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Early Interaction of Rhinoviruses with Host Cells

K. LONBERG-HOLM AND B. D. KORANT

Central Research Department,[1] Experimental Station, E. I. du Pont de Nemours and Company, Wilmington, Delaware 19898

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The rate of attachment of type 2 virions to suspensions of HeLa cells is much greater than that of type 14, but the number of receptor sites per cell is similar for each type. The receptor sites may be partly saturated with excess virions; attachment is greatly reduced after about 10^4 particles have been taken up per cell. A lack of saturation of type 14 receptors by excess type 2 indicates that their receptor sites are separate on the cell surface. Excess of type 2 blocks attachment of type 1A, however, and excess of type 14 blocks type 51. Attachment of the human rhinoviruses is temperature-dependent with a Q_{10} of 2.7. The eclipse reaction is also temperature-dependent. At 34.5 C, the irreversible eclipse of cell-associated rhinovirus type 2 requires only a few minutes, whereas the rate of eclipse of cell-associated type 14 is considerably slower. The eclipse product of type 2 rhinovirus has been recovered from infected cells. It sediments at about 90% of the rate of the infective virions and is missing virus polypeptide 4 (the smallest of the capsid polypeptides). Upon being subjected to CsCl gradient centrifugation, virus polypeptide 2 is also lost but the product still contains ribonucleic acid and bands at about 1.45 g/cc.

Knowledge of how picornaviruses attach to and react with host cells is largely limited to the enteroviruses. The specific receptors for poliovirus and coxsackievirus group B have been shown to have characteristic sensitivities to inactivation by individual proteolytic enzymes (32) but are not inactivated by neuraminidase (14, 32). The absence of receptors may limit the host range of picornaviruses (18), but other factors such as the ability of the cells to permit eclipse, uncoating or penetration (7, 13, 29), or viral replication (4, 30, 31) are equally important.

It has generally been observed that picornaviruses attach efficiently to host cells in the cold (1, 3, 14, 22). However, some moderate degree of temperature dependence has been reported for some strains of virus under various conditions (14, 15, 23). Mandel has shown that early after attachment cell-associated poliovirus can be recovered in infective form by disruption of the membrane-virion complex with the detergent SDS (sodium dodecyl sulfate, reference 20). At physiological temperature, the cell-associated particles also undergo an irreversible loss of infectivity or "eclipse." Eclipse (or irreversible eclipse) will be defined in the present report as the loss of recoverable infectivity of the cell-associated virus as determined after treatment with SDS.

A substantial portion of virions of poliovirus type 1 attached to cells in the cold have been observed to "elute" upon subsequent incubation at physiological temperature (9, 15). The particles which have eluted are noninfective, and their infectivity cannot be restored by treatment with acid or detergents (21), despite the fact that the eluted virions contain infective ribonucleic acid (RNA; reference 15). Although there is no direct evidence for it, the eluted virions may be structurally identical to the cell-associated eclipsed virions. Eluted poliovirus particles are resistant to ribonuclease (15, 21), unstable to CsCl gradient centrifugation (15), and sediment at a slightly slower rate than infective virions (9). A similar phenomenon occurs in the case of coxsackievirus B3, and the eluted particles have been shown to lack the small capsid polypeptide VP4 (6). Loss of infectivity of poliovirions has also been equated with loss of VP4 (2).

The early interaction of the cardioviruses with host cells has also been recently described. Plaque variants of mengovirus have differing rates of attachment and uncoating (19). Other cardioviruses have been examined, and these appear to be subject to an abortive early step leading, instead of to elution, to fragmentation of the capsid (11).

The rhinoviruses have been examined only very briefly. Human rhinovirus type 2 (HRV-2) and human rhinovirus type 14 (HRV-14) are both reported to attach to WI-26 cells, whereas only

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HRV-2 attaches to monkey kidney cells; this difference is in keeping with the host range of the viruses (10). The uptake of HRV-14 (27) and HRV-2 (26) infectivity by cell suspensions has been reported, and in the latter case it was also reported that the rate is greater at 33 than at 4 C.

We have examined the interaction of HRV-2 and HRV-14 with HeLa and other cells and report here on the kinetics of attachment, the number and nature of the receptor sites, the temperature coefficient for the attachment reaction, and the existence of receptor groups within the rhinovirus family. In addition, we have examined the rate of the eclipse reaction and the nature and composition of the eclipse product.

MATERIALS AND METHODS

Virus and cells. HRV-2 and HRV-14 strains and their growth in roller bottle cultures of HeLa cells (rhino-HeLa cells) will be described elsewhere. Human rhinovirus type 51 (HRV-51), strain FO1-4081, was obtained from W. L. Davies of Stine Laboratory (Newark, Del.), and human rhinovirus type 1A (HRV-1A), strain 2060, was obtained from R. Grunert of the same laboratory. HRV-2 and HRV-14 were plaque-purified, and the identity of all types was confirmed with standard antisera. Poliovirus type 2 of the attenuated strain 712-Ch-2ab was obtained from R. Bablanian of the State University of New York and was propagated in our line of HeLa cells. The labeled pools of virions employed in all experiments had been purified, as will be described elsewhere, by sucrose gradient centrifugation and CsCl gradient banding and were stored at 4 C in the presence of 5% heat-inactivated fetal calf serum. These pools contained a known number of particles per milliliter as determined by the ratio of extinction at 260 nm (E_{260}) to counts per minute after CsCl gradient centrifugation. In the case of HRV-1A only, the CsCl gradient centrifugation step preceded sucrose gradient centrifugation and the latter employed solutions of sucrose containing 1 M NaCl and 0.02 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (*p*H 8.1); the E_{260} was then determined on the sucrose-purified virions.

HeLa cells (rhino-HeLa cells) were also used to study attachment and eclipse. These were grown in Spinner culture in Eagle's minimal essential medium (8) modified for Spinner cells (Grand Island Biological Co., Grand Island, N.Y.) and containing 7% horse serum at 3×10^5 to 6×10^5 cells per ml. The cells were chilled and washed twice by centrifugation at $300 \times g$ for 10 min with cold Spinner medium containing 5% heat-inactivated fetal bovine serum.

Attachment of virus. Unless otherwise stated, the standard conditions for virus attachment were as follows. HeLa Spinner cells (10^7 per ml) were mixed with purified virions at 0 C in Spinner medium containing 5% heat-inactivated fetal bovine serum. The suspension was divided into portions of 0.4 to 0.5 ml, and these were distributed into plastic tubes (12 by 75 mm) which were then tightly stoppered. At zero time, the tubes were placed in a water bath with a rotary shaker and agitated with 240 strokes/min at 34.5 C (or another temperature if so indicated). The reaction was stopped by removal of the tube to ice temperature and addition of 2.0 ml of ice-cold Spinner medium or phosphate-buffered saline. The cells were pelleted at $300 \times g$ for 10 min, washed one or more additional times, resuspended in a measured volume of 0.05 M Tris (*p*H 8.1), and sampled for infectivity and radioactivity determinations.

Control experiments with both HRV-2 and HRV-14 have shown that it does not matter if cells are washed with either phosphate-buffered saline (minus magnesium and calcium) or complete Spinner medium. The simple inorganic medium was thus preferred in cases in which large volumes of cell supernatant fluid were to be counted to reduce quenching of radioactivity in the scintillation spectrometer.

Determination of infectivity. Infectivity was determined by a plaque assay which will be reported elsewhere. Cell-associated (noneclipsed) infectivity was determined by diluting the cell suspension in SDS, buffered at *p*H 8.1, to a final concentration of 0.4% SDS. This mixture was left for 10 min at room temperature, diluted 10-fold with Spinner medium containing 5% heat-inactivated fetal bovine serum, and then frozen at −20 C until the day of the assay.

Digestion of samples with ribonuclease. To determine the fraction of RNA-labeled virions which had been uncoated, samples were digested for 30 min at 34.5 C with 25 μg of ribonuclease A (Sigma Chemical Co., type 1-A) per ml in 0.02 M Tris buffer (*p*H 7.5). If the samples had been purified by gradient centrifugation, additional protein as 5% heat-inactivated fetal bovine serum was added to protect the integrity of intact particles. Digestion under these conditions rendered more than 90% of the RNA from boiled virus soluble in trichloroacetic acid.

Assay of radioactivity. Samples to be assayed for acid-precipitable counts were precipitated with a final concentration of trichloroacetic acid of 8 to 10% (w/v) in the presence of 2 mg of bovine serum albumin (fraction V). The samples were pelleted at low speed after 16 hr in the cold, and the trichloroacetic acid supernatant fluid was aspirated. The pellets were then dissolved in 1.0 ml of 0.15 M NaOH (non-acid-precipitated samples were dissolved in 1 ml of 0.1 M NaOH) and were counted with 10 ml of added Hayes solution number 3 (12). This solution also contained about 60% (v/v) Cabosil to serve as a thixotropic gel in the presence of the alkali. ^{32}P-containing samples were sometimes counted, without scintillator, by Čerenkov radiation (5); colored samples were first bleached with Clorox.

Recovery of eclipse products from infected cells. Infected cells were washed twice and then resuspended in 0.05 M Tris(*p*H 8.1) at 2×10^7 cells per ml. These cells were treated for 15 sec with the Biosonik III ultrasonic generator fitted with a 4-mm probe (Bronwill Scientific, Rochester, N.Y.). In most experiments, deoxyribonuclease was then added to 200 μg/ml, and the mixture was left for 10 min on ice. The detergent Nonidet P-40 (NP-40; Shell

Chemicals Ltd., London, U.K.) was then added to 0.5%, and the mixture was layered onto gradients for ultracentrifugation.

Linear CsCl gradients having a volume of 5 ml were made from 1.48 and 1.32 g/cc CsCl solutions in 0.02 M Tris buffer (*p*H 7.5). Up to 0.5 ml of cell homogenate and a layer of mineral oil were layered onto these gradients. The gradients were then centrifuged for 15 hr at $2.2 \times 10^5 \times g$ at 4 C in a Spinco L-2 ultracentrifuge (Beckman Instruments Inc., Palo Alto, Calif.), and fractions were taken from the bottom of the gradients and were always precipitated with trichloroacetic acid before counting. The density of selected fractions was determined with a pycnometer. Sucrose gradients, 4 or 28 ml, were made with 10 and 25% sucrose in 1 M NaCl containing 0.02 M Tris-hydrochloride (*p*H 8.1) (16). Extracts of infected cells were layered on top of the gradients and then were subjected to $1.7 \times 10^5 \times g$ for 1 hr (small gradients) or to $9 \times 10^4 \times g$ for 5 hr (large gradients) at 4 C. Fractions were taken from the bottom. After the liquid contents were removed, the empty tubes were rinsed with 0.1 M NaOH. The radioactivity which was thus recovered both from pellet and from the walls of the tube is designated as P in several of the figures.

RESULTS

Attachment of HRV-2 to HeLa cells. Figure 1a shows the disappearance of infectivity and radioactivity from the cell supernatant fluid when purified ^{32}P-labeled HRV-2 virions were incubated at 34.5 C with 10^7 HeLa spinner cells per ml. In this experiment, the trichloroacetic acid-soluble radioactivity was determined by Čerenkov radiation, and the sum of the corrected Čerenkov counts plus the acid-precipitable counts was represented as total counts per minute. The total counts and acid-precipitable counts were parallel during the first 40 min, and only after this time was there an accumulation of acid-soluble ^{32}P.

The initial uptakes of infectivity and radioactivity were parallel, but loss of infectivity was more complete so that virtually all extracellular infectivity was gone by about 30 min, whereas roughly 10% of the radioactivity remained. This residual fraction probably represents altered, possibly eluted virions which were generated during the interaction with cells. This is likely because it was shown in other experiments that after a short (5-min) period of attachment of labeled virions, cell-associated acid-insoluble radioactivity was eluted to a variable extent (up to about 20%) when the cells were washed in the cold and then reincubated at 34.5 C for 20 min. Although it is also not shown in Fig. 1a, the ribonuclease-resistant fraction of the acid-precipitable counts in the supernatant fluid coincided with the total acid-precipitable counts, and this indicates that the noninfective extracellular virions were not uncoated (i.e., their RNA is not susceptible to ribonuclease). It was found in other experiments that the incubation of virus in medium without cells for 1 hr at 34.5 C did not produce significant loss of infectivity.

Figure 1b shows the cell-associated infectivity and radioactivity from the same experiment as Fig. 1a. The cell-associated infectivity was rapidly lost; it was observed in a number of experiments that about half of the cell-associated HRV-2 had been eclipsed by 5 min after the start of incubation. The total radioactivity and the trichloroacetic acid-precipitable radioactivity diverged after about 20 min, indicating that there was extensive digestion of cell-associated virions. The ribonuclease-resistant counts were similar to the

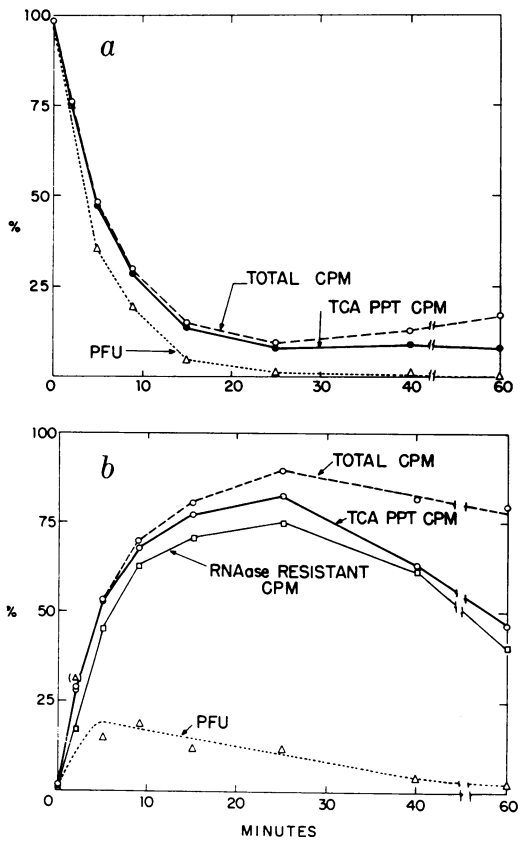

FIG. 1. *Uptake of ^{32}P-labeled HRV-2 by HeLa-O Spinner cells at 34.5 C, 10^4 virions per cell. (a) Supernatant total radioactivity, acid-precipitable radioactivity, and infectivity. (The infectivity in the supernatant fraction was determined after treatment with SDS.) (b) The cell-associated total radioactivity, acid-precipitable radioactivity before and after treatment with ribonuclease and the infectivity recovered after treatment with SDS. Per cent ^{32}P or plaque-forming units added at zero time.*

total acid-precipitable counts which indicates that there was no major accumulation of uncoated RNA inside the cells; i.e., digestion rapidly followed any uncoating in or on the cell. The relatively slow loss of acid-precipitable counts either before or after ribonuclease digestion indicates that the eclipse reaction (loss of infectivity) was much more rapid than uncoating.

Attachment of HRV-14 to HeLa cells. Figure 2 shows a typical attachment of labeled HRV-14. The process of interaction of ^{32}P-HRV-14 and HeLa cells in Spinner culture was qualitatively similar to that with HRV-2, except that both uptake and eclipse were slower. The initial rate of uptake of HRV-14 was usually 20 to 40 times slower than that of HRV-2 under comparable conditions. It appears that there was a greater proportion of noninfective particles produced in the supernatant fraction relative to the eclipsed virions produced in the cell fraction than was the case with HRV-2 (Fig. 2). [Again, as with HRV-2, elution of cell-associated virions was also demonstrated by washing and reincubating cells to which radioactive virus had been attached (*data not shown*).] Although it is not shown as clearly as with HRV-2, the eclipse of cell-associated virions of HRV-14 appeared to precede the uncoating step.

Effect of temperature on the attachment of HRV-2. Figure 3 shows that the attachment of

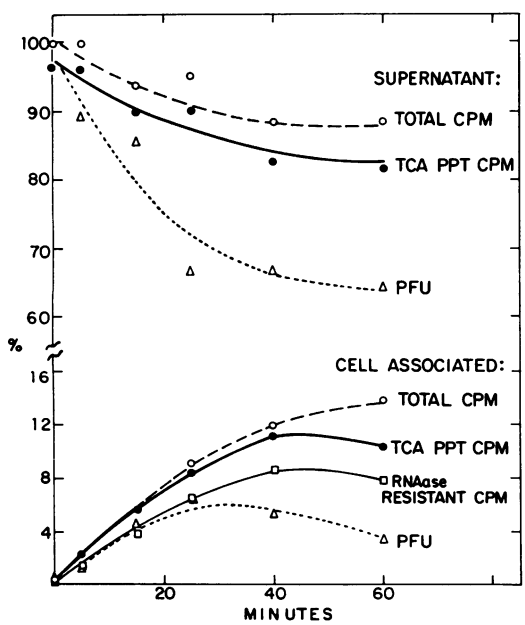

FIG. 2. *Uptake of ^{32}P-labeled HRV-14 by HeLa-O cells at 34.5 C, 3.6 × 10^4 particles per cell. See Fig. 1. Note the break in the scale for per cent uptake.*

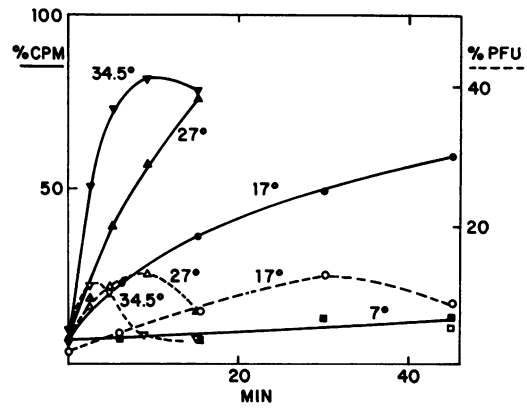

FIG. 3. *Attachment and eclipse of ^{3}H-uridine-labeled HRV-2 by HeLa-O cells at various temperatures, 10^4 virions per cell. Trichloroacetic acid-precipitated radioactivity associated with the cells is given as per cent of the added acid-insoluble counts (solid curves, scale at left), and cell-associated infectivity after SDS treatment is given as per cent added plaque-forming units (dashed curves, scale at right).*

labeled HRV-2 was strongly dependent upon temperature. The attachment of poliovirus type 2 of the attenuated strain 712-Ch-2ab was also found to be dependent upon the temperature. The data for both viruses are represented as the logarithm of the initial attachment rate versus the temperature (Fig. 4). The attachment of HRV-14 was also temperature-dependent to a similar degree, but in this case the uptake data at low temperatures were not precise enough to permit calculation of the Q_{10}.

In the experiment shown in Fig. 3, the cell-associated infective virus was also measured, and it is represented as per cent of the total added infectivity. For example, at 17 C about two-thirds of the cell-associated virus had undergone eclipse in the sample taken 30 min after the start of incubation. It can be deduced from this and other data that the rate of eclipse was reduced when the temperature was decreased but that the eclipse reaction was not reduced sufficiently to have permitted attachment in the absence of eclipse. The results of this and other experiments suggest that decreased temperature reduces the rates of attachment and eclipse of HRV-2 to approximately the same extent.

Saturation of receptors on the cell surface. The uptake of virus is a function of the particle multiplicity (particles per cell). When more virions are added to the system, a decreasing fraction of particles are able to attach and this variation indicates that there is a limited number of specific receptor sites on each cell. Figure 5 shows the adsorption isotherm for HRV-2 and HRV-14 on

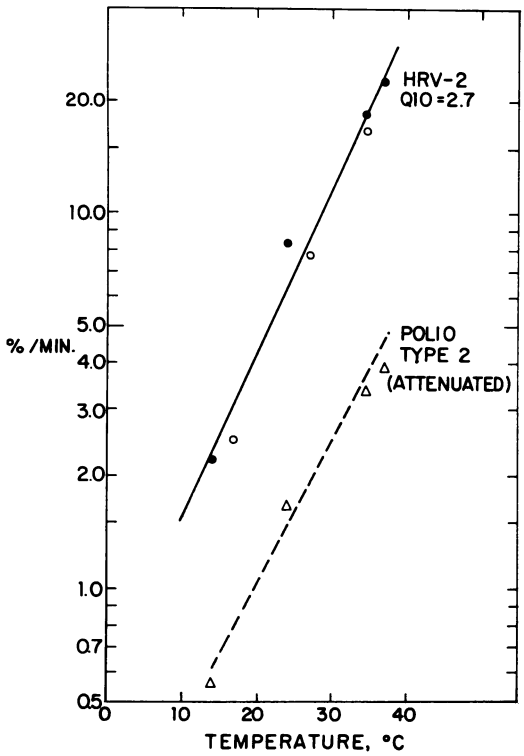

FIG. 4. *Relationship between the rate of attachment and temperature for HRV-2 and poliovirus 2 (strain P712-Ch-2ab). The initial rates were estimated from Fig. 3 and also from the data of other experiments.*

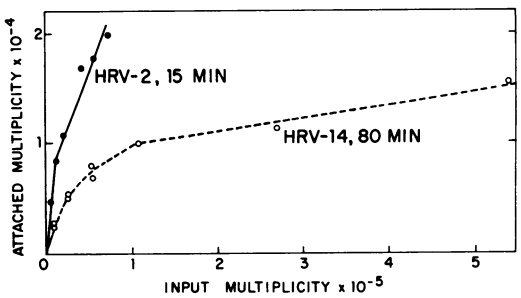

FIG. 5. *Adsorption isotherms for HRV-2 and HRV-14 on HeLa-O cells at 10^7 cells per ml and at 34.5 C. ^{32}P-labeled HRV-2 was attached for 15 min or ^{3}H-uridine-labeled HRV-14 was attached for 80 min. To calculate the attached multiplicities, the per cent cell-associated trichloroacetic acid-precipitable counts per minute at the end of the incubation was multiplied by the added ratio of particles per cell.*

the same scale. The number of particles attached per cell was plotted against the number of particles per cell added to the suspension. With both types of virus, there was a break in the curve at about 10^4 particles per cell. (The break in the curve for HRV-2 is not displayed very convincingly because of the scale employed in Fig. 5; see, however, Fig. 6.) The change in slope of the curves at about 10^4 particles adsorbed per cell indicates that the capacity of the cells to adsorb virus can be partially saturated. Although higher input multiplicities, in particles per cell, are needed to achieve this with HRV-14 than with HRV-2, the number of adsorbed particles per cell at which this saturation occurs appears to be similar in both cases.

The adsorption periods employed in Fig. 5 were different for the two viruses because the attachment rates differ; they represent compromises dictated by the systems. In the case of type 2 virus, maximal uptake, expressed as per cent cell-associated and trichloroacetic acid-precipitable counts, occurred at about 15 min (for multiplicities of about 10^4 particles per cell). If other times were chosen, as in Fig. 6, the results still appeared to give a break at about 10^4. (Note that the scale changes from Fig. 5 to Fig. 6.) In the case of HRV-14, however, it was found that shorter periods did not give very satisfactory data over a practical range of input multiplicities because of the slow rates of attachment.

Cross-saturation and rhinovirus receptor families. If the same receptor site on the cell is used for either of two types of virion, excess of one should block the attachment of the other and vice versa. We shall refer to such a phenomenon as cross-saturation. A number of attempts of various kinds were made to discover cross-saturation between HRV-2 and HRV-14, and all attempts failed. Figure 7 shows that the attachment of ^{32}P-HRV-14 was not blocked by excess ^{3}H-uridine-labeled HRV-2. The top panel gives the per cent uptake of uridine-labeled HRV-2 virus at low and high particle multiplicities; this confirmed that HRV-2 saturated its own receptors under the conditions of the experiment. In the lower panel, cells were incubated with a relatively low multiplicity of ^{32}P-labeled HRV-14, and the attachment was not affected by the presence or absence of the high multiplicity of HRV-2 employed in the top panel. Other similarly unsuccessful experiments, which are not shown here, involved the use of excess ultraviolet light-inactivated HRV-2 in attempts to block the attachment of infective particles of HRV-14. Also, the preincubation of cells with large excesses of HRV-14 had no effect on their ability to attach HRV-2.

HRV-2 did saturate the receptors for HRV-1A, however, and HRV-14 did saturate receptors for HRV-51. In the experiment of Fig. 8, an excess of HRV-2 was found to reduce the attachment of ^{14}C-labeled HRV-1A when added together with HRV-1A before the start of the incubation. In

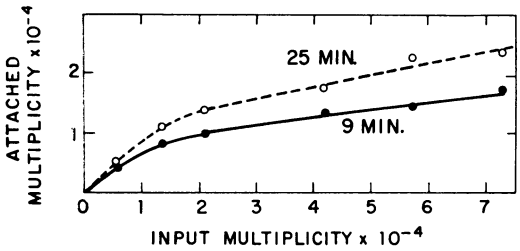

FIG. 6. *Adsorption isotherms for ^{32}P-HRV-2 as determined by 9 and 25 min of incubation.*

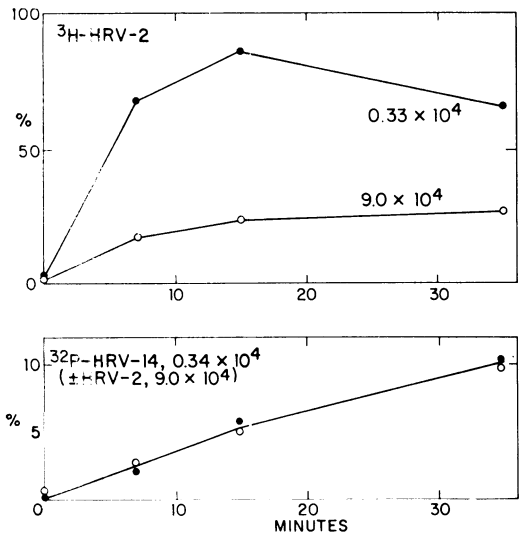

FIG. 7. *Lack of effect of excess HRV-2 particles on the uptake of HRV-14. (Top) Kinetics of uptake of ^{3}H-uridine-labeled HRV-2 at 0.33 × 10^4 and 9.0 × 10^4 particles per cell. (Bottom) Kinetics of uptake of ^{32}P-labeled HRV-14 at 0.34 × 10^4 particles per cell without (●) or with (○) the additional presence of 9 × 10^4 particles per cell of HRV-2.*

the experiment shown in Fig. 9, samples of cells were first incubated for 30 min with or without an excess of HRV-14. After this first incubation, ^{32}P-HRV-51 was added, and those samples which contained cells partially saturated with HRV-14 were able to attach only about one-third as much ^{32}P as those untreated with HRV-14. In another experiment, not shown, excess ^{3}H-HRV-2 had no effect at all on the attachment of HRV-51.

CsCl gradient analysis of HRV-2 eclipse product. Attempts to isolate the product of eclipse of HRV-2 by CsCl buoyant density centrifugation showed that both the cell-associated infective virions and also the eclipsed virions were largely bound to cellular membrane material (Fig. 10). If cells were infected with ^{3}H-uridine-labeled HRV-2 and then were broken by sonic treatment

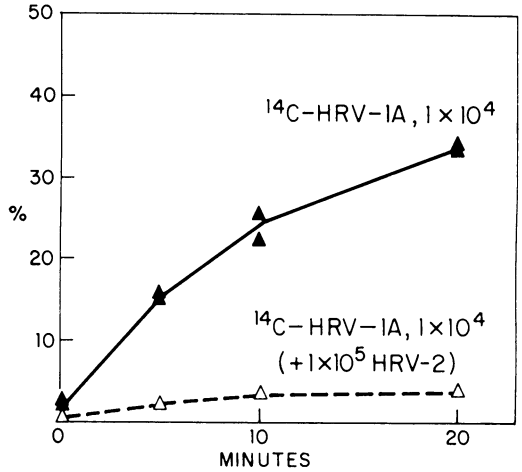

FIG. 8. *Saturation of HRV-1A receptors by excess HRV-2. Solid curve: ^{14}C-amino acid-labeled HRV-1A attachment at 10^4 particles per cell as per cent added radioactivity in the cell fraction. Dashed curve: Attachment of ^{14}C-amino acid-labeled HRV-1A at 10^4 particles per cell admixed with 10^5 HRV-2 particles per cell.*

and layered directly onto CsCl preformed gradients, relatively little radioactivity was recovered in the gradient. When the cell extract was first treated with 0.5% NP-40, a detergent used in other studies to release the membrane-bound products of human adenovirus-cell interaction (16), the eclipse product appeared to band at about 1.45 g/cc in CsCl. The noneclipsed cell-associated virions (1.40 g/cc) could also be recovered. Similar results were obtained in another experiment with ^{14}C-amino acid-labeled HRV-2, except that a portion of the radioactivity was also recovered at about 1.29 g/cc as would be expected for free virion protein.

After 5 min of attachment, 63% of the cell-associated virus underwent eclipse in the experiment of Fig. 10 (*data not shown*). At the same time, 60% of the cell-associated radioactivity found in the gradient became associated with the dense component at about 1.45 g/cc (middle gradient). After 20 min of additional incubation, 98% of the cell-associated virus was eclipsed but some radioactivity still trailed into the 1.40 g/cc region of the gradient (bottom gradient, Fig. 10).

The approximate correlation between the extent of eclipse and the distribution of radioactivity by isopycnic banding was confirmed in the experiment of Fig. 11, in which ^{32}P-labeled HRV-2 was permitted to interact with HeLa cells before CsCl gradient centrifugation. In this and subsequent experiments, the sonic extracts of the cells were treated with deoxyribonuclease as well as NP-40

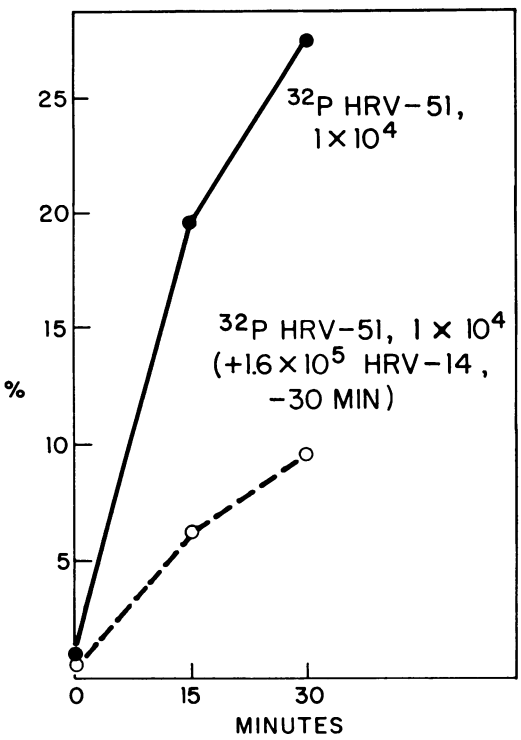

FIG. 9. *Saturation of HRV-51 receptors by excess HRV-14. Cells were incubated for 30 min at 34.5 C with either 0 or 1.6 × 10^5 particles per cell of HRV-14. After this preincubation, 10^4 particles per cell of ^{32}P-HRV-51 were added. Solid curve: Uptake of ^{32}P-HRV-51 by cells incubated in the absence of HRV-14. Dashed curve: Uptake of ^{32}P-HRV-51 by cells treated with HRV-14.*

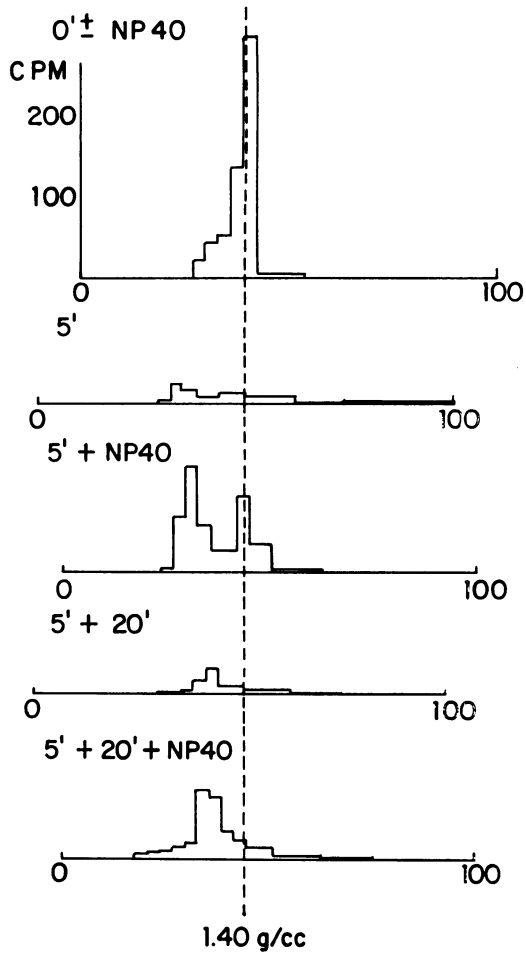

FIG. 10. *Separation of an eclipse product of ^{3}H-uridine-labeled HRV-2 by CsCl gradient centrifugation. Deoxyribonuclease was not employed. Extract of noninfected cells was mixed with marker virus in the top panel. Cells exposed for 5 min at 34.5 C to HRV-2 and washed and sonically treated are shown in the next two panels. Half of the cells were also washed and reincubated in fresh medium for 20 min before analysis in the last two panels. As indicated, NP-40 was added to the sonically treated material to 0.5% before gradient centrifugation. Only the first 100 drops of each gradient are shown. The densities of several fractions of each gradient were determined by pycnometry, and the results are represented so as to align the position of intact infectious virus (1.40 g/cc) under the dotted line. The same number of cell equivalents (5 × 10^6) was employed in each gradient, and the scale for radioactivity is the same in each. The total recovery of radioactivity (including tube washings which are not shown) for the non-detergent-treated samples was within 87% of that of the detergent-treated samples.*

to improve the resolution and increase the capacity of the gradients (*see above*). The attachment of HRV-2 was also permitted to continue for 30 min without washing the cells so that the results could be compared directly with Fig. 1. Again, within the limits of the accuracy of the method, the formation of the dense component appeared to coincide with the eclipse of cell-associated virus. However, after 30 min of incubation, all but a small fraction of the cell-associated infectivity was eclipsed, but a relatively larger fraction of the cell-associated radioactivity remained in the region of intact virions (1.40 g/cc). The shape of the curve indicates that this may have been largely the result of heterogeneity in the density of the eclipse product in the CsCl gradient rather than the result of the presence of unaltered virions.

Figure 12 shows the cell supernatant fractions from the experiment shown in Fig. 11. The dense component was produced during the period of

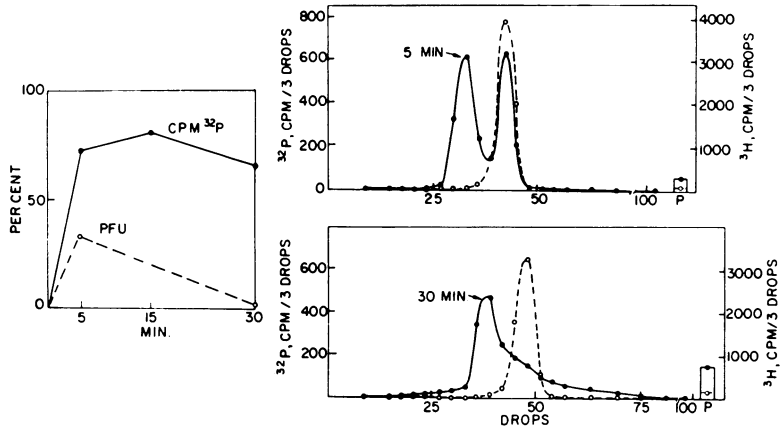

FIG. 11. *Cell-associated radioactivity and infectivity during the attachment of ^{32}P-labeled HRV-2 and the CsC gradient separation of the eclipse product. The uptake of trichloroacetic acid-precipitable radioactivity and the cell-associated infectivity are given as per cent of that added at zero time in the panel to the left. The CsCl gradient analysis of the cell-associated ^{32}P at 5 min and 30 min after the start of infection is shown in the panels to the right (solid curves). ^{3}H-uridine-labeled HRV-2 has been added to each gradient as a marker (dashed curves). NP-40 and deoxyribonucleic acid were employed in each sonically treated preparation. P designates radioactivity recovered from the drained tubes.*

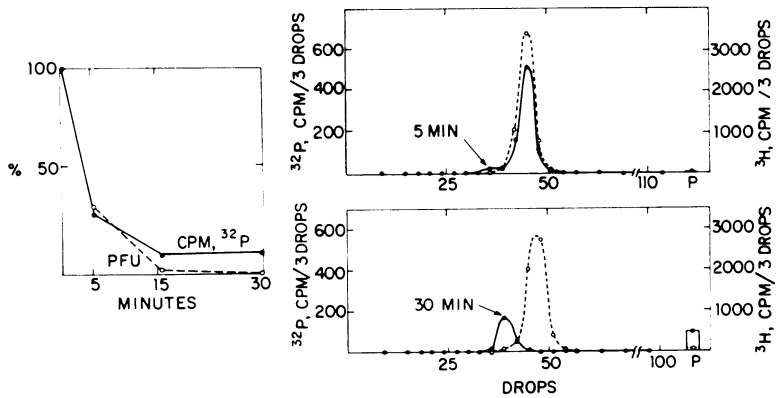

FIG. 12. *Cell supernatant radioactivity and infectivity during attachment of ^{32}P-labeled HRV-2 and CsCl gradient separation of the noninfective extracellular material. This is the extracellular material from the same experiment as shown in Fig. 11. Neither NP-40 nor deoxyribonuclease was used before gradient centrifugation, and SDS was not used in the determination of infectivity. Marker ^{3}H-labeled HRV-2 is shown by the dashed curves in the gradients.*

time in which virtually all supernatant infectivity was lost. A large portion of the nonattached trichloroacetic acid-precipitable radioactivity was composed of dense component. Although it is not shown in the figure, control gradients on the starting virus preparation showed that most of this material could not have been present as minor contamination of the starting virus. It should also be noted that the supernatant fractions were not treated with NP-40, which suggests that the extracellular noninfective particles formed during interaction of HRV-2 with the cells are not bound to membrane fragments. Other experiments have shown that incubation of virions in the absence of cells does not produce detectable amounts of dense component.

Sucrose gradient separation of HRV-2 eclipse products. Sucrose gradients can also resolve an eclipse product of HRV-2. Figure 13 shows the separation of material from HeLa cells incubated for 5 min with ^{32}P-virions. The infected cells were subjected to sonic disintegration and treated with deoxyribonuclease and NP-40, and the sucrose gradients used for the separation contained 1 M NaCl (*see above*). The slow component, peak C, had a sedimentation rate of 135*S* in this and other

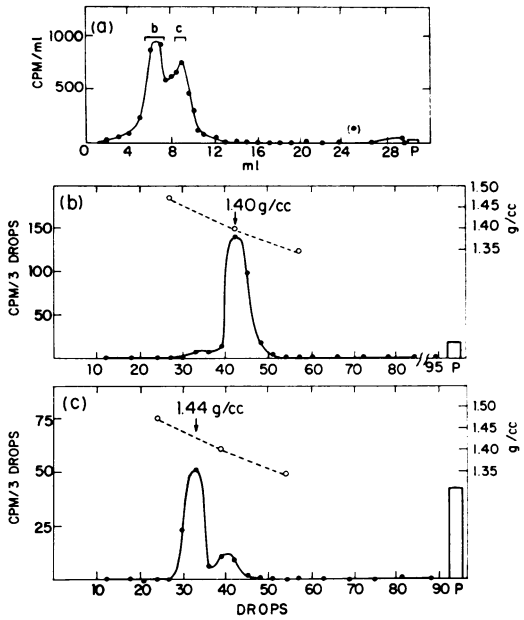

FIG. 13. *Separation of the eclipse product of HRV-2 on a sucrose gradient and its subsequent banding in CsCl. HeLa cells were exposed to 1.8 × 10⁴ particles per cell of ³²P-labeled HRV-2 for 5 min. The cell fraction was sonically treated and treated with deoxyribonuclease and NP-40, and 1.3 × 10⁷ cell equivalents were separated on a 28-ml sucrose gradient containing 1 M NaCl. The fractions were counted by Čerenkov radiation, and pools b and c were analyzed on CsCl gradients (Fig. 13b and c). The recoveries of radioactivity in b and c, including tube washings, were 90 and 80% of the applied counts.*

experiments (as based on assigning 150S to the intact virions). The peaks designated as b and c were rebanded on CsCl gradients as shown in the second and third panels. Component b corresponded to infective virus (1.40 g/cc), and component c corresponded to the dense component (approximately 1.45 g/cc). However, under the conditions for isolation and rebanding in CsCl, a sizable portion of the radioactivity of c appeared to be lost and was found in the washings of the CsCl gradient (P). The recovery of radioactivity in parallel gradients was variable (*not shown*).

In a separate experiment, it was found that the RNA of the 135S material, like that of the intact virions, was largely resistant to 25 μg of ribonuclease per ml for 15 min at 37 C in the presence of 5% fetal bovine serum (*not shown*). Other experiments which are not reproduced here showed that much of the eluted virion material also sedimented at about 135S. When amino acid-labeled HRV-2 was employed, the eclipse product also sedimented largely at 135S, but a small amount of an 80S material which presumably represents empty capsids was also produced (see, for example, Fig. 14).

The cell-associated eclipse product, as obtained by sucrose gradient centrifugation, was also subjected to polypeptide analysis. Figure 14a shows the separation of particles from cells exposed to

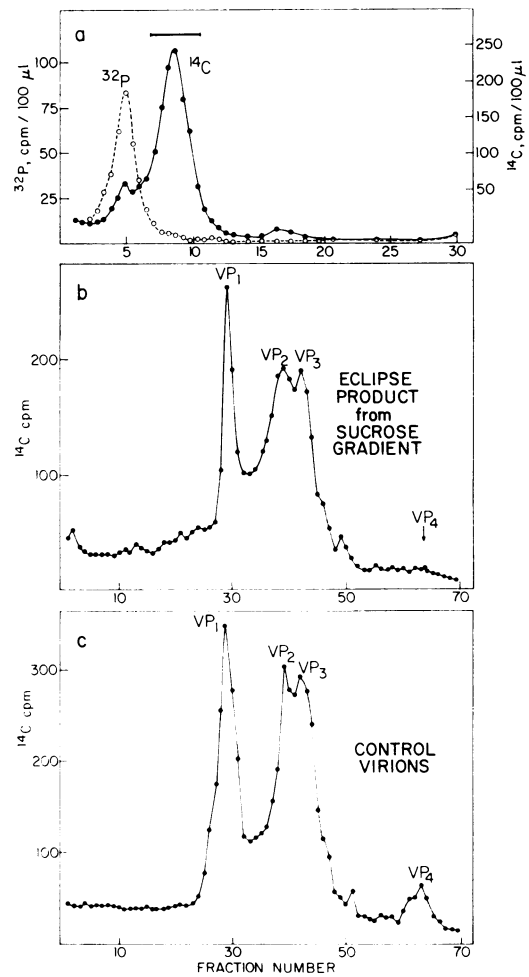

FIG. 14. *Polypeptides of the HRV-2 eclipse product. (a) Sucrose gradient separation of the eclipse product of HRV-2. This experiment is similar to that of Fig. 13, except that cells were exposed for 15 min to 2.8 × 10⁴ particles per cell of ¹⁴C-amino acid-labeled HRV-2. The gradient volume in milliliters is given on the abscissa. (b) Eclipse product as indicated by the bar in Fig. 14a and equivalent portions from two other gradients run in parallel were pooled and concentrated and then analyzed by polyacrylamide gel electrophoresis as will be described elsewhere. The position expected for VP4 is indicated. (c) Uneclipsed ¹⁴C-labeled virus from the same pool employed in Fig. 14a was mixed with sucrose, concentrated, and analyzed in the same way as described above.*

^{14}C-amino acid-labeled HRV-2 for 15 min. Figure 14b shows the polypeptides of the slow component which was obtained from fractions pooled as indicated in Fig. 14a. The pooled material was dialyzed against 0.1% SDS in 0.01 M phosphate buffer (*p*H 7.0) and was concentrated by dialysis against solid polyethylene glycol. A control sample of intact virions was also dialyzed and concentrated in the same manner.

The polypeptides were separated by polyacrylamide gel electrophoresis in the presence of 0.1% SDS. As will be shown elsewhere, four major polypeptides can be detected in the virion of HRV-2, providing that the smallest of these (VP4) is not lost during the preparation of the sample for electrophoresis by the dialysis of partially disrupted virions in the absence of SDS. Two of the polypeptides, VP2 and VP3, can only be clearly resolved if the sample is permitted to run so long that VP4 is lost from the end of the gel. The analysis of the eclipse product of HRV-2 (Fig. 14b) shows that VP4 is absent from its expected position (Fig. 14c).

Eclipse product of HRV-14. The attachment and eclipse of HRV-14 were slower than with HRV-2 (Fig. 2). Figure 15 shows that, after 60 min of attachment to HeLa cells and 60 min of additional incubation, ^{32}P-labeled HRV-14 virions were recovered sedimenting at about 135*S* relative to marker ^{3}H-uridine-labeled HRV-14. (In another experiment, which is not shown, eluted HRV-14 particles were also found to sediment at 135*S*.) In addition to the 135*S* material in Fig. 15, a portion of the radioactivity was found at the top of the gradient and probably corresponds to degraded RNA; another portion sedimented at 40*S*.

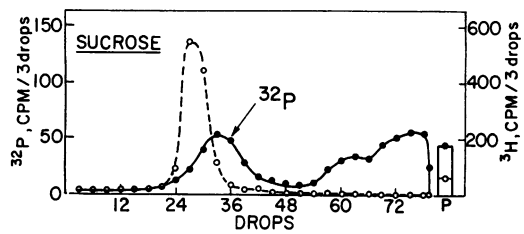

FIG. 15. *Modification of HRV-14 virions by incubation with HeLa cells. Cells were incubated for 60 min with 10^5 particles per cell of ^{32}P-HRV-14, and 13.5% of the added trichloroacetic acid-precipitable counts became cell-associated. The cells were washed and incubated again for 60 min in fresh medium after which 5.7% of the initial acid-precipitable counts remained cell-associated. (The supernatant fluid contained 2.3% eluted acid-precipitable counts.) The cells were sonically treated, treated with deoxyribonuclease and Nonidet P-40, and subjected to gradient centrifugation on a 4-ml sucrose gradient. Total counts per minute per three drops are plotted against drop number. ^{3}H-uridine-labeled HRV-14 was added, as a marker, before separation.*

Unlike the case of HRV-2, there were no new components seen by CsCl gradient analysis of cells infected with RNA-labeled HRV-14, except that much of the total radioactivity could be recovered in the tube washings and probably corresponded to naked RNA. It seems probable then that the HRV-14 slow component is unstable in CsCl and dissociates to free RNA and protein. As of the present time, we have not analyzed the polypeptides of the HRV-14 slow component and we can only conjecture that it, like HRV-2 slow component, is missing only VP4.

DISCUSSION

The attachment of HRV-2 and HRV-14 as well as of attenuated poliovirus type 2 shows a surprising temperature dependence with a greatly reduced attachment rate at 0 to 4 C. The Q_{10} for attachment of HRV-2 and attenuated poliovirus type 2 has been calculated to be 2.7 (Fig. 4). Whether this reflects a high activation energy for the step or one of the steps leading to a firm linkage between virion and receptor or alternatively a temperature-dependent requirement for a configurational or energetic state of the plasma membrane before the attachment can occur is not clear at the present time. A similar temperature dependence for attachment has been found for coxsackieviruses B1 and B3 (R. L. Crowell, *personal communication*). Not all rhinoviruses appear to share the temperature requirement for attachment, and, in other experiments which are not reported in this paper, it was found that equine rhinovirus (Plummer) readily attaches to HeLa cells in the cold.

The rate of attachment of the human rhinoviruses to HeLa cells depends upon the serotype; HRV-2 attaches 20 to 40 times as rapidly as HRV-14, whereas HRV-51 and HRV-1A have intermediate rates. The serotypes which have been examined thus far fall into two receptor families by cross-saturation experiments. HRV-2 and HRV-1A share receptors as do HRV-14 and HRV-51. Experiments with excess HRV-2 and excess HRV-14 show that virions of one family cannot block independent attachment of virions of another family (Fig. 7). Thus, the receptors are spatially separated from each other on the plasma membrane. Cross-saturation does occur within a family (Fig. 8 and 9). It has been suggested to us that this grouping of rhinovirus serotypes within receptor families may be related to host range, since HRV-1A and HRV-2 are both able to replicate in monkey cells as well as in human cells, whereas HRV-14 and strain F01-4081 of HRV-51 both require human cells (E. J. Stott, *personal communication*; see also reference

10). Further data on other rhinovirus serotypes will be required to demonstrate if there is such a correlation between the *M* and *H* character [ability to grow in both monkey and human cells or only in human cells (28)] and membership in a receptor family.

Some of the biochemical characteristics of the receptors are known from experiments on the inhibition of attachment after various treatments of the cells, and these will be reported elsewhere. However, it should be stated here that the human rhinovirus receptors, like some other picornaviruses (14, 31), are insensitive to neuraminidase but are sensitive to proteolytic enzymes and that the attachment of HRV-2 but not HRV-14 can be inhibited by 5 mM ethylenediaminetetraacetic acid added to the incubation medium.

Although there is a competition for the available rhinovirus receptors on the cell membrane, the adsorption isotherm is not ideal since there does not appear to be a maximum number of particles which can become cell-associated. Instead, a break in the adsorption isotherm is observed (Fig. 5 and 6), but, above this point, as more virus is added, additional attachment occurs at a lower efficiency. The break occurs at roughly 10^4 particles per cell for both HRV-2 and HRV-14 [approximately the same number of adenovirus receptor sites have also been seen on HeLa cells (24)]. The attachment of 10^4 virions covers about 1% of the HeLa cell surface if the virions are assumed to have a diameter of about 30 nm and if the HeLa cell is spherical with a diameter of 15 μm. Thus, the shape of the adsorption isotherm is not the result of saturation of the entire cell surface. The lack of ideality may be the result of one or more factors. (i) The receptors may be recycled since subsequent steps of interaction are also proceeding during the period required for the attachment reaction. (ii) There may be subpopulations of receptors or cells which show a decreased affinity towards virions. (iii) The virions might contain one or more minor subpopulations with regard to receptor affinity. These subpopulations could even be generated during the process of virus-cell interaction. (iv) Some other, nonspecific mechanism for uptake such as pinocytosis might also occur in addition to the specific receptor mechanism.

Figure 16 gives a schematic representation of the components which have been separated by gradient centrifugation of the eclipsed virions of HRV-2. On the bottom is placed the dense component shown in Fig. 10 to 13 as banding at approximately 1.45 g/cc. This is probably identical with a dense component which can be isolated from crude HRV-2 preparations during virion purification, and both of these dense components have been shown to contain mainly VP1 and

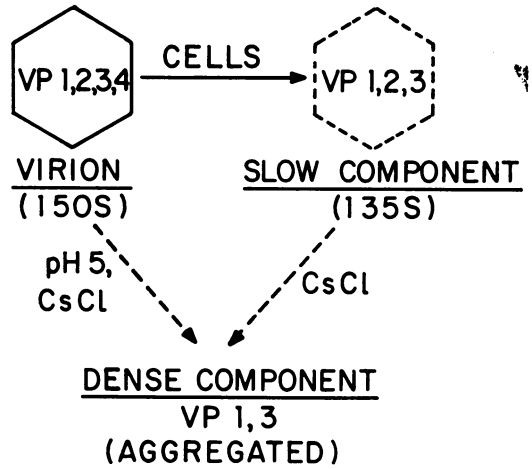

FIG. 16. *Schematic relationship between various HRV-2 components obtained after eclipse.*

VP3 after isolation from CsCl gradients (*unpublished data*).

The dense component itself is not the eclipse product but is probably formed when the eclipse product is centrifuged into a CsCl preformed gradient. This is another way of saying that the eclipse product is unstable in CsCl, and, in the case of HRV-2, it is converted to the dense component by loss of VP2. This conversion occurs in variable yield; most of the RNA of eclipsed virions can be recovered as the dense component when cell extract is placed directly on CsCl (Fig. 11 and 12), but less is recovered if the slow component (135*S*) is isolated by sucrose gradient centrifugation and then rerun on CsCl (Fig. 13).

The slow component, which sediments at 135*S* and contains VP1, 2, and 3 (Fig. 14), is probably closely related to the eclipse product as it is formed in or on the cell. This component is associated with the cell membrane fraction, but there is no evidence at present which can permit us to decide whether the eclipsed virus is located on, imbedded in, or inside the plasma membrane. Despite the fact that it is missing a portion of its capsid protein, the slow component appears to be ribonuclease-resistant and thus it probably does not resemble the particles which have been reported to be produced in vitro from human rhinovirus by heat or acid treatment and which can be seen to have holes in the capsid by electron microscopy (17, 25).

The rhinovirus cell-associated eclipse product and the noninfective rhinovirus eluted particles are both similar in that they have a sedimentation rate which is about 10% less than that of infective virions. A similar reduction in sedimentation has also been reported for the noninfective eluted particles of poliovirus type 1 (9), and these also

are resistant to ribonuclease (15, 21). The eluted particles of coxsackievirus B3 have also recently been shown to share these properties with poliovirus and in addition have been shown to lack VP4 (6) as does the rhinovirus eclipse product. The rhinovirus type 2 eclipse product differs from the eluted particles from poliovirus and coxsackievirus in that it is partly stable to CsCl gradient centrifugation and in CsCl produces a component which is lacking both VP2 and VP4. HRV-14 resembles the enteroviruses more in this respect, since the 135*S* particle produced during eclipse appears to be completely unstable in CsCl.

Although we cannot now present evidence for it, we would like to suggest that the eclipse which is observed to occur with the major portion of the infecting virions in these studies may be similar in nature to a biochemical step which is undergone by each infecting particle during the process of productive infection. In the HRV-2 system, this represents one particle in 2,200 (*unpublished data*).

Uncoating of the cell-associated rhinovirus virions can occur only after eclipse (Fig. 1), and many of the uncoated particles probably enter an abortive pathway leading to digestion of the virion RNA to trichloroacetic acid-soluble products. Since the ratio of infecting particles to plaque-forming units is very high, only a very small fraction of the eclipsed particles may ultimately enter into a productive sequence leading from uncoating to translation of the viral genome.

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