteins (19, 25, 26) and the lipid of the virus; the core contains the viral RNA complexed with a nucleocapsid protein (25, 26). 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, 25), being encoded in the viral RNA (22). 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 have used this technique in the current paper to study the insertion of Sindbis envelope glycoproteins into the cell surface. Infected cells are labeled with rabbit IgG specific for Sindbis virus followed by labeling with a conjugate of 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 chick embryo fibroblasts were seeded in 35 mm petri plates containing a 12 mm coverglass, using Eagle's minimal essential medium supplemented with 10% fetal calf serum. After 18-24 hr at 37 C, when the cells were still subconfluent, they were infected with approximately 50 plaque-forming units 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, 25). 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 µg of Sindbis mixed with an equal volume of

complete Freund's adjuvant. Three weeks later the same amount of virus with adjuvant was injected intraperitoneally. Three days after the second injection 250 µg 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 chick embryo fibroblasts (about 10^8 cells/bottle). Anti-Sindbis activity in the serum was monitored by hemagglutination inhibition, using pigeon red blood cells (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 (3). Purified rabbit IgG (Miles Laboratories) was crosslinked 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 (Busycon canaliculatum). Particulate material was removed by centrifugation at 12,000 g for 10 min, and the hemocyanin was then collected by centrifugation at 150,000 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 g for 10 min at 4 C. The supernatant was applied to a column (2.5 x 100 cm) of agarose (A - 1.5 m; Bio-Rad Laboratories) in PBS. The void volume fractions, which contain the hemocyanin, were pooled, filtered through a .22 μm Millipore filter 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 mg/ml 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 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, coverglasses with cells on them were washed several times in PBS and treated in one of the following ways:

a) 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 µg/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.

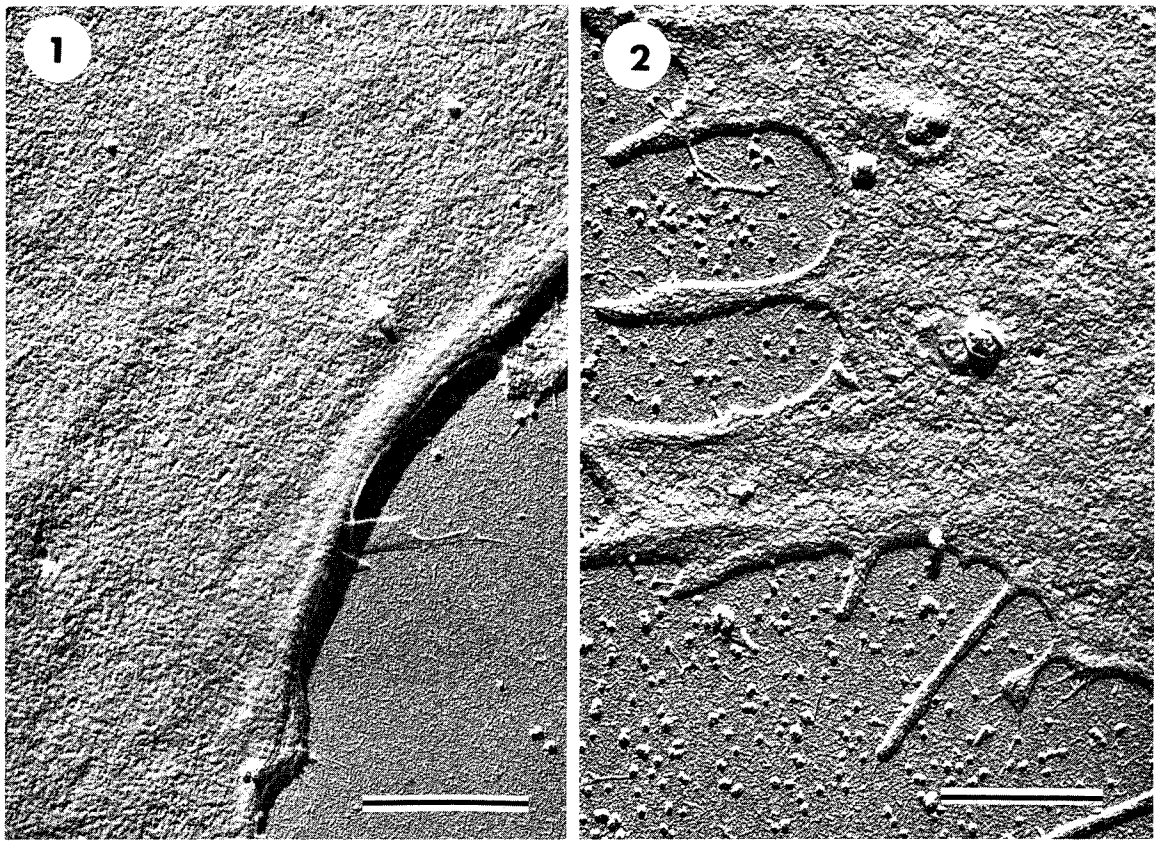
b) No prefixation. Cells were treated as above, except that the prefixation and glycine treatments were omitted. For labeling at 4 C, cells were rinsed with cold PBS, then treated with anti-Sindbis IgG for 10 min at 4 C followed by G-Hcy for 10 min at 4 C. For labeling at 37 C, all operations were performed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

Electron microscopy. Fixation of cells and preparation of surface replicas for electron microscopy have been previously described (4). Replicas were examined on a Philips E.M. 300.1. 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, as shown in Fig. 1 and Fig. 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) is quite variable from experiment to

FIGS. 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. X20,000. Scale bars are 1 μm.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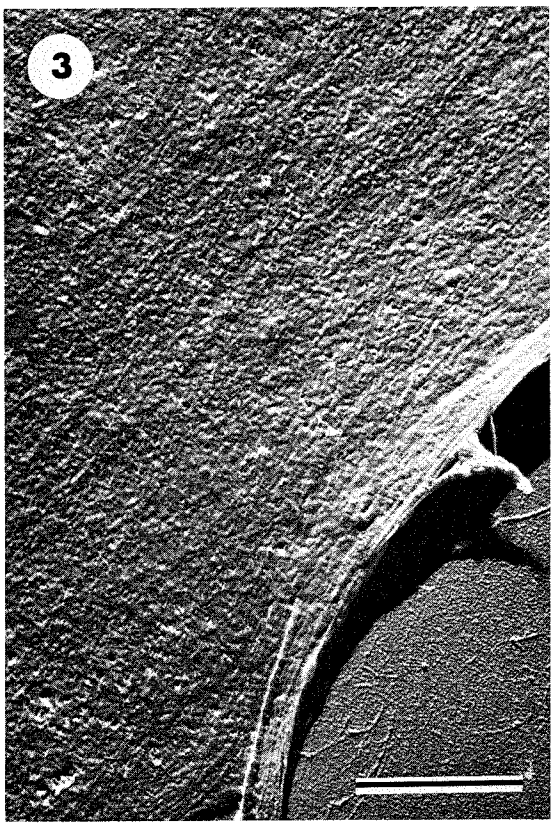
experiment. This background label appears to be nonspecific, although its origins are unclear. A similar nonspecific binding to the background was found when hemocyanin was used to study the distribution of concanavalin A binding sites on the surface of cells (18, 24). Thus, although this background labeling is poorly understood, it does not interfere with the interpretation of results of binding to the cell surface and has been ignored for the purposes of these experiments.

Time of appearance of Sindbis virus glycoproteins at the cell surface. All of the labeling experiments discussed in this section were done on prefixed cells, to prevent clustering artifacts caused by the use of multivalent reagents.

At 1 hr after infection (Fig. 3), there is little or no detectable hemocyanin on the cell surface, illustrating that under our conditions the antigens contributed by the inoculum do not interfere with the assay. However, by 2 hr after infection, significant amounts of hemocyanin are seen (Figs. 4 and 5). The cell in Fig. 4 is lightly labeled, and represents a very early stage in the insertion of the viral glycoproteins. The hemocyanin label is fairly evenly distributed over the surface of the cell, although close inspection reveals that the distribution is 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

FIGS. 3-6. Surface replicas of prefixed cells treated at 37 C with anti-Sindbis IgG and G-Hcy at 1 hr (Fig. 3), 2 hrs (Figs. 4 and 5), and 2.5 hrs (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, X20,000; Fig. 4, X26,000; Fig. 5, X24,000; Fig. 6, X20,000. Scale bars are 1 μm.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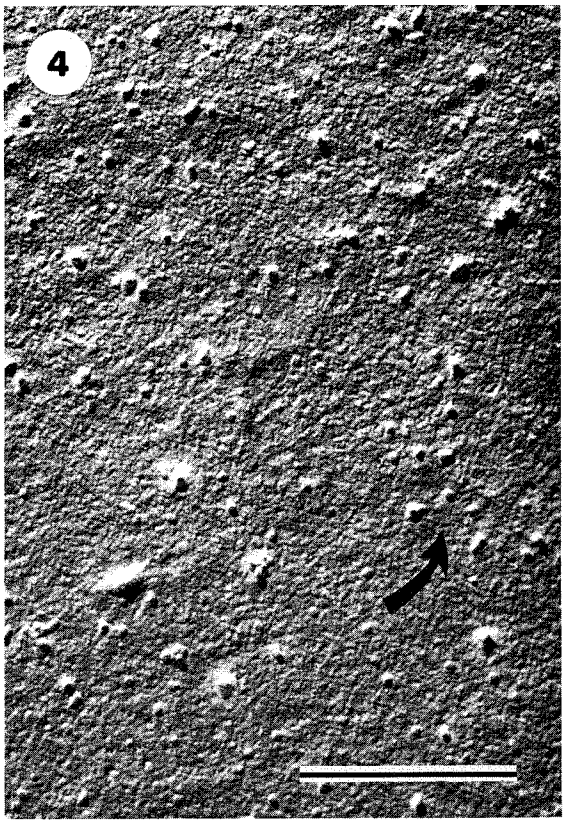
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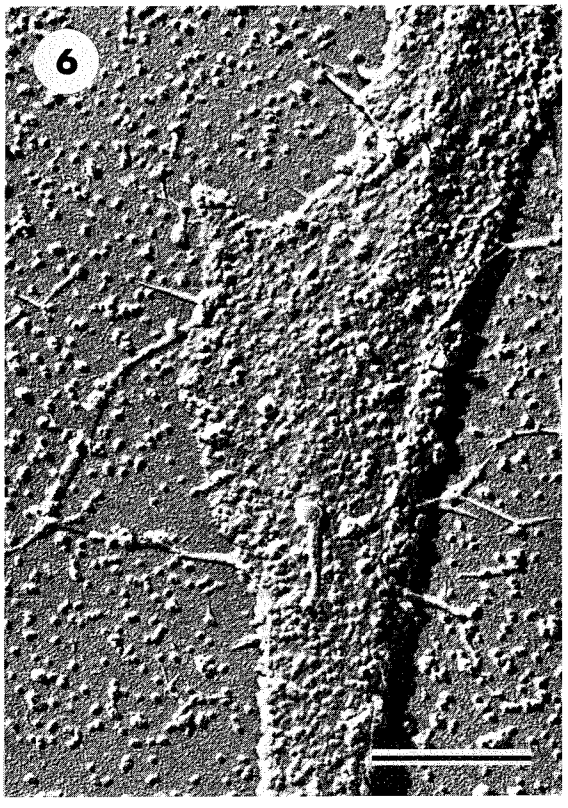
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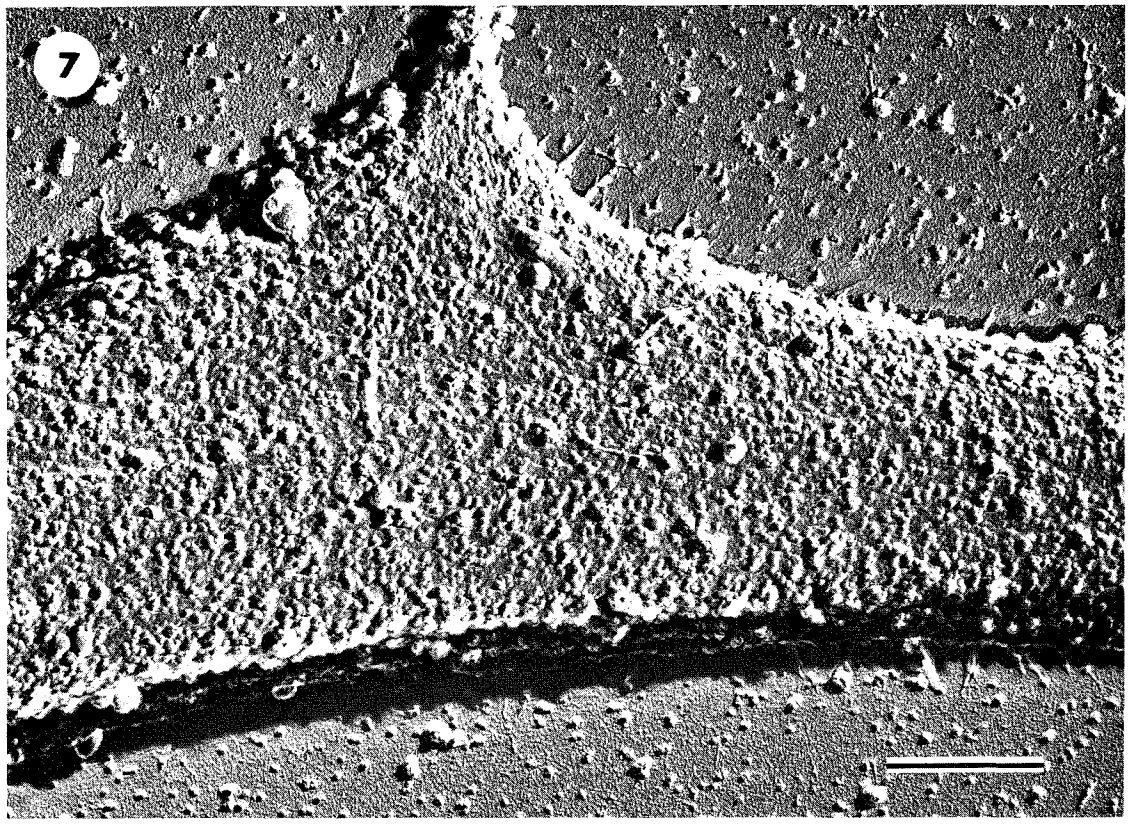
devoid of hemocyanin. The cell in Fig. 5 is more heavily labeled. Again the label is fairly evenly distributed over the surface, but there is some clustering of the hemocyanin. This is illustrated by two adjacent circles of equal size on the picture. One encircles about 10 hemocyanin molecules, the other none. Several possible explanations for this slight clustering exist. Firstly, the topographical organization of the cell surface could exclude the virus glycoproteins from certain areas. Secondly, a Sindbis virus particle contains several hundred molecules of glycoproteins, and a region on the cell surface with enough glycoprotein for one virion represents a small patch of glycoprotein; clustering of hemocyanin early after infection could represent an early sign of virus patch formation. Thirdly, this clustering may arise by insertion of glycoprotein at certain sites, followed by diffusion of the glycoprotein molecules away from the site.

The cell in Fig. 6 had been infected for 2.5 hr, and illustrated that at this time a few of the cells are heavily labeled. By 3 hr after infection most cells are heavily labeled; a representative example is given in 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 has been infected for 5.5 hr. Virus-specific processes where most virus release occurs are evident (arrow) and are solidly packed with hemocyanin, as expected, since these processes contain a linear array of maturing virions (4). The remainder

FIGS. 7 and 8. Surface replicas of prefixed cells treated at 37 C with anti-Sindbis IgG and G-Hcy at 3 hrs and 5.5 hrs after infection with Sindbis virus, respectively. The arrow in Fig. 8 indicates a virus-specific process. X20,000. Scale bars are 1 μm.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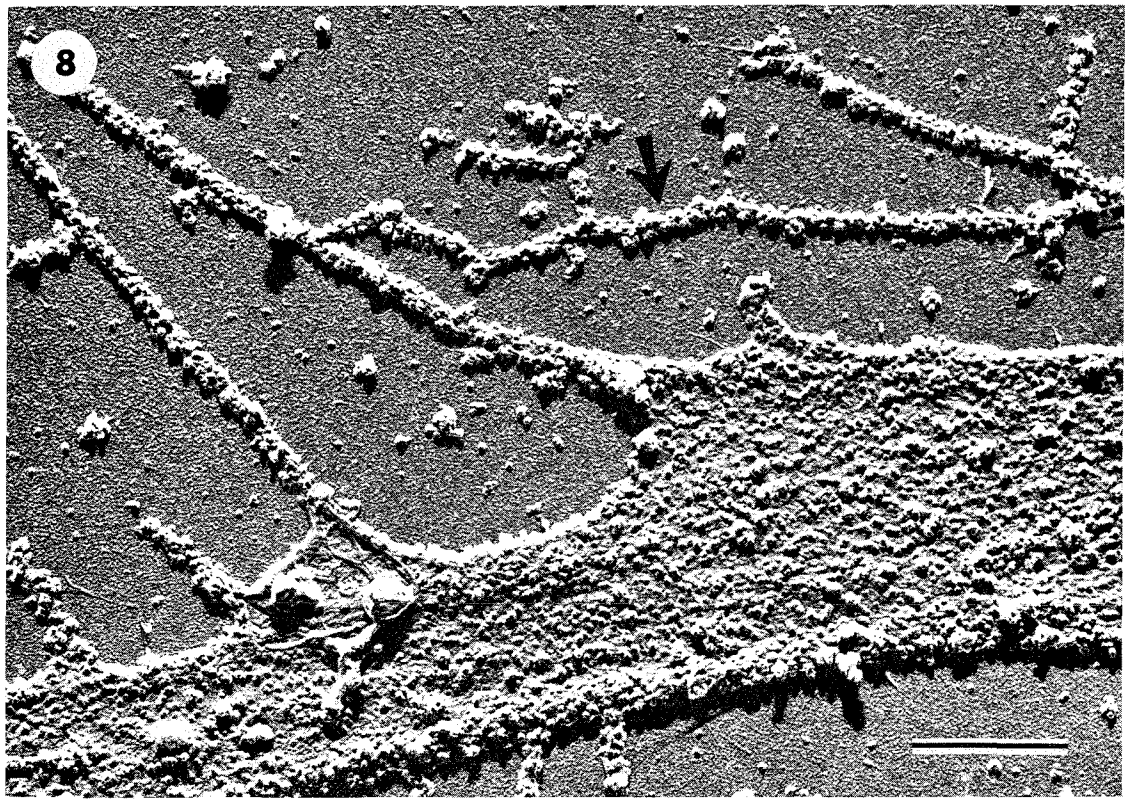
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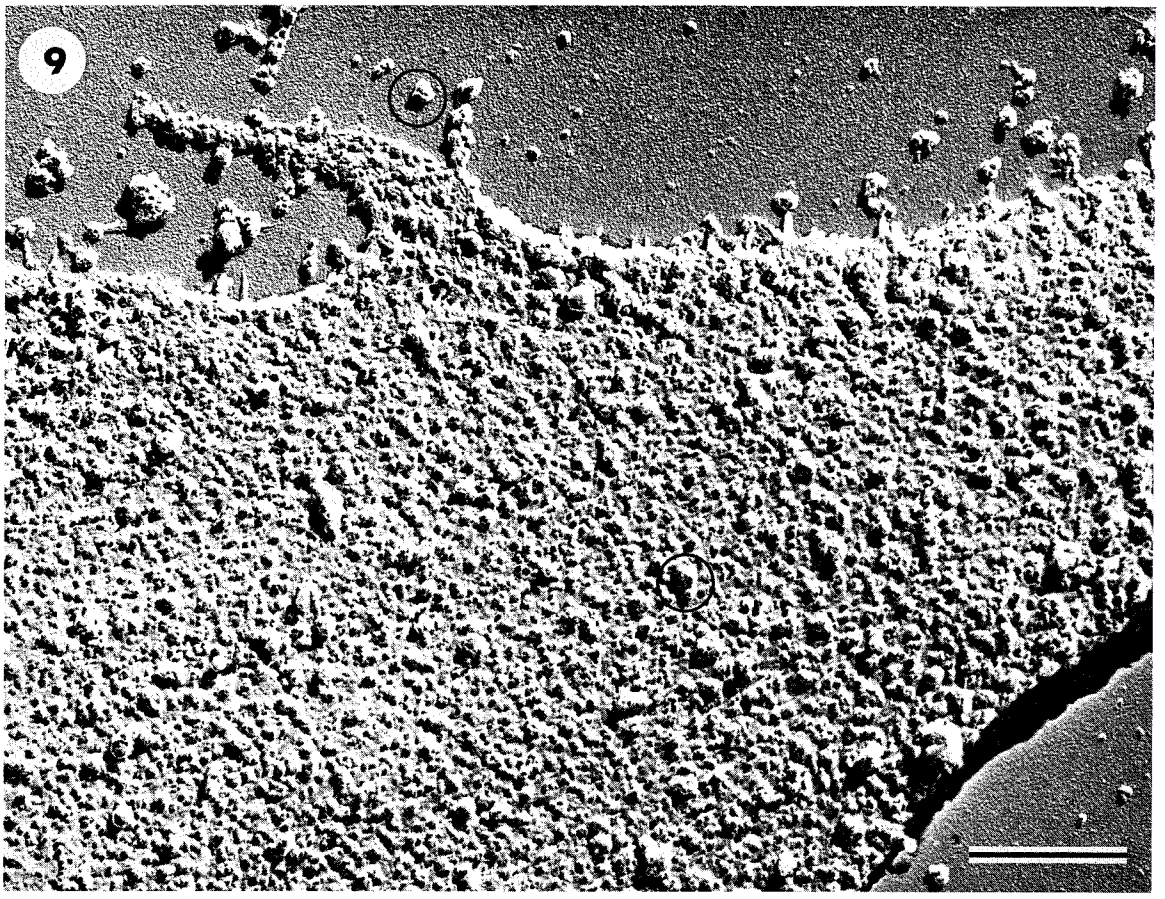
of the cell is also heavily labeled, however, although somewhat less so than the processes. In Fig. 9 is shown a cell at 6.5 hrs after infection. This cell is very heavily labeled with hemocyanin over its entire surface. Several virus particles are seen lying on the background, coated with hemocyanin; 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 hr after infection the few cells that were releasing virus had only a few budding figures on the cell surface. Substantial amounts of budding virions were not detected until 3.5 to 4 hr after infection, in agreement with our earlier studies (4). Even by 5.5 hr 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 hrs.

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,

FIG. 9. Surface replica of a prefixed cell treated at 37 C with anti-Sindbis IgG and G-Hcy at 6.5 hrs after infection with Sindbis virus. Circles indicate aggregates of hemocyanin molecules which are probably attached to released virions. X19,000. Scale bar is 1 μm.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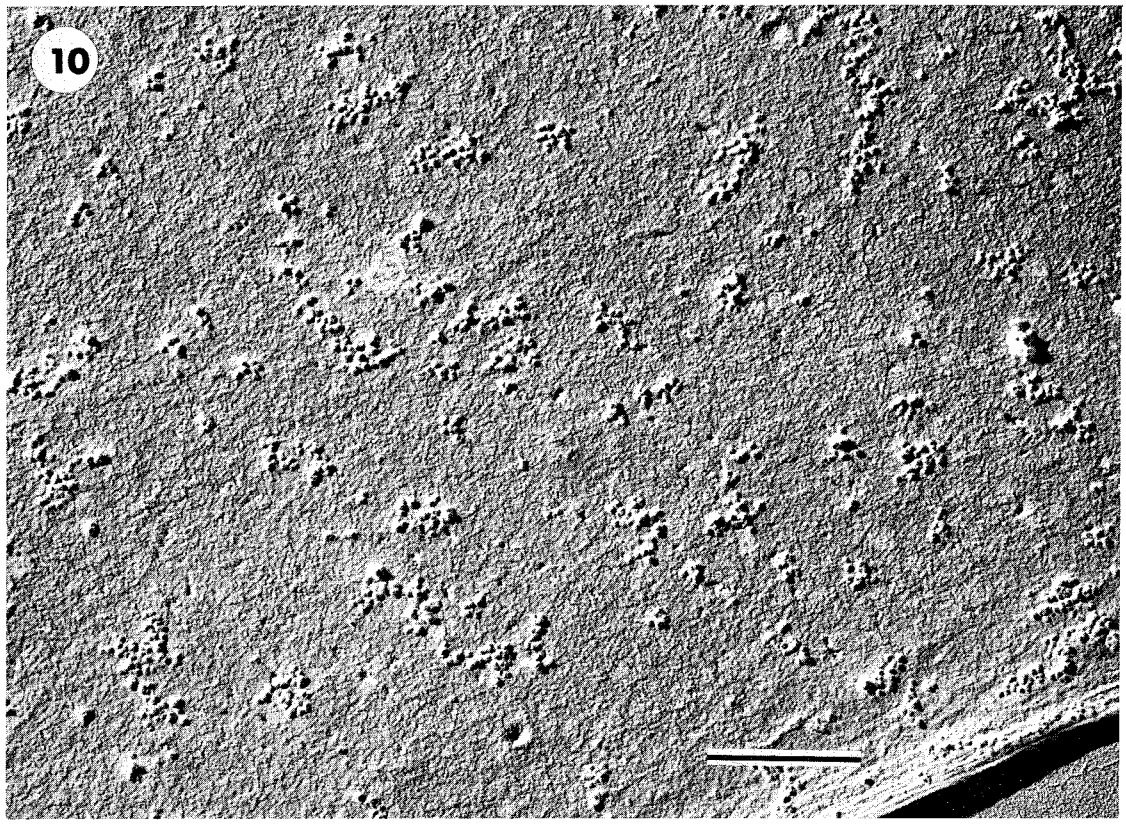
when unfixed cells were treated at 37 C, the hemocyanin label was clustered. Fig. 10 shows a cell with clustered labeling at 2 hr after infection. This cell is lightly labeled, so the clustering is quite obvious. At 2.5 hr after infection, when more glycoprotein has been inserted into the cell surface, many more clusters of hemocyanin were found on the cell than at 2 hr after infection. Later in infection, treating unfixed cells at 37 C did not produce discrete clusters but rather large patches of hemocyanin 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 crosslink the Sindbis virus glycoproteins which have been inserted into the cell surface and cause them to become clustered.

DISCUSSION

The results reported in this paper show that the insertion of Sindbis virus envelope glycoproteins into the plasma membrane of an infected cell begins as early as 2 hr after infection and appears to occur fairly evenly over the entire cell surface (Figs. 4 and 5). By 3 hr 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

FIGS. 10 and 11. Surface replicas of unfixed cells treated at 37 C with anti-Sindbis IgG and G-Hcy at 2 hrs and 3 hrs after infection with Sindbis virus, respectively, shcwing clustering of hemocyanin on the cell surface. Fig. 10, X20,000; Fig. 11, X18,000. Scale bars are 1 μm.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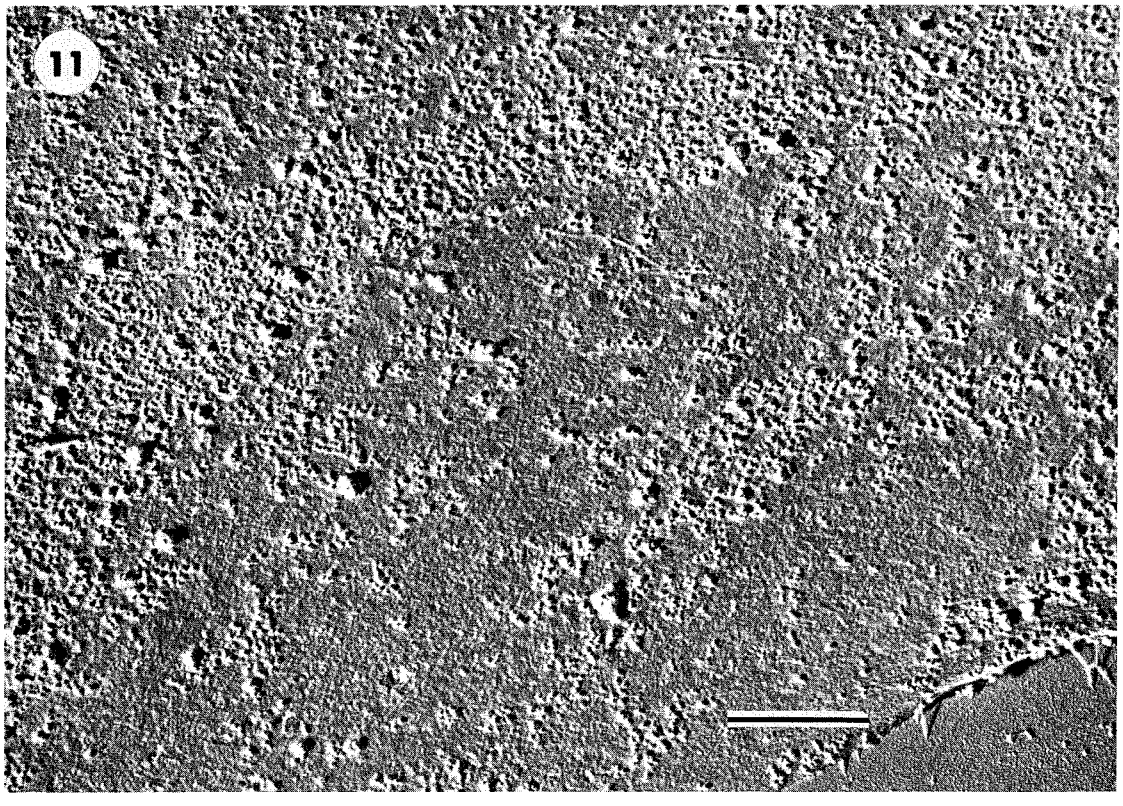
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able to move laterally through the membrane (Figs. 10 and 11).

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 2-1/2 hr after infection, at least an hour 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 3-1/2 hours 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 three hours after infection, until by seven hours after infection the amount of tightly bound virus is only 10-20% of that to uninfected cells (16).

All of these effects begin before the production of mature virions. Although a few virions are produced at 3-1/2 hours after infection, maximal linear release of infectious particles begins at about 4 to 4-1/2 hours 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 in 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, 24). Using either fluorescein 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, 24). 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., in press). Thus, as a cell surface marker the Sindbis glycoproteins show similarities to other surface markers that have been studied.

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AGGLUTINATION OF SINDBIS VIRUS AND OF CELLS INFECTED WITH SINDBIS VIRUS BY PLANT LECTINS

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ABSTRACT

We have examined the agglutination of Sindbis virus and of chick and hamster cells infected with Sindbis virus by two of the plant lectins, concanavalin A and Ricinus communis agglutinin. Both lectins agglutinate the virus by binding to the polysaccharide chains of the envelope glycoproteins. Both chick and hamster cells exhibit increased agglutination by the lectins after infection by Sindbis virus. In the case of chick cells infected with Sindbis virus, this increase in agglutinability occurs between 3 and 5 h after infection. Infected and mock-infected cells bind the same amount of ^{3}H-labeled concanavalin A, which suggests that the increase in agglutination after infection is due to rearrangements at the cell surface rather than to insertion of new lectin binding sites per se.

It is clear from work done in several laboratories that cells transformed by tumor viruses and carcinogens or treated lightly with proteases are agglutinated by much smaller amounts of the plant lectins than is required to agglutinate normal (untreated) cells (9, 13, 15, 16, 26). Most of the recent experimentation has been done with four of the lectins: (i) concanavalin A (Con A), which binds to α-D-glucose and α-D-mannose-like sites (2); (ii) wheat germ agglutinin, which binds to N-acetyl-D-glucosamine-like sites (9); (iii) soy bean agglutinin, which binds to N-acetyl-D-galactosamine and α-D-galactose-like sites (26); (iv) Ricinus communis agglutinin (RCA), which binds to α-D-galactose and L-rhamnose-like sites (19). The mechanism for this change in agglutination appears to be a rearrangement or clustering of binding sites, since transformed cells bind the same amount of radioactively-labeled Con A (4, 10, 21) and wheat germ agglutinin (21) as normal cells, indicating that increased agglutination is not due to an increase in the total number of binding sites. Moreover, electron microscope studies with ferritin-conjugated Con A show a clustering of the conjugate on the surface of transformed cells, as opposed to the uniform distribution of Con A sites on normal cell surfaces (18).

Similar changes in agglutination have also been observed in cells infected by enveloped viruses (5, 23). We have been studying the agglutination of the Sindbis virion, a group A arbovirus, and of cells infected with Sindbis virus.

MATERIALS AND METHODS

Lectins. Con A was purchased from Miles-Yeda, 3X crystallized, or purified by the method of Agrawal and Goldstein (2). RCA was a gift of G. L. Nicolson.

Agglutination of Sindbis virus. Purified Sindbis virus, grown in chicken embryo fibroblasts and labeled with ^{14}C-methionine (28, 29) was dialyzed extensively at 4 C against phosphate-buffered saline, pH 7.4 (PBS of Dulbecco and Vogt [12] but lacking Ca and Mg). After dialysis the protein concentration was 60 μg/ml, as determined by the method of Lowry et al. (17). Bovine serum albumin was then added to a final concentration of 1 mg/ml to stabilize the virus. To 0.8 ml of the virus suspension (2,500 counts/min) was added 0.2 ml of various concentrations of Con A and RCA in PBS; after 2 h at room temperature, the reaction mixtures were centrifuged at 1,000 × g for 15 min at 4 C, and half of the supernatant fluid was assayed for radioactivity. The precipitates were washed once and then dissociated by a 0.5-ml wash of 0.1 M α-methyl-D-mannoside or 0.2 M α-D-galactose, respectively. After centrifugation to remove any remaining aggregates, the solubilized precipitates were assayed for radioactivity.

Agglutination of cells. The agglutination procedure used followed closely that of Sela et al. (26). The cells were removed from the petri plates with a solution of 1 mM EDTA in PBS and were washed three times in PBS. The cells were filtered through 20-μm nylon mesh to eliminate clumps of cells, and the concentration was adjusted to 10^6/ml. Various concentrations of the lectins in 0.1 ml

were mixed with an equal volume of cells in Disposo-trays (Lindbro). This suspension was incubated at room temperature for 30 min and was then scored for agglutination using an inverted phase microscope, with half-maximal agglutination defined as in Eckhart et al. (13).

Cell infection. Confluent monolayers of chicken embryo fibroblasts or of BHK-21 cells in 60-mm petri plates were infected with 50 plaque-forming units per cell at 37 C in 0.5 ml of PBS (with 0.9 mM Ca^{2+} amd 0.5 mM Mg^{2+}) containing 1% fetal calf serum and 1 µg of actinomycin D per ml. One hour after infection the inoculum was removed, the plates were washed, and 3 ml of Eagle minimal essential medium containing 3% fetal calf serum and 1 µg of actinomycin D per ml was added. Two hours after infection the medium was changed to medium lacking actinomycin D. Mock-infected cells were treated identically, except without virus.

The use of actinomycin D improves the virus yield, presumably due to suppression of interferon production. However, virus stocks stored at -50 C in Eagle medium containing 3 to 5% fetal calf serum inactivate to some extent if actinomycin D is present, whereas virus stocks are stable under these conditions in the absence of actinomycin D. Thus, actinomycin D is routinely used only in the early stages of virus infection.

Preparation of labeled Con A. ^{3}H-Con A was prepared by the acetylation procedure of Agrawal et al. (3) using ^{3}H-acetic anhydride (specific activity 50 mCi/mmol), except that 0.1 M α-methyl-D-mannoside was present during the reaction to protect the binding

sites. ^{3}H-Con A was identical to native Con A in migration on a Sephadex G-100 column in 0.2 M sucrose and in agglutination of cells; also, native Con A competed with ^{3}H-Con A for binding to cells. The specific activity of the ^{3}H-Con A used for binding experiments was 165 counts per min per μg.

Binding of labeled Con A to cells. Monolayers of chick cells (in 60-mm petri plates), either infected or uninfected, were washed three times with PBS and then various amounts of ^{3}H-Con A in 1 ml of PBS were added. Control plates were treated with ^{3}H-Con A in the presence of 0.1 M α-methyl-D-mannoside. After incubating for 45 min at room temperature, the monolayers were washed three times with PBS and then dissolved in 2 ml of 1% sodium dodecyl sulfate (SDS); 0.2 ml of each dissolved monolayer was assayed for radioactivity. There was an average of 3.5×10^{6} cells per monolayer, determined by trypsinizing several mock-infected and infected monolayers and counting them in a hemocytometer.

Pronase digestion and affinity chromatography. Sindbis virus, labeled with radioactive glucosamine or fucose, was prepared as previously described (28, 29). Under the conditions used, greater than 97% of these labels migrated with the membrane glycoproteins of the virus on SDS-polyacrylamide gels, and greater than 90% remains in the original chemical form (28). Polysaccharide chains from the glycoproteins were prepared by Pronase digestion of the virus (8) (>95% of the label rendered acid-soluble) followed by dialysis against PBS. The polysaccharides were then tested for adsorption on a Con A-

Sepharose 4B affinity column, prepared by the method of Cuatrecasas and Anfinsen (11). The Con A on the column was biologically active in that ^{14}C-glycogen could be adsorbed to the column and eluted with 0.1 M α-methyl-D-mannoside. Columns containing approximately 1 ml of Con A-Sepharose were poured at room temperature and washed extensively with PBS. Digests of either ^{3}H-glucosamine- or ^{3}H-fucose-labeled Sindbis virus in 0.5 ml of PBS were then applied and allowed to equilibrate with the column for 2 h at room temperature. The column was washed with PBS until no label was detected in the effluent and finally eluted with 0.1 M α-methyl-D-mannoside.

RESULTS

Agglutination of Sindbis virus by Con A and RCA. Since it has been shown that Con A binds to Semliki Forest virus (5, 20), we expected that Con A and perhaps other lectins would bind to and agglutinate Sindbis virus. The results in Table 1 indicate that both Con A and RCA agglutinate Sindbis virus. No agglutination is observed in the presence of 0.1 M α-methyl-D-mannoside for Con A or 0.2 M α-D-galactose for RCA, and Sindbis virus-lectin precipitates are dissociated by the appropriate inhibitor. Thus, the agglutination is specific and reversible. We have also found that wheat germ agglutinin will agglutinate the virus.

To test if the Con A was bound through the envelope glycoproteins of Sindbis virus (28), the polysaccharide chains of the virus were prepared by Pronase digestion. These polysaccharides, labeled

TABLE 1. Agglutination of Sindbis virus with Con A and RCA

Con A			RCA		
Concentration (μg/ml)	Counts/min (% of total)		Concentration (μg/ml)	Counts/min (% of total)	
	Supernatant	Precipitate after dissociation		Supernatant	Precipitate after dissociation
0	100	--[a]	0	100	--
5	95	--	1.5	50	42
10	96	--	3	11	85
25	41	55	6	4.5	91
50	7.5	88	10	3.2	90
75	2.6	91	25	1.7	93
100	2.3	90	50	1.3	92
150	1.5	92	100	0.6	94
150[b]	100	--	100[c]	100	--

[a] No precipitate.

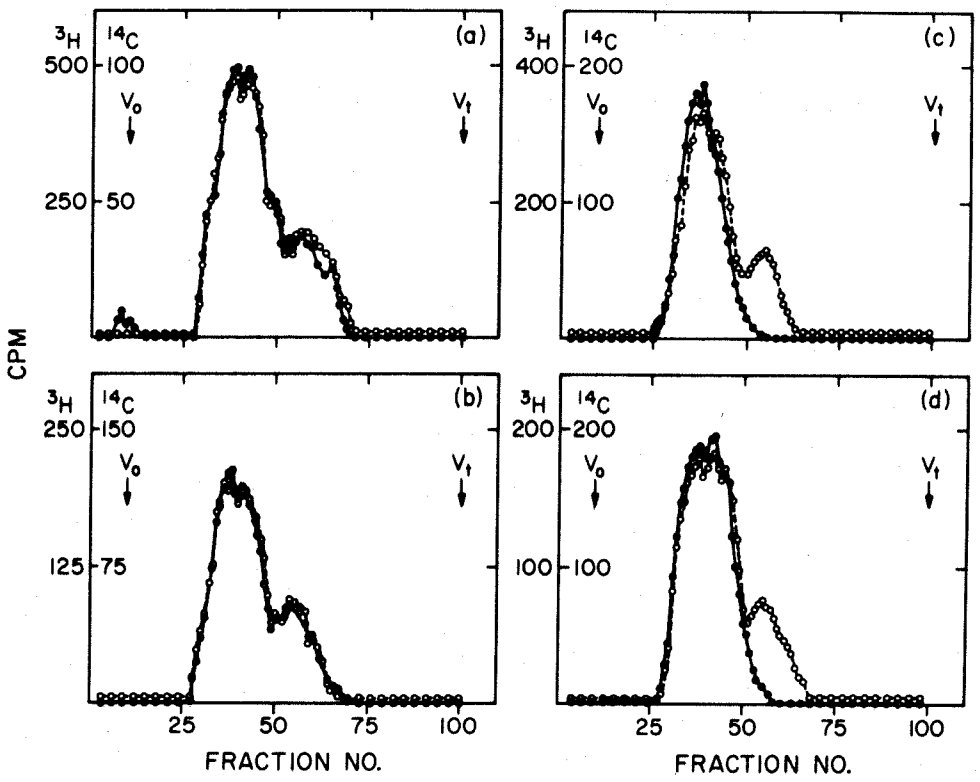
[b] Contained 0.1 M α-methyl-D-mannoside.

[c] Contained 0.2 M α-D-galactose.

with either ^{3}H-glucosamine or ^{3}H-fucose, were tested for binding to a Con A-Sepharose 4B affinity column. For either label, about 75% of the total label added was adsorbed to the columns; all of the bound label could be eluted with 0.1 M α-methyl-D-mannoside. Most of the nonadsorbed label could be adsorbed if applied to another Con A-Sepharose column, and in this way over 95% of the total label could be adsorbed. No label was adsorbed to a column of Sepharose 4B alone, and 0.1 M N-acetyl-D-glucosamine could not elute any of the label adsorbed to the Con A-Sepharose columns.

The envelope glycoproteins of Sindbis virus contain at least 3 polysaccharide chains which differ in length and composition (8). From the previous results, it appears that all three chains are bound by Con A. As a confirmation of this, the various polysaccharide chains were separated by Biogel P-6 chromatography before and after adsorption to Con A-Sepharose columns (Fig. 1). In each panel of Fig. 1, the ^{14}C-glucosamine-labeled polysaccharide chains (open points) have not seen Con A and are used as a reference. As is shown in panels (a) and (b), polysaccharide chains labeled with ^{3}H-glucosamine migrate identically with the marker regardless of whether the label is present in a total digest (Fig. 1a) or if the label is present in chains specifically bound to Con A (Fig. 1b). Similarly, no difference in pattern is detectable for fucose-labeled chains after specific adsorption to Con A columns (Fig. 1c, d). Thus, all three classes of the polysaccharide chains of the envelope glycoprotein of the virus are bound specifically by Con A.

FIG. 1. Polysaccharide chains of Sindbis virus on a Biogel P-6 column. Pronase digests of Sindbis virus, labeled with ^{3}H-fucose, ^{3}H-glucosamine, or ^{14}C-glucosamine were applied to a 0.9- by 60-cm column of Biogel P-6, equilibrated with 0.1 M phosphate (pH 7.8) containing 0.1% SDS. Ten milliliters of effluent was collected into a graduate cylinder, and then 0.22-ml fractions were collected directly into liquid scintillation vials. Digests of ^{14}C-glucosamine-Sindbis virus which were not adsorbed to Con A - Sepharose columns were used as markers. (a) and (b), Before and after adsorption to Con A-Sepharose, using ^{3}H-glucosamine-Sindbis virus; (c) and (d), before and after adsorption to Con A-Sepharose, respectively, using ^{3}H-fucose-Sindbis virus. Symbols: (•), ^{3}H; (o), ^{14}C; V_o, void volume; V_t, total volume.


(a)
(b)
(c)
(d)
^3H
^{14}C
V_o
V_t
500
100
250
50
250
150
125
75
400
200
200
100
200
200
100
100
CPM
25
50
75
100
FRACTION NO.
FRACTION NO.

Agglutination of infected cells. Figure 2 shows the agglutination of chicken embryo fibroblasts at various times after infection by Sindbis virus. Between 3 and 4 h after infection, when approximately 1 plaque-forming unit per cell has been released, infected cells begin to exhibit increased agglutination with Con A and RCA. Agglutinability of the cells continues to rise until 5 h after infection and then levels off. At 5 hr after infection, approximately 100 plaque-forming units per cell have been released, or about 1% of the final virus yield (Fig. 2). No agglutination was ever observed in control incubations containing the inhibitor α-methyl-D-mannoside or α-D-galactose for Con A and RCA, respectively. The same result was obtained when hamster cells (BHK-21) were infected with Sindbis virus, except that the change in agglutinability begins about 5 h after infection and is complete at 6 h. These results do not agree with those of Becht et al. (5), who did not see a consistent increase in agglutination of chick cells after infection with Sindbis virus but did see an increase in the agglutination of hamster cells after Sindbis virus infection.

Binding of ^{3}H-Con A to infected and mock-infected cells. Figure 3 shows the amount of ^{3}H-Con A bound to infected and uninfected cell monolayers when various amounts of the lectin are added. The control incubations (dotted lines) were identical except for the presence of 0.1 M α-methyl-D-mannoside. Mock-infected cells (solid points) appeared to bind slightly more Con A than infected cells, but this difference appears to be due to nonspecific adsorption, since

FIG. 2. Agglutination of chick cells after infection by Sindbis virus. The growth curve applies to both Con A and RCA experiments. Symbols: (●), mock-infected; (o), infected.

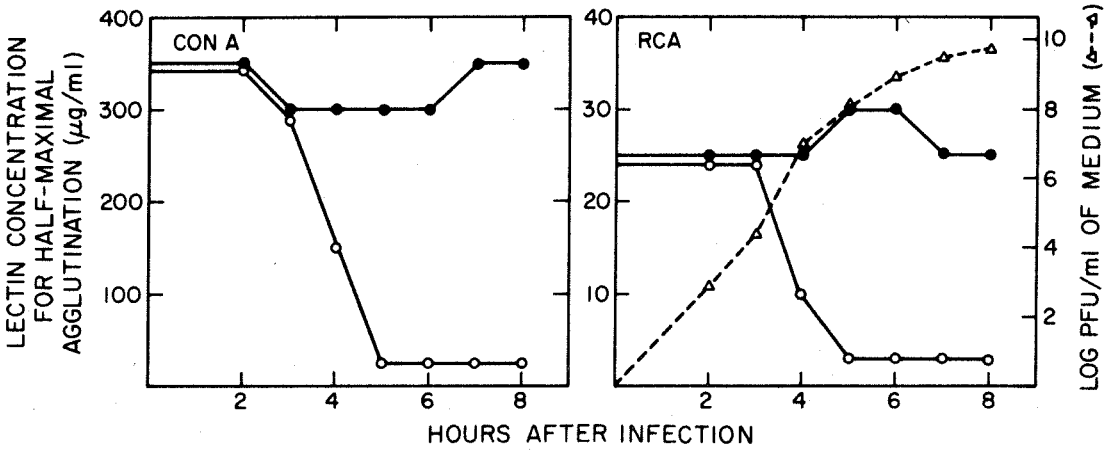
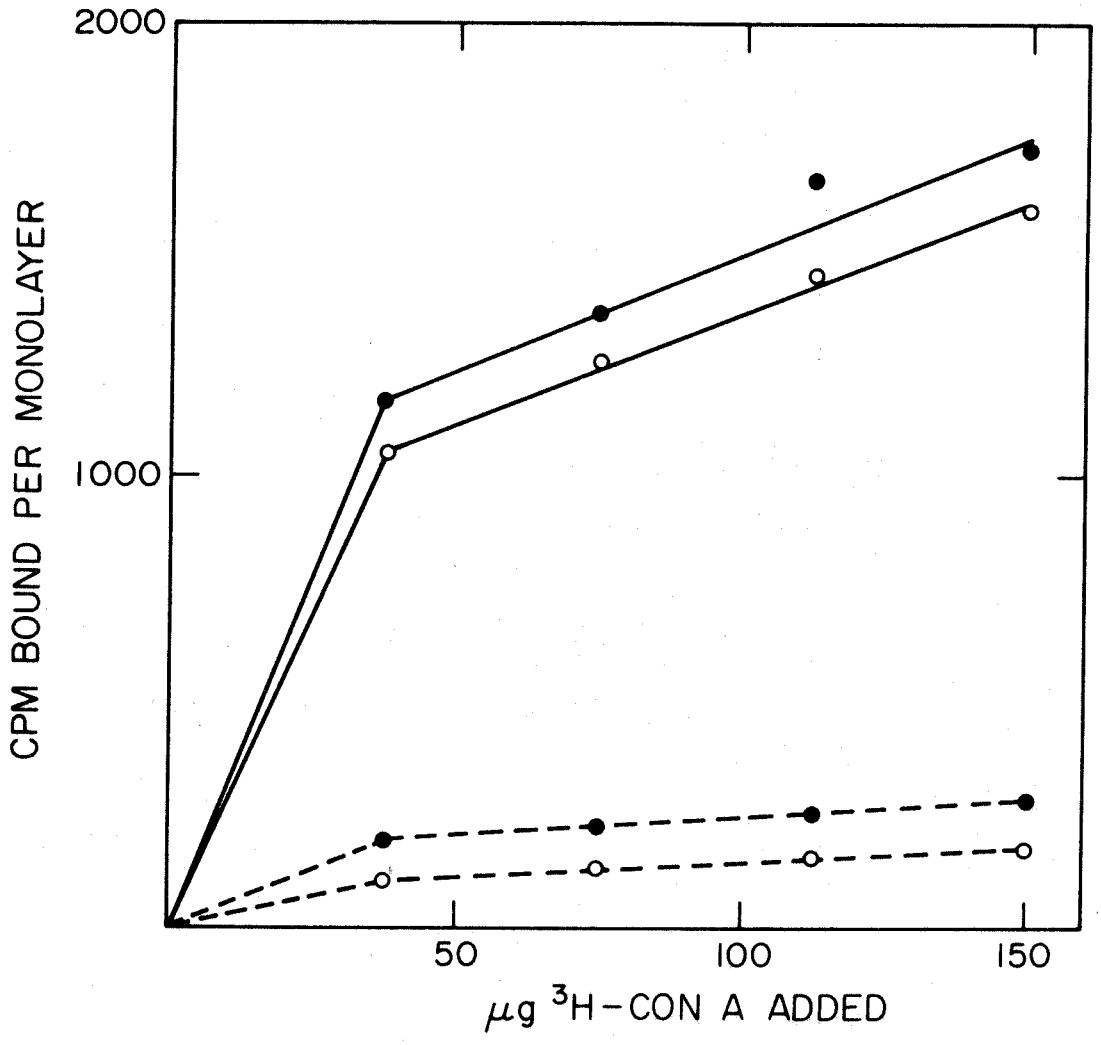

LECTIN CONCENTRATION FOR HALF-MAXIMAL AGGLUTINATION (μg/ml)
CON A
RCA
LOG PFU/ml OF MEDIUM (△--△)
HOURS AFTER INFECTION
400
300
200
100
40
30
20
10
10
8
6
4
2
2
4
6
8

FIG. 3. Binding of ^{3}H-Con A to mock-infected chick cells and to cells infected by Sindbis virus. Symbols: (●), mock-infected; (o), infected; solid lines, no inhibitor; dotted lines, binding in the presence of 0.1 M α-methyl-D-mannoside. Procedures for infecting cells, preparation of ^{3}H-Con A, and determination of ^{3}H-Con A bound to monolayers have been described in Materials and Methods.


2000
1000
CPM BOUND PER MONOLAYER
50
100
150
μg ³H-CON A ADDED

uninfected cells also bind more Con A in the presence of the specific inhibitor. In this experiment the cell monolayers had been infected or mock-infected for 7 h. At this point in infection, approximately 400 plaque-forming units of Sindbis virus have been released per cell (Fig. 2), but the monolayers show only minimal cytopathic effect. From this experiment we can conclude that the marked increase in agglutinability of cells after infection is not due to an increase in the total number of available binding sites.

The number of Con A molecules specifically bound per cell was calculated from the data in Fig. 3, using the specific activity of the ^{3}H-Con A (165 counts per min per μg), the number of cells per plate (3.5×10^{6}), and assuming a molecular weight of 10^{5} for Con A (24). Each cell, whether infected or uninfected, binds 1.5×10^{7} molecules of Con A. This value agrees reasonably well with values previously obtained with other cells (4, 21).

DISCUSSION

We have shown that Sindbis virus is agglutinated by several of the plant lectins, including Con A, RCA, and wheat germ agglutinin, and that this agglutination is effected through polysaccharide chains covalently linked to the surface (glyco) proteins of the virion. It was somewhat surprising to us that Con A would attach to all of the polysaccharide chains of the virion, because Con A is reported to bind specifically to a terminal mannose, glucose, or glucosamine (14, 27). The specificity of the other lectins has not been explored in as much

detail, but if RCA and wheat germ agglutinin also bind to terminal carbohydrates, the virus polysaccharides must exhibit a high degree of branching. We note that the larger virus polysaccharides also contain fucose and sialic acid which are always terminal. Thus these chains, no greater than 16 to 18 residues in length, may possess five or more termini.

After infection of cells by Sindbis virus or other group A arboviruses, the two envelope glycoproteins (25, 29) of the virion are synthesized de novo and inserted into the cell surface; the virus nucleocapsid buds only through regions modified to contain the virus-specific proteins (1, 22, 30). Thus, many new receptor sites for Con A and other lectins are inserted into the cell plasma membrane. It was interesting to find that the infected cells bound the same amount of Con A as did the uninfected cells. It is possible that the number of new binding sites inserted into the cell surface is small compared to the number of preexisting sites so that an increase after infection would not be seen. This seems unlikely, however, in view of the large amount of virus glycoprotein made after infection (up to 2% of the total cell protein late in the infection cycle is virus glycoprotein [29]) and in view of the large areas of budding seen in the electron microscope (6; Birdwell and Strauss, manuscript in preparation). It seems more likely that preexisting sites are lost during virus alteration of the cell surface. It is possible that part of the effect is due to steric hindrance among the Con A molecules; i.e., the Con A binding sites can be packed more tightly than can Con A molecules. The unit cell of crystalline Con A contains the

equivalent of two Con A tetramers and has a volume of 5×10^{-19} cm^3 (24). Thus, in the crystal, each Con A molecule requires the equivalent of 4×10^{-13} cm^2 in cross-sectional area, and the 5×10^{13} Con A molecules bound per petri plate would occupy at least 20 cm^2 of cell surface area. Since the area of the petri plate used is 22 cm^2, it is clear that a significant fraction of the cell surface area is occupied by Con A molecules. Furthermore, a budding virus particle represents a cluster of Con A binding sites, since there is room on the surface of a virion for several hundred Con A molecules. Thus, in those areas that bind Con A, the Con A molecules must be fairly tightly packed, and differences in the absolute number of potential binding sites available would thus be obscured.

Since infected cells bind the same amount of Con A as do the uninfected cells, the increased agglutinability of the infected cells must be due to changes in the topology of the cell surface (perhaps caused by budding virus) rather than to the insertion of the viral glycoproteins per se. In addition, this change occurs early in infection, after commencement of budding but when less than 1% of the final virus yield has been produced; virus-specific protein synthesis becomes detectable only after 3 h of infection (29). It is interesting to note that this stage of Sindbis virus life cycle is characterized by rapid changes in two other cell surface properties as well; an increase in the ability of the infected cells to hemadsorb (7) and a decrease in the ability of infected cells to adsorb Sindbis virions under certain conditions (Pierce, Strauss, and Strauss, manuscript in

preparation). An electron microscope study of these topological changes, using surface replica techniques, is in progress.

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Part III

Electron Microscopic Study of Sindbis Virus Maturation

INTRODUCTION

Previous electron microscopic studies of the maturation of group A arboviruses have been restricted to examining thin-sections of infected cells, with little concern for the relationship between the cell and its substrate. While thin-sections of infected cells can show in detail the interactions of budding virions with the plasma membrane (Acheson and Tamm, 1967), such studies reveal little about the topography of the infected cell surface. Furthermore, to test the possibility that virus-specific modifications of the plasma membrane vary from one region of the cell surface to the next, it is necessary to study virus maturation by a technique which reveals large regions of the cell surface. For this reason, I have been studying the maturation of Sindbis virus with the surface replica technique, which does reveal large areas of the top or underside of cells grown as monolayers (Smith and Revel, 1972; Revel and Wolken, 1973). This technique involves in situ fixation of cells grown on a glass substrate, followed by dehydration and critical-point drying. The cells are then shadowed with platinum and carbon to form a replica, which is then separated from the cells and examined in the electron microscope.

In the following section, studies are presented on the release of Sindbis virus and some of its temperature-sensitive mutants from the top and underside of infected cells; also, colchicine and cytochalasin B, which disrupt cell microtubules and microfilaments,

respectively, have been used to test their effect on the release of Sindbis virus from infected cells. For comparison, the release of vesicular stomatitis virus, an enveloped virus unrelated to the arboviruses, has been studied.

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Smith, S. B., and J. P. Revel. 1972. Mapping of concanavalin A binding sites on the surface of several cell types. Develop. Biol. 27:434-441.

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REPLICATION OF SINDBIS VIRUS

III. AN ELECTRON MICROSCOPIC STUDY OF VIRUS MATURATION USING THE SURFACE REPLICA TECHNIQUE

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ABSTRACT

We have examined surface replicas of chick embryo cells infected with Sindbis virus. In some cells the virus buds over extensive areas of the cell surface, although there are areas where budding virus is clustered and other regions almost devoid of budding figures. In other cells, virus budding occurs primarily or exclusively at the periphery of the monolayer cells. Budding in these cells often occurs in long processes extending from the cell, which have widths from one to several virion diameters and lengths up to 7 μm. These processes have also been examined by means of thin sections to visualize their internal features, especially the presence of nucleocapsids in the process of budding.

INTRODUCTION

Several electron microscopic studies have been carried out on group A arboviruses and on cells infected with them. The final stage in the maturation of these enveloped viruses occurs when the nucleocapsid buds through a modified cellular membrane, acquiring its envelope in the process. Using thin sections of infected cells it has been shown that budding occurs primarily at the plasma membrane, although some virions may be produced at cytoplasmic membranes surrounding vacuoles (Acheson and Tamm, 1967; Morgan et al., 1961; Higashi et al., 1967; Grimley et al., 1968, 1972; Friedman et al., 1972); group B arboviruses, on the other hand, appear to mature predominantly at internal membranes (Filshie and Rehacek, 1968; Matsumura et al., 1971). Electron microscopy of cells infected with Sindbis virus and prepared by freeze-etching have supplied further information on the mechanics of virion maturation. It has been shown that virus does not bud uniformly from all areas of a cell surface. Moreover, as the nucleocapsid interacts with the plasma membrane, the inner layer of membrane fuses around the "core" before the outer layer does (Brown et al., 1972; Waite et al., 1972). Other electron microscopic investigations have concentrated on the morphology of the completed virions (Acheson and Tamm, 1967; Morgan et al., 1961; Simpson and Hauser, 1968a,b).

We have chosen the surface replica technique to examine virus budding from cells infected with Sindbis virus in order to visualize as large a portion of the cell surface as possible.

Complementary studies of thin sections have been included to show the relationship of viral nucleocapsids in the process of maturation to the surface features seen in the replicas.

MATERIALS AND METHODS

Cells and virus. Primary cultures of chicken embryo fibroblasts were seeded in 60-mm Petri plates containing one or two 12-mm cover glasses. When cells on the cover glasses were subconfluent, they were infected with approximately 50 plaque-forming units per cell of Sindbis virus (HR strain) at 37°, as previously described (Birdwell and Strauss, 1973), except without actinomycin D.

Fixation and electron microscopy. Between 3 and 7 hr after infection the cover glasses were removed from the Petri plates and washed in phosphate-buffered saline, pH 7.4 [PBS of Dulbecco and Vogt (1954) but lacking Ca and Mg]. The cells were then fixed on ice for 10 min with 2.5% glutaraldehyde, 0.5% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4. After dehydration in a series of ethanol washes, the cells were dried by the critical point method (Cohen et al., 1968) and surface replicas were prepared (Smith and Revel, 1972). Replicas were examined in a Siemens Elmiskop I.

For thin-sectioning, cells were grown on coverglasses sprayed with Teflon (Fluoroglide, Chemplast, Inc.), infected as usual and fixed by the method of Karnovsky (1965). Flat-embedding was done in Epon-Araldite (Chang, 1971; Mollenhauer, 1964). Cells were stained en bloc with uranyl acetate, and sections were subsequently stained on

grids with lead citrate (Reynolds, 1963; Venable and Coggeshall, 1965). Sections were examined in a Phillips E.M. 300.1 or E.M. 301.

RESULTS

Electron micrographs of surface replicas are shown in the contrast such that the shadows appear dark.

Budding Virus

Figure 1 shows a surface replica of a chick cell which is releasing Sindbis virus over a large proportion of its surface. However, the virions are being released nonuniformly; in particular, the region of the cell in the lower half of the figure appears to have few budding particles. Comparable conclusions have been reached by Brown et al. (1972) with freeze-etched cells.

Examination of Fig. 1 appears to reveal particles in various stages of maturation, some just beginning to bud, others almost completely mature. Moreover, the cell periphery appears smooth, with few projections. The type of virus release illustrated by Fig. 1 was commonly encountered.

Figure 2 shows a surface replica of two chick cells at 6 hr after infection. The cell on the right appears to have little virus associated with its surface. At this time during the Sindbis virus replication cycle roughly one out of three cells appeared to produce very little virus. The presence of such cells illustrates that the virus seen on the surface of producing cells, such as the one on the left of Fig. 2, must originate at that cell and do not arise from

FIG. 1. Surface replica of Sindbis virus-infected cell at 4.5 hr after infection. × 20,000. Scale bar is 1 μm.

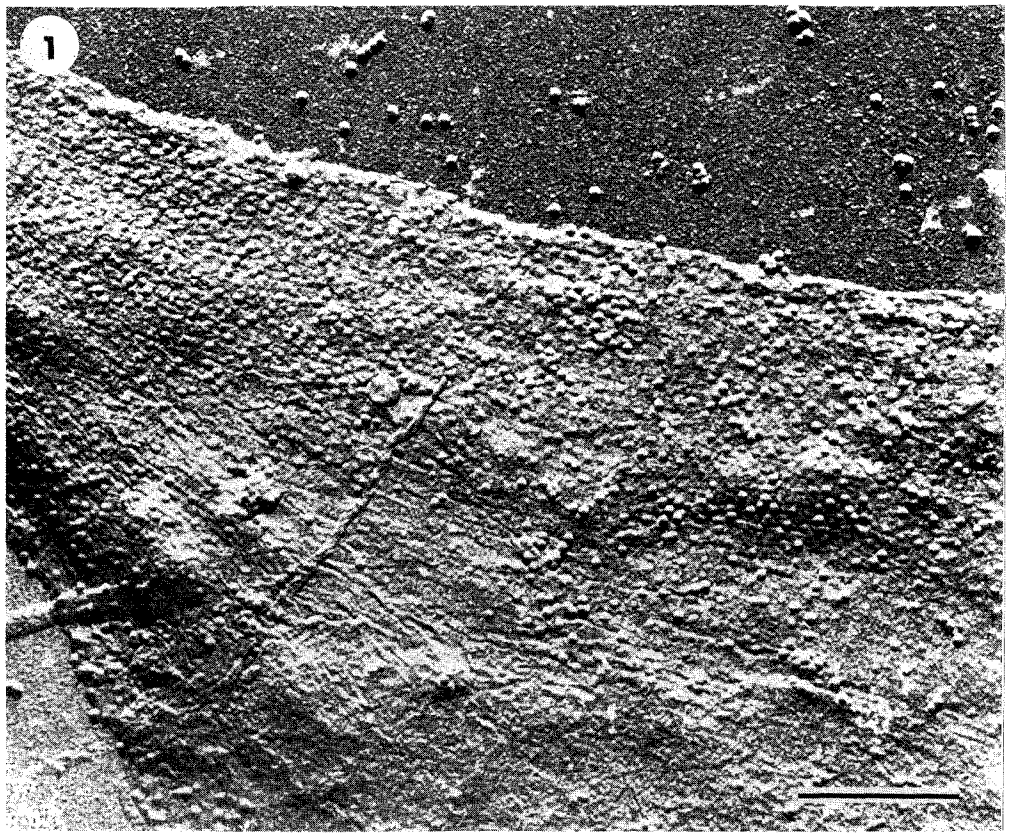
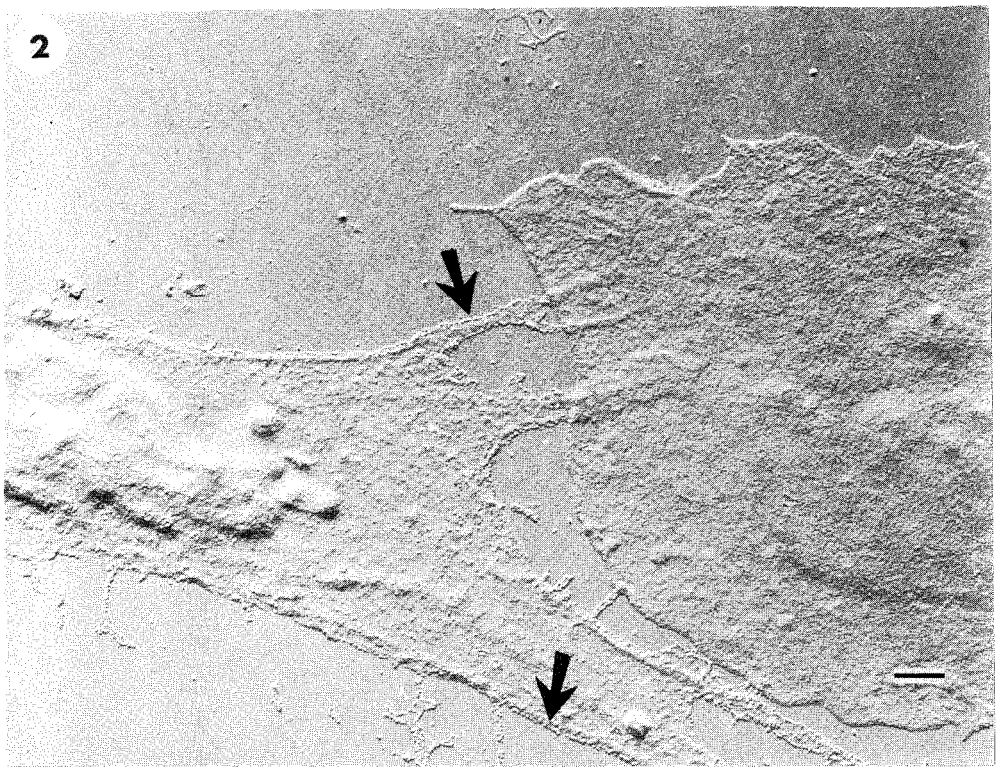

1

FIG. 2. Surface replica of Sindbis virus-infected cells at 6 hr after infection. The cell on the left is actively releasing virus, especially at its periphery (arrows), whereas the cell on the right appears to be releasing few virions. × 5,800. Scale bar is 1 μm.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random adsorption of released virus from the medium, nor from the original inoculum. As a control, not illustrated, large amounts of virus have been adsorbed to monolayers at 4°. Under these conditions, all cells examined had large numbers of particles adhering to them.

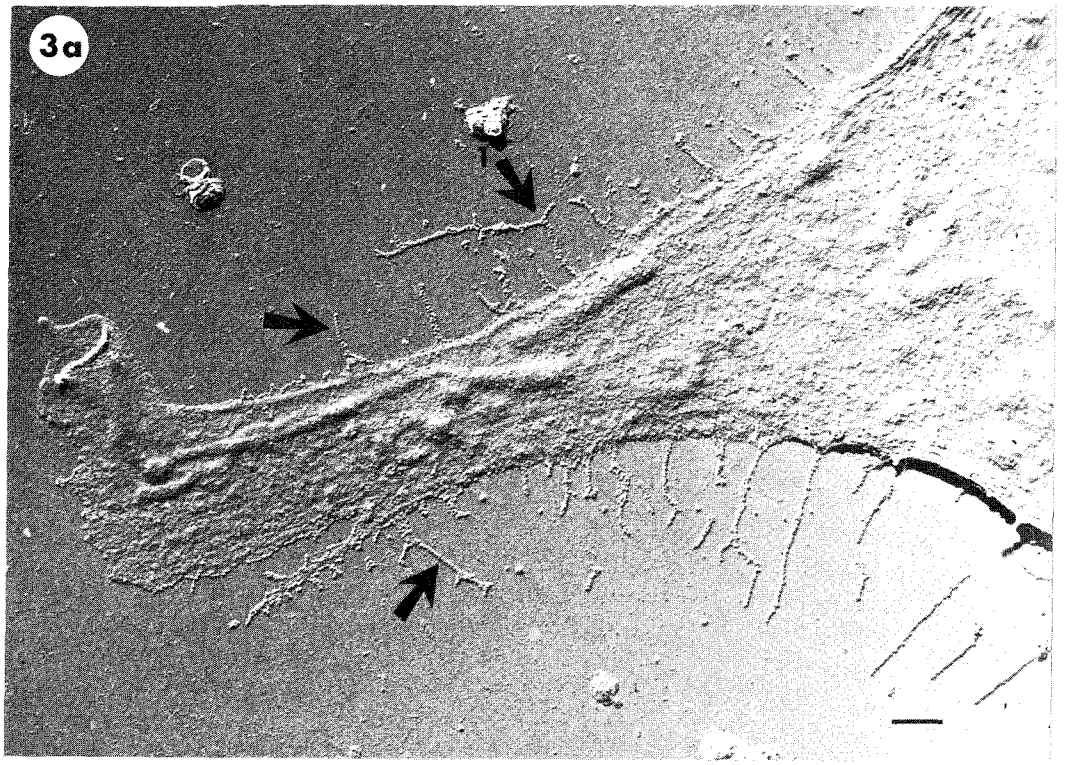
The cell on the left in Fig. 2, while releasing large amounts of virus over extensive regions of its surface, also shows concentrations of budding figures at the periphery (arrows).

Virus Release through Cell Processes

In contrast to the cell in Fig. 1, infected cells are frequently observed in which the majority of the budding virus is seen at the periphery, and in most cases in processes extending from the edges of the cell. Surface replicas of such cells are seen in Figs. 3 and 4. In some cells the processes appear to consist of a single row of budding nucleocapsids (arrows on Figs. 3a, 4a, and 4b); most of the processes from the cell in Fig. 3a fall in this category. In others these cellular extensions appear to contain double, triple, or multiple rows of budding virus (black and white arrows, Figs. 3b and 4c); note especially Fig. 4c in this regard. Figure 3b illustrates a cell in which most of the processes are more than one particle wide, but the budding virus figures are found primarily at the edges of the processes with little budding virus seen at the center. Whatever the thickness of the processes, they often branch (e.g., arrow 1 in Fig. 3a).

The lengths of the various processes vary greatly. Some of those illustrated in Fig. 3a, for example, are more than 4 μm long,

FIG. 3. Surface replicas of Sindbis virus-infected cells at 6 hr after infection showing the release of virus through processes extending from the cell periphery. In (a), processes with single rows (black arrows) and multiple rows of virions are seen. Arrow 1 indicates a branching process. In (b) mostly multiple rows (black and white arrows) are seen, (a) × 6,500; (b) × 15,400. Scale bars are 1 μm.


3a

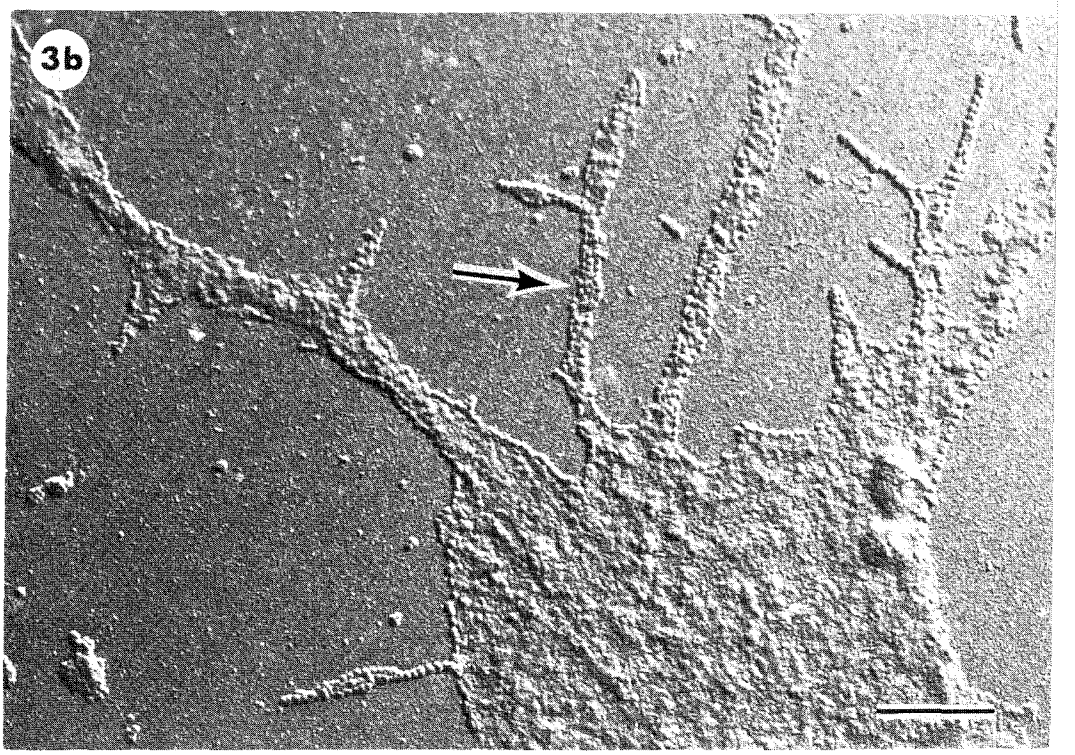
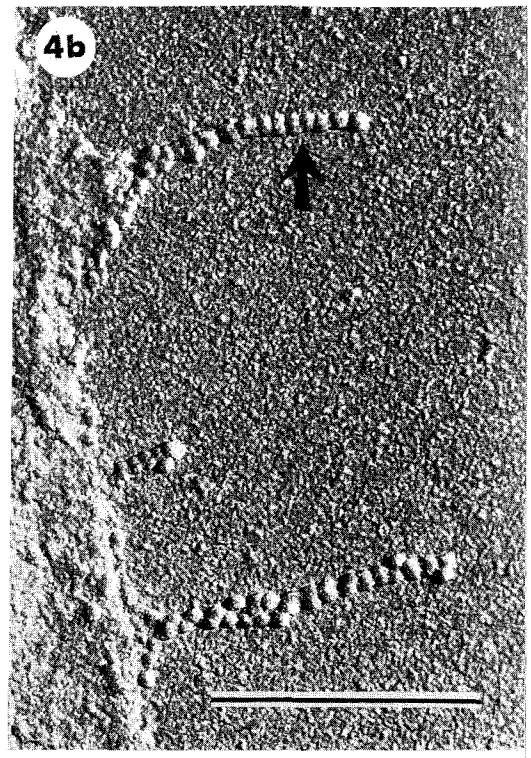
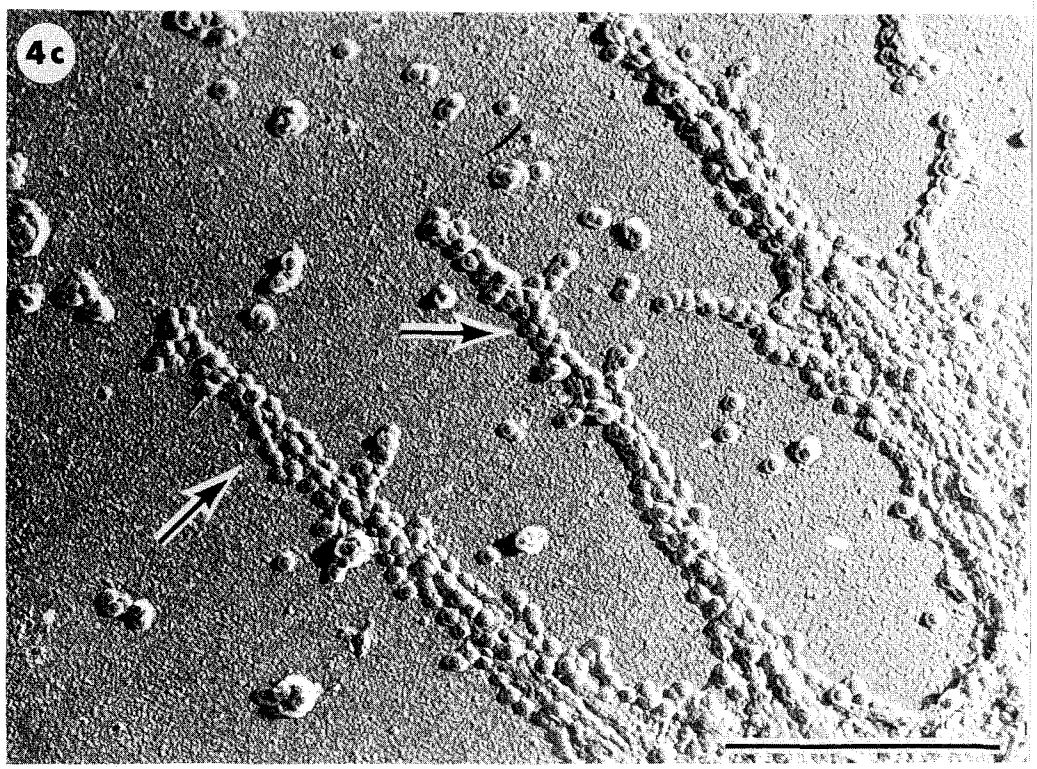

3b

FIG. 4. Surface replicas of Sindbis virus-infected cells at 6-7 hr after infection. The meaning of the arrows is the same as in Fig. 3. (a) × 25,000; (b) × 35,000; (c) × 35,000. Scale bars are 1 μm.


4a


4b


4c

and lengths up to 7 μm have been observed. Other processes are quite short. Notice that in the cells shown in Figs. 2 and 3 there is a clustering of budding figures along the edges of the cell as well as in the processes. Moreover, these processes always arise from the edges of the cell, never from the upper surface.

Processes were rarely observed at 3 hr after infection, but by 7 hr after infection most cells which showed budding virus had these extensions. Similar results were obtained with baby hamster kidney cells infected with Sindbis virus, but the processes did not appear until later in the life cycle. No such appendages were seen on mock-infected cells.

This mode of virus release, with the great majority of maturing particles only at the cell edge and in these long extrusions extending from the cell edge seems to represent a different type of maturation figure from that seen in Fig. 1. However, all combinations of the two modes can and do exist. We have also examined replicas of the underside of infected cells (Revel and Wolken, 1973). Both types of maturation are also observed from the underside, indicating that virus release is not restricted to the top of the cell.

Thin Sections of the Processes

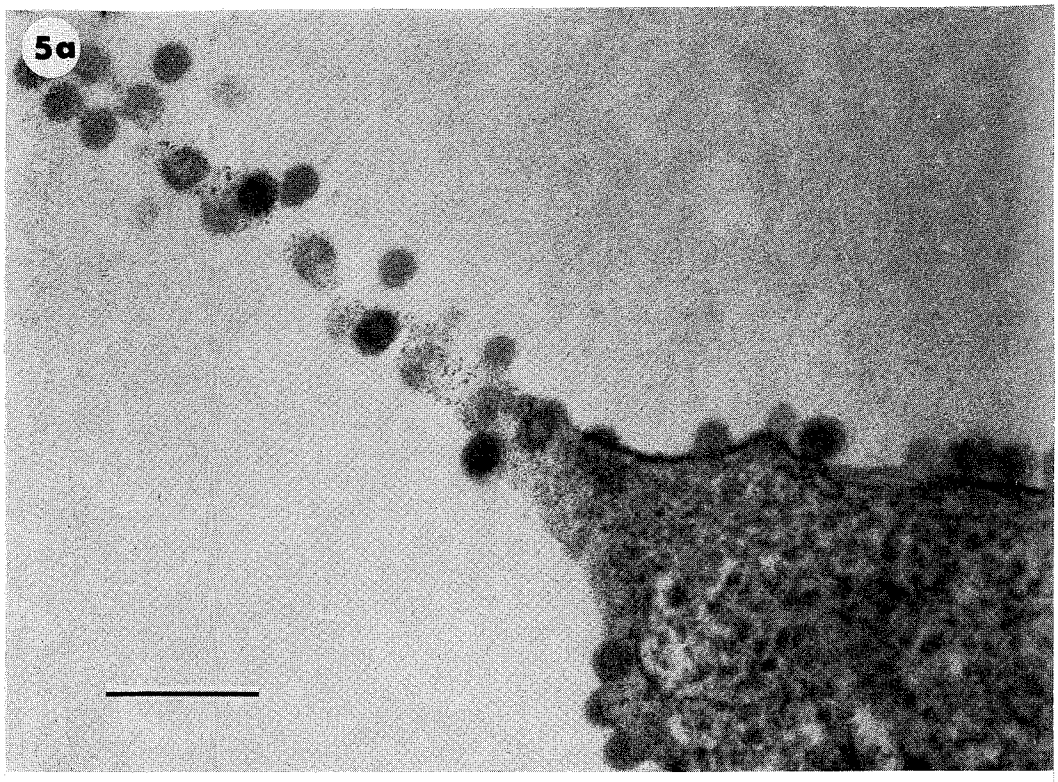
To confirm that the processes observed by the surface replica technique were active sites of virus maturation, containing nucleocapsids in the process of budding, infected cells were examined after thin-sectioning. To avoid possible disruption of these processes during treatment, infected cells were embedded as a monolayer to

preserve cell surface morphology as much as possible. Figures 5 and 6 show thin sections of processes chosen to represent the various configurations observed.

Figure 5a shows a section through a process which is more than one virion diameter in width. This cell also shows an array of nucleocapsids lined up along what is presumably the periphery of the cell (these nucleocapsids appear to be in completely or almost mature virions whose membranes are not visualized under these conditions). Figure 5b shows a section through a process (top half of figure) which is only one virion diameter in width at the distal end and which appears to branch. In addition, the "budding" figure shown by the arrow probably represents a cross section through a process with multiple rows of budding nucleocapsids, although it could also be interpreted as a section through a virus particle containing several nucleocapsids.

It was difficult to find long processes which were completely in the plane of sectioning. Figures 6a and 6b show processes which are in the plane of sectioning for more than 2 μm. The thickness of these processes appears to vary from one nucleocapsid in diameter to several, but this could be due to the plane of sectioning not being centered in the process throughout. Figures 6c, 6d and 6e show three processes at higher magnification. The process in Fig. 6e appears to average one virion diameter across, whereas those in 6c and 6d are several virion diameters in width. All these processes show clearly that nucleocapsids are maturing by budding through the sides of these

FIG. 5. Thin sections of Sindbis virus-infected cells at 6.5 hr after infection. Rows of core particles are seen in structures that may be similar to the processes seen in surface replicas. In (a), the process leaves the plane of sectioning at the upper left-hand corner. In (b), the arrow indicates a "pleomorphic" form of Sindbis, which may be a cross-section of a process. (a) × 97,000; (b) × 106,000. Scale bars are 200 nm.


5a

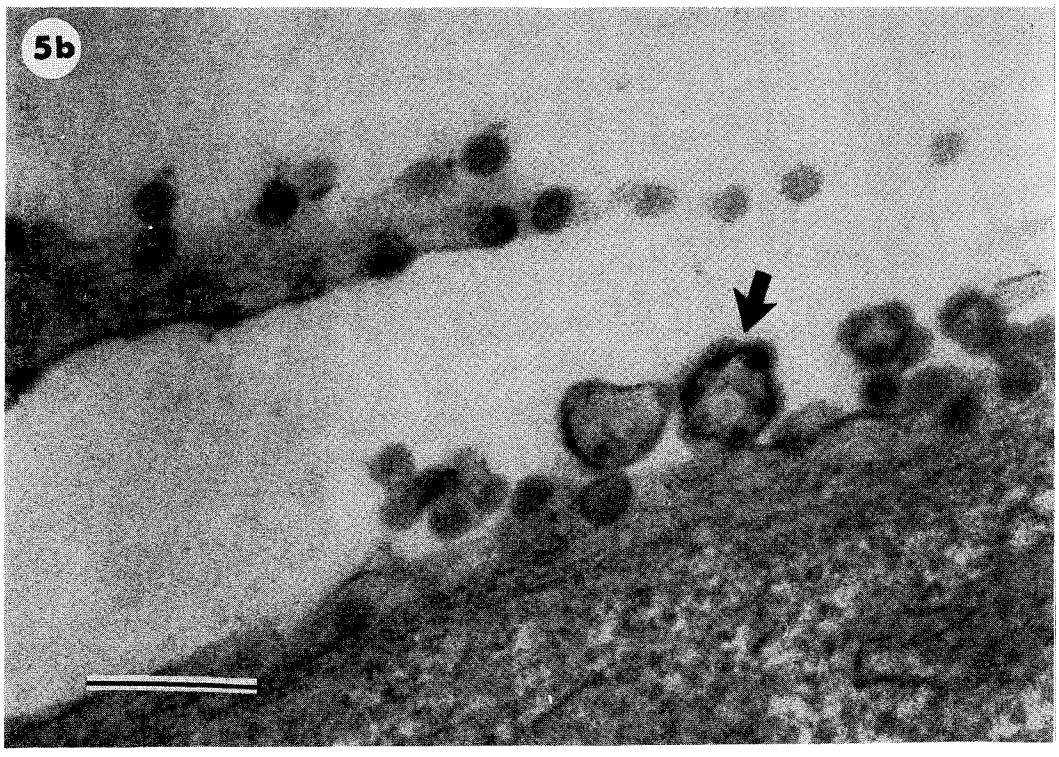
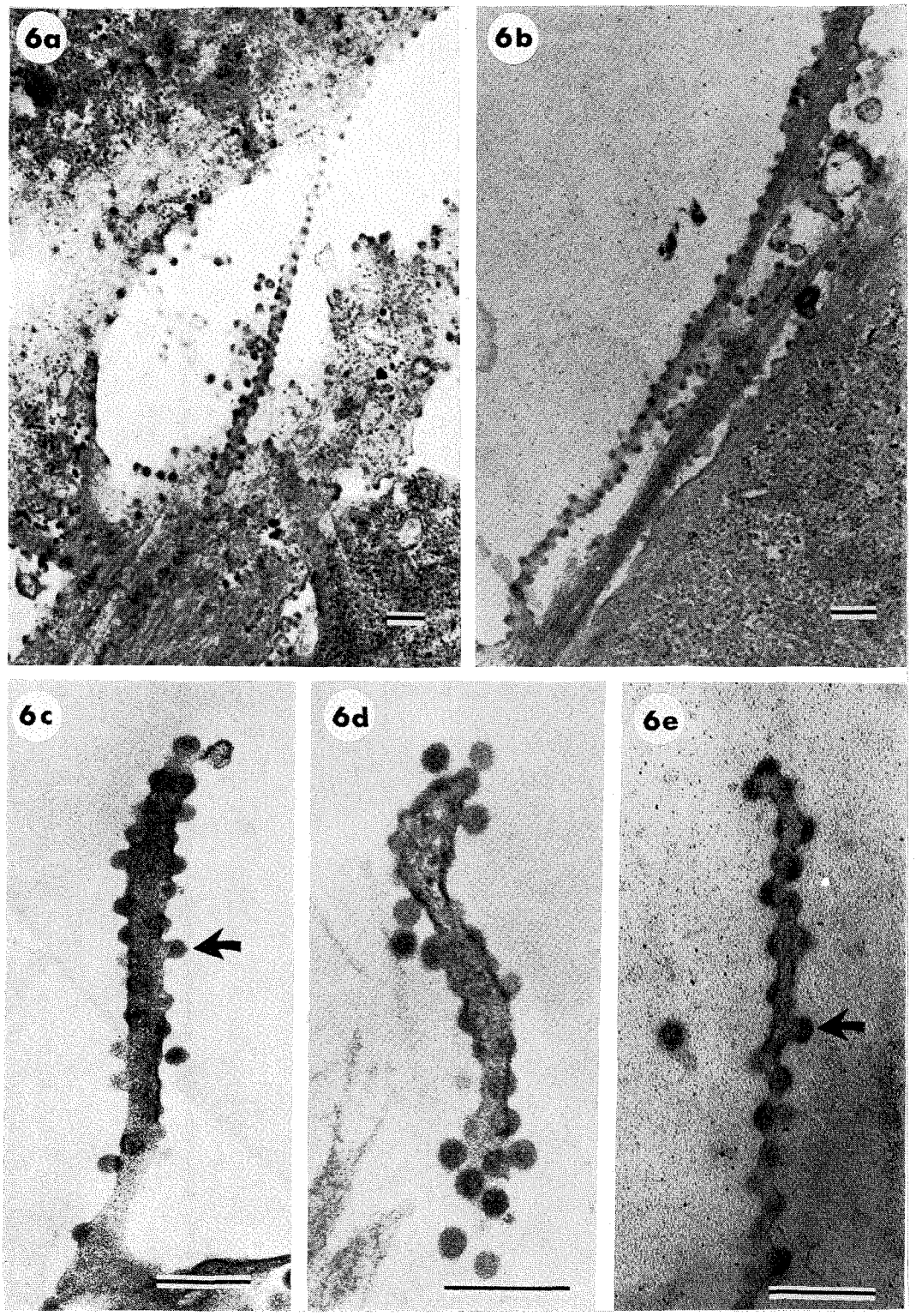

5b

FIG. 6. Thin sections of infected cells. In (a) and (b), long processes are seen which may be attached to the cell. In (c), (d), and (e), selected processes are shown at higher magnifications. Arrows indicate particles budding through the sides of the processes. (a) × 31,000; (b) × 36,000; (c) × 70,000; (d) × 95,000; (e) × 80,000. Scale bars are 200 nm.


6a
6b
6c
6d
6e

processes (e.g., arrows on Figs. 6c and 6e), as well as at the presumptive ends of the processes.

DISCUSSION

Examining surface replicas of cells which have been fixed *in situ* enables one to study a large area of the outer surface of a cell, while the cell is still in the same position relative to its substratum. This should provide a better approximation of the natural state of the cell surface as it was in the living monolayer than sample preparations which require mechanical harvesting of cells.

The findings of this study on Sindbis virus maturation confirm results reported from freeze-etched preparations (Brown *et al.*, 1972) that Sindbis virus can be seen to bud from the infected cell preferentially in certain areas or patches. In some cells the virus appears to mature predominantly at the cell periphery, as in Figs. 3 and 4, while in other cells, large areas of the cell surface show virus release (such as Fig. 1). It is difficult to estimate what proportion of the cells show clusters, or patches, of budding virus, since many cells are lost during replica preparation and there is no assurance that the remainder are completely representative, but probably more than half of the cells display this type of nonuniform budding.

One of the most interesting results of the present study was the finding of processes on many cells which are apparently major

sites of virus maturation. These processes are always seen to emanate from the edges of the cells; however, many cells show preferential virus maturation at the periphery of the cell even in the absence of such structures.

As shown in both the surface replicas and the thin sections, budding virions often bud through the sides of the processes; in the replicas branching of the processes is often seen. Some processes are observed which, although still attached to the cell, contain over 100 immature virus, while much shorter processes are often seen broken off from the cell. These detached processes may be due to the handling of the cells rather than to the normal mode of viral release. Thus it is not clear whether nucleocapsids bud exclusively from processes as they extend from the cell or from processes which break off in pieces from the cell. The release of virions through processes may be responsible for the pleomorphic forms of Sindbis virus (Matsumura et al., 1971) and Chikungunya virus (Higashi et al., 1967) which are sometimes observed in thin sections. The replicas often show released particles which appear to contain more than one nucleocapsid (e.g., Figs. 1 and 4c).

The molecular basis for preferential maturation at the cell periphery and in particular at these processes is not clear at present. It is possible that the plasma membrane at the edges of a cell in a monolayer culture is more fluid or more metabolically active than at other regions of the cell surface. Such fluidity might facilitate both the insertion of virus-specific elements into the membrane as well

as the actual budding event. Monolayer cells have been shown, by time-lapse photography, to be in constant motion at the edges, appearing to "ruffle," extending and retracting cellular "pseudopods." This ruffling may indicate a greater fluidity at the periphery.

Another possibility is that once a nucleocapsid has budded partially through an area of membrane, this area of altered membrane serves as a center of nucleation to attract other nucleocapsids and the chain reaction destabilizes the configuration of the membrane in a certain "patch." As virus matures, nucleocapsids gathered at this favored site follow one another through the plasma membrane, with processes resulting when these particles bud faster than the membrane can fuse around them.

Further studies of the budding process, utilizing temperature-sensitive mutants of Sindbis virus, are in progress.

ACKNOWLEDGMENTS

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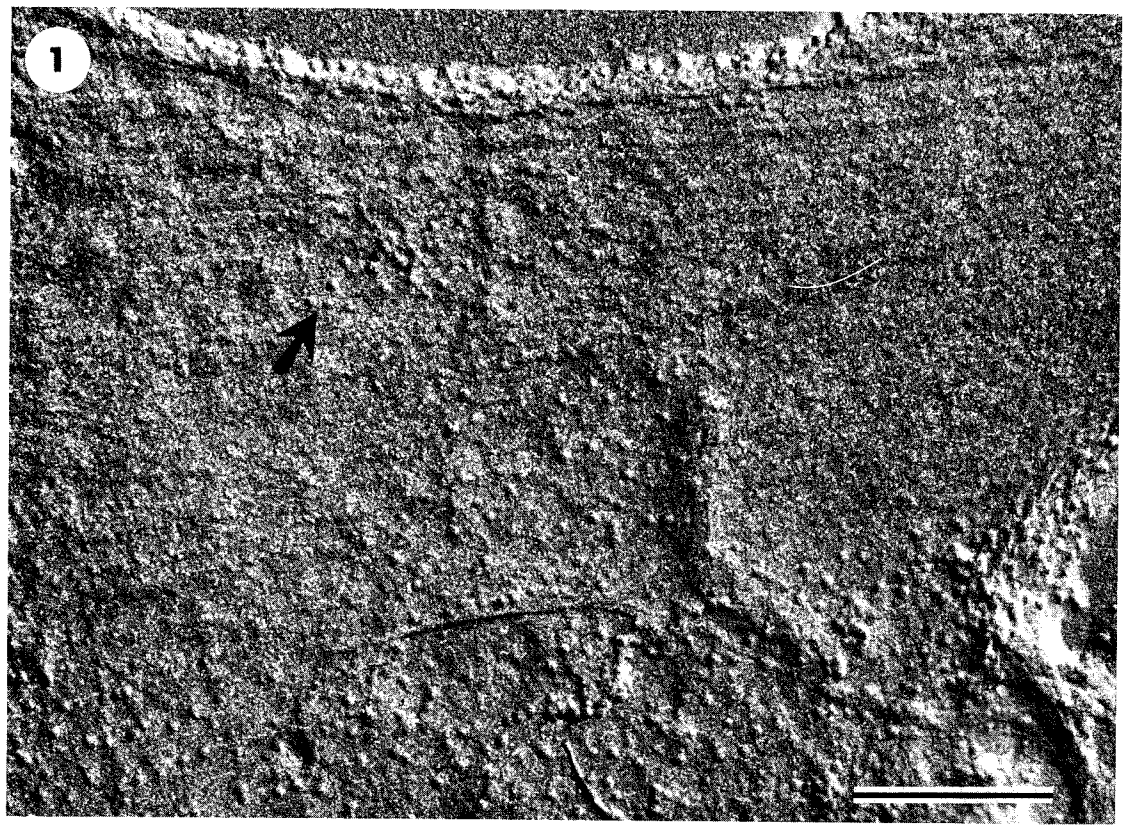
RELEASE OF SINDBIS VIRUS FROM THE UNDERSIDE OF INFECTED CELLS

Techniques have been developed for examining the undersides of cells grown as monolayers in tissue culture (Revel and Wolken, 1973). Cells grown on plastic petri dishes are fixed and dehydrated *in situ*. Sections of the monolayer are then removed from the plastic by treatment with amyl acetate and then picked up on coverglasses in an upside-down orientation. After air drying, surface replicas are prepared as previously described (Smith and Revel, 1972; Revel and Wolken, 1973).

In Figures 1 and 2 are shown surface replicas of the underside of chick embryo fibroblasts at 6 hrs after infection with Sindbis virus (HR strain). From these electron micrographs, it is clear that Sindbis virus is released from the undersides of cells grown in monolayers; the release of Sindbis virus from the tops of cells grown in monolayers has been previously reported (Birdwell *et al.*, 1973). The cell in Figure 1 has released only a few virus particles (arrow), whereas the cell in Figure 2 has budding virions over much of its underside.

FIGURES 1 and 2. Surface replicas of the undersides of Sindbis virus-infected cells. The arrow in Fig. 1 points to a few budding virions on the underside of the cell. X25,000. Scale bars are 1 μm.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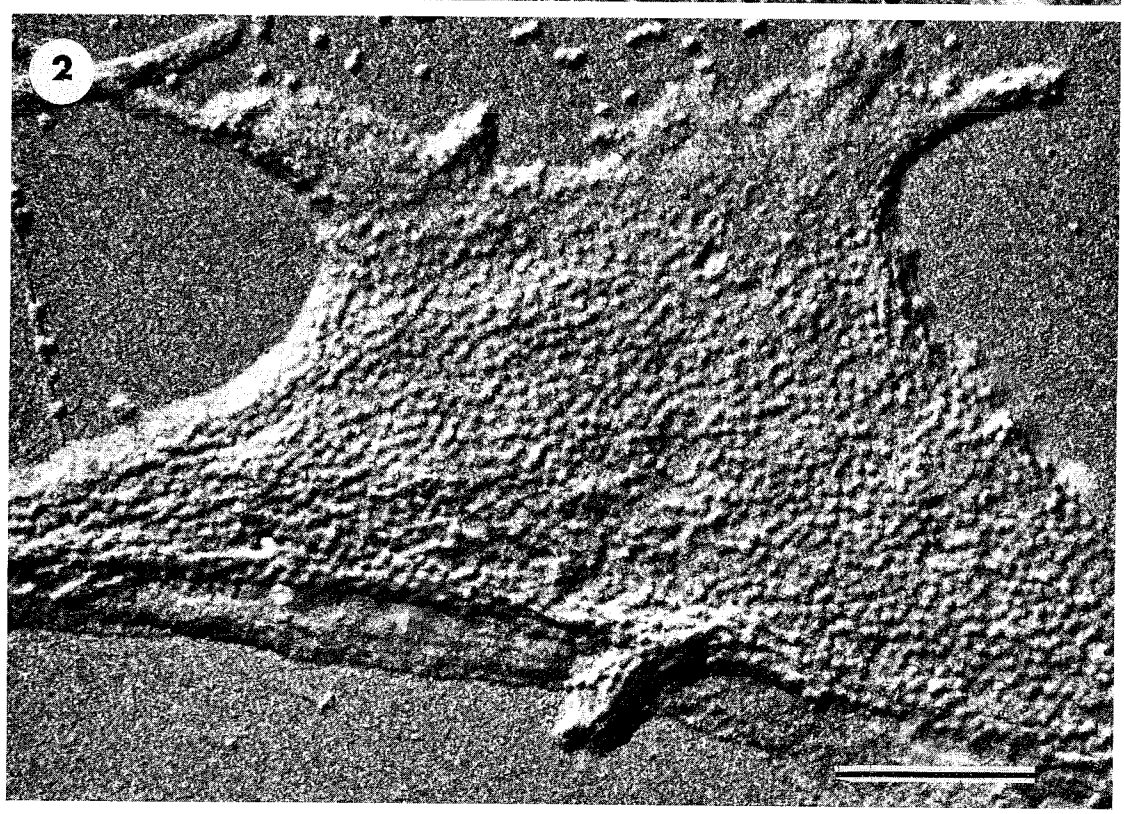
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The following material has been accepted for publication in Virology.

MATURATION OF VESICULAR STOMATITIS VIRUS: ELECTRON MICROSCOPY OF SURFACE REPLICAS OF INFECTED CELLS

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There have been several studies on the maturation of enveloped animal viruses from the surfaces of infected cells. In many cases a similar mechanism seems to be involved: insertion of virus-specific envelope glycoproteins into the surface and budding of subviral nucleocapsids through these altered regions. During this process of budding, we have found that Sindbis virus, a Group A arbovirus, is often released from infected cells through virus-specific "processes" originating at the cell periphery (1). These processes, which are up to 7 μm long, may reflect a difference in the cell surface between the periphery and center of cells grown on artificial substrata. To determine if similar processes were formed in cells infected by other enveloped viruses, we examined cells infected with the unrelated virus, vesicular stomatitis virus (VSV), using the surface replica technique (2).

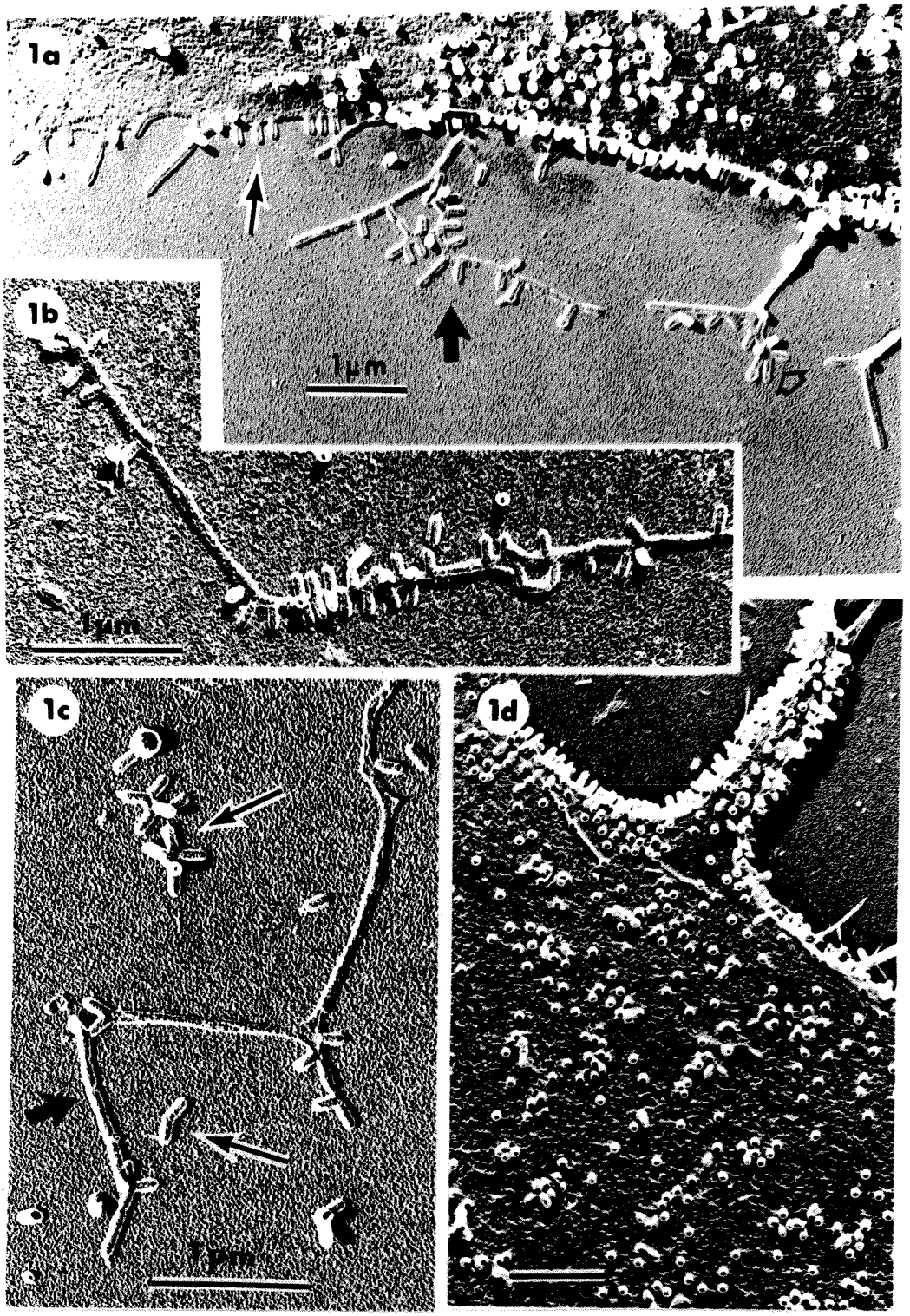
Chick embryo fibroblasts and baby hamster kidney cells (BHK-21) were grown on coverglasses as previously described (1, 3). Cells were infected with 50 plaque-forming units per cell of VSV (Indiana serotype) at 37°, using the procedure previously described for Sindbis virus, except without actinomycin D (3). Between 4 and 7 hours after infection the cells were treated for electron microscopy as previously described (1) and surface replicas were prepared. Replicas were examined in a Phillips electron microscope 300.1; electron micrographs are presented here in reverse contrast.

Processes analogous to those found in Sindbis-infected cells were also found in VSV-infected cells. Figure 1a shows part of a

VSV-infected chick embryo fibroblast with processes extending from the cell periphery containing budding VSV particles (solid arrow, open arrow). These processes became more common as the infection progressed. By 7 hours after infection, approximately 10% of cells releasing virus exhibited these extensions. Some isolated processes are shown in Figs. 1b and 1c; these have apparently broken off from the cell during the process of preparation for electron microscopy. Budding is preferentially through the sides of the process shown in Fig. 1b. In Fig. 1c long rods are seen with several (immature) virions linked end-to-end (solid arrow). Figure 1c shows other aberrant forms which consist of two or more particles fused at their base (black and white arrows). These presumably arise by incomplete separation of budding viruses.

The cell in Fig. 1a is also releasing considerable amounts of virus through the top of the cell and along the edges. There appears to be a slight clustering of virus release at the cell edge, although this is not as marked as in Sindbis-infected cells (1). Figure 1d shows a cell which is releasing quantities of virus but which lacks processes. There may be a very slight clustering of virus release along the edge of the cell. Also seen on the surface of this cell (circles) are figures that appear to be progenitors of fused forms of the virus similar to those seen in Fig. 1c. Alternatively, these fused forms could result from the breakage of branched areas of the processes into small pieces (such as Fig. 1a, open arrow).

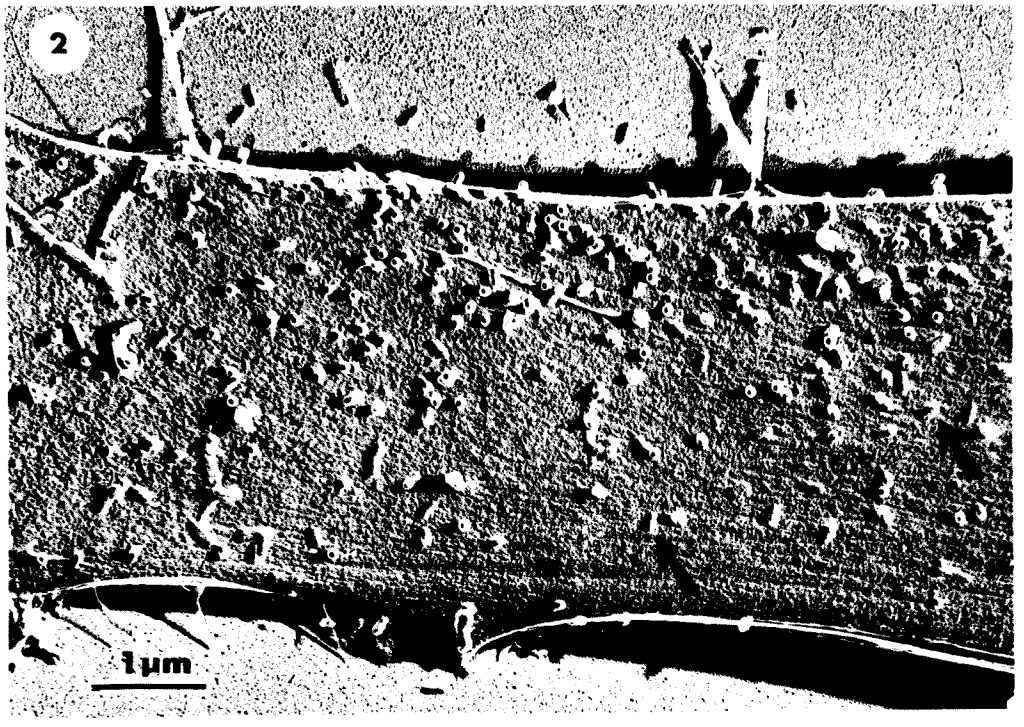
FIG. 1. Surface replicas of VSV-infected chick embryo fibroblasts. In (a), × 16,000, both processes of budding virions (solid arrow, open arrow) and single particles (black and white arrows) can be seen at the cell edge. In (b), × 25,000, a process is seen which has broken off from the cell. In (c), × 24,000, fused forms of VSV (black and white arrows) are seen next to a process which contains many virions linked end-to-end (solid arrow). In (d), × 14,000, the cell is releasing many single particles through its surface, as well as some fused forms (circles). (a)-(c) are at seven hours after infection; (d) is at six hours after infection.


1a
1µm
1b
1µm
1c
1µm
1d

For comparison, a VSV-infected BHK cell is shown in Fig. 2. No virus-specific extensions were observed in BHK cells, although such structures might appear later in the growth cycle (replication in BHK cells is slower than in chick cells). In BHK cells, however, a significant fraction of the virions on the surface of the cell appear to be on their sides (arrows). These are probably virions which are budding through sideways, but could be mature virus which has readsorbed to the cell in this orientation. Such figures are rare in chick cells.

Our findings indicate that as in the case of Sindbis virus-infected cells (1) the production of virus-specific processes at the cell periphery does occur in cells infected with VSV. As in Sindbis virus-infected cells, they are highly branched and are found only at the cell periphery. In contrast to the results for Sindbis infection, however, in VSV-infected cells these processes represent only a minor mode of virus release and have been seen in only a minority of cells; in general, virions are released singly from all regions of the cell surface. These processes are probably related to the long rods containing virions seen earlier in thin sections of cells infected with rabies virus (4) and in replicas of cells infected with VSV (5).

FIG. 2. Surface replica of a VSV-infected BHK cell at six hours after infection, × 15,000. Many virions are seen lying on their sides at the cell surface (arrows).


2
1 µm

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INHIBITION OF SINDBIS VIRUS RELEASE BY COLCHICINE AND CYTOCHALASIN B

It has been shown that when cells grown as monolayers are infected with Sindbis virus, much of the virus is released through long processes which always originate at the cell periphery (Birdwell et al., 1973). The mechanism for this particular mode of virus release is not known, but release in such a manner may be due in part to some of the known properties of the cell edge. For example, when monolayer cells are observed by time-lapse photography, the leading edge of the cell periphery is actively "fluttering" or "ruffling," suggesting that certain regions of the cell edge may be more fluid or metabolically active than other parts of the cell surface. These ruffling regions may facilitate the formation of virus-specific processes because of a rapid turnover of membrane components; for a more detailed discussion, see Birdwell et al., 1973.

The nature of the cell edge has been further investigated using the drugs colchicine and cytochalasin B, which disrupt cell microtubules and microfilaments, respectively. When fibroblasts growing in monolayers are treated with colchicine, all regions of the cell edge ruffle and the cell no longer has directional locomotion (Vasiliev et al., 1970). When glial cells are treated with cytochalasin B, the cells no longer migrate and ruffling activity stops at all regions of the cell periphery (Spooner et al., 1971). Thus, microfilaments appear necessary for ruffling and cell migration, but microtubules somehow provide direction for the cell as it migrates. Since some of the effects of colchicine and cytochalasin B are clearly manifest at the cell edge, it seemed worthwhile to test the

effects of these drugs on the release of Sindbis virus from infected cells, especially in the form of virus-specific processes at the cell edge.

The culturing of chick embryo fibroblasts in 60 mm petri dishes or on 12 mm coverglasses, and infection with Sindbis virus, have been previously described (Birdwell and Strauss, 1973; Birdwell et al., 1973). At 2 hrs after infection, the medium was removed from the cells and replaced with medium containing various concentrations of either colchicine or cytochalasin B, as shown in Table 1. At 6.5 hrs after infection, the medium was removed and saved for titering by plaque assay. The drugs were then washed out of the cells by washing them five times with fresh medium, and fresh medium was added to the cells. One hr later this medium was removed and saved for titering. Appropriate controls were used in these experiments, including cells infected in the absence of drugs, as shown in Table 1.

As can be seen from Table 1, all concentrations of both drugs inhibit the release of Sindbis virus, and the inhibition continued for at least one hr after the drugs were removed. As explained in Table 1, this inhibition was not due to a nonspecific binding of either drug to virus particles. To see if there was any effect on the release of Sindbis virus through virus-specific processes extending from the cell periphery, cells treated as above with colchicine and cytochalasin B were examined by the surface replica technique (Smith and Revel, 1972; Revel and Wolken, 1973). Colchicine had no effect on the formation of these processes; the

Table 1

Infection of Chick Embryo Fibroblasts with Sindbis Virus in the Presence of Colchicine and Cytochalasin B

Concentration of Drugs	Titer (% of Control[c])	
	6.5 Hrs After Infection[d]	7.5 Hrs After Infection (One Hr After Removal of Drugs)
Colchicine[a]		
10 µg/ml	30%	32%
5 µg/ml	35%	37%
1 µg/ml	35%	38%
Cytochalasin B[b]		
10 µg/ml	45%	49%
5 µg/ml	50%	51%
1 µg/ml	65%	62%

Legend to Table 1

[a] Colchicine dilutions were prepared from a stock solution of 5 mg/ml in phosphate-buffered saline (PBS, of Dulbecco and Vogt, 1954, without Ca and Mg).

[b] Cytochalasin B dilutions were prepared from a stock solution of 5 mg/ml in DMSO.

[c] Controls, either in the absence of the drugs or in the presence of 0.2% DMSO, had the same titers: 3×10^9 and 5.5×10^9 plaque-forming units per ml at 6.5 hrs and 7.5 hrs after infection, respectively.

[d] After dilution of these samples for plaque assays, the highest concentration of either drug was 1 pg/ml. As a control, a high-titer stock of Sindbis virus was diluted to 3×10^9 plaque-forming units per ml in the presence of 10 µg/ml of either drug. This virus dilution was then further diluted to give drug concentrations of 1 pg/ml and was then titered by plaque assay. There was no effect on infectivity by this low concentration of drug remaining in the virus inoculum.

morphology of these cells was slightly altered by colchicine, changing from a fibroblast shape to a polygonal one. The failure to inhibit process formation is not too surprising if the ruffling activity of the cell edge is necessary for process formation, since colchicine does not inhibit ruffling (Vasiliev et al., 1970). Cytochalasin B-treated cells had fewer virus-specific processes, but the morphology of these cells was greatly altered; the central body of the cell rounded up into a ball and long, thin cytoplasmic processes were formed. Therefore, the reduction in the number of virus-specific processes may not result solely from disruption of microfilaments but also as a secondary effect of gross morphological changes. Likewise, the inhibition of Sindbis virus release seen in plaque assays is subject to the same criticism.

However, the inhibition of Sindbis virus release by colchicine, as determined by plaque assays, is in general agreement with the work of others which suggests that microtubules may play some role in maintaining the topography of the cell surface. During the phagocytosis of inert particles by polymorphonuclear leucocytes, large areas of the cell surface are internalized but five different membrane transport systems can still function (Tsan and Berlin, 1971). However, when phagocytosis occurs in the presence of colchicine, adenine and lysine transport systems are inhibited by about 40% (Ukena and Berlin, 1972). These results suggest that transport sites on the membrane of polymorphonuclear leucocytes are separated from each other, and that microtubules are necessary for normal transport

function, possibly by maintaining the normal topographical distribution of membrane components. Additional evidence that microtubules aid in preserving the integrity of the cell surface comes from the finding that the agglutination of polymorphonuclear leucocytes by Con A, which is certainly a cell surface phenomenon, is inhibited by colchicine (Berlin and Ukena, 1972). Since Sindbis virus matures predominantly at the cell surface (Acheson and Tamm, 1967), the inhibition of virus release by colchicine may be caused by a breakdown in the organization of the cell surface.

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ELECTRON MICROSCOPIC EXAMINATION OF CELLS INFECTED WITH TEMPERATURE-SENSITIVE MUTANTS OF SINDBIS VIRUS

When cells grown as a monolayer are infected with wild type Sindbis virus, virus-specific "processes," which contain maturing virions, are formed at the cell periphery (Birdwell et al., 1973). Such processes are also seen in cells infected with vesicular stomatitis virus, although not as often as in Sindbis virus-infected cells (Birdwell and Strauss, Virology, 1974, in press). How these processes are formed is not known, but process formation must be involved in the budding mechanism. Temperature-sensitive mutants of Sindbis virus have been isolated (Burge and Pfefferkorn, 1966), and it was hoped that by studying mutants defective in virus assembly, more could be learned about the nature of the virus-specific processes formed at the cell edge. Two mutants were used in this study: ts-23 (isolated by Burge and Pfefferkorn, 1966), which is presumably defective in the viral envelope glycoproteins (Burge and Pfefferkorn, 1968), and ts-103 (isolated by E. G. Strauss in our laboratory), which is suspected of having a budding defect. The mutant ts-23 is a temperature-sensitive conditional-lethal mutant because it grows at 30° (the permissive temperature) but not at 39° (the nonpermissive temperature). The mutant ts-103 will grow at 39°, albeit poorly (E. G. Strauss, personal communication).

MATERIALS AND METHODS

Cells and virus. The culturing of chick embryo cells and infection with Sindbis virus have been previously described (Birdwell and Strauss, 1973; Birdwell et al., 1973), except that infections were carried out at 30° and 39°.

Electron microscopy. Preparation of surface replicas and thin sections of infected cells fixed as a monolayer has been previously described (Birdwell et al., 1973). Replicas are presented in reverse contrast.

RESULTS

Figure 1 shows a surface replica of a cell at 12 hrs after infection with ts-23, at 30°. Virus-specific processes extending from the cell periphery are clearly visible (open arrow); these processes are similar to those seen in wild type-infected cells (Birdwell et al., 1973). At 39° no virions are seen in cells infected with ts-23, either as single particles or in processes. Wild type Sindbis virus grows at both 30° and 39°, and will produce virus-specific processes at both temperatures. Thus, ts-23 is released from infected cells in the same way as wild type Sindbis virus.

The release of ts-103 from infected cells differs markedly from that of wild type Sindbis virus. In wild type-infected cells many virions are often released in patches from certain regions of the cell surface, but this mode of virus release was rarely seen in replicas of ts-103 infected cells. Most of the virus in cells infected with ts-103 is released through an assortment of processes extending from the cell periphery. Figures 2-5 show replicas of such processes found in cells at 16 hrs after infection with ts-103, at 30°. As with wild type-infected cells, the processes originate only

FIG. 1. Surface replica of a cell infected with ts-23 at 30 C. The open arrow indicates a virus-specific process. X21,000. Scale bar is 1 μm.

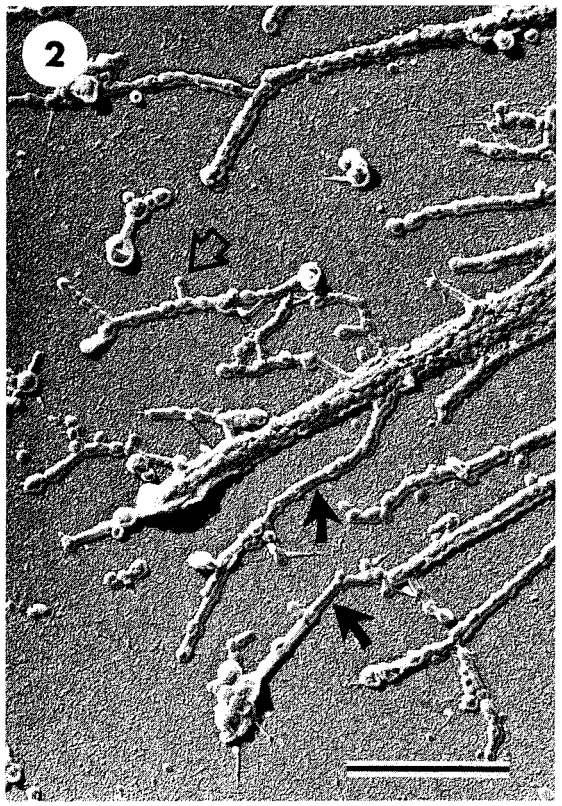

1

at the cell edge, but the morphology is quite different from the wild type process.

In Figures 2, 3, and 5 for example, the processes do not appear evenly packed with virions. Many of the processes in Figures 2 and 3 contain regions which seem to be devoid of any budding particles (solid arrows) and which have a diameter of 50-55 nm. On the other hand, many of the particles in the processes have diameters of 100-150 nm, as measured in replicas; these larger particles are often found at the end of processes (Fig. 3, black-and-white arrow). The processes in Figure 5 have wide bulges in many regions (black-and-white arrows), which may represent the formation of larger particles. Particles with 70 nm diameters, the diameter of a wild type particle, are also found in ts-103-infected cells, sometimes in processes similar to those found in wild type-infected cells (e.g., open arrow, Fig. 5) and sometimes in the abnormal processes (e.g., open arrows in Figures 2 and 3). Figure 4 shows a highly branched process with a diameter of 50-55 nm which is almost completely devoid of particles, except for 70 nm particles at the tips of the processes (open arrow). Processes similar to those shown in Figures 2-5 are also found in cells infected with ts-103 at 39°, although less frequently.

To further examine these processes, ts-103-infected cells were examined by thin-sectioning. Figures 6-9 show thin-sections of cells infected with ts-103 at 16 hrs after infection at 30°. Many multi-cored particles are seen, apparently detached from the cell (black-and-white arrows, Figs. 6-8); such particles are rarely seen in

FIGS. 2-5. Surface replicas of virus-specific processes seen in cells infected with ts-103 at 30 C. Open arrows indicate 70 nm virus particles, black-and-white arrows indicate 100-150 nm virus particles, and solid arrows indicate sections of processes which appear to be devoid of virus particles. Fig. 2, X20,000; Fig. 3, X26,000; Fig. 4, X20,000; Fig. 5, X13,000. Scale bars are 1 μm.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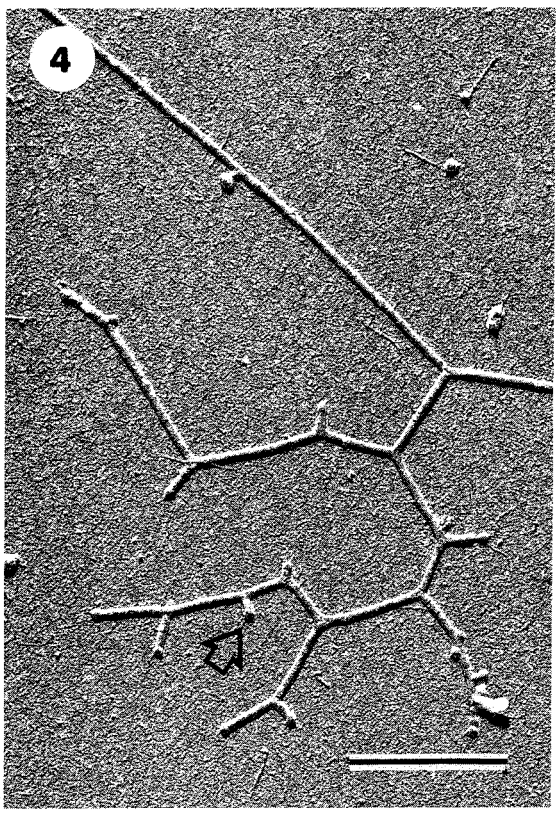
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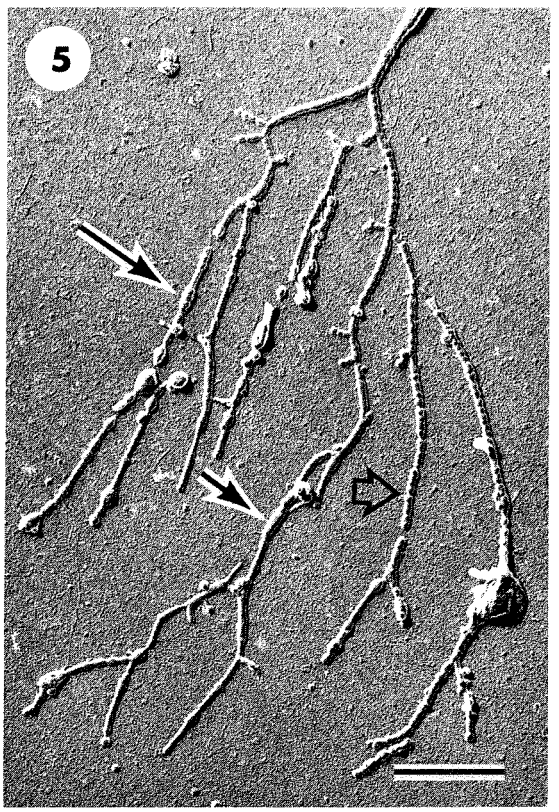
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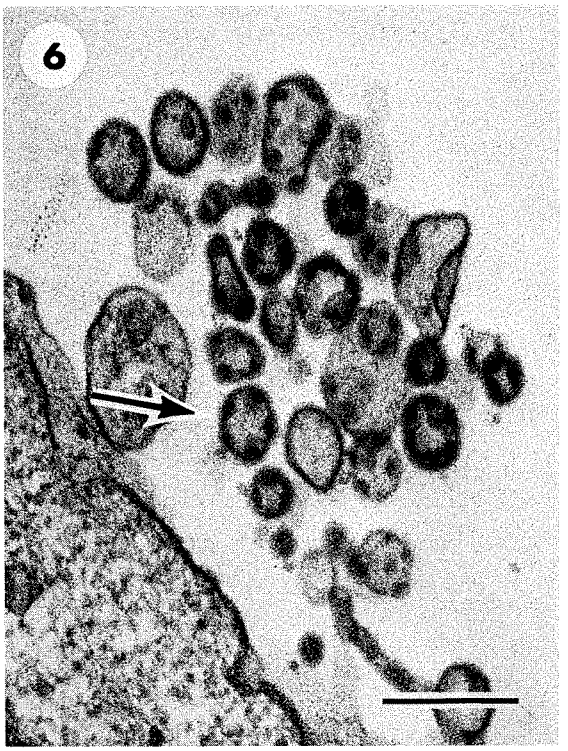
FIGS. 6-9. Thin-sections of virus-specific processes seen in cells infected with ts-103 at 30 C. The meaning of the arrows is the same as in Figs. 2-5. Fig. 6, X78,000; Fig. 7, X54,000; Fig. 8, X75,000; Fig. 9, X73,000. Scale bars are 200 nm.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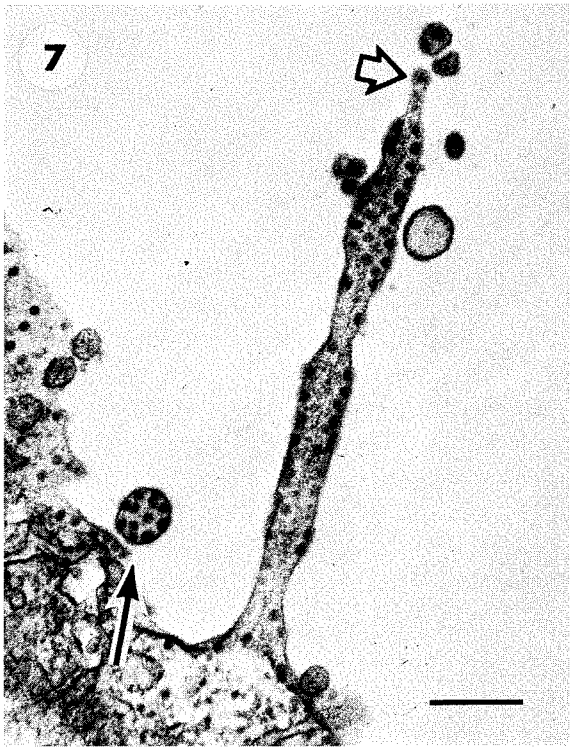
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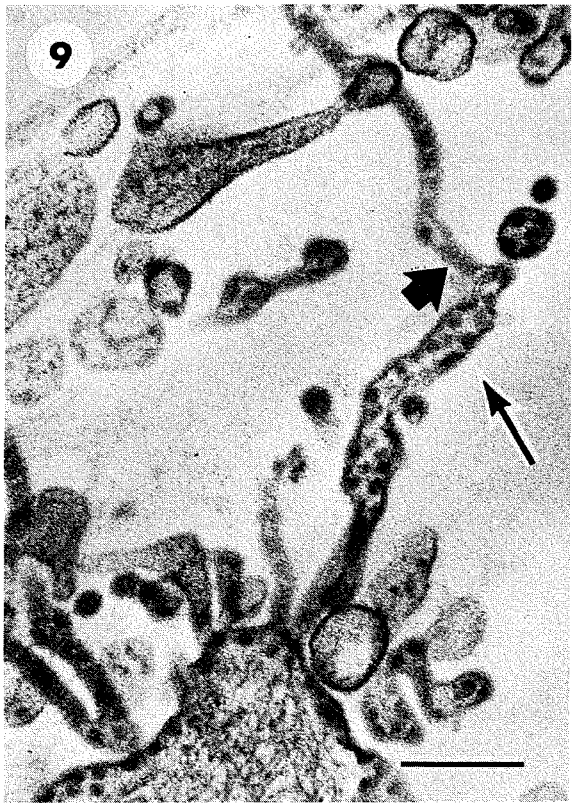
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wild-type-infected cells. Some of these multi-cored particles have diameters of 200 nm and contain more than 10 nucleocapsids. In many of these large particles, the nucleocapsids appear to reside preferentially along the inner side of the membrane surrounding the particle, although some particles appear to have nucleocapsids in the center. The appearance of nucleocapsids in the center could be due to the plane of sectioning occurring near the edge of such a vesicle, however. Large processes such as in Figure 7 are probably the source of the multi-cored particles seen in thin-section, since a cross-section through such a process would produce a multi-cored particle which would seem to be detached from the cell. Note also that this process appears to be terminated by a normal budding virus particle (open arrow) with a single nucleocapsid and a diameter of 65-70 nm. Figure 8 shows a thin-section of what is probably a wide, multi-branched process containing many nucleocapsids throughout its length. One branch of this process has a diameter of 50-55 nm and appears to be devoid of nucleocapsids (arrow). This process branch also has a multi-cored particle at its end, and may correspond to the types of processes shown in Figure 3. One of the processes shown in Figure 9 (black-and-white arrow) has a wide region with many nucleocapsids which appears to narrow to approximately 50-55 nm (solid arrow). The wider regions of processes, as in Figures 8 and 9, may be the bulging areas seen in replicas of the processes (Fig. 5).

DISCUSSION

Whereas the release of ts-23 from infected cells at 30° resembles that of wild type Sindbis virus, the release of ts-103 is quite different. Most of the virus is released in aberrant processes which contain particles larger than the wild type particles seen in surface replicas. The many multi-cored particles seen in thin-sections are probably sections through these larger particles, since no particles are seen with abnormally large nucleocapsids. It is not clear how much virus is released as large particles, since single-cored particles can bud through the sides of processes containing many nucleocapsids (Birdwell et al., 1973). When ts-103 released from infected cells is purified on sucrose gradients, most of the viral infectivity is associated with material which sediments faster than 70 nm particles (E. G. Strauss, personal communication). Experiments are in progress to determine if this faster sedimenting material contains large, multi-cored particles. Nevertheless, the location of large particles at the ends of processes, as in Fig. 3 (black-and-white arrow), implies that some large particles are probably released as such from the processes.

The exact nature of the defect which causes the abnormal release of ts-103 is not known. The production of multi-cored particles appears to involve a defect in the mechanism by which the virus acquires its envelope as it buds through the plasma membrane, so that nucleocapsids bud together instead of singly. The defect lies

presumably in one of the three structural proteins of the virion so that either the nucleocapsids of the virus are altered or the cell surface through which the nucleocapsids bud contains altered viral glycoproteins.

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