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Physical and Metabolic Requirements for Early Interaction of Poliovirus and Human Rhinovirus with HeLa Cells

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Attachment, "tight binding," and eclipse of radioactive poliovirus 2 (P2) and human rhinovirus 2 (HRV2) were investigated. The activation energy for attachment of both HRV2 and P2 was about 13 kcal/mol. HRV2 differed from P2 in two respects: the Arrhenius plot for attachment of HRV2 showed a break at 15 to 19°C when the cells were first treated several hours at 0°C, and attachment of HRV2 was inhibited by treatment of cells with metabolic poisons able to reduce cellular ATP by more than 90%. Tight binding was determined by isolation of a specific P2-membrane complex or by loss of EDTA dissociability of HRV2. Tight binding of both viruses was slowed by 0.01 M iodoacetamide but not by 0.02 M F^-; F^- plus 0.002 M CN^- slowed tight binding of HRV2 but not of P2. Eclipse, the irreversible alteration of parental virions, was detected by isolation of cell-associated subviral particles or by loss of cell-associated infectious virus. Eclipse of both viruses is slowed by iodoacetamide or F^-. It seems likely that the early steps of infection with picornaviruses may be sensitive to alterations in the cell membrane produced by metabolic inhibitors or by treatment at low temperature.

It has been established that picornaviruses attach to specific cellular receptors (3, 21, 27, 36). Receptors can attach picornaviruses in vitro, as reviewed by McLaren et al. (28), and "eclipse" (irreversible alteration) of picornaviruses also appears to be catalyzed by membrane fragments in vitro (2, 10, 28, 35, 39). These studies give the impression that the host cell plays a passive role in these early steps and that eclipse may simply be a consequence of the interaction between receptor and virion.

Other research has indicated that there are sequential steps that lead from a loose to a tight bond between a picornavirus and the cell surface (1, 7, 19, 24, 29) and that most infecting virus particles are shielded from neutralizing antibodies before they are eclipsed (7, 24). The loss of neutralizability of cell-associated virus has been used as the operational criterion for "penetration" (5, 7, 10, 12, 23, 24). In the case of poliovirus, the progression of loosely to tightly bound virions leads to a complex between native virus and cell membrane components that can be isolated by gradient centrifugation after the disruption of the cell. This complex is resistant to the detergents Nonidet P-40 (NP-40), Triton X-100, and even deoxycholate, but is disrupted by 0.2% SDS (20).

Recent work has identified cell-associated subviral particles that are the products of irreversible alteration (eclipse) of human rhinoviruse type 2 (HRV2) (21, 22) and poliovirus type 2 (P2) (19, 20) and shows that complete uncoating of the viral RNA probably follows eclipse.

The experiments reported here show that the attachment of either P2 or HRV2 depends strongly on temperature and that the attachment processes of both viruses are inhibited by metabolic poisons, albeit in different ways. Some of the effects discovered suggest that virus attachment may be a sensitive indicator of subtle alterations in membrane structure.

MATERIALS AND METHODS

Virus and cells. HRV2 strain HGP and P2 strain P-217 Ch2ab were plaque-assayed, propagated, labeled with ^3H- or ^{14}C-amino acids, and purified as already described (15, 20). ^{35}S-labeled virus was prepared by infecting cells in methionine-free medium, containing dialyzed serum, and 10 μCi of [^{35}S]methionine per ml. Virus particles per milliliter in each pool were measured (15). Typical pools of purified labeled virus contained 2×10^6 cpm and 10^{13} particles per ml.

A line of "rhino-HeLa cells" was propagated in suspension culture, washed twice with, and resus-

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pended in, Eagle minimal essential medium modified for spinner cells (Grand Island Biological Co., Grand Island, N.Y.) containing 5% heat-inactivated fetal calf serum ("medium") (21). Suspension cultures of mouse L cells were handled as above.

Attachment of virus to cells. The initial rate of attachment of radioactive virus was usually measured by filtering and washing cells after short periods of incubation with virus. Cells in medium (10^7/ml, or as specified) were either held at bench temperature for 2 to 4 h, or at 0°C for 3.5 to 6 h ("cold treatment") before use. Samples of 2.2 ml were transferred to a 5-ml jacketed glass vessel and stirred with a magnetic bar. The temperature of the vessel was maintained to ±0.01°C by a circulating water bath; the half-time for temperature equilibration of the sample was 17 s as determined with a Thermistor probe. Samples were permitted to equilibrate for 3.0 min, unless otherwise stated. Thirty seconds before adding radioactive virus to the incubated cells, 2×10^6 cells were removed and injected into 4.0 ml of ice-cold medium, which already contained diluted radioactive virus. This zero incubation time sample was filtered and washed in the same way as samples of infected cells. Radioactive virus (10 to 20 μl) was then added to the remaining cells and, at various times, samples of 2×10^6 cells each were removed and injected into tubes containing 4.0 ml of ice-cold serum-containing medium. After the last sample was taken, all of the samples were filtered and washed (five times, with 2 ml each time) on prewashed membrane filters (SSWPO2500, Millipore Corp., Bedford, Mass.) with the aid of a 10-place filtration manifold (Hoefer Scientific Instruments, San Francisco, Calif.). Samples (50 μl each) of the unfiltered mixture of virus and cells were also placed on filters in vials and counted to calculate the percentage of cell-associated virus.

In a few experiments, attachment of radioactive virus was measured by direct sedimentation of infected, undiluted cells. Samples of 200 μl were placed into conical plastic tubes and were centrifuged for 5 s in a Microfuge (Beckman Instruments, Inc., Fullerton, Calif.). The beginning of centrifugation was recorded as the time of the sample. The supernatant fluid was carefully removed from the cells with a Pasteur pipette and counted. The cells were drained further by aspiration and then resuspended in 200 μl of medium. This was withdrawn with a Pasteur pipette and counted. Losses in transfer were thus approximately equal for both fractions. Control experiments with cells lacking receptors for poliovirus showed that 1.5 to 2% of radioactive poliovirus in the supernatant fraction contaminated the cell fraction (see Results).

Calculation of the initial rate of attachment. Method (i). The fraction of cell-associated virus was plotted versus time, and the rate was estimated from the initial slope of the curve. (In a few experiments, a smoothed curve was drawn and the rate was calculated from the difference between the values at 0 and 20 s.) Initial rate constants were expressed as the fraction of virus attached per minute per cell.

Method (ii). In one experiment that used high multiplicities of virus, the rate of attachment decreased rapidly during the early part of the reaction, and the initial rate was obtained as the coefficient of the first-order term of a third-order polynomial that best fitted the data by the criterion of least squares. A computer program for doing this was provided by John S. Fok of the Du Pont Central Research & Development Department.

Tight binding of HRV2. Radioactive virus was dissociated from infected cells at 0°C with medium containing 10 mM EDTA as already described (29). The percentage of the total radioactivity that was recovered with the cells was plotted as a function of time.

Eclipse of cell-associated infectivity. Purified infectious virus was permitted to attach, at 0°C, to cells poisoned with metabolic inhibitors. Unattached virus was removed by washing twice with cold medium, and the infected cells were resuspended in medium containing inhibitors. The infected cells were incubated at 34.5°C (HRV2) or 37°C (P2), and at various times 50-μl portions were removed, lysed with 0.4% SDS, and diluted with serum-containing medium for eventual plaque assay (21).

Cellular ATP. Each sample of 10^6 cells was diluted with 2 ml of medium at 0°C and sedimented immediately for 1 min in a clinical centrifuge. The pelleted cells were drained and mixed with 1.5 ml of cold 0.4 M perchloric acid. After 30 min at 0°C the suspension was neutralized with ice-cold 0.6 M $KHCO_3$ and centrifuged for 10 min at 7,000 rpm. The supernatant fluid was stored at −70°C and ATP was assayed with the Du Pont model 760 Luminescence Biometer (Du Pont Instruments, Wilmington, Del.).

Recovery of the particles from cells infected with radioactive poliovirus. Infected cells were washed, subjected to ultrasonic vibration, treated with DNase and detergents, and analyzed by sucrose gradient centrifugation in the presence of 1 M NaCl, as already described (19). Samples of 0.6 ml (10^7 cell equivalents) were sedimented on gradients of 16-ml volume. Reproducible recovery of subviral particles in cell homogenates after treatment with SDS required strict adherence to the following steps. Ice-cold homogenates of cells in 0.02 M (pH 8.1) Tris containing 0.5% NP-40 were pipetted at room temperature into a tube containing 50 μl of 4% SDS per ml of sample. The mixture was then layered carefully onto a prechilled sucrose gradient and subjected promptly to ultracentrifugation at 4°C.

Preparation of crude membranes with attached poliovirus. Cells were washed and resuspended at 5×10^7 cells/ml in ice-cold buffer containing 0.14 M NaCl, 0.003 M KCl, 0.01 M (pH 7.2) HEPES (*N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid), 0.5 mM $MgCl_2$, and 1% bovine serum albumin. Purified poliovirus was added at 5×10^3 particles/cell, and the mixture was stirred 2 to 3 h at 0°C. The cells were then washed twice and resuspended in the same buffer and incubated for 10 min at 24°C to permit tight binding. The cells were washed again, resuspended (2×10^7/ml) in 0.01 M Tris buffer (pH 7.4) containing 1 mM EDTA, permitted to swell for 5 min at 24°C, and broken in an ice-cold steel Dounce

homogenizer. $MgCl_2$ was added to obtain a concentration of 3 mM, and the mixture was centrifuged for 1 min in a tabletop clinical centrifuge to sediment nuclei. The supernatant fluid was adjusted to 5 mM $CaCl_2$ and centrifuged for 30 min at 8,000 rpm in a Sorvall HB4 rotor at 0°C (Du Pont Instruments, Wilmington, Del.). The pellet containing crude membranes was resuspended in medium (2.5×10^7 cell equivalents/ml) and incubated at 37°C in the presence or absence of metabolic inhibitors. Samples of 50 μl were treated with SDS and diluted as described for eclipse of cell-associated infectivity.

Radioactive samples. Samples were diluted with 0.5 ml of 1.6 M NH_4OH and mixed with 10 ml of a toluene-Triton X-100-based scintillation solution. Membrane filters were placed in vials and wetted with 1 ml of 0.2 M NaOH and counted in a dioxane-containing counting solution that has been described previously (21).

Materials. [^{35}S]methionine (0.1 Ci/mmol) was purchased from Amersham-Searle (Arlington Heights, Illinois). Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was obtained as a gift from P. G. Heytler of Du Pont Central Research & Development Department. HEPES and bovine serum albumin, fraction V, were purchased from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Dependence of the initial rate of attachment on cell concentration. The techniques used to measure the initial rate of attachment gave very precise results when multiple determinations were made on 1 day. For example, the K value for the initial rate of attachment of ^{14}C-labeled P2 (10^4 virions/cell) to HeLa cells (10^7/ml) at 37°C determined seven times was 7.31×10^{-9} cm^3/min with a standard deviation of 0.17×10^{-9} cm^3/min. Typical standard deviations with HRV2 were also about 2 to 4% of the rate.

However, the initial rate of attachment depended to some extent on uncontrollable variables, presumably related to changes in the properties of the cells from day to day. Over a year, the average initial rate of attachment of HRV2 (10^4/cell) to HeLa cells under physiological conditions was $2.3 \pm 0.8 \times 10^{-8}$ cm^3/min at 34.5°C. Because of this 35% day-to-day variation, experimental comparisons were always made with one preparation of cells.

The effect of cell concentration and virus multiplicity of the initial rate of attachment on HRV2 is illustrated in Fig. 1 and Table 1. At the higher multiplicities, there was considerable uncertainty in assigning an initial rate of attachment by the manual graphic method usually employed (method i). For this reason, the initial rate was also estimated by a computer least-squares method for fitting a cubic equation to the observed data points (method ii).

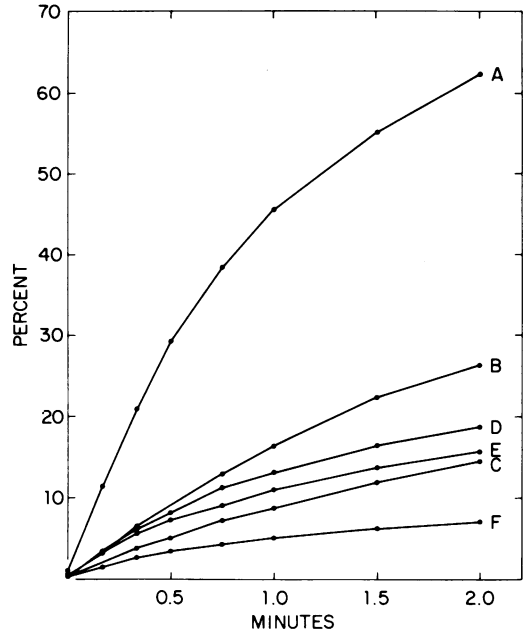

FIG. 1. *Attachment of ^{35}S-labeled HRV2 to HeLa cells at 30C. Virus was added to cells at 0 min and samples were removed, diluted, and filtered as described in Materials and Methods. The cell concentration and the number of virus particles per cell varied as described in Table 1. Percentage of added virus which has become cell-associated is shown as a function of time in minutes.*

The initial rates obtained by these two methods were in reasonable agreement (Table 1). The K values for the initial rates did not depend upon cell or virus concentration with multiplicities of 10^4 particles per cell or less.

Irreversibility of attachment. To determine whether the dilution and washing of cells systematically eluted a loosely bound population of virus, the total cell-associated virus, elutable plus nonelutable, was measured by direct sedimentation of the infected cells (see Materials and Methods). There was little evidence for reversibly bound virus. Figure 2a compares the attachment of HRV2 to HeLa cells at 34.5°C measured by dilution, filtration, and washing versus by direct sedimentation. Experiments at 0°C also indicated that no more than about 1 to 2% of the total virus was cell associated and in an elutable form during the initial 1 to 2 min of interaction (not shown).

Cell-associated poliovirus, measured by direct sedimentation, did not greatly exceed irreversibly bound virus during min 1 of the reaction (Fig. 2b). In similar experiments with mouse L cells, only 1.5 to 2% of the total poliovirus was recovered with the cells by direct

TABLE 1. *Rate constants for attachment of ^{35}S-labeled HRV2 to HeLa cells at 30°C*

Incubation[a]	Cells/ml ($\times 10^{-7}$)	Particles/cell ($\times 10^{-3}$)	K ($cm^3/\times min$) $\times 10^{7}$ [b]	
			Method i	Method ii
A	3.2	0.78	0.187	0.221
B	1.0	2.5	0.185	0.186
C	0.5	5.0	0.206	0.222
D	1.0	10.0	0.183	0.200
E	1.0	16.2	0.183	0.168
F	0.5	32.4	0.146	0.161

[a] HeLa cells were incubated with virus and samples were removed, diluted, and counted as described in Materials and Methods. The attachment of ^{35}S-labeled virus to cells is shown in Fig. 1.

[b] Initial rate of attachment was estimated manually (method i) or computed (method ii), and the rate constant is listed as the fraction of virus attached per cell per minute.

sedimentation (not shown). We assume that this represents unattached virus remaining in the cell pellet and on the walls of the centrifuge tube after direct sedimentation. The data of Fig. 2a and b were not corrected for this background. Even if this correction were made, a small amount of reversibly bound poliovirus may have been detected after 2-min incubation (Fig. 2b), as indicated by differences between the dashed line and continuous curves.

Effect of temperature on the initial rate of attachment. Cells were allowed to equilibrate to the temperature of a water-jacketed vessel for 3.0 min (Materials and Methods), and then virus was added and the initial rate of irreversible attachment was measured by removing, diluting, filtering, and washing samples of cells at short intervals. The stock suspension of HeLa cells (1.0×10^7/ml) used at different temperatures in a single experiment was prepared and kept either at room temperature or at 0°C for 3.5 to 6 h before use.

Figure 3a illustrates the effect of temperature on the K values for initial attachment of HRV2 to cells that had not been cold treated. An energy of activation, calculated from the slope of the line in this Arrhenius plot, was 12.5 kcal. The average from four experiments was 13 kcal (range, 10.7 to 16.0 kcal).

The attachment of HRV2 to cold-treated cells is qualitatively different (Fig. 3b). Below 15 to 19°C, the rate of attachment was very slow, and this made the calculation of the energy of activation very difficult. In two experiments we obtained values of 39 kcal (Fig. 3b) and 56 kcal (not shown). The apparent activation energy calculated from the slope above 20°C was 10.1 kcal (Fig. 3b) or 16.0 kcal in a second experiment that is not shown; these values average 13 kcal, which is approximately the same average obtained with non-cold-treated cells.

Attachment of P2 to cells that had not been treated at 0°C showed an average activation energy of 13 kcal (values of 12.8 and 14.1 kcal were calculated from the results of two experiments that are not illustrated). This is similar to the average value for attachment of HRV2. Treatment of cells at 0°C did not produce a significant break in the Arrhenius plot for attachment of P2 (Fig. 3c).

The effect of treatment at 0°C ("cold treatment") on the subsequent ability of cells to attach HRV2 at a temperature below the break in the Arrhenius plot was investigated in separate experiments. Several hours of cold treatment were required to produce a maximal impairment of attachment of virus at 8°C (Fig. 4a). In contrast, cold-treated cells were reactivated by incubation at 34.5°C in only 1 min (Fig. 4b).

Metabolic inhibitors and virus attachment. Treatment of HeLa cells for 15 min at 34.5°C with 0.01 M iodoacetamide or 0.02 M fluoride plus 0.002 M cyanide had no effect on subsequent attachment of poliovirus (not shown). However, attachment of HRV2 was strongly inhibited by metabolic poisons. Incubation of cells with iodoacetamide at 34.5°C gradually reduced their ability to attach HRV2, but treatment of virions with iodoacetamide before adding cells did not inhibit subsequent attachment to unpoisoned cells (Fig. 5).

Fluoride alone (0.02 M) or cyanide alone (0.002 M) had relatively little effect on the ability of cells to attach HRV2 (approximately 25% or less inhibition after 13 min at 34.5°C; data not shown). The combination of fluoride and cyanide, however, produced a rapid inhibition of the ability of cells to attach HRV2. Maximal inhibition was completed within 8 min (Fig. 6). A similarly rapid inhibition was produced by 0.02 M fluoride plus 2×10^{-5} M FCCP (not shown). FCCP is a potent uncoupler of oxidative phosphorylation.

The effects of metabolic inhibitors on attachment of HRV2 appeared to parallel their effects on the cellular levels of ATP. Cellular ATP was depleted by 95% within 6 min after treatment

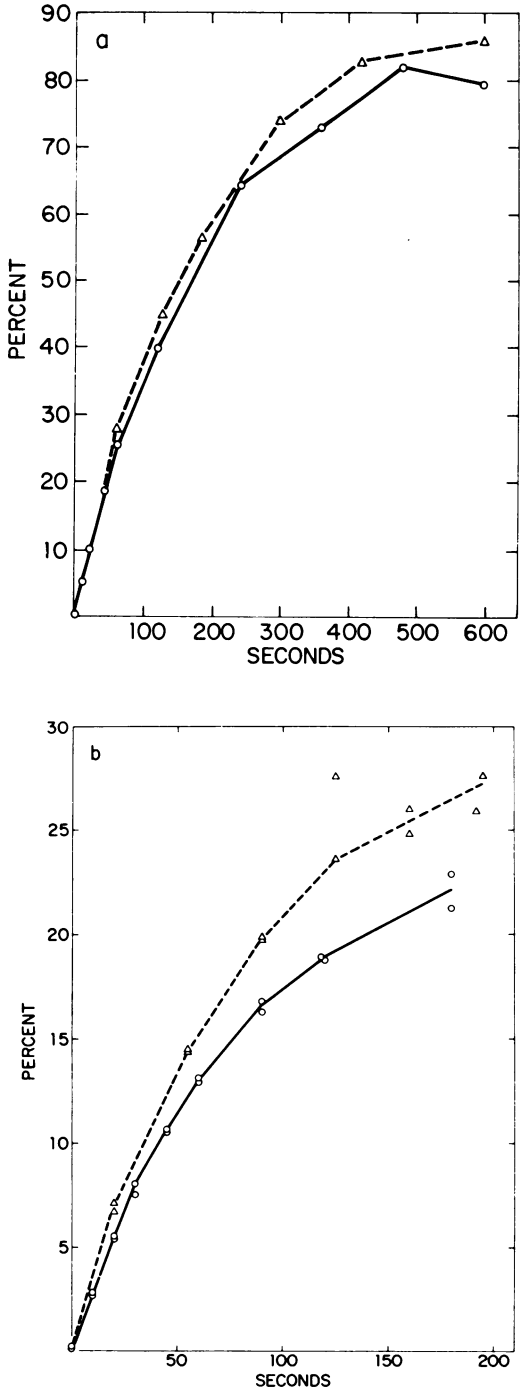

FIG. 2. *Attachment of labeled virus to HeLa cells measured by direct sedimentation (△) or filtration of cells after dilution and washing (○). (a) ^{14}C-labeled HRV2, 10^4 particles per cell, 10^7 cells per ml, 34.5°C. (b) ^{14}C-labeled P2, 6×10^3 particles per cell, 2×10^7 cells per ml, 37°C (duplicate attachments made on the same day). See Materials and Methods.*

with F^- plus CN^-, whereas treatment with iodoacetamide required a longer time to deplete ATP to a similar extent (Fig. 7). Fluoride alone produced only a 63% depletion in ATP during 10 min of incubation.

Tight binding of HRV2. Experiments have shown that attachment of HRV2 to HeLa cells requires Ca^{2+}, Mn^{2+}, or Zn^{2+}, but that Mg^{2+} cannot substitute (N. M. Whiteley, unpublished data). After attachment, HRV2 passes from a state in which it can be dissociated from the cell by EDTA, to a more tightly bound form that cannot be dissociated with EDTA; in the absence of EDTA, little if any infectious virus dissociates from the cells (29). The effect of metabolic inhibitors on the progression of virus to a form that cannot be dissociated was tested by adding the inhibitors for a short period so that they did not strongly block virus attachment. This is illustrated in Fig. 8: cells were incubated at 34.5°C for 8 min with inhibitors and labeled HRV2. The cells were then washed and incubated at 0°C with excess EDTA, and virus that had not progressed to the tightly bound form was dissociated and recovered in the supernatant fraction. Virus was also permitted to attach to cells at 0°C at which temperature the progression to the tightly bound form is slowed; approximately half of the virus attached at 0°C could be eluted by EDTA in 1 h (lower curve). Only about 7% of virus permitted to attach to cells at 34.5°C in the absence of inhibitors was dissociated during 1-h incubation with EDTA (top curve). Iodoacetamide and fluoride plus cyanide significantly reduced the amount of labeled virus that had progressed to the tightly bound stage of attachment.

Progression of poliovirus to a tightly bound form was measured by a different method (see below) and was strongly inhibited by iodoacetamide, but not by F^- plus CN^-.

Effect of metabolic inhibitors on cell-mediated eclipse. Virus was permitted to attach to poisoned or nonpoisoned cells at 0°C, and the infected cells were then incubated at 34.5°C (HRV2) or 37°C (P2) in the presence or absence of metabolic inhibitors, and cell-associated infectious virus (PFU) was recovered after lysis of the infected cells with SDS. Figures 9a and b illustrate the effects of iodoacetamide and fluoride on cell-mediated eclipse of HRV2 and P2, respectively. In these experiments, about 90% of the cell-associated infectivity was rapidly inactivated by unpoisoned cells during the first 15 min of incubation. (There may have been a lag of about 2 min before eclipse proceeded efficiently.) Poisoned cells were less efficient at inactivating virus, and after 10 to 20 min, the rate of eclipse by fluoride-treated cells de-

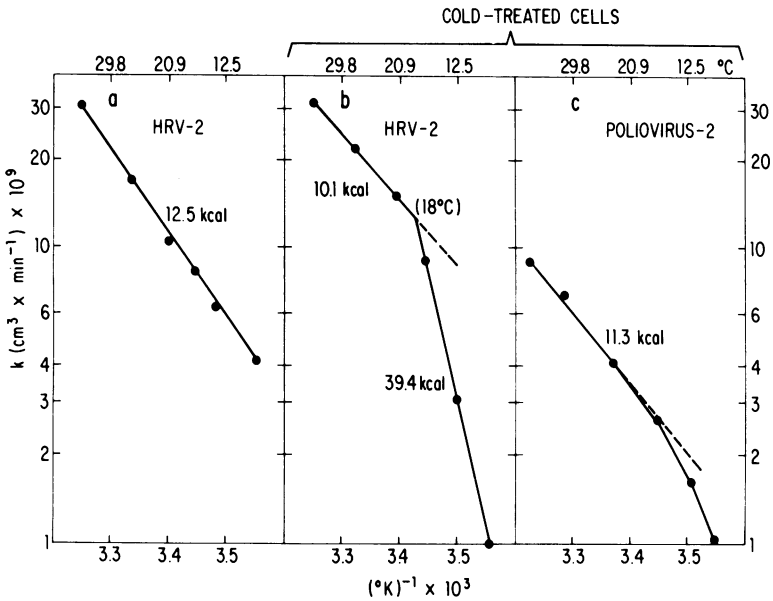

FIG. 3. *Effect of temperature on the initial rate of attachment of labeled virus to HeLa cells. (a) ^{14}C-labeled HRV2 and cells not exposed to treatment at 0°C. (b) ^{14}C-labeled HRV2 and cells treated for more than 3.5 h at 0°C. (c) ^{14}C-labeled P2 and cells treated more than 3.5 h at 0°C. In each experiment, cells were at 10^7 per ml, and virus was added at a multiplicity of 10^4 particles per cell. Cells were equilibrated to the indicated temperature for 3.0 min, and the initial rate of attachment was measured by the filtration method.*

creased further. Iodoacetamide appeared to be more effective at blocking eclipse than fluoride alone, and this was confirmed in other experiments (data not shown).

The in vitro eclipse of membrane-associated P2 was also investigated. Virus was attached to cells at 0°C, permitted to become tightly bound at 24°C; the cells were then broken and a crude membrane fraction was prepared and incubated at 37°C in the presence or absence of inhibitors (Materials and Methods). At 37°C, membrane-associated virus was inactivated about 10 times more rapidly than the spontaneous inactivation of virus in the absence of membranes (data not shown). This system was not, however, comparable with intact cells because iodoacetamide enhanced the rate of virus inactivation, either on membranes, or in the absence of membranes, by 50 to 100%, and because fluoride (or fluoride plus cyanide) had little effect (not shown).

Effect of inhibitors on cell-associated P2. Cells were poisoned with metabolic inhibitors and then infected with ^{14}C-labeled P2. Figure 10 illustrates the separation of cell-associated viral particles after 45 min of incubation at 37°C. The left panels show the sedimentation patterns of radioactive virus when the cell homogenate was treated with only a nonionic detergent. The pattern for control unpoisoned cells is similar to the pattern for fluoride-poisoned cells. Most cell-associated ^{14}C-labeled P2 was converted to material that sedimented about 15% more slowly than native virus, and a second more slowly sedimenting component was also detected. The pattern of ^{14}C-labeled virus from cells infected in the presence of iodoacetamide (lower left) showed that very little P2 was converted to the component(s) that sedimented more slowly than native virus. The recovery of radioactivity was also relatively low, and there was evidence for aggregated material that sedimented more rapidly than native virus.

The right-hand panels of Fig. 10 show the gradient sedimentation of extracts after addition of 0.2% SDS (Materials and Methods). This treatment destroys complexes between native virions and cellular membranes that sediment 15% more slowly than marker virus, but "A-particles" continue to sediment approximately 15% more slowly than native virions. The A-particles are thought to represent the major product of cell-mediated eclipse of P2 (20). Since little native virus was recovered after SDS treatment of the control, most of the cell-associated ^{14}C-labeled virus must have been in the form of A-particles. In fluoride-poisoned cells, a large amount of native virus was recovered, and in the iodoacetamide-poisoned cells most of the radioactivity recovered co-sedimented with native virus.

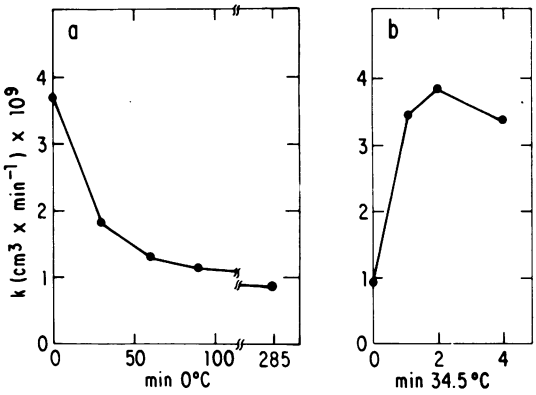

FIG. 4. *(a) Effect of incubation at 0°C on the subsequent ability of HeLa cells to attach HRV2 at 8°C. Cells (10^7/ml) were incubated 10 min at 34.5°C and then for various periods at 0°C. At the indicated times, portions were transferred to a vessel at 8°C, permitted to equilibrate for 3.0 min, and then ^{14}C-labeled HRV2 (10^4 particles/cell) was added. The initial rate of attachment was measured by the filtration method. (b) Effect of incubation at 34.5°C on the ability of cold-treated HeLa cells to attach HRV2 at 8°C. Cells (10^7/ml) were treated at 0°C for 4.5 h, or longer, and portions were then incubated for various periods at 34.5°C and transferred to a vessel at 8°C. After 3 min of equilibration, ^{14}C-labeled HRV2 (10^4 particles/cell) was added. The initial rates of attachment were measured by the filtration method.*

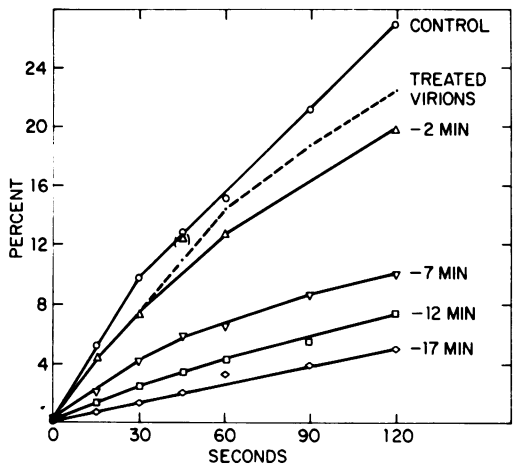

FIG. 5. *Effect of incubation of HeLa cells with 0.01 M iodoacetamide upon their subsequent ability to attach HRV2. Cells were incubated for a total of 15 min at 34.5°C before addition of ^{35}S-labeled HRV2 (10^4 particles/cell). At the indicated time before addition of virus, a solution of iodoacetamide (in medium) was added to bring the final concentration of cells to 10^7/ml. In one incubation (treated virions), virus was incubated 15 min with 0.02 M iodoacetamide and then mixed with an equal volume of 2×10^7 cells/ml. Percentage of cell-associated virus was measured by the filtration method.*

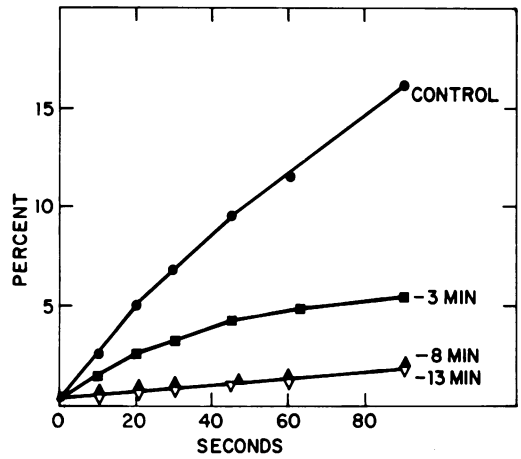

FIG. 6. *Effect of incubation of HeLa cells with fluoride plus cyanide upon their subsequent ability to attach HRV2. Cells incubated for a total period of 13 min at 34.5°C before addition of ^{35}S-labeled HRV2 (10^4 particles/cell). At indicated times before addition of virus, a solution of inhibitors was added to bring the final concentration to 0.02 M NaF and 0.002 M KCN and the final cell concentration to 10^7/ml. Percentage of cell-associated virus was measured by the filtration method.*

From the results of Fig. 10, we suggest the following interpretation. Iodoacetamide, and to a lesser extent fluoride, inhibited cell-mediated alteration of poliovirus. (In this experiment, inhibition appeared to be more complete than in the experiments of Fig. 9b.) Iodoacetamide also slowed the tight binding of cell-associated poliovirus to the complex which sediments 15% more slowly than marker virus but which is disrupted by 0.2% SDS (Fig. 10, lower left panel). Fluoride did not significantly slow the formation of tightly bound poliovirus-membrane complex. It was found in separate experiments that fluoride plus 0.002 M cyanide also did not greatly slow formation of the poliovirus-membrane complex, even when samples were compared after only 15 min of incubation (data not shown).

Iodoacetamide poisoning also tended to enhance the amount of P2 that eluted from cells after initial attachment. In the experiment of Fig. 10, ^{14}C-labeled P2 was permitted to attach for 15 min, excess virus was removed by washing, and the cells were incubated 30 min longer (Fig. 10, legend). Samples from each incubation were counted to determine the cell-associated radioactive virus at each step in the experiment. From these data, it was calculated that about 35% of the added virus attached to the cells during the initial period of 15 min, regardless of the presence of inhibitors. However, dur-

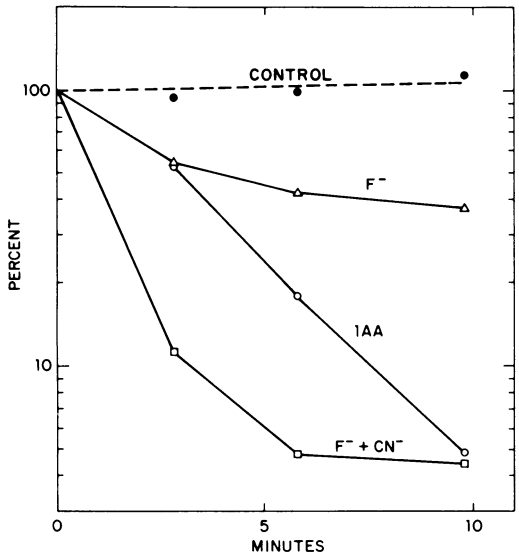

FIG. 7. *Cellular levels of ATP during incubation with metabolic poisons. HeLa cells were incubated at 34.5°C for 10 min and then inhibitors were added: iodoacetamide (IAA), 0.01 M; sodium fluoride (F^-), 0.02 M; fluoride plus potassium cyanide ($F^- + CN^-$), 0.02 M and 0.002 M. Portions of the cells (10^6) were removed 2.75, 5.75, and 9.75 min after addition of inhibitors. The results are represented as percentages of ATP detected in control samples removed 0.25 min before introduction of the inhibitors (approximately 1 pg of ATP/cell). The final concentration of cells in the incubations was 10^7/ml, and ATP was measured as described in Materials and Methods.*

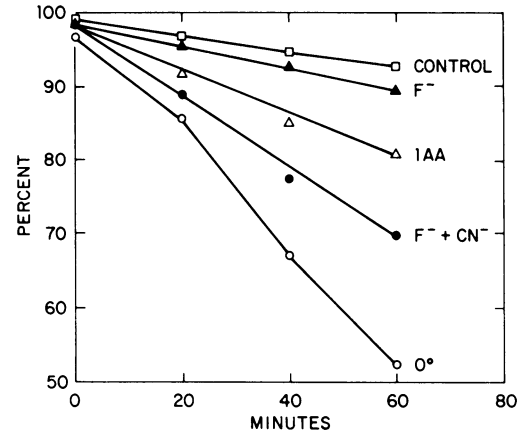

FIG. 8. *Effect of inhibitors on the tight binding of HRV2. Cells were permitted to attach ^{35}S-labeled HRV2 under various conditions (below) and were washed and resuspended at 0°C in medium containing 10 mM EDTA. Samples were removed after periods of incubation, and the percentage of the attached virus that was not dissociated by EDTA was determined (Materials and Methods). Percentage of cell-associated virus during dissociation with EDTA is given versus time of elution. In the absence of inhibitors (control), 76% of the virus attached to the cells during 8 min at 34.5°C. In the presence of 0.02 M fluoride (F^-), 0.01 M iodoacetamide (IAA), 0.02 M fluoride plus 0.002 M cyanide ($F^- + CN^-$) the respective attachment was 69, 69, and 40%, and these values were normalized to 100% for representation. Virus was also permitted to attach for 75 min at 0°C (lower curve), and 17% became cell associated.*

ing the subsequent 30-min incubation, of the virus which had attached, 21% eluted in the control, 26% eluted in the presence of fluoride, and 50% eluted in the presence of iodoacetamide (data not shown). This enhanced elution from iodoacetamide-poisoned cells was confirmed in other experiments in which it was also found that the eluted labeled particles were noninfectious and sedimented in sucrose gradients as A-particles. Treatment with 0.2% SDS did not release native virus (data not shown).

Figure 11 illustrates the sedimentation behavior of poliovirus A-particles eluted from HeLa cells during normal infection. This sample was treated with 0.2% SDS to destroy any contaminating tight complexes between native virions and membrane components, and it can be seen that native virions were largely absent. The pattern obtained without SDS treatment was identical (not shown).

All of these results may be summarized as follows. Iodoacetamide slows, but does not block, eclipse of cell-associated poliovirus (Fig. 9b) and also slows tight binding of virus and the formation of a complex with membrane components (Fig. 10, lower left panel). Most of the virus that is eclipsed in the presence of iodoacetamide is eluted as free A-particles.

Effect of temperature on the cell-mediated eclipse of virus. Separate experiments on HRV2 and P2 are compared in the form of Arrhenius plots in Fig. 12. These measured the rate of eclipse in the temperature range 25 to 36°C by the method used in Fig. 9a and b. Cell-associated HRV2 was altered more rapidly than poliovirus under these conditions, and with a lower apparent energy of activation: 32 kcal, as shown in Fig. 12 (and 25 kcal in a second experiment that is not illustrated).

Poliovirus was eclipsed by cells at a very low rate at 25°C, and hence there was considerable uncertainty about the slope of the Arrhenius plot. The activation energy for cell-mediated eclipse of P2 was calculated to be 74 kcal from the data of Fig. 12, and 50 kcal in a second experiment (not shown).

DISCUSSION

HRV2 and P2 showed both common and different features of their early steps in infection. The results will be discussed using a hypotheti-

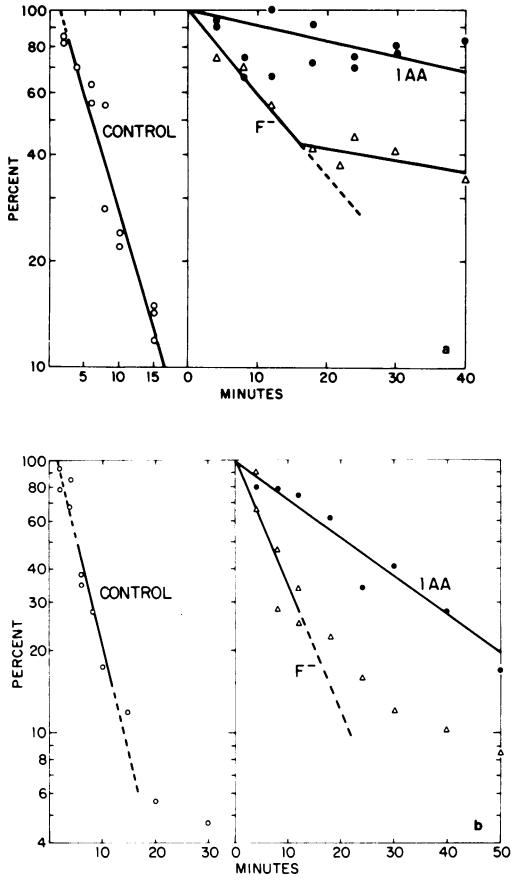

FIG. 9. *Effect of metabolic inhibitors on cell-mediated eclipse. Purified HRV2 (a) or P2 (b) was permitted to attach to treated cells (see below) for 1 h at 0°C (5 × 10⁷ cells/ml; 10⁴ particles/cell). Unattached virus was removed by washing, and the infected cells were resuspended to 10⁷/ml in the appropriate medium and incubated at 34.5°C (HRV2) or 37°C (P2). Portions were removed, lysed with SDS, diluted, and assayed for infectivity (Materials and Methods). The cells employed for attachment of virus were first incubated 3 min at 34.5°C (HRV2) or 10 min at 37°C (P2) in the presence of 0.02 M fluoride (F⁻), 0.01 M iodoacetamide (IAA), or without inhibitor (Control). After unattached virus was removed by washing, the cells were resuspended in medium with inhibitors and cell-associated PFU in unincubated samples was normalized to 100%. The rates of cell-mediated eclipse (estimated from the linear portion of the curves during the initial 15 min of incubation) were inhibited 75% (HRV2) and 64% (P2) by F⁻, or 94% (HRV2) and 80% (P2) by IAA. Similar results were found in other experiments, which are not shown.*

cal model shown in Fig. 13. Some features shown only by HRV2 are illustrated in the lower part of the figure.

Kinetics of attachment. HRV2 and P2 attached rapidly to HeLa cells under our conditions. Attachment did not follow pseudo first-order kinetics. When 10^4 virus particles were used per cell, first-order kinetics were not expected, since each cell contains only about 10^4 receptor sites (21). Even with low multiplicities of virus, attachment data revealed significant deviation from first-order kinetics (unpublished). This may be attributed to one of three causes. (i) Receptor sites for the same virus may differ from each other in affinity, and "better" sites may be occupied first. (ii) Viruses may be altered and eluted during incubation. (iii) Although homogeneous by criteria of gradient centrifugation, radioactive viruses may exist in different conformational states (25) with different biological properties.

The initial rate of virus attachment was directly dependent upon the concentration of cells, as shown for HRV2 (Table 1). Therefore, the initial rate constant for attachment could be expressed in cubic centimeters per minute, i.e., the fraction of virus attached per cell per minute. Under physiological conditions, HRV2 attached to HeLa cells with a K value of about 2×10^{-8} cm³/min. This is significantly less than a theoretical maximum for picornaviruses of 1.7×10^{-7}, which can be calculated from the diffusion of virus particles to the cell surface (42), as discussed elsewhere (K. Lonberg-Holm and L. Philipson, *in* H. Bough and J. M. Tiffany, ed., *Cell Membranes and Viral Envelopes*, in press). The significance of this discrepancy is that there must be a very large number of unsuccessful collisions between virus and cell for every collision leading to a permanent bond (14, 32).

Most cell-associated HRV2 or P2 is not dissociated by dilution and washing of infected cells, and hence there is little or no evidence that a significant fraction of these viruses attaches first to host cells by a reversible bond that then progresses slowly to an irreversible bond during prolonged incubation (Fig. 2a and b). In this we distinguish between irreversibly bound virus, which does not dissociate upon dilution, and tightly bound virus, which is resistant to dissociation by treatment with nonphysiological agents such as EDTA or nonionic detergents. (Note that in the past the term irreversible attachment was used sometimes to refer to virus eclipse or irreversible alteration, rather than to loss of ability to undergo spontaneous dissociation [33]). In Fig. 13, attachment of virus (step 1) is shown as irreversible. It is also possible that a virus attaches in a reversible manner but that most virus is then very rapidly converted to an irreversibly bound form. For example, reaction with one receptor subunit

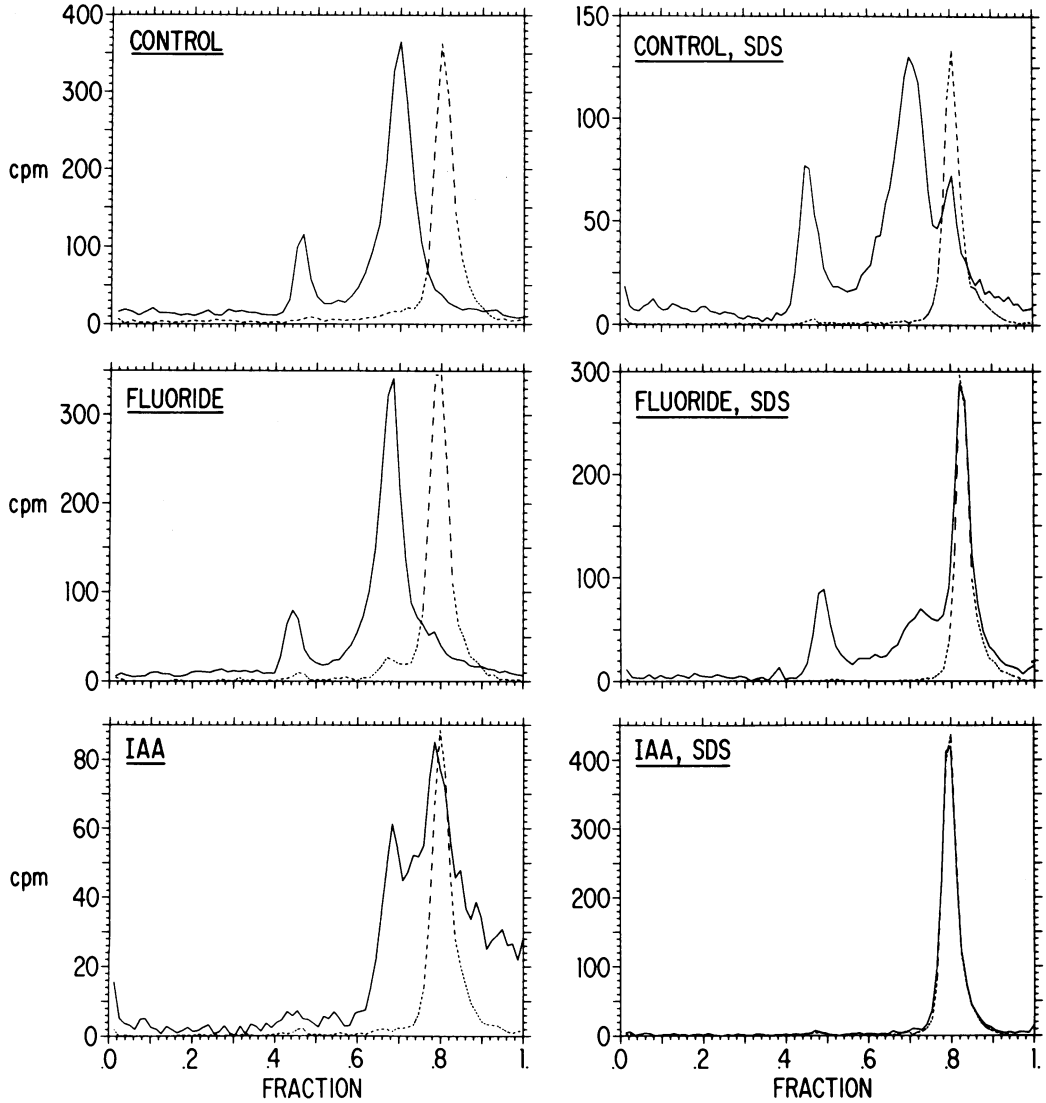

FIG. 10. *Sedimentation of cell-associated products of infection of HeLa cells wtih ^{14}C-labeled P2 for 45 min at 37°C. Cells (10^7/ml) were incubated 10 min at 37° in medium alone (control), or medium containing 0.02 M fluoride or 0.01 M iodoacetamide (IAA). ^{14}C-labeled P2 was then added and permitted to attach for 15 min. The cells were washed with medium, resuspended (10^7/ml) with inhibitors, and incubated for 30 min longer. The infected cells were then washed, disrupted, mixed with marker ^{3}H-labeled P2. NP-40 alone (left panels) or NP-40 plus SDS (right panels) were added as described in Materials and Methods. Sedimentation is from left to right. Continuous lines, ^{14}C-labeled, and dashed lines, marker ^{3}H-labeled virus. In the control gradients, the recoveries of applied ^{14}C were 93 and 73% with or without SDS. Extracts of fluoride-treated or IAA-treated cells gave recoveries of 92 and 72% without SDS, and 70% and 88% with SDS.*

might give a weak bond with a binding constant that favors dissociation. In contrast to P2 and HRV2, cardioviruses (discussed in Lonberg-Holm and Philipson, in press) and some bacteriophages (9) attach rapidly to host cells in a form that dissociates upon dilution.

Attachment at different temperatures. The initial rate of attachment of HRV2 and P2 doubles for a 10°C increase in the range 20 to 39°C. This is somewhat less than the factor of 2.7 measured earlier by a less precise method (21), but is still much greater than a factor of 1.3 expected from the effects of temperature on the diffusion coefficients of virus and cell (42). An older study of the attachment of poliovirus to fragments of brain tissue (16), and more recent

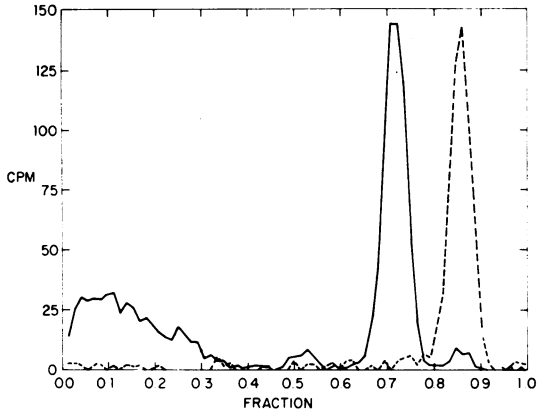

FIG. 11. *Sedimentation of eluted P2 particles. Cells were infected 10 min at 37°C with ^{14}C-labeled P2, washed, and incubated for an additional 30 min. The supernatant fraction was mixed with ^{3}H-labeled marker virus (dashed curve), subjected to ultrasonic vibration, treated with 0.5% NP-40, and 0.2% SDS, and sedimented into a sucrose gradient (Materials and Methods).*

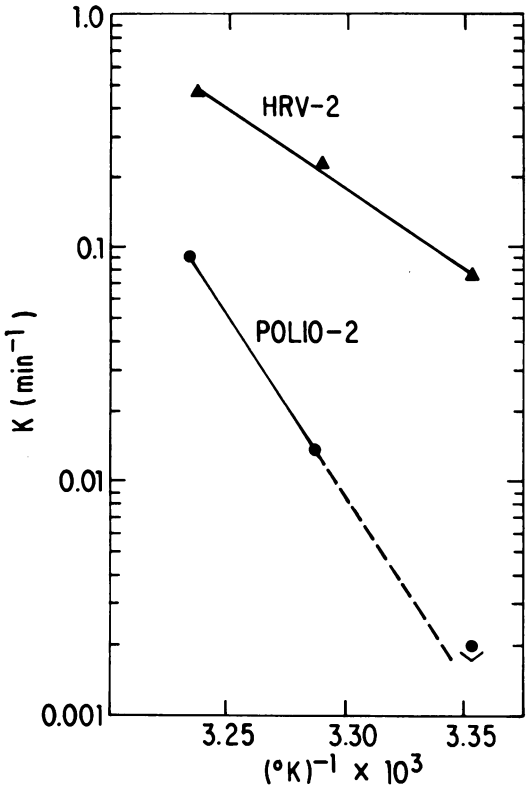

FIG. 12. *Effect of temperature on the rate of cell-mediated eclipse of HRV2 and P2. The rates were determined by the method employed in Fig. 9.*

studies on attachment of coxsackievirus B3 (R. L. Crowell, 9th Miles Int. Symp., Baltimore, Md., 1975, in press) and adenovirus 2 (34), have also shown a strong dependence on temperature. In contrast, a low temperature coefficient has been reported for attachment of some viruses in other systems (reviewed in K. Lonberg-Holm and L. Philipson, in press).

The activation energy for attachment of HRV2 and P2 is approximately 13 kcal per mol of virus under physiological conditions (Results). One explanation for the high activation energy for attachment might reside in the effect of temperature on cell lipids, assuming that lipids provide the bilayer continuity of the cell membrane (41). If more than one receptor subunit is required for the irreversible attachment of virus, the correct positioning of the subunits may require lateral or rotational diffusion in the membrane. Diffusion of membrane components has been shown to be strongly dependent upon temperature (8). In Fig. 13, attachment of

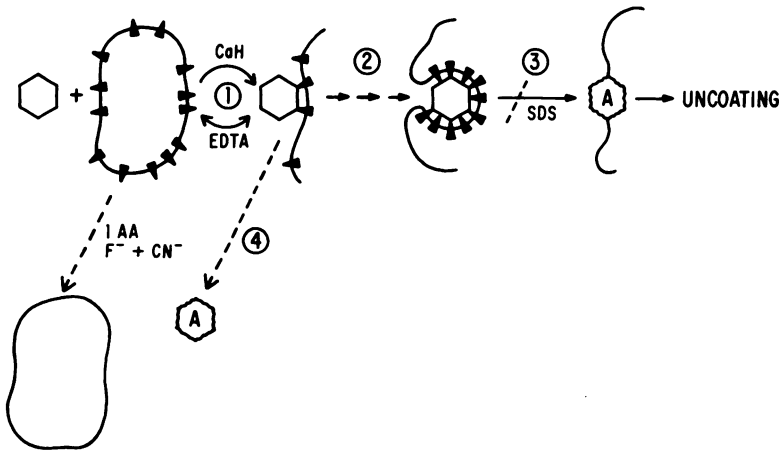

FIG. 13. *Hypothetical model for early steps of infection (see text).*

virus (step 1) is illustrated as employing two receptor subunits. Alternatively, it is also possible that the activation energy for attachment results from conformational alterations in proteins required for attachment. However, it is unlikely that covalent bonds are formed between the virus and the cell.

An unusual effect of temperature was detected in attachment of HRV2 to cells treated at 0°C for several hours (Fig. 3b). Attachment at temperatures below 15°C could be enhanced if cells were warmed briefly and then cooled again before addition of virus (Fig. 4). This phenomenon suggests the "melting" of an inactive structure at temperatures above the break in the Arrhenius curve. It is possible that HRV2 receptors must be located in a fluid phase (40) of the membrane lipids, and that prolonged cold treatment transforms the lipids that are associated with the HRV2 receptors into a solid phase. Phase separation of membrane lipids may influence the availability of some membrane receptors (37, 38) and has clearly been shown to affect a step after loose or reversible attachment of an enveloped virus to host cells (17).

There may be alternative explanations for the observed break in the Arrhenius plot for attachment of HRV2 to cold-treated cells. Prolonged cooling could also cause alterations in membrane structures not directly related to the lipids. The ultrastructural topology of the surface of lymphocytes has been shown to be altered by cold treatment (18); it would be interesting to determine whether or not changes in HRV2 receptors can also be correlated with alterations in the surface of HeLa cells.

Effects of metabolic poisons on attachment of HRV2. Attachment of HRV2, but not P2, was sensitive to inhibitors able to reduce the level of cellular ATP by at least 90% (Fig. 5–7). It is a priori unlikely that ATP participates directly in attachment. Energy deprivation may lead to a loss of an active configuration of HRV2 receptors at the cell surface (Fig. 13, step 6). Such a model has a precedent in the effect of metabolic poisons on attachment of certain bacterial viruses. Poisoned bacterial cells lose or retract F pili and simultaneously lose ability to attach phages whose receptors reside on the pili (26, 30, 31). It would also be interesting to look for ultrastructural correlates for the loss of HRV2 receptors on poisoned HeLa cells.

Effects of inhibitors on the tight binding of cell-associated HRV2 and P2. Calcium or certain other divalent cations are required for attachment of HRV2, but not P2 (21). When EDTA is added to infected cells, some HRV2 may be dissociated (29) (Fig. 13). The dissociable virus particles progress during several minutes of incubation to a tightly bound nondissociable state eluted. In Fig. 13 this tightly bound virus is depicted as multivalently bound, but it is also possible that it has undergone either engulfment or penetration. Progression of HRV2 to a nondissociable form was slowed but not blocked by treatment of cells with concanavalin A (18) and has now also been shown to be retarded by fluoride plus cyanide, or by iodoacetamide (Fig. 8). Cells were treated with these inhibitors only briefly during attachment of virus to minimize their ability to block attachment, and hence cells were incompletely poisoned (Fig. 7). Therefore, the observed reduction in tight binding should be considered only as an indication of the sensitivity of this step to metabolic inhibitors.

Evidence for tight binding of P2 was obtained by a different method. A tightly bound complex of native virions and membrane components is formed after attachment, during several minutes of incubation. The complex may be isolated from disrupted infected cells in sucrose gradients; it sediments at about 85% the rate of native virions and can be distinguished from A particles, with which it co-sediments, by releasing native virions after it is treated with 0.2% SDS (20). The formation of this complex is retarded in the cold, or by treating the cells with concanavalin A (19). Iodoacetamide, but not fluoride, inhibits the formation of this complex. Fluoride plus cyanide also had no effect on this tight binding, as described in Results.

Effect of temperature on the eclipse of cell-associated virus. The activation energy for cell-mediated eclipse of HRV2 is less than that for P2 (Fig. 12). In two experiments, the average value for HRV2 was 29 kcal; for P2, it was 62 kcal. The value of 62 kcal is uncertain because of the relatively slow rate of eclipse of P2 at temperatures below about 30°C. It is close to the value of 70 kcal calculated for the cell-mediated process leading to the alteration of photosensitive P1 to a photoresistant form during early infection (24). Mandel has already shown that "penetration" of P1 (to an antibody-resistant state) has an activation energy of approximately 14 kcal (24), and we suggest that this step may be equivalent to "tight binding" by the criterion we have employed. If this is so, tight binding would not be the rate-limiting step in the pathway leading to eclipse of P2 at temperatures below 37°C. We do not have good evidence to decide whether or not tight binding of HRV2 (to a form resistant to dissociation by EDTA) is the rate-limiting step in eclipse of HRV2 at temperatures below 34.5°C, but we do not believe it to be so. Tight binding does pro-

ceed rapidly at 25°C (29). The difference in activation energy for cell-mediated eclipse of HRV2 and P2 suggests that different cellular processes are used but, alternatively, the difference may reflect the different activation energies for direct thermal alteration of HRV2 and polioviruses (6).

Effect of inhibitors on cell-mediated eclipse. Specific antiviral compounds such as the thiopyrimidine S-7 (20) and SDS (22) block cell-mediated eclipse by interacting with and stabilizing picornaviruses in their native unaltered conformations. Experiments that are not illustrated here have shown that fluoride or iodoacetamide probably does not inhibit eclipse by this mechanism. Uninfected cells were incubated with the inhibitors, and the medium was saved and used as a diluent for infectious P2 virus which was then heat-inactivated at 47°C. The poisoned media did not slow the rate of heat inactivation, implying that the inhibitors, or other substances released from poisoned cells, did not stabilize the virions (K. Lonberg-Holm, unpublished data).

Our results showing inhibition of cell-mediated eclipse (Fig. 9a and b) are in apparent contradiction to an earlier report that eclipse of P1 was insensitive to metabolic poisons (10). It is possible that the method employed in the earlier study to recover uneclipsed cell-associated virus (treatment with 6 M LiCl for 3 min) was unable to release "tightly bound" virus. We found that P2 progressed to the tightly bound state in the presence of fluoride (Fig. 10) or fluoride plus cyanide, as already discussed. These results also imply that the effect of metabolic inhibitors on eclipse cannot be attributed entirely to an ability to block tight binding, by assuming that tight binding is a prerequisite for cell-mediated eclipse.

The basis for inhibition of eclipse by metabolic poisons also brings into question a view that eclipse is a direct consequence of a sequence of interactions between virus and receptor subunits. Eclipse has been described as "irreversible attachment" (reviewed by Philipson [33]). It has also been found that eclipse of picornaviruses can be catalyzed in vitro by membrane fragments (2, 10, 11, 39), and this makes it unlikely that ATP participates directly (although metabolic processes may be active in such in vitro systems). It is possible that active cellular metabolism enhances the rate of eclipse above that which can occur in a poisoned system, by maintaining structural features or ionic environments that contribute to the labilization of native virions. Pertinent to this, we have found that eclipse of poliovirus on broken cell membranes proceeds at a rate slower than on intact healthy cells and at approximately the same rate as observed with poisoned cells (K. Lonberg-Holm and N. M. Whiteley, unpublished data). We conclude that the cell is not passive in the process leading to eclipse of HRV2 and P2.

Cell-associated and eluted virus particles during early infection. Progression of cell-associated poliovirus to the tightly bound complex can occur in the absence of eclipse (19, 20). It is likely that this occurs before eclipse during successful infections (Fig. 13, step 2). A certain amount of P2 is also eluted or "sloughed" from infected cells in an irreversibly altered form (7, 13). We have found that these particles sediment as A-particles with or without treatment with 0.2% SDS (Fig. 11). These particles also lack the virion polypeptide VP4 (K. Lonberg-Holm and N. M. Whiteley, unpublished data), in agreement with published reports of eluted particles of coxsackievirus B3 (4). This process of elution of P2 was enhanced in cells treated with iodoacetamide, as described in Results. This is interpreted in the model of Fig. 13 (step 4): eclipse of virus that has not progressed to the stage of tight binding results in elution. In general, eclipse may either precede or follow tight binding, and the ratio of eluted to cell-associated virus may be determined by the relative rates of step 4 and step 2.

A-particles are the major products of cell-mediated eclipse of HRV2 (21, 22) and P2 (20). Fluoride and iodoacetamide slowed the formation of A-particles from cell-associated P2 (Fig. 10). Fig. 13 shows formation of A-particles from tightly bound virus leading directly to intercalation in the cell membrane (step 3). A-particles of both P2 and HRV2 have lipophilic properties and may be able to interact with membrane lipids directly (20a), and subsequent uncoating of the viral RNA might proceed while the A-particles remain associated with the membrane. An alternative model would require a mechanism for the dissolution of the complex between tightly bound virions, or A-particles, and the cell membrane and the release of viral particles into the cytoplasm.

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