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Interactions Between Sendai Virus and Human Erythrocytes

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Concentrated Sendai virus, when adsorbed to erythrocytes at 4 C, caused invaginations in the plasma membrane. Following elevation of the temperature to 37 C, the plasma membrane became fused with the viral envelope before dissolution of the virions and rupture of the cells. Cell lysis was accompanied by rapid and total loss of hemoglobin to the extracellular space. Following aqueous pyridine extraction, the hemoglobin-free ghosts remaining were found to be devoid of *N*-acetylneuraminic acid and to have solubility properties different from those of normal erythrocyte ghosts. By the action of viral neuraminidase, bound *N*-acetylneuraminic acid was also liberated from purified virus receptor substance whose electrophoretic mobility was thereby substantially reduced. Cu^{++} selectively inhibited hemolysis and neuraminidase without interfering with hemagglutination and attachment. Neuraminidase appeared to be essential for Sendai virus hemolysis; viral particle size may also be a critical factor in this process.

The penetration of Sendai virus into susceptible cells is associated with complex and rapid interactions between viral envelope and plasma membrane (13). Similar interactions have been observed to follow the adsorption of Sendai virus to erythrocytes with which, under appropriate conditions, the end result is hemolysis. The aim of the present investigation was to correlate morphological changes with the chemical alterations induced in the erythrocyte membrane by viral action. The results, which form the basis of this report, suggest that the hemolytic process, as it occurs with this virus, may be due to several interdependent phenomena rather than to a "hemolysin" as hitherto supposed.

MATERIALS AND METHODS

Virus. Type 1 parainfluenza (Sendai) virus was propagated in the chorioallantoic cavity of 11-day-old chicken embryos. Infective fluid was harvested after 48 hr of incubation at 37 C. After preliminary low-speed centrifugation, the fluid was subjected to ultracentrifugation at 100,000 × *g* for 2 hr. A 100- to 200-fold concentration of hemagglutinin and infectivity was thereby achieved. Virus so concentrated was used without further purification.

Hemolysis and hemagglutination. Hemolytic activity was measured with group O human erythrocytes, washed three times in 0.9% NaCl and resuspended to a concentration of 0.5% in phosphate-buffered saline (PBS), *p*H 7.2. Dilutions of virus in 0.2 ml of PBS were mixed with 1 ml of erythrocytes in the cold and allowed to react for 10 min. The tubes were then held at 37 C for 30 min. After being chilled by the addition of 3 ml of cold PBS and centrifuged at 4 C for 10 min at 500 × *g*, hemoglobin in the supernatant fluids was measured spectrophotometrically by absorption at 540 nm. The highest dilution giving 100% hemolysis in 30 min under standard conditions was taken as the end point, and the corresponding amount of virus was used as the test system for measuring inhibition by serum or other substances. Hemagglutination and hemagglutination-inhibition assays were performed with chicken erythrocytes by the pattern technique as previously described (8).

Antisera. Rabbits were immunized by the administration of three weekly footpad injections of concentrated Sendai virus in complete Freund's adjuvant for a total dose of 120,000 hemagglutinating units (HAU). Animals were bled 10 days after the last injection and were bled out 2 weeks later. Antisera were coupled with fluorescein and ferritin according to methods described elsewhere (C. Howe et al., Progr. Med. Virol., *in press*).

Analytical methods. Neuraminidase activity of Sendai virus was assayed as previously described (4) with either Collocalia mucoid (8) or neuraminyllactose (Calbiochem, Los Angeles, Calif.) as substrate. Free *N*-acetylneuraminic acid (NANA) was measured by the thiobarbituric acid method (23).

Erythrocyte preparations. Erythrocytes from freshly drawn blood samples were separated on the basis of bouyant density, according to the method of Danon and Marikovsky (2). For comparison of susceptibility to hemolysis by Sendai virus, samples were adjusted to

equal packed cell volumes and total hemoglobin content. Purified virus receptor substance (VRS) was prepared from erythrocytes in freshly drawn blood of group O by methods previously described (9).

Preparations for electron microscopy. Cells or membranes were centrifuged into a pellet, fixed for 30 min in 1% glutaraldehyde, washed, fixed for 30 min in 1% osmium tetroxide, dehydrated, and embedded in epoxy resin (Epon 812). Sections were stained with uranyl acetate and lead citrate before being examined in a Phillips 200 electron microscope.

RESULTS

Inhibition of hemolysis. Rabbit antibody produced in response to the injection of whole Sendai virus inhibited hemolysis at dilutions of 1:160 to 1:320 (Fig. 1) in the standard test system. The same antiserum inhibited hemagglutination to dilutions of 1:400 (4 to 5 HAU). Preimmunization serum from the same animal contained insignificant amounts of antihemagglutinin and antihemolytic activity. When coupled with fluorescein, the globulin from viral antiserum caused characteristic specific cytoplasmic staining reactions after it was applied to acetone-dried preparations of chick embryo fibroblast cultures infected with Sendai virus. These are not otherwise dealt with in this report.

Sendai virus neuraminidase was examined for sensitivity to inhibition by heavy metals and other metabolic inhibitors, as in earlier studies with type 2 parainfluenza virus (4). The pertinent results, to be reported in detail in a separate communication, may be summarized. With crystalline neuraminyl-lactose as substrate, the enzyme in 1,000 HAU was completely blocked by 3×10^{-4} M copper acetate. We found that 10^{-3} M 2,4-dinitrophenol (DNP) did not inhibit neuraminidase, hemagglutinin, or hemolysis. Hemolytic activity was inhibited completely by 6×10^{-5} M copper acetate and partially by half that concentration (Fig. 1). The neuraminidase of Sendai virus was also active against VRS as noted below.

Effect of Sendai virus on constituents of erythrocyte membranes. To prepare membranes of erythrocytes lysed by Sendai virus, 30 ml of washed cell suspension (1%) was mixed with 175,000 HAU of virus at 4 C. After 10 min, when heavy clumping had become pronounced, the cells were brought to 37 C. Lysis was complete within 10 to 15 min; the preparation was held at 37 C for 30 min with frequent mixing. The suspension was centrifuged at 25,000 $\times$ *g* for 1 hr, and the sediment was washed once by resuspension in 20 milliosmolar phosphate buffer containing ethylenediaminetetraacetic acid (10^{-4} M) and by a second centrifugation at 19,000 $\times$ *g*. The colorless ghosts were suspended in 2 ml of water, to which 2 ml of pyridine was added (1). After dialysis for 36 hr against water, an insoluble residue appeared which was separated by centrifugation from soluble material. On lyophilization, the weight of the former was 30 times that of the latter. Hydrolysis of both fractions with 0.1 N H_2SO_4 for 30 min yielded no detectable thiobarbituric acid chromogen, indicating the absence of NANA from the ghost material. Hemoglobin-free ghosts prepared by hypotonic lysis of normal erythrocytes (5) contain 2 to 3% bound NANA (*unpublished data*). All of the NANA may be accounted for in the nondialyzable

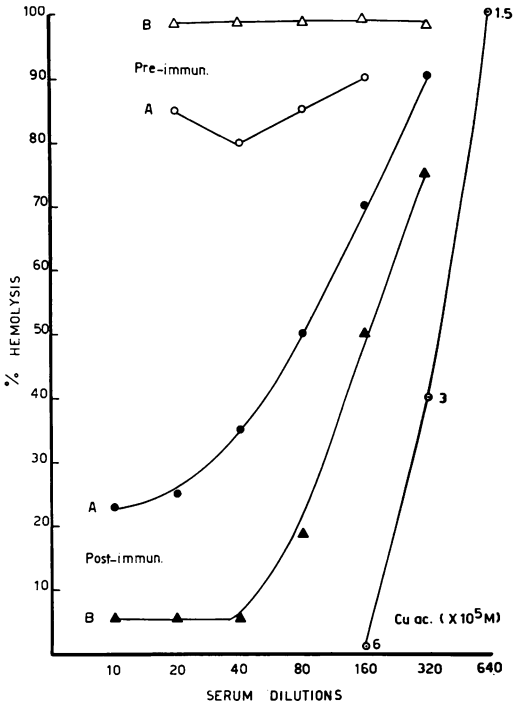


FIG. 1. *Inhibition, by specific antiviral antibody, of hemolysis caused by Sendai virus. Curves marked A: dilutions of pre- and postimmunization sera were mixed with samples of erythrocytes to which virus had already been adsorbed at 4 C. Curves marked B: dilutions of pre- and postimmunization sera incubated for 20 min at room temperature with a 100% hemolytic dose of virus; erythrocytes were then added at 4 C. Dilutions of copper acetate (CuAc) were added to a 100% hemolytic dose of virus prior to mixing with cells. All reaction mixtures, in a final volume of 1 ml, were held at 4 C for 10 min, then at 37 C for 30 min; 3 ml of cold saline was added, the mixtures were centrifuged, and the supernatant fluids were read at 540 nm. Values are given as the per cent of hemoglobin released from standard volume of erythrocytes lysed in distilled water.*

residue remaining after pyridine treatment, which results in a solubility ratio strikingly different from that of the Sendai-treated membrane material (Table 1). VRS from human erythrocytes (9) was treated at 37 C with concentrated Sendai virus. The mixture was then analyzed for free NANA. About one-third of the NANA of the VRS had been split off by viral action. Both treated and untreated samples of VRS were subjected to immunoelectrophoretic analysis. The patterns, developed with rabbit antibody to hemoglobin-free erythrocyte ghosts, are shown in Fig. 2.

Electron microscopy of the hemolytic reaction. Samples of human 0 cells (0.5 ml, 1%) were mixed with 25,000 HAU of active Sendai virus at 4 C. After 10 min, three samples were brought to 37 C for 1, 2, and 10 min, respectively. Each was then quickly chilled and centrifuged in the cold to separate ghosts and any remaining unlysed cells. The pellets were fixed in glutaraldehyde. Hemolysis was partial in the 1-min sample and complete in the 2-min sample. Cells in a fourth sample, held at 4 C for 45 min and showing no hemolysis, were also pelleted and fixed in glutaraldehyde. All four samples were then processed for examination by electron microscopy as described.

Figure 3a illustrates, at low magnification, cells from the control sample held at 4 C for 45 min. Numerous virions are seen attached to the surface of the cells, occasionally forming intercellular bridges. Higher magnification (Fig. 3b) reveals that some virions have produced indentations of the cell surface. In these indentations, the intact viral particles are separated from the cell membranes by a distance equal to the width of the peripheral spikes of the viral envelope. Figure 3c illustrates a single virion attached to two erythrocytes. Such configurations presumably account for visible hemagglutination.

As already noted, hemolysis was complete in these preparations by the end of 2 min; the pellet of ghosts was colorless. Figure 4 shows, at low magnification, membranes to which virions are attached and the complete absence of hemoglobin. The indentations noted in Fig. 3 are still evident and their appearance suggests the continued firm attachment of virions at these sites. It should be noted that the larger viral particles, for the most part, are found in these invaginations of the membrane. Occasional bridging particles, also of relatively large size, are still discernible. Figure 5 shows a virion in close proximity to a red cell ghost on the left; the cell membrane and viral envelope still appear to be intact. In some instances, there was fusion of the cell membrane with the viral envelope resulting in the establishment of continuity between the interior of the virion and the interior of the erythrocyte ghost (Fig. 6). Figure 7 illustrates a large viral particle with nucleoprotein filaments which are in the process of passage into the erythrocyte at the bottom. Disruption of the viral envelope has occurred at numerous small loci. In Fig. 8, a virion is seen to have ruptured outward from the site of fusion with the cellular membrane at the bottom; the viral nucleoprotein is being discharged into the extracellular space. At the top of the micrograph, virions are seen attached to another erythrocyte. Figure 9 shows nucleoprotein filaments in the process of release both into the cell on the left and outwardly into the extracellular space on the right. A neighboring viral particle is attached to the cell but has not fused with it. In Fig. 10, little remains of the virion except a few nucleoprotein filaments, several of which extend into both the intra- and extracellular spaces. Figure 11 illustrates a dis-

TABLE 1. *Effect of concentrated Sendai virus on hemoglobin-free erythrocyte ghosts*[a]

Ghosts	Ratio of insoluble to soluble	Per cent NANA[b]
Sendai-treated..	30	0
Normal.....	2.3	3

[a] Extracted with 50% pyridine as described in text.

[b] After hydrolysis in 0.1 N H_2SO_4, at 80 C for 30 min. Sum of NANA in soluble and insoluble fractions recovered after pyridine treatment of membranes.

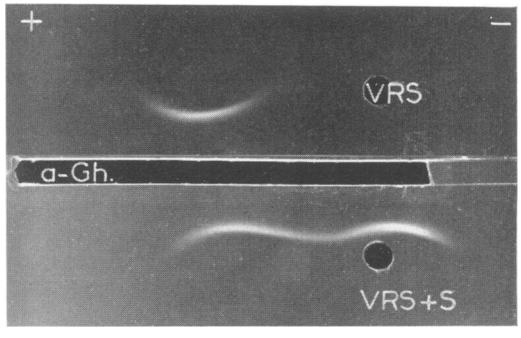


FIG. 2. *Immunoelectrophoretic patterns, developed with antibody to hemoglobin-free ghosts, of virus receptor substance from human erythrocytes before (VRS) and after (VRS + S) treatment with concentrated Sendai virus (50 μg of VRS per 2,000 HA units of virus; 37 C). Thiobarbituric acid analysis (23) on VRS + S showed 32% free NANA; there was no free NANA with VRS.*

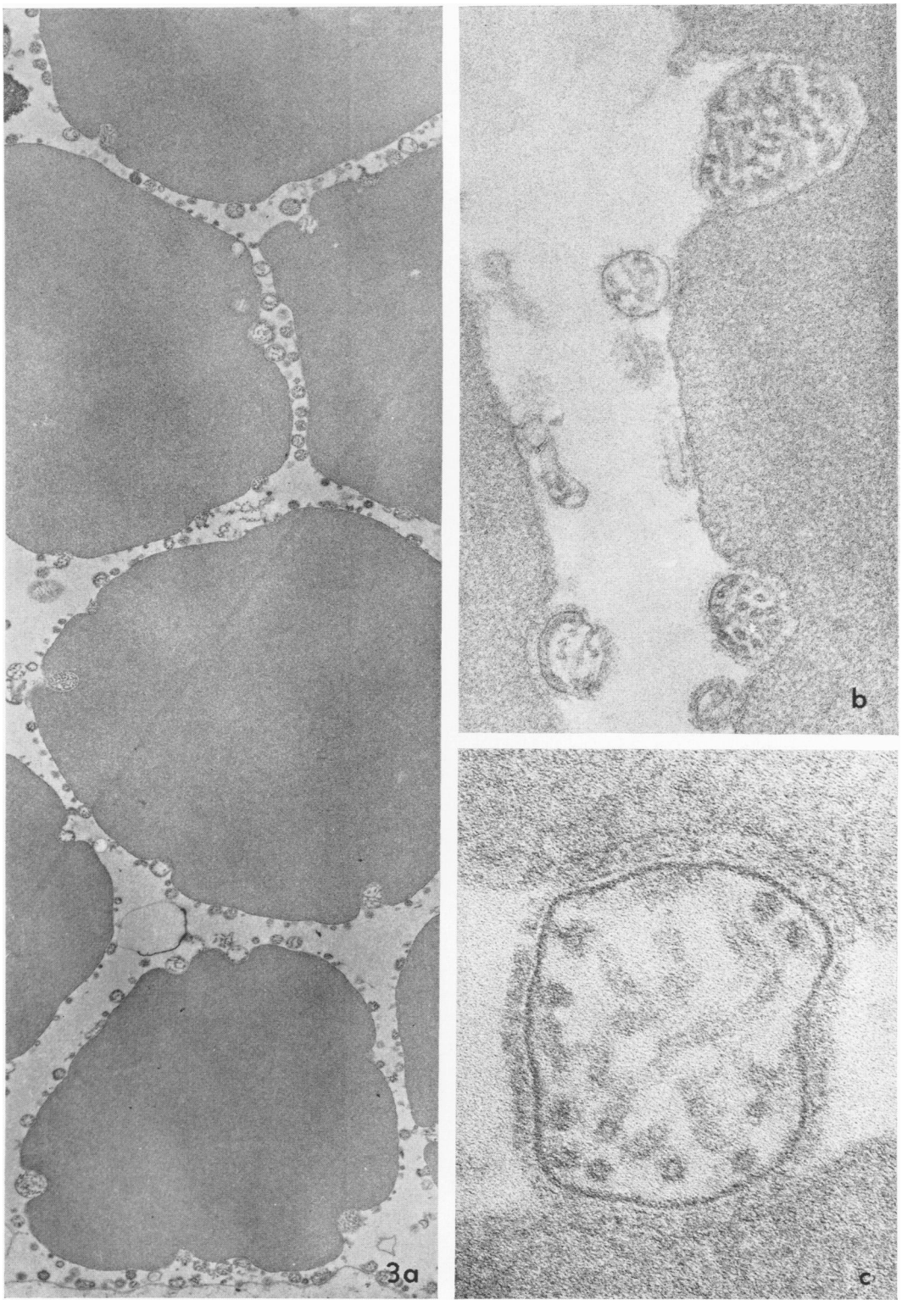


FIG. 3. *Human erythrocytes mixed with concentrated Sendai virus and held at 4 C for 45 min.* (*a*) × *12,000;* (*b*) × *72,000;* (*c*) × *200,000.*

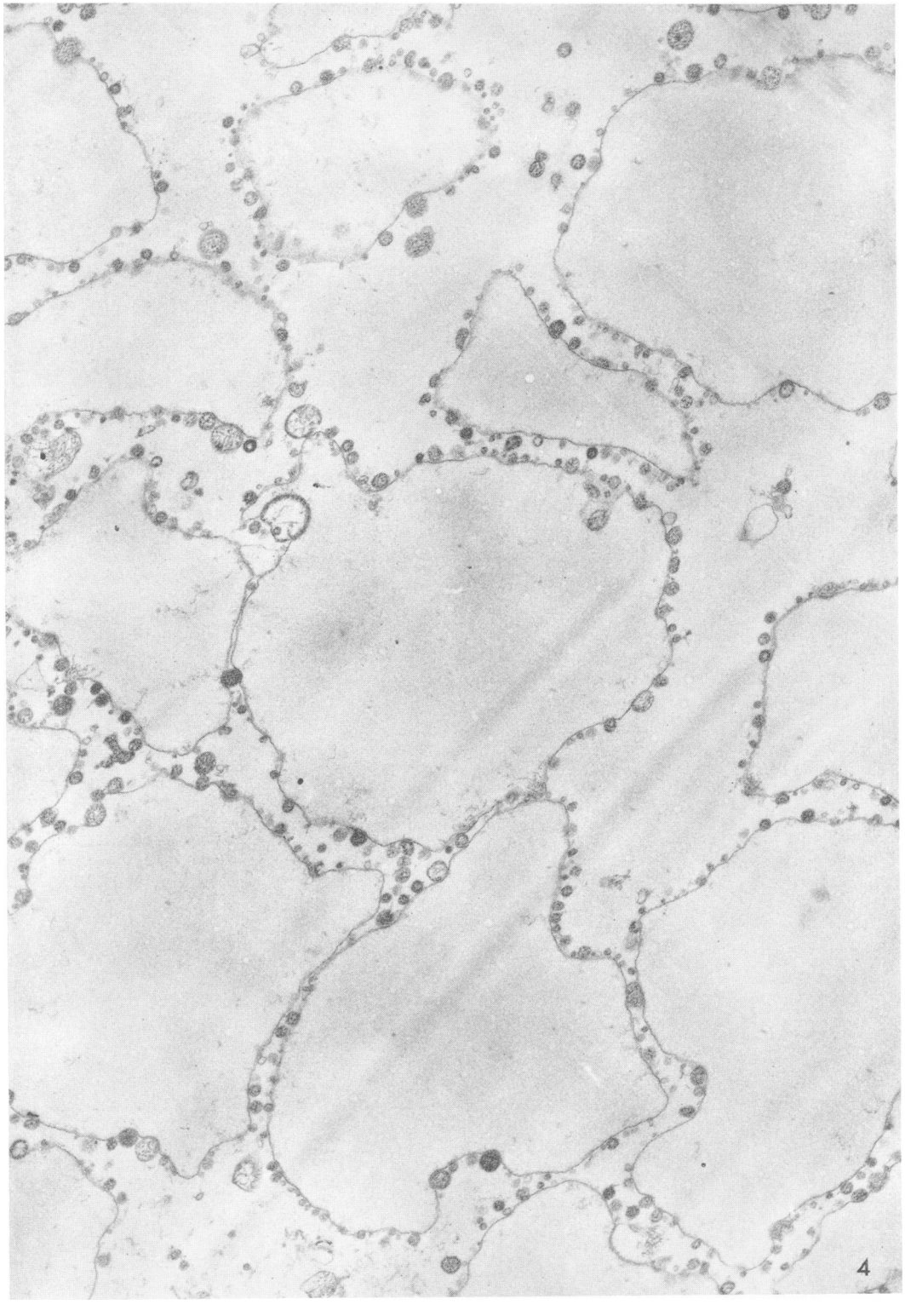
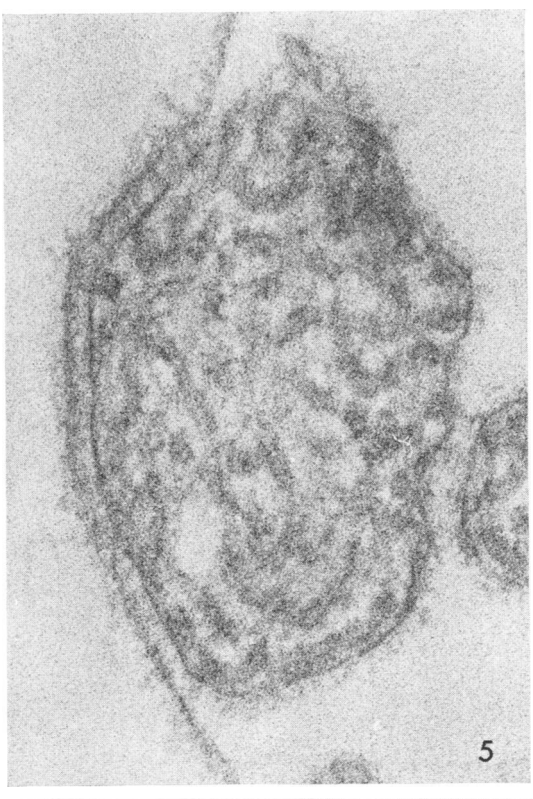


FIG. 4. *Erythrocyte ghosts rendered hemoglobin-free by the lytic action of Sendai virus after 2 min at 37 C.* × *12,000.*



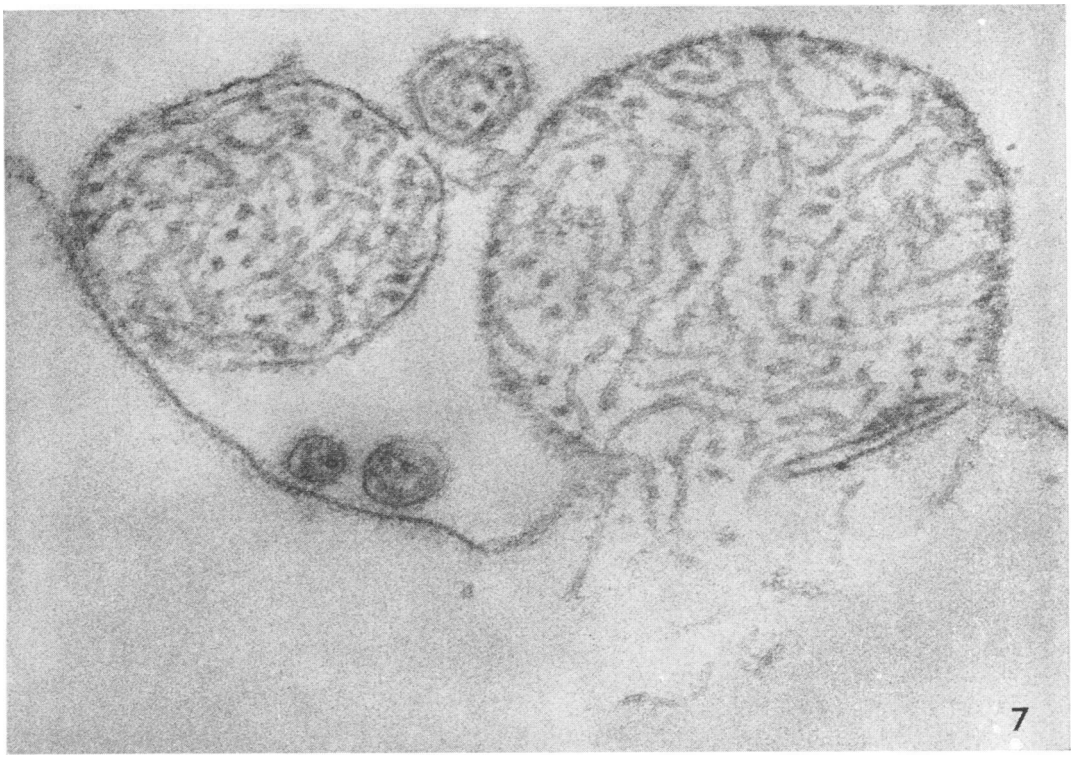
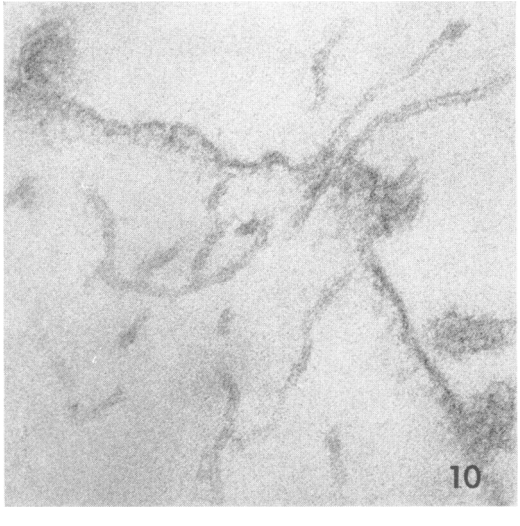


FIG. 5–7. *Sendai virions in various stages of fusion with erythrocyte membrane. Fig. 5 and 6, × 150,000; Fig. 7. × 86,000.*



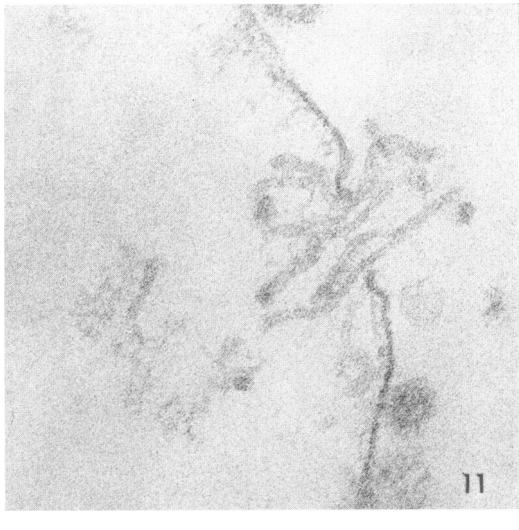


FIG. 8–11. *Disruption of virions with liberation of nucleoprotein to the extracellular space and into hemoglobin-free ghosts. Fig. 8, × 130,000; Fig. 9, × 170,000; Fig. 10 and 11, × 100,000.*

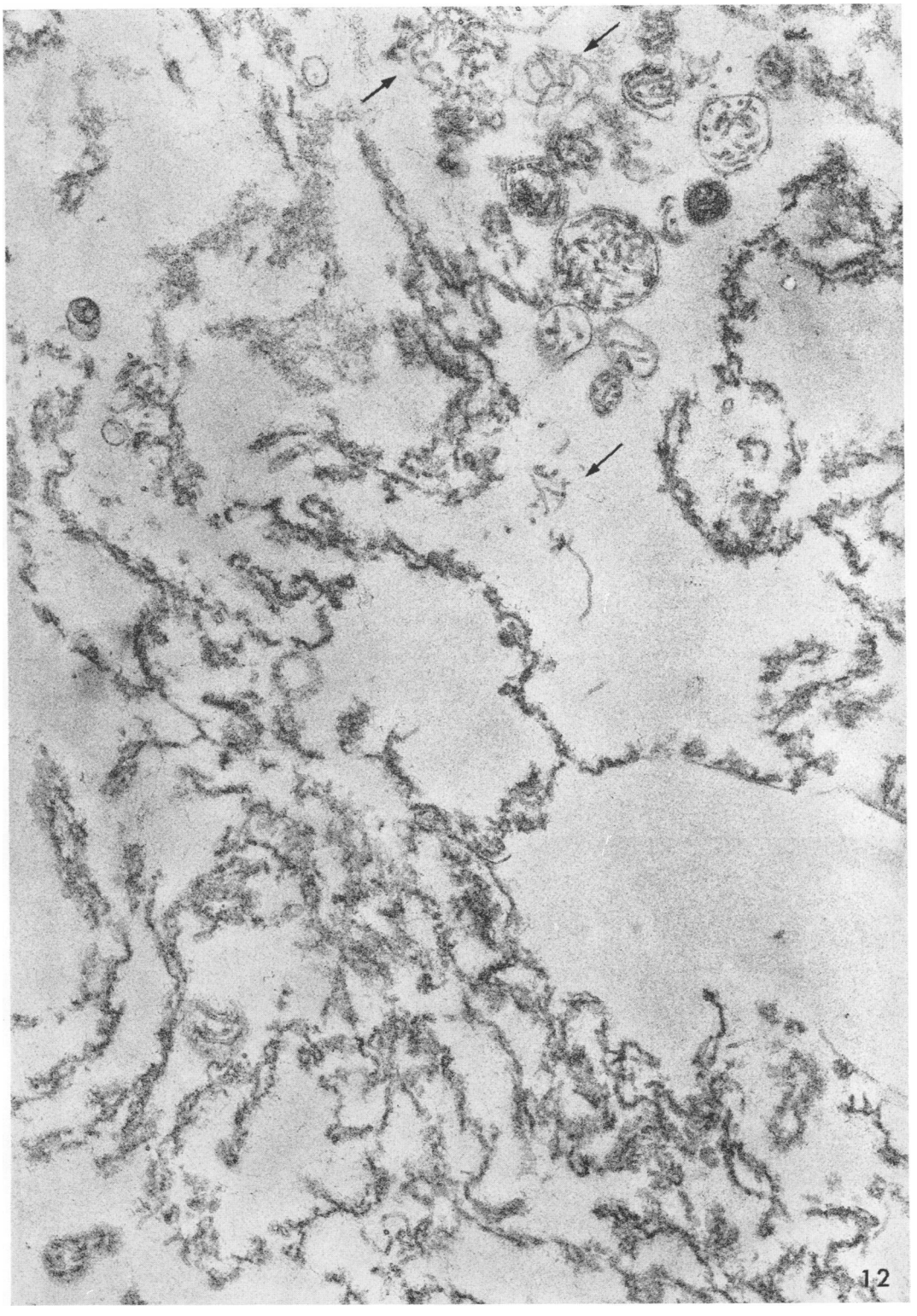


FIG. 12. *Cell membranes remaining after the exposure of erythrocytes to concentrated virus for 10 min at 37 C. × 50,000.*

continuity in the cell wall through which nucleoprotein filaments protrude. This is presumably one site at which a virion became attached and then disrupted. In these last two micrographs, the extracellular space is to the right.

In the sample taken after 10 min at 37 C, the erythrocyte membranes appeared fragmented and distorted, as seen in Fig. 12. Among these folded membranes, a few large viral particles appeared (upper portion of micrograph) to be intact along with others that were partially disrupted. In several areas, remnants of free nucleocapsid were encountered (arrows).

As already noted, viral attachment to, and lysis of, erythrocytes was accompanied by the partial or complete disruption of many virions, whose nucleoprotein cores were thus liberated. These altered viral particles and fragments were examined for their reactivity with viral antibody. After adsorption of virus to erythrocytes in the cold, the mixture was warmed to 37 C for 3 min and then quickly chilled and centrifuged at low speed to form a loosely packed pellet. This pellet was fixed in buffered Formalin for 2 min, the Formalin was then removed by one centrifugation in saline, and the pellet was resuspended in ferritin-conjugated antiviral antibody. Excess antibody was removed by three successive centrifugations and resuspensions in buffered saline, and the final pellet was fixed in glutaraldehyde and osmium as described above. As shown in Fig. 13, two large particles are adsorbed to membranes of adjacent cells. Each particle is tagged only around the segment of its envelope that is not in close apposition to membrane. Two smaller virions are shown to be tagged around their entire circumference. In addition, the envelope of one of the larger particles (left) shows defects through which ferritin antibody has penetrated. This penetration is even more pronounced in the virion illustrated by Fig. 14, in which much of the cell membrane seems still to be intact at the site of attachment. In Fig. 15, however, there has been not only pronounced penetration of ferritin antibody but also disintegration of both viral and erythrocyte membranes. Scattered ferritin granules are also seen within the cell. In Fig. 16, a gap is evident where the interior of the virus communicates with the interior of the cell, and ferritin is present throughout the confines of both virus and cell. This virion appears to be undergoing dissolution as judged by the appearance of nucleoprotein filaments, which are less tightly packed and of lower density than those shown in the preceding figures. The viral envelope also exhibits discontinuities. The striking permeability of the attached viral particles (Fig. 15 and 16) and the concomitant disintegration of the plasma membrane appear to afford the avenue for escape of hemoglobin. Figure 17 shows the remnants of a viral envelope, to which nucleocapsid remains attached, and which is annealed into a segment of erythrocyte membrane, thus leaving only a "footprint" which still binds ferritin antiviral antibody.

DISCUSSION

The action of Sendai virus on erythrocytes may be envisaged as comprising several related steps leading to hemolysis. To begin with, intact virions appear to be essential, since it has been shown that Sendai virus, as well as Newcastle disease and mumps viruses, lose hemolytic activity on disruption with detergents or fat solvents (15, 18); loss of hemolytic activity also follows digestion of the viral particles with trypsin (16, 20). The first step in the reaction, attachment, is dependent on the presence at the cell surface of specific NANA-containing receptors for viral hemagglutinin. Pretreatment of erythrocytes with viral subunits possessing neuraminidase interferes with the hemolytic activity of intact viral particles, presumably as a result of receptor destruction (22). In our preparations, the reaction of attachment, occurring at 4 C, was often accompanied by the formation of marked invaginations of the membrane into which individual virions became enfolded (Fig. 3). Attachment and hence hemolysis were prevented by antiviral antibody, provided the latter was combined with virus before its adsorption to erythrocytes (Fig. 1B). Antiserum added after attachment of virus to cells in the cold, however, still showed some capacity to block hemolysis when the temperature of the reaction was raised to 37 C (Fig. 1A). This finding suggests that antibody, under these circumstances, by combining with distally exposed portions of attached virions, blocked their further enfolding into invaginations of the membrane and hence limited the extent of their contact with cell receptors.

Fusion of parainfluenza virions with the membrane of metabolizing cells was found to be a necessary prelude to the penetration of viral nucleocapsid; moreover, membranes were found to reform at the sites where penetration had occurred (13). In the present study, fusion was likewise observed between the erythrocyte membrane and the viral envelope. After attachment and concomitantly with hemolysis, the structural integrity of attached viral particles was altered, as shown by discontinuities in the viral envelope and the penetration of ferritin antibody into the disintegrating internal structure (Fig. 14 to 16).

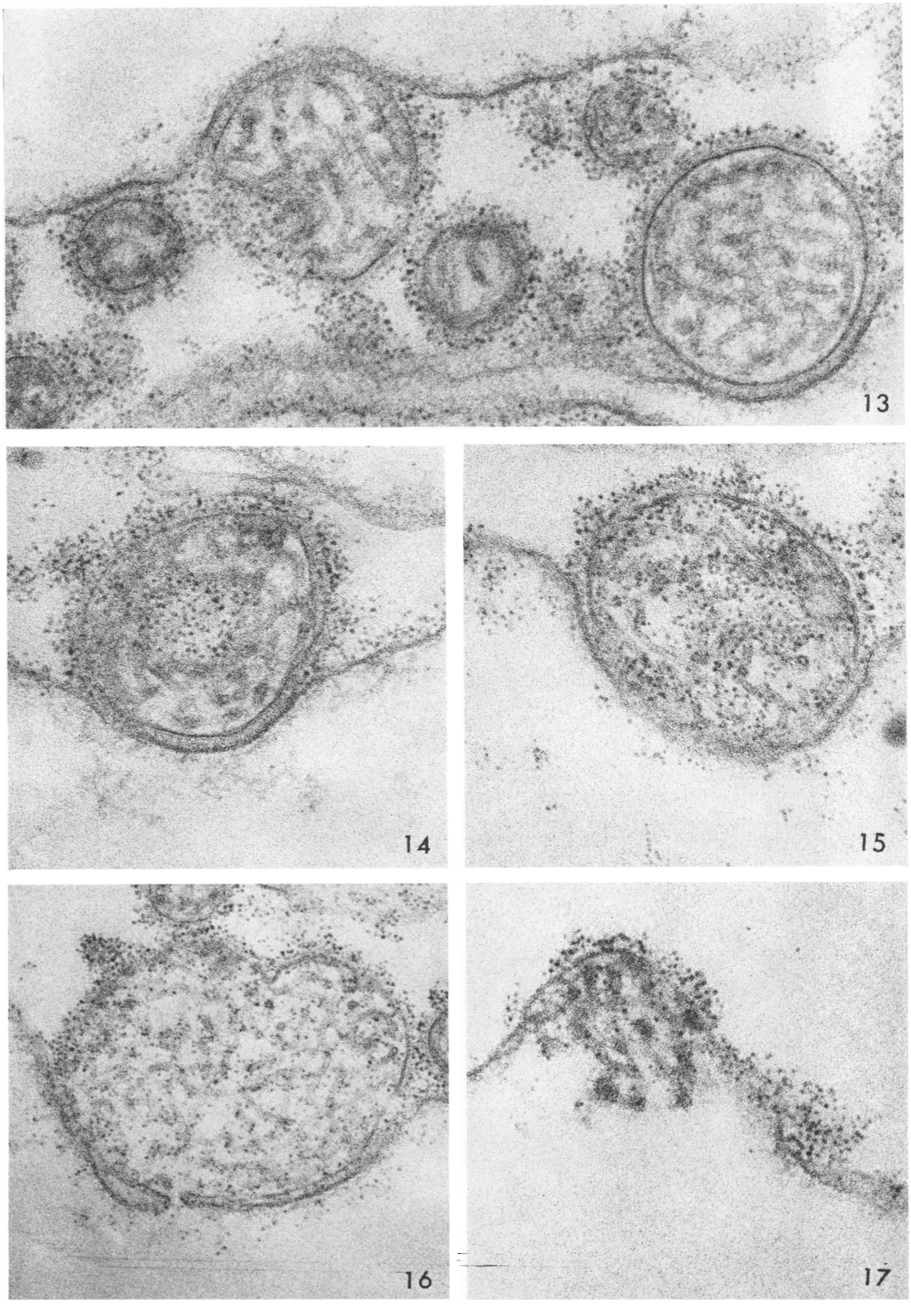


FIG. 13–17. *Reaction of ferritin-conjugated antiviral antibody at different stages of fusion. Fig. 13–16, × 90,000; Fig. 17, × 76,000.*

The end result was liberation of the nucleocapsid into empty ghosts and the extracellular space. Rupture of the attached virions may have resulted from shearing action caused by transient attachment to other cells or ghosts. However, a more subtle triggering mechanism may exist for the release of viral nucleocapsid during the hemolytic reaction. This possibility has not been excluded.

The lysis of erythrocytes by Sendai virus bears certain similarities to the first stages of polykaryocyte formation after the application of high input multiplicities of virus to metabolizing cells in suspension. In both instances, the cellular membrane becomes invaginated around the envelope of attached viral particles, as shown with erythrocytes in Fig. 3 and with Ehrlich ascites tumor cells in the micrographs of Hosaka and Koshi (7). In the latter instance, when the temperature was raised to 37 C, fusion of cells was initiated by the formation of intercellular bridges, which occurred by rejunction of disrupted membrane leaflets in proximity to the virus. The fusion of one cell membrane with another took place without notable loss of cellular contents to the exterior. In accord with this observation, Kohn (11) showed that isolated cells in monolayers, when exposed to high concentrations of Newcastle disease virus, did not lyse. Okada et al. (19), as well as Hosaka et al. (7), believed the essential steps in the process of membrane fusion to be first the "disconnection," or degradation, of the membrane at the point of contact with the virion and then "reconnection," i.e., repair, of the disconnected sites or their fusion with neighboring sites on the same or different cells. Only the latter step was energy-dependent, requiring nucleotide-triphosphate (NTP). The "disconnection" was assumed to be mediated by the virus. Inhibition of cell fusion as a result of NTP deficiency (blockade with DNP) or removal of Ca^{++} ions (14) resulted in lysis of the cells after adsorption of virus and incubation at 37 C (11). Such findings suggest that continued "disconnection" of the cell membrane, without repair, took place as a result of viral action. An analogous situation may be presented by the erythrocyte membrane, which, although it fuses with the viral envelope, lacks the energy necessary for its own repair. The disconnecting agent of the virus is thus allowed to continue acting at the sites of viral attachment, and our data suggest that this agent may be neuraminidase. This hypothesis is supported by several observations. The mechanism of attachment could be readily dissociated from neuraminidase activity, and thus, from hemolytic activity, by the action of Cu^{++}. Copper ions did not inhibit hemagglutination or attachment of viral particles to erythrocytes; cells agglutinated by high concentrations of virus previously mixed with 10^{-3} M copper acetate presented an electron microscopic picture indistinguishable from that shown in Fig. 3a to c. Hemolysis, however, was blocked by copper acetate (Fig. 1) as was the action of viral neuraminidase on crystalline neuraminyl-lactose. The specific action of viral enzyme on erythrocytes was evident after their lysis with high concentrations of Sendai virus. The hemoglobin-free membranes, markedly altered with respect to solubility in water, were depleted of NANA (Table 1). Similarly, enzymatic alteration of purified receptor substance was demonstrated by cleavage of NANA from the macromolecule. The loss of NANA may account for the marked change in mobility of the virus-treated substance when compared to the untreated sample in immunoelectrophoretic analysis (Fig. 2). The biphasic shape of the immune precipitate is explained by differences in the degree of NANA depletion among the molecules of mucoid. The latter, however, retained its intrinsic antigenic homogeneity, as evidenced by the complete continuity of the precipitin line. Terminally bound NANA, which normally accounts for 15 to 20% of the dry weight of the intact acidic mucoid (3), apparently contributed little to the antigenic determinants reactive with this precipitating antibody.

Our electron microscopic findings suggest that the larger viral particles are more effective than smaller ones in causing invagination of the cell membrane and hence in achieving correspondingly wider contact with cell receptors. When many viral particles become attached to and act upon a single cell, as seen in Fig. 3a, profound changes in permeability characteristics of the membrane undoubtedly result; these changes allow the escape of intracellular contents at the sites of viral attachment. The importance of viral particle size to the hemolytic reaction is also suggested by comparison with influenza virus which, even in high concentration, does not cause the lysis of erythrocytes. One exception is the A_2 strain of influenza virus described by Neurath (17). It is to be noted, however, that A_2 strains not only display greater neuraminidase activity but also elute less readily from erythrocytes than do other strains of influenza virus. These two factors would, of themselves, enhance the discrete localized action of neuraminidase and therefore contribute to the hemolytic activity of these strains.

The close association between the hemolytic activity of paramyxoviruses and their capacity for inducing polykaryon formation has been repeatedly noted. There is some evidence that both

properties are somehow related to the relatively higher lipid content of paramyxoviruses, as compared with the influenza viruses (10, 11, 21). Moreover, both activities presumably depend upon the high lipid content of cellular membranes. In the erythrocyte, virtually all the lipid of the cell is known to reside in the membrane and to account for 40% of the weight of hemoglobin-free ghosts (5). Some sort of interaction of lipoprotein constituents has therefore been suggested as a basis for fusion of the viral membrane with the cell membrane, such interaction itself not requiring enzymatic mediation (11). Changes related to red cell lipid content have been reported to result from hemolysis by the paramyxoviruses. Thus, Hosaka (6) and Moberly (12) reported the loss of sphingomyelin and alterations in the membrane lipoproteins as a result of hemolysis by mumps virus.

The fatty acid composition of erythrocyte membranes was shown to undergo changes as the cells age in vivo (G. B. Phillips et al., *in preparation*). This observation may be related to preliminary results in our laboratory which have suggested that susceptibility to lysis by Sendai virus may also vary with cellular age. Thus, old and young cells, separated on the basis of their bouyant densities (2), displayed quantitative differences in both degree and rate of hemolysis when tested in equivalent concentration with the same amount of virus. In accord with these findings, electron microscopic examination of these preparations also revealed distinct differences between old and young cells treated with Sendai virus for 2 min at 37 C. Cells in the old fraction retained more hemoglobin and showed fewer virions fused to their membranes than did cells in the young fraction; only rarely were ribonucleoprotein filaments seen in process of release. This aspect of the problem is currently under investigation.

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