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Effect of Ionic Strength on the Binding of Sindbis Virus to Chick Cells

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Sindbis virus can adsorb to chicken embryo fibroblasts in two different ways. "Loosely" bound virus can be washed off the cell with buffers of ionic strength 0.2 or greater, whereas "tightly" bound virus remains attached under these conditions. When Sindbis virus is adsorbed to chick cells at 4 C from a buffer of ionic strength 0.17, 40 to 50% of the adsorbed virus is loosely bound, the remainder tightly bound. Infection of chick cells by Sindbis virus has only small effects on the total amount of virus that can be bound to the cells. However, the amount of Sindbis virus that can be tightly bound declines rapidly beginning at 2 to 3 h after infection. By 7 h after infection, the amount of virus that can be tightly bound is only 10 to 20% of the amount bound to uninfected cells. The adsorption (and penetration) of virus at 37 C is most efficient at an ionic strength of 0.15 to 0.17; at this ionic strength most of the adsorbed virus is tightly bound. At higher ionic strengths the virus adsorbs poorly. At lower ionic strengths most of the virus is loosely bound. A second enveloped virus, vesicular stomatitis virus, has been studied for the purposes of comparison; its adsorption behavior differs from that of Sindbis virus.

The first step in the infection of a cell by a virus is the adsorption of the virus to a specific receptor on the cell surface. This is followed by a configurational rearrangement of the virus particle that leads to the loss of particle infectivity ("eclipse") and, in some way, to the introduction of the virus nucleic acid into the cell. Many of the details of these early events have been established for bacteriophage (1, 18, 20), but relatively little is known in the case of animal viruses. Studies on the adsorption of poliovirus to HeLa cells in suspension culture demonstrated that the adsorption of virus followed classical kinetics (2, 8); adsorption occurs efficiently at 0 C. Eclipse and penetration, however, do not occur in the cold; temperatures of 20 C or higher are required, and the eclipse event is separate from the adsorption event. After eclipse of the virus particle, a noninfectious particle can be eluted from the cell that still contains all of the nucleic acid and protein of the virion (8, 11, 14). Furthermore, the virus particle may eclipse upon reaction with subcellular fractions, which presumably contain the virus receptor.

Enveloped animal viruses also appear to adsorb to specific receptors on the surface of the susceptible cell. The receptors for the myxoviruses and paramyxoviruses involve sialic acid, because removal of sialic acid from otherwise sensitive cells renders these cells unable to adsorb the virus (12, 16). In the case of enveloped viruses, however, the event corresponding to eclipse of the picornaviruses appears to be a fusion of the virus envelope with the surface membrane of the cell (17).

We have been studying the adsorption of Sindbis virus, a group A arbovirus, to chicken embryo fibroblasts and the influence of ionic strength upon this early event in infection.

MATERIALS AND METHODS

Virus strains. Sindbis virus was the HR strain of Burge and Pfefferkorn (6). Vesicular stomatitis virus (VSV) (Indiana serotype) was obtained from Phil Marcus.

Chick cells. Chicken embryo fibroblasts were prepared from 10-day-old embryos. Cells were seeded at a concentration of 1.5×10^6 cells per ml in Eagle minimal essential medium (10) containing Earle salts and 2% fetal calf serum. Medium and serum were supplied by Grand Island Biological Company. The volumes seeded were 4.5 ml per 60-mm petri plate and 150 ml per 800-cm^2 roller bottle. Cell monolayers were used 3 to 5 days after seeding.

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Preparation of ^{14}C-labeled Sindbis virus. Virus was grown in medium of low ionic strength where no virus release occurred and subsequently harvested in medium or buffer of high ionic strength, a technique suggested by the results of Waite and Pfefferkorn (22).

Medium bathing a roller-bottle monolayer was changed 3 h before infection with 50 ml of Eagle medium containing 2% fetal calf serum. The cells were infected with virus at a multiplicity of 20 PFU per cell from 10 ml of phosphate-buffered saline (PBS) (9), containing 1% dialyzed fetal calf serum and 1 μg of actinomycin D per ml. After 1 h, the virus inoculum was removed and 40 ml of modified Eagle medium was added. This medium was of low ionic strength and contained (i) 0.06 M NaCl instead of the normal 0.116 M, (ii) one-tenth of the normal concentration of all amino acids except glutamine, which was present at the normal concentration, and methionine, which was present at one-twentieth of the usual concentration, (iii) 2% dialyzed fetal calf serum, and (iv) 1 μg of actinomycin D per ml. At 3 h after infection the medium was changed with 40 ml of the same medium, but lacking actinomycin D and containing 0.25 μCi (50 μCi/mM) of [^{14}C]methionine (Schwarz/Mann) per ml.

At 12 to 14 h after infection the culture fluid was removed and discarded, and the monolayer was washed twice with 10-ml volumes of low-ionic-strength medium. The virus was then harvested by washing the monolayer for 15 min at 37 C with 5 or 10 ml of a solution of high ionic strength (either Eagle medium containing 0.216 M NaCl, or Tris-sodium chloride buffer of ionic strength 0.25). The monolayers were usually washed a second time with 5 ml of the same high-ionic-strength solution, and the virus eluates were pooled. Occasionally, an eluate was used as a virus stock without further purification. Usually, however, the virus was purified by sucrose gradient sedimentation. Up to 75% of the radioactivity in an eluate sedimented with the sedimentation coefficient of intact virus (Fig. 1). Peak fractions were pooled and dialyzed for 2 h at 4 C against a large volume of PBS.

In some cases the virus was concentrated by precipitation with polyethylene glycol (13, 15) and then layered onto a sucrose gradient. The ^{14}C-virus was mixed with an equal volume of nonradioactive carrier virus (prepared by infecting cells at low multiplicity in Eagle medium containing 3% fetal calf serum), and one-fourth volume of 40% polyethylene glycol in 2 M NaCl was added. After 1 h at 0 C, the precipitate was collected by centrifugation at 10,000 rpm for 45 min or at 15,000 rpm for 15 min, resuspended in a small volume of 0.2 M NaCl containing 50 mM Tris, pH 7.4, and EDTA, and clarified by centrifugation at 2,000 rpm for 10 min. The recovery of virus was greater than 90% by this procedure. (It is important, however, that the ionic strength of the resuspending buffer be greater than 0.2; otherwise, virus adsorbs to cellular debris and is lost.) For some experiments the virus was precipitated without addition of carrier virus; in these cases the recovery of virus was variable, averaging about 50%. The carrier virus preparation is most probably effective because of membrane fragments and other cellular debris present that coprecipitate with the virus.

Preparation of labeled VSV. The conditions for labeling VSV were similar to those used for Sindbis virus, but the radioactive medium had an ionic strength of 0.16. Approximately 12 h after infection the entire culture fluid was harvested; and the virus was then concentrated and purified by polyethylene glycol precipitation (no carrier virus was necessary) and sucrose gradient centrifugation as described for Sindbis virus. (The time and speed of centrifugation were adjusted for the higher sedimentation coefficient of VSV.)

Infection of cells for adsorption experiments. Chick cells in 60-mm petri plates were infected at a multiplicity of 30 to 50 PFU per cell from 1 ml of PBS containing 1% fetal calf serum. After a 1-h adsorption

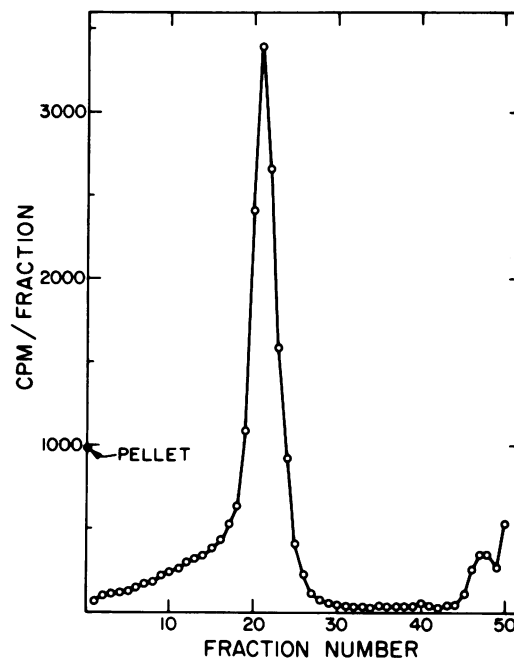

FIG. 1. *Sucrose gradient sedimentation of ^{14}C-labeled virus released from chick cells into high-ionic-strength medium. Sindbis virus was grown in chick cells in low-ionic-strength (0.10) medium containing [^{14}C]methionine. The monolayers were washed with medium of the same ionic strength 13 h after infection, and then incubated briefly in medium of ionic strength 0.25. An amount of virus equivalent to the yield for 25 cm^2 of monolayer was subjected to sedimentation in a 15 to 30% sucrose gradient containing 0.2 M NaCl, 0.05 M Tris, pH 7.4, 0.001 M EDTA, 0.3% fetal calf serum. Centrifugation was for 58 min at 36,000 rpm and 23 C in the SW 41 rotor. Fractions were collected by drops from the bottom of the tube and counted with 1 ml of water in 10 ml of a dioxane-naphthalene-based scintillation fluid. Sedimentation is from right to left.*

period at 37 C, the inoculum was removed and 3 ml of Eagle medium containing 2% fetal calf serum was added. Mock-infected cells were treated similarly but without virus.

Adsorption of ^{14}C-labeled virus. For experiments at 4 C, petri plates were removed to the cold room and allowed to chill. The medium was removed by aspiration, the plates were allowed to drain tipped on edge, and any residual medium was removed. A 1- or 0.5-ml portion of PBS containing 1% fetal calf serum and 10^3 to 10^4 counts per min of ^{14}C-labeled virus was added to each plate. The plates were rocked at 15-min intervals to redistribute the inoculum. After 90 min of adsorption, the inoculum was removed by aspiration, and the monolayer was washed twice with 5-ml portions of the appropriate buffer. The plates were drained well, and the monolayers were dissolved in 2 ml of 1% sodium dodecyl sulfate (SDS).

Monolayers to be pretreated with high-ionic-strength buffer before virus adsorption were treated similarly, but after removal of the culture fluid 5 ml of high-ionic-strength buffer (usually 0.2 M NaCl containing 0.01 M phosphate, pH 7.4) was added. This buffer was removed, and the monolayers were washed once with 5 ml of 0.15 M NaCl containing 0.01 M phosphate before the virus inoculum was added.

Adsorption experiments at 37 C were similar in design, but manipulations were performed at room temperature and adsorption steps at 37 C. The monolayers were also incubated for 10 min at 37 C in the wash solutions used to remove unadsorbed virus.

Assay of bound virus. Monolayers dissolved in 1% SDS were precipitated with trichloroacetic acid (20% final concentration). The precipitates were collected on DA membrane filters (Millipore Corp.), dried thoroughly at 60 C, and counted in 5 ml of toluene-fluor.

RESULTS

Effect of ionic strength upon the adsorption of Sindbis virus to uninfected cells at 37 C. Radioactive Sindbis virus was adsorbed to monolayers of chick cells for 75 min at 37 C from PBS solutions containing different concentrations of NaCl to vary the ionic strength. After adsorption the monolayers were washed at 37 C with either PBS of the same ionic strength as used for adsorption, or with a solution of high ionic strength (0.25). The results presented in Fig. 2 show that the optimal ionic strength for virus adsorption under these conditions is 0.15 to 0.17, where approximately 20% of the input radioactivity was adsorbed to the cells. Eighty to 90% of the adsorbed virus was resistant to elution with buffer of ionic strength 0.25. This probably represents virus that has successfully initiated an infection cycle, because the particle to plaque-forming unit ratio is 3 to 6 under the conditions of virus preparation and adsorption to cells (unpublished results).

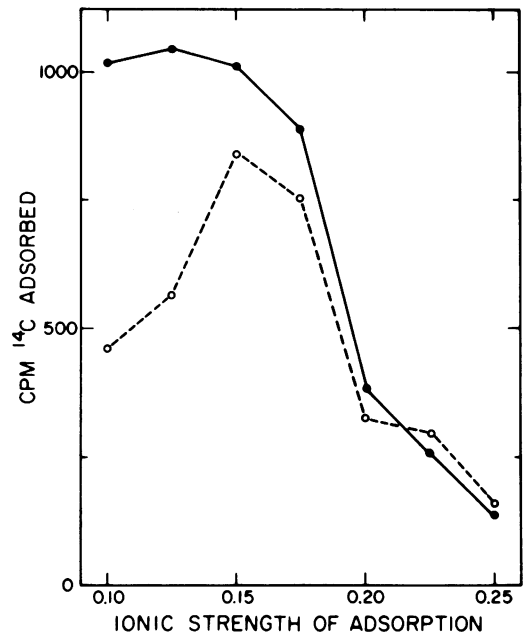

FIG. 2. *Adsorption of ^{14}C-labeled Sindbis virus to chick monolayers in solutions of different ionic strengths. Sindbis virus, labeled with [^{14}C]methionine, was diluted into buffers of differing ionic strength. These buffers consisted of PBS (9), but the NaCl concentration was varied so that the final ionic strength ranged from 0.1 to 0.25; the buffers also contained 1% fetal calf serum. Virus was adsorbed to monolayers of chick cells in 60-mm petri plates from 0.5 ml of buffer (containing 4,400 counts/min) for 75 min at 37 C. The inocula were removed and the monolayers incubated for 20 min at 37 C in 2.5 ml of buffer of the same ionic strength (●) or of ionic strength 0.25 (○). The monolayers were then dissolved in 1% SDS, precipitated with trichloroacetic acid, and assayed for radioactivity.*

At ionic strengths greater than 0.17, relatively little adsorption occurred, but nearly all of the virus that did adsorb resisted elution with buffer of ionic strength 0.25. At ionic strengths less than 0.15, virus adsorbed well, but 50% of this could be eluted subsequently with high-ionic-strength buffer. Thus, much of the virus adsorbed at low ionic strength is "loosely" bound, whereas most of the virus adsorbed at ionic strengths greater than 0.15 is "tightly" bound.

Effect of ionic strength upon the elution of virus adsorbed to cells at 4 C. ^{14}C-labeled Sindbis virus was adsorbed to monolayers of chick cells from PBS (ionic strength 0.17) for 90 min at 4 C. The monolayers were then washed with sodium chloride-phosphate solutions of various ionic strengths, and the radioactivity

remaining adsorbed to the monolayer was assayed. The results are shown in Fig. 3.

Under these conditions, about 50% of the virus is adsorbed to the cells. As the ionic strength of the wash solution is raised above 0.17, however, much of the adsorbed virus is eluted. The dependence upon the ionic strength of the wash solution is very steep. When the monolayers were washed with a solution of ionic strength 0.20 approximately one-half of the adsorbed virus is eluted, indicating that much of the virus adsorbed at 4 C is only "loosely" bound. Thus, only 20 to 25% of the applied virus is adsorbed to cells in a manner resistant to washing with an ionic strength of 0.20; after washing with an ionic strength of 0.2, an amount of virus is left that is equivalent to that which can bind and initiate infection at 37 C.

In some experiments, the amount of virus adsorbed to the cells is greater when the wash solution has an ionic strength of 0.25 than when the ionic strength is 0.2. This effect is not always seen.

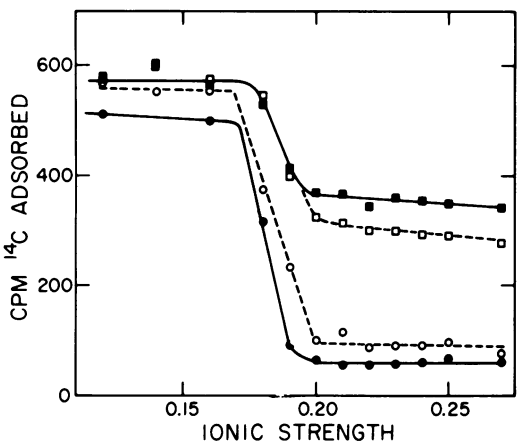

FIG. 3. *Elution of Sindbis virus adsorbed to chick cells by washing with buffers varying in ionic strength. ^{14}C-labeled Sindbis virus was diluted into PBS (9) containing 1% fetal calf serum and adsorbed to monolayers of chick cells out of 1 ml (containing 900 counts/min) for 90 min at 4 C. The inocula were removed, and the monolayers were washed twice at 4 C with sodium chloride-phosphate buffers of various ionic strengths. These wash buffers contained 0.01 M phosphate, pH 7.4, and NaCl from 0.1 M (ionic strength 0.12) to 0.25 M (ionic strength 0.27). The monolayers were then assayed for bound radioactivity. Symbols: ■, uninfected cells, not prewashed; □, uninfected cells, prewashed with sodium chloride-phosphate of ionic strength 0.2 followed by sodium chloride-phosphate of ionic strength 0.17 before virus adsorption; ●, cells 7.2 h after infection by Sindbis virus, not prewashed; ○, cells 7.2 h after infection by Sindbis virus, prewashed with sodium chloride-phosphate of ionic strength 0.2 followed by sodium chloride-phosphate of ionic strength 0.17 before virus adsorption.*

The behavior of virus bound to infected or to uninfected cells is similar in response to ionic strength of the wash solution, but less virus is bound to infected cells. This effect is further explored in the following sections.

Prewashing the cells, either infected or uninfected, with sodium chloride-phosphate solutions of ionic strength 0.20 to 0.25 had little effect on the subsequent adsorption and elution of Sindbis virus.

Decline in tight binding of the virus after Sindbis infection. The experiment shown in Fig. 3 demonstrates that the amount of virus bound to infected cells or to uninfected cells is approximately the same when the monolayers are washed with buffers of ionic strength 0.17 or less after virus adsorption. However, the amount of virus "tightly" bound, in a fashion resistant to washing with ionic strength 0.2, is markedly less for infected cells. In the experiment in Fig. 3, the amount of virus tightly bound to cells infected for 7 h by Sindbis virus was only about 20% of that which was tightly bound to uninfected cells.

To determine the time of loss of tight binding by infected cells, chick cells were examined for

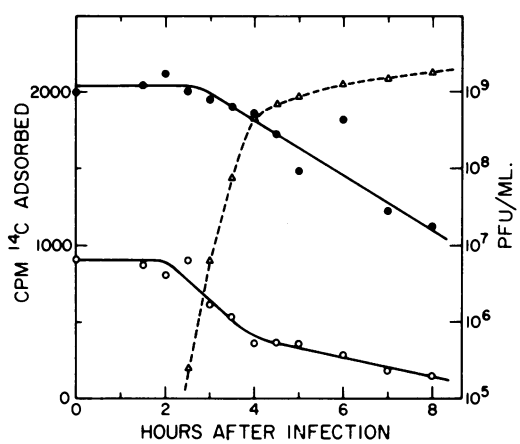

FIG. 4. *Adsorption of Sindbis virus to chick cells at various times after infection with Sindbis virus. ^{14}C-labeled Sindbis virus in PBS containing 1% fetal calf serum was adsorbed to monolayers of chick cells at various times after Sindbis virus infection. The adsorption period was 90 min at 4 C out of 0.5 ml containing 5,400 counts/min. The monolayers were then washed twice at 4 C with sodium chloride-phosphate buffers of ionic strength 0.17 (●) or 0.27, (○), and the radioactivity remaining bound to the monolayers was assayed. A growth curve of the virus is shown for the same experiment (△).*

their ability to adsorb Sindbis virus at various times after Sindbis infection. Radioactive virus was adsorbed to the cells for 90 min at 4 C, and the cells were then washed with sodium chloride-phosphate solutions that were either 0.17 or 0.27 in ionic strength. The results are shown in Fig. 4. Also plotted is a growth curve of the virus performed at the same time to indicate the course of the infection.

Most of the loss in tight binding occurs between 2 and 4 h after infection, with a slower, continued decline thereafter. At 3 h after infection, fewer than 10 PFU/cells have been released into the culture fluid. Thus, the reduction in binding begins when less than 1% of the 8-h virus yield has been produced. Virus is still being actively produced at 8 h; cells continue to yield virus under these conditions until about 12 h after infection.

In the experiment of Fig. 4, there is also a progressive decline in the total amount of virus bound by the infected cells. The total amount of virus bound by infected cells has been variable from experiment to experiment, but most often results comparable to Fig. 3 have been obtained, where the total amount of virus bound to infected cells is only slightly less than that bound by uninfected cells.

The decrease in binding of Sindbis virus to Sindbis virus-infected cells is accompanied by an increase in the binding of an unrelated virus, VSV, (Table 1) implying that loss of Sindbis binding is not due to changes in cell surface area but rather to configurational changes occurring at the cell surface.

Binding of VSV to cells. VSV, a bullet-shaped virus, is another enveloped virus that matures by budding, but is quite different in structure from Sindbis virus. For the purposes of comparison, the binding of VSV to uninfected and infected cells as a function of ionic strength was examined. For reference, a growth curve of VSV under our experimental conditions is shown in Fig. 5. This may be compared with the growth curve of Sindbis virus in Fig. 4.

The binding of VSV to cells as a function of ionic strength is shown in Fig. 6. In contrast to the results with Sindbis virus, VSV binds equally well to cells infected for 8 h with VSV as it does to uninfected cells. In addition, the dependence upon ionic strength for elution of the virus is not as marked.

Sindbis virus, however, binds somewhat less readily to VSV-infected cells than to uninfected cells (Table 2). This difference in binding is small in comparison with that between Sindbis virus-infected and uninfected cells, especially when the ionic strength of the wash buffer is 0.25.

TABLE 1. *Binding of ^{14}C-labeled VSV to chick cells after Sindbis virus infection*[a]

Chick cells	^{14}C-VSV adsorbed (counts/min)	
	After 0.17[b] wash	After 0.25[b] wash
Uninfected	620	410
Sindbis-infected	1,010	520

[a] VSV (11,000 counts/min in 1 ml) was adsorbed for 90 min at 4 C to chick monolayers of either uninfected cells, or cells 5 h after infection with Sindbis virus. The monolayers were then washed with buffers of ionic strength 0.17 or 0.25, and the amount of bound virus was assayed.

[b] Ionic strength.

DISCUSSION

Sindbis virus appears to adsorb to chick cells in two different ways, which can be referred to as "loose" binding and "tight" binding. At 4 C loose binding occurs at an ionic strength of 0.17 or less with both infected and uninfected cells,

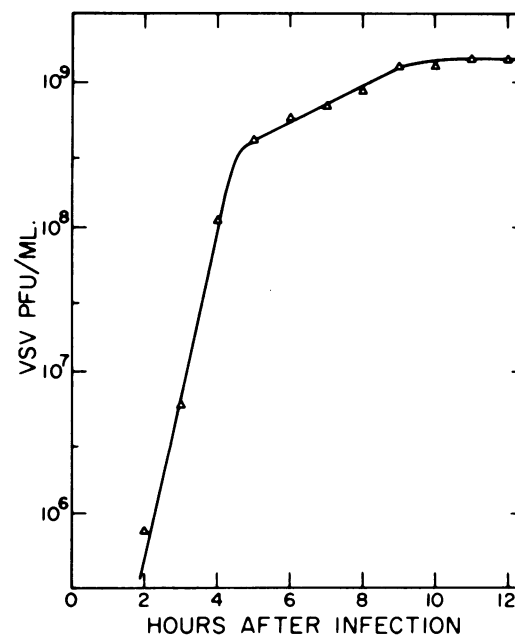

FIG. 5. *Growth curve of VSV. Monolayers of chick cells were infected with VSV at a multiplicity of 17. After various times at 37 C the number of plaque-forming units of virus released into the culture fluid was assayed.*

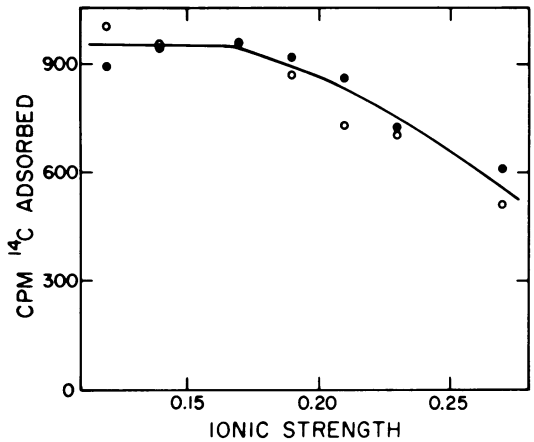

FIG. 6. *Effect of ionic strength upon elution of VSV adsorbed to chick cells. VSV, labeled with [^{14}C]-methionine, was diluted to 5,550 counts per min per ml in PBS containing 1% fetal calf serum. One-ml portions were adsorbed for 90 min at 4 C to monolayers of uninfected cells (●) or to cells which had been infected for 8 h by VSV (○). The monolayers were then washed twice with sodium chloride-phosphate buffers of various ionic strengths, and the amount of radioactivity remaining bound was assayed.*

TABLE 2. *Binding of ^{14}C-labeled Sindbis virus to chick cells after VSV infection*[a]

Chick cells	^{14}C-Sindbis adsorbed (counts/min)	
	After 0.17[b] wash	After 0.25[b] wash
Uninfected	2,300	1,190
VSV-infected	1,780	880

[a] Sindbis virus (4,400 counts/min in 1 ml) was adsorbed for 90 min at 4 C to chick monolayers of either uninfected cells or cells 8 h after infection with VSV. The monolayers were then washed with buffers of ionic strength 0.17 or 0.25, and the amount of virus bound was assayed.

[b] Ionic strength.

and is readily reversed by exposure to ionic strengths in excess of 0.2. At 37 C, adsorption of virus is complicated by penetration events. However, a loose binding does occur when adsorption occurs at ionic strengths less than 0.15. At an ionic strength of 0.17, loose binding either does not occur or is unstable.

A tight binding of virus to cells can also occur at 4 C. Virus bound in this way remains attached even when the cells are washed with buffers of high ionic strength (although very little virus will attach at ionic strengths greater than 0.17). In the case of uninfected cells, tightly bound virus accounts for approximately 50% of the virus adsorbed, but only about 10 to 20% of the virus adsorbed to Sindbis virus-infected cells is tightly bound. Virus adsorbed at 37 C that resists elution into buffers of high ionic strength is probably virus that has eclipsed and penetrated.

The sites on the cell surface responsible for tight binding of Sindbis virus decline early after infection, beginning at a time when only a small fraction of the final virus yield has been produced. Loose binding of virus is affected relatively little. We have considered the possibility that loss of these sites is due to saturation by progeny virus particles, but this seems unlikely because the loss begins early and loose binding is not appreciably affected. In addition, one would expect a similar phenomenon to occur with VSV, which also releases progeny virus into the medium, but it does not. The loss of tight binding sites coincides with several other events reflecting changes in the cell surface at 3 to 5 h after infection. Cells become agglutinable at this time by several of the plant lectins (4), cells acquire the ability to hemadsorb red blood cells (7), budding virus can be detected in the electron microscope (3), and the cells will adsorb increased quantities of the unrelated virus VSV.

The loose binding of Sindbis virus may be a nonspecific adsorption of the virus, unrelated to virus infection. Alternatively, adsorption of the virus may be a two-stage event. Virus is first loosely bound, then a certain fraction of this bound virus undergoes a second event that converts the viruses to being tightly bound. Tight binding of the viruses could be due to attachment of the virus to several receptors simultaneously. In this case, the loss of tight binding after Sindbis infection could be due either to a general reduction of virus receptors, making attachment to multiple sites less probable, or to an alteration in the clustering of the sites on the cell surface.

Waite and Pfefferkorn (22) have shown that the interaction of Sindbis virus with cells is sensitive to ionic strength in another way. Virus does not complete the budding process at ionic strengths less than 0.15, although the earlier events in the virus life cycle occur normally. In addition, Pfefferkorn and Clifford (19) have shown that the virus is insoluble at ionic strengths less than 0.15. It is unclear at the present time what relationship exists between these phenomena and the ionic-strength dependence of virus adsorption, but it is clear

that ionic surface charges play an important role in the interaction of Sindbis virus with the host cell.

ACKNOWLEDGMENTS

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