Mode of Host Cell Penetration by Bacteriophage ϕ X174

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Received for publication 1 March 1971

Bacteriophage ϕ X174 is an icosahedral phage which attaches to host cells without the aid of a complex tail assembly. When ϕ X174 was mixed with cell walls isolated from the bacterial host, the virions attached to the wall fragments and the phage deoxyribonucleic acid (DNA) was released. Attachment was prevented if the cell walls were treated with chloroform. Release of phage DNA, but not viral attachment, was prevented if the cell walls were incubated with lysozyme or if the virions were inactivated with formaldehyde. Treatment of the cell walls with lysozyme released structures which were of uniform size (6.5 by 25 nm). These structures attached ϕ X174 at the tip of one of its 12 vertices, but the viral DNA was not released. The virions attached to these structures were oriented with their fivefold axis of symmetry normal to the long axis of the structure. No virions were attached to these structures by more than one vertex. Freeze-etch preparations of ϕ X174 adsorbed to intact bacteria showed that the virions were submerged to one half their diameter into the host cell wall, and the fivefold axis of symmetry was normal to the cell surface. A second cell could not be attached to the outwardly facing vertex of the adsorbed phage and thus the phage could not cross-link two cells. When the virions were labeled with ^3H -leucine, purified, and adsorbed to *Escherichia coli* cells, about 15% of the radioactivity was recovered as low-molecular-weight material from spheroplasts formed by lysozyme-ethylenediaminetetraacetic acid. Other experiments revealed that about 7% of the total parental virus protein label could be recovered in newly formed progeny virus.

The bacteriophages may be grouped morphologically into three major classes: (i) those possessing a complex tail assembly which serves as the organelle of attachment (e.g., T phages and lambda), (ii) those which are filamentous in shape (e.g., f1 and fd), and (iii) those without apparent tail structures (e.g., MS2 and ϕ X174; reference 16). Members of all three morphological groups have their genetic information stored in the form of nucleic acid buried within a proteinaceous capsid. To be expressed, this genetic information must be released from the protein capsid and penetrate into the host cell. The process by which the complex "tailed" phages release their deoxyribonucleic acid (DNA) into the host cell is fairly well understood. The tail structures of these virions provide a passageway through which the phage genome leaves the head and is "released" into the host cell. The filamentous phages and certain of the tailless phages find their receptor sites on specific bacterial pili and flagella. These pili behave as tail structures for the tailless phages which apparently release their nucleic acid directly into the pilus or flagellum from where it is transferred into the host cytoplasm (16).

Trenkner, Bonhoeffer, and Gierer (23) have shown that the filamentous bacteriophage fd, after attaching to the bacterial pilus, is drawn in toto into the confines of the host cell. The exact mechanism by which the viral genome is released from the helical capsid is not known, but it may be that the virion capsid is depolymerized as it is drawn with the pilus structure into the host cell. Trenkner and co-workers further demonstrated that the protein of the infecting fd bacteriophage is reutilized to form the structural proteins of the progeny particles.

The icosahedral bacteriophage ϕ X174 does not require a pilus or flagellum for attachment but rather attaches directly to the surface of the host cell (22). Electron microscopy of ϕ X174-infected cells reveals ghosted particles on the outside surface of the cell envelope, indicating that the phage DNA penetrates the host cell envelope by some kind of uptake mechanism. ϕ X174 is apparently composed of four structural proteins (4). One of these proteins is the major capsid component, whereas the other three are believed to comprise 12 structurally equivalent spikes which are located at each of the vertices of the icosahedron.

dral virion. Since the viral capsid is not completely broken down during the process of infection (20), some mechanism must exist by which a passageway is opened in the virion upon contact with the cell surface allowing for the release of the DNA. Two mechanisms can be suggested. On the one hand, an opening in the capsid could occur by virtue of a configurational change in one or more of the structural proteins that interact with the cell. Alternatively, an opening in the capsid structure could result from the removal of one or more of its protein components.

Fujimura and Kaesberg (10) have shown that the interaction of ϕ X174 particles with isolated cell wall fragments results in (i) a loss of phage titer and (ii) the susceptibility of the phage DNA to deoxyribonuclease. This result confirms the observation that the phage-cell fragment interaction leads to empty capsids but does not describe the mechanism for the DNA release. Newbold and Sinsheimer (19, 20) further characterized this process dividing the early events of the infectious cycle into three steps, attachment, eclipse (herein referred to as DNA release), and DNA penetration. The initial attachment stage involved a reversible binding of the virion to a host cell receptor. This interaction is without apparent permanent alteration of the virion. During the eclipse stage, the virion binds more tightly and undergoes an irreversible change in configuration which exposes the DNA before its penetration of the host cell. These authors suggested that nearly 100% of the parental protein remained at or near the cell surface, as it could be almost quantitatively eluted from the infected complex by treatment with a solution of sodium tetraborate and ethylenediaminetetraacetic acid (EDTA).

In this paper, we report further results related to the mechanism of infection by ϕ X174 and propose a sequence of events leading to the uptake of the ϕ X174 genome through the host envelope.

MATERIALS AND METHODS

Bacteria and bacteriophage. *Escherichia coli* C is the usual host for ϕ X174. *E. coli* HF 4714 is a permissive host for ϕ X174 amber mutants. Both strains were obtained from Robert Sinsheimer. *E. coli* K₁₂W₆ (from David Denhardt) can support ϕ X174 growth when it is spheroplasted and infected with isolated ϕ X174 DNA. This strain cannot, however, be infected by intact virus.

ϕ X174 *am3*, a lysis-defective amber mutant, was used throughout these studies and was a gift of Robert Sinsheimer.

General methods. Procedures for handling bacteriophages were generally those described by Adams (1). High-titer stocks of ϕ X174 *am3* were prepared in the 3XD medium of Fraser and Jerrell (9). The proteins of ϕ X174 *am3* were radioactively labeled in TCG medium (14), in which vitamin-free Casamino

Acids were replaced with ³H-labeled L-leucine (New England Nuclear no. net 135) at a concentration of 0.5 mCi/ml and other essential amino acids at concentrations of 5 μ g/ml each. Cells were infected with a multiplicity of 5 to 10 plaque-forming units (PFU) per cell in cold TCG. After a 5-min adsorption period, the infected cells were spun ($4,000 \times g$, 5 min) and resuspended into the radioactive medium. After 2 hr of incubation with aeration of 37 C, the cells were concentrated by centrifugation and lysed by the lysozyme-EDTA procedure of Hutchinson and Sinsheimer (12). The radioactive phage were then banded twice on cesium chloride and sedimented once on 5 to 20% sucrose gradients. The 114S fraction was collected, dialyzed against saturated sodium tetraborate, and stored at 4 C.

Density-labeled ϕ X174 phages were prepared as described, with the exception that the infected cells were resuspended in the radioactive (leucine) TCG medium supplemented with 5 μ g of 5'-fluorodeoxyuridine per ml (a gift of Hoffman LaRoche, Inc.), 50 μ g of uracil per ml, and 10 μ g of 5'-bromodeoxyuridine per ml (Calbiochem). When these heavy labeled virions were purified (as described above) and banded together with light ¹⁴C-reference phage, they were found to separate clearly from the light virions ($\rho = 1.43$) and banded at a density of 1.589 m/m. ³H-thymidine-labeled ϕ X174 phage were prepared in the TCG medium of Kozinski and Lin (14) supplemented with 50 μ Ci of ³H-thymidine per ml (New England Nuclear Corp.) and purified as described above. ³²P-labeled *E. coli* DNA was provided by Joseph Schwartzman.

Preparation of *E. coli* cell walls. Cell walls were prepared from cells growing logarithmically in 3XD medium. Cells were cooled to 0 C in an ice bath and concentrated in 1/100 their original volume by centrifugation at 3 C. The concentrated suspension was passed twice through a French pressure cell (American Instrument Co., Inc., Silver Spring, Md.) at 30,000 psi, being careful to keep the temperature of both the cell and the treated material below 4 C. The resulting paste was centrifuged at $5,000 \times g$ for 10 min at 3 C to remove unbroken cells and large pieces of debris. The supernatant of this spin was centrifuged twice at 23,000 rev/min ($80,000 \times g$) for 45 min at 3 C in the Spinco SW 25.1 swinging bucket rotor. The pellet was resuspended and quickly frozen at -78 C. The preparation was stored at -60 C until used. The concentration of cell walls in the final preparation represents the yield from 5×10^{11} to 10^{12} cells/ml. Electron microscopy of the preparation revealed cell walls and fragments of various sizes but showed no intact cells.

Electron microscopy. Negative staining was carried out with the procedure described by Anderson (2). Freeze-etching was done on Balzers BA 360 M unit by using essentially the procedures outlined in the operators manual. Specimens were examined in a Siemens Elmiskop 1A electron microscope.

RESULTS

Interaction of ϕ X174 with isolated cell walls. The ability of various preparations of isolated cell

TABLE 1. Interaction of ϕ X174 with untreated and variously treated preparations of isolated *E. coli* cell walls

Expt, ϕ X174 plus	Percentage of total ϕ X174 titer lost in reaction mixture	Percentage of total ϕ X174 DNA label made acid-soluble by deoxyribonuclease	Percentage of total <i>E. coli</i> B DNA made acid-soluble by deoxyribonuclease
F medium (control).....	0	0	80
<i>E. coli</i> C cell walls.....	80	65	78
<i>E. coli</i> C cell walls pretreated by incubation at room temperature for 2 hr.....	60	30	81
<i>E. coli</i> K ₁₂ W ₆ cell walls..	0	0	88
<i>E. coli</i> C cell walls pretreated by shaking with chloroform...	<10	0	80
<i>E. coli</i> C cell walls pretreated with 2×10^{-4} g of lysozyme per ml for 15 min at 37 C.....	72	10	80
Formaldehyde-treated ϕ X174 ^a mixed with <i>E. coli</i> C cells.....	75		
Formaldehyde-treated ϕ X174 mixed with <i>E. coli</i> C cell walls.....		<10	78

^a Percentage of titer lost was determined by measuring the amount of ¹⁴C or ³H label bound to whole bacteria as described in the text.

walls to inactivate ϕ X174 and to make the viral DNA sensitive to deoxyribonuclease is shown in Table 1. Purified ³H-thymidine-labeled (total of 9×10^4 counts/min) or unlabeled ϕ X174 phage (total of 10^9 PFU) were mixed with a variety of pretreated cell wall preparations (1 ml of suspension prepared as described above) and incubated for 10 min at 37 C. The ability of a preparation to make the viral DNA deoxyribonuclease-sensitive was determined by incubating the mixture in the presence of 10^{-4} g of deoxyribonuclease per ml (Worthington Biochemical Corp.). The amount of DNA degraded by the enzyme was determined by measuring the percentage of the total input counts which became soluble in cold 0.3 M trichloroacetic acid in the presence of 2% bovine serum albumin as carrier. The deoxyribonuclease activity of the reaction mixture was tested by the addition of ³²P-labeled *E. coli* DNA. The data (Table 1) show that the bacteriophage preparation loses neither titer nor does its nucleic acid become acid-soluble in the absence of *E. coli* cell walls. However, 80% of the *E. coli* DNA was

degraded under the same conditions. The addition of *E. coli* C cell walls to this mixture (Table 1) resulted in an 80% inactivation of the phage population accompanied by a 65% enzymatic degradation of the viral DNA. When this preparation of C cell walls was allowed to stand at room temperature for 2 hr, its ability to inactivate the phage and to expose the DNA to nuclease activity was reduced (Table 1), suggesting that the ability of the *E. coli* cell wall to bind ϕ X174 and cause it to release its DNA is labile, possibly due to the presence of autolytic enzymes in the isolated walls.

Similar experiments were carried out with cell walls prepared from *E. coli* K₁₂W₆ (Table 1), which does not possess receptors for ϕ X174. The results are comparable to those obtained with the control; i.e., infectivity was not lost nor was viral DNA made sensitive to enzymatic degradation. This result is not surprising since *E. coli* K₁₂W₆ does not bind ϕ X174 when the intact bacterium is used. Therefore, this experiment serves as a control for other experiments described in this table and demonstrates that the sensitivity or insensitivity of the bacterial cell wall with respect to the virion is maintained in the isolated preparation.

When the isolated *E. coli* C cell walls were pretreated by shaking for 5 min at room temperature with a droplet of chloroform (Table 1), nearly all adsorbing capability was lost, as was the ability to sensitize the viral DNA to nuclease. Intact *E. coli* C cells also lost their ability to adsorb ϕ X174 when treated with chloroform. This result supports the notion that the ϕ X174 receptor is composed, in part at least, of the lipid layers of the *E. coli* cell envelope, as these layers are extracted by organic solvents (21).

Incubation of the cell wall preparation with lysozyme (Worthington code lysf) at a concentration of 2×10^{-4} g/ml for 15 min at 37 C before the addition of phage greatly reduced the ability of the cell walls to cause the viral DNA to become deoxyribonuclease-sensitive but had little effect on their ability to reduce the titer of the phage suspension (Table 1). Lysozyme, a β -1-4-glucosaminidase (17), attacks the muramic acid layer of the *E. coli* cell walls; thus the integrity of this structure appears to be essential to the release of DNA but not to the binding of the virion to the cell wall structure. A similar result (attachment without DNA release) was obtained in an experiment with ³H-thymidine-labeled phages which were treated for 4 hr at room temperature with 4% formaldehyde buffered at pH 7.2 and then dialyzed exhaustively against F medium. Treatment of ϕ X174 with 4% formaldehyde at 20 C in 3XD medium at a pH of 6.5

reduced the plaque-forming ability within 60 sec by 10^5 PFU/ml and within 5 min by more than 10^7 PFU/ml. Although fixation had destroyed plaque-forming ability, these phages were still able to adsorb to *E. coli* C cells (Table 1). When these fixed virions were mixed with *E. coli* C cell walls, the phage DNA remained deoxyribonuclease-insensitive, whereas ^{32}P -labeled *E. coli* DNA fixed and dialyzed in the same manner was 78% degraded (Table 1). The host specificity of the phage was apparently unaffected by formaldehyde treatment since these phages did not bind to *E. coli* K₁₂W₆ cells. This result indicates that formaldehyde treatment does not affect that component of the ϕ X174 particle which is responsible for binding the virion to the host cell but does prevent the release of DNA.

Electron microscopy of adsorbed ϕ X174. Attempts to visualize the events described above in the electron microscope gave the following results. The phages attached to lysozyme pretreated cell wall fragments appeared generally full, whereas those attached to untreated walls appeared generally "empty." It was not possible, however, to determine by these experiments how many of these virions were made empty during preparation for the microscope.

The orientation of ϕ X174 adsorbed to whole cells and cell wall fragments was studied in the electron microscope with material prepared by the technique of negative staining and freeze-etching. A preparation of ϕ X174 was mixed with *E. coli* C cell walls which had been treated with lysozyme as described in the preceding section. Figure 1 shows an area of a negatively stained preparation of this mixture. Lysozyme treatment seemed to release small fragments of relatively uniform size (6.5 by 25 nm) from the cell walls to which ϕ X174 was found attached. The phage particles appeared to be able to adsorb to both sides of these fragments; however, the electron density of these attached virions indicated that this interaction did not result in the release of DNA. The arrows in this electron micrograph point out the six vertices one would expect to see when viewing from above an icosahedron which is lying on its threefold axis of symmetry. The particles appear to attach to the fragments by the tip of one of these vertices.

Figure 2 shows a freeze-etched preparation of intact *E. coli* C cells which have adsorbed ϕ X174 bacteriophages. Subunits (capsomers) can be identified on many of the virions. The adsorbed virions appear to be buried to about half their diameter within the cell surface material and have one of their fivefold axis of symmetry normal to the surface of the cell. One of these particles is enlarged and its symmetrical arrangement is

pointed out by arrows. This fivefold configuration is the expected arrangement when viewing from above an icosahedron standing on one of its 12 vertices. These results obtained by freeze-etching confirm the observations obtained by negative staining, namely, that adsorption occurs through the interaction of one of the bacteriophage's vertices and the host cell wall. This observation is in agreement with the view of Edgell et al. that the vertices of ϕ X174 represent the organelles of attachment (7). If these 12 vertices are identical, any one of them would be expected to be capable of attaching the phage to the host cell upon collision. It might be expected that a virus with several (12) equivalent organelles of attachment might be capable of binding to more than one cell wall fragment. Electron microscopic observation of phage-cell wall mixtures was focused on detecting the multiple attachment of a phage particle to wall fragments. Such a survey failed to reveal virions adsorbed to wall fragments by more than one of their vertices. If all 12 vertices were equivalent and capable of binding the phage to the host cell, one would have expected to see virions bind to cell structures by more than one spike. Further, if, as shown in Fig. 2, one of the vertices faces away from the host cell, it might also be possible to cross-link host cells by the addition of high multiplicities of virus. This possibility was tested in the following experiment. *E. coli* C cells at a concentration of 5×10^8 cells/ml were infected with ϕ X174 at multiplicities of 250 phage per cell. The mixture was incubated at 37 C for 5 min and then washed twice in 3XD medium to remove unadsorbed phage. The infected cells were then divided into two equal volumes. One of these received enough ϕ X174 antiserum to inactivate 99% of the phage population in 1 min. The other received no further treatment or was mixed with an equal number of uninfected cells. Both of the mixtures were incubated at 37 C for 20 min. At these conditions of time and temperature, the steps of attachment, DNA release, and penetration occur (20). A control was carried out with uninfected cells and antiserum to be certain that antiserum could not clump cells in the absence of phage. Clumping of cells was viewed in the phase-contrast microscope. Photomicrographs of the three reaction mixtures are shown in Fig. 3. Clumping was observed only in the preparation containing the infected cells which had been treated with ϕ X174 antiserum. Antibodies to ϕ X174 can bind more than one virion per molecule and can therefore establish a bridge between infected cells and thus lead to cell clumping. The ϕ X174 virus itself might be expected to behave similarly if it were able to attach simultaneously to two cells through

FIG. 1. Electron micrograph of a negatively stained preparation of ϕ X174 mixed with lysozyme-treated cell wall fragments (A). The virions attach to these fragments by one of their vertices. Arrows point out the sixfold symmetrical appearance of one of the attached particles. The inset shows a 12-subunit icosahedron positioned in an attitude which would reveal this sixfold appearance. Bar represents 50 nm.

FIG. 2. Electron micrograph of a freeze-etch replica revealing ϕ X174 particles attached to the surface of an *E. coli* C cell. The viewing angle is roughly perpendicular to the surface of the cell. The subunits of the particles are visible, and it is apparent that the particles are buried to about one-half their diameter in the cell surface with one of their fivefold axis of symmetry normal to the surface. The insert (right) shows an enlarged attached particle on which this fivefold arrangement is pointed out. The insert (left) shows a 12-subunit icosahedron positioned in an attitude which would reveal this fivefold appearance. Bar represents 50 nm.

FIG. 3. Phase-contrast light micrographs of (a) uninfected *E. coli* C cells plus anti- ϕ X174 serum, (b) ϕ X174-infected *E. coli* C cells plus anti- ϕ X174 serum, (c) ϕ X174-infected cells without antiserum. Cells are observed to clump only when virus, bacteria, and antiserum are present at the same time.

any pair of opposite vertices; the lack of clumping in the presence of ϕ X174 indicates that this is not the case. This finding is in agreement with the studies by electron microscopy. The ability of ϕ X174 to clump susceptible cells at 15 C was tested in an experiment similar to that described above. At 15 C, attachment of the virion to the host-cell occurs but DNA release and penetration are prevented (20). The result of this experiment was identical to that obtained at 37 C: clumping was only observed when ϕ X174-infected cells were treated with ϕ X174 antiserum. If the failure to undergo multiple attachment is the result of a configurational change resulting from a phage-host interaction, it must be concluded that the attachment step alone is adequate to produce this change. Phage which have undergone only the attachment step may be recovered from the host virus complex and will adsorb to host cells with a normal rate (19); therefore, such a configurational change must also be reversible.

Removal of ϕ X174 parental protein from the phage-cell complex by lysozyme EDTA treatment. The preceding experiments indicate that ϕ X174 DNA is released from a virion which is embedded into the host cell envelope to about one-half its diameter with one of its vertices oriented toward the "rigid layer" of the cell. The transport of the viral genome into the cell might be preceded by the removal of some material of the viral capsid. We have investigated the possibility that this capsid material may accompany the phage DNA into the cell.

ϕ X174-infected cells were treated with lysozyme and EDTA in an attempt to remove the parental

ϕ X174 protein from the host virus complex by removal of the outer layers of the infected *E. coli* cell wall. These experiments were carried out in 16% sucrose solutions to prevent osmotic shocking of the cells and to allow for conditions which would provide for normal phage production.

Cells were infected with a multiplicity of 30 3 H-leucine-labeled phages per cell. After allowing 5 min at 37 C for adsorption, the cells were washed three times by spinning and resuspending to remove unadsorbed phage. The infected cells were then converted to spheroplasts by spinning and resuspending into 3XD medium containing 16% sucrose (w/v), 10^{-4} g of lysozyme per ml, and 10^{-3} M EDTA, at pH 7.2. Spheroplast formation was followed morphologically in the phase-contrast microscope. The cells were incubated in this solution at room temperature for about 20 min. At the end of the incubation period, the spheroplasts were washed on a step gradient similar to that described by Guthrie and Sinsheimer (11) at 5,000 rev/min ($6,000 \times g$) for 15 min in an SW 50L swinging bucket rotor. The gradient was collected dropwise through a hole punched in the bottom of the tube, and samples were assayed for radioactivity. The average result of 28 such experiments revealed a loss of about 70% ($\pm 5\%$) of the labeled coat protein from the virus spheroplast complex. When the spheroplasts collected from this gradient were osmotically shocked by dialysis against sucrose-free buffer [0.5 M tris(hydroxymethyl)aminomethane (Tris), pH 7.2] and then sedimented on a 5 to 20% sucrose gradient placed above a 1.75 g/ml CsCl layer [22,000 rev/min ($50,000 \times g$) for 14 hr in

SW 50L rotor; Fig. 4], only about 17% of the input counts were found to sediment to the salt-sugar interface. The remaining counts stayed near the top of the gradient. This result is interpreted to mean that most of the radioactive phage protein found attached to the spheroplasts is present as a low-molecular-weight component, as intact phage and empty phage capsids (ghosts) would have moved well away from the top of the gradient under these conditions.

It is generally accepted that removal of gram-negative cell walls by lysozyme-EDTA treatment during spheroplast formation is not a quantitative process. The possibility thus exists that the radioactivity in the spheroplast fraction is bound to residual (undigested) cell wall and not associated with the cell membrane or cytoplasm of the cell. Since it was demonstrated that formaldehyde-treated ϕ X174 could adsorb to host cells without releasing their DNA (Table 1), it was reasoned that such treated phage could serve as a control for this experiment as their DNA would not be expected to penetrate the cell wall. Whereas about 30% of protein label of untreated ϕ X174 remained with the host cell, the formaldehyde-treated phage left only 15% of their protein label with the spheroplasts. When these spheroplasts were disrupted and centrifuged as described above, the radioactivity was found to sediment almost quantitatively to the CsCl interface (Fig. 4). This result suggests that the fixed viral protein found sedimenting with the spheroplasts is in the form of intact virions attached to cell wall material not removed by lysozyme-EDTA treatment. If the 15% of viral protein associated with the spheroplasts in this experiment does indeed represent the limit of enzymatic digestion of the host cell wall during spheroplast formation, then approximately one-half of the unfixed parental protein which cosedimented with the spheroplast fraction in the preceding experiment may be associated with cell membrane or cytoplasm. Sucrose gradient sedimentation analysis of this fraction indicates that much of this protein exists as a slow sedimenting low-molecular-weight component (Fig. 4).

The above results suggest that after infection a portion of the phage protein either enters the cytoplasm of the host cell or remains bound to the cytoplasmic membrane and that this component is of low-molecular-weight compared to whole phage or phage ghosts. Attempts were made to determine if this cytoplasm or membrane-associated material represented only one or two of the four proteins making up the phage capsid. The protein label sedimenting with the spheroplast fraction was analyzed by sodium dodecyl sulfate-acrylamide gel electrophoresis with the procedure

FIG. 4. Sedimentation profile of osmotically shocked spheroplasts produced from *E. coli* C cells infected with radioactive formaldehyde-fixed (dashed line) or unfixed (solid line) ^3H -leucine-labeled ϕ X174. The shocked spheroplasts were layered onto a 5 to 20% sucrose gradient (0.2 M NaCl-0.05 M Tris-0.003 M EDTA, pH 7.4) which was layered above a CsCl shelf ($\rho = 1.76 \text{ g/ml}$) and sedimented at 22,000 rev/min for 14 hr in SW 50L. Equal-volume fractions were collected from the bottom of the tube, and each fraction was assayed for radioactivity. The CsCl sucrose interface occurs at fraction 4.

of Burgess and Denhardt (5). No difference was found in the profile or relative proportion of spheroplast-associated radioactivity compared to whole phage or the material removed by lysozyme.

Incorporation of parental ϕ X174 protein into progeny virions. The preceding experiments raise the possibility that some of the infecting viral protein may accompany the viral nucleic acid into the host cell. This possibility was further examined in an experiment designed to detect the presence of parental protein in the progeny phage populations. Figure 5 shows the results of such an experiment.

Logarithmically growing *E. coli* C cells were infected with heavy ($5'$ -bromodeoxyuridine) radioactive (^3H -leucine) ϕ X174 (3XD medium at a concentration of 5×10^8 cells/ml with a calculated multiplicity of approximately 30 PFU/cell). The culture was incubated at 37 C with aeration for 2 hr and was then lysed by the addition of lysozyme and EDTA and by three successive freeze-thawings. After lysis was apparent, a small amount of ^{14}C (leucine) light reference phage was added to the mixture. Cesium chloride was then added directly to the mixture to bring the mean density to about 1.39 g/cm^3 (4.5-ml final volume), and the mixture was centrifuged for 72 hr at 37,000 rev/min ($140,000 \times g$) in the SW 50L rotor. The gradient was collected dropwise from the bottom of the tube directly into scintillation vials containing GFA fiberglass filter discs. After drying, the samples were assayed for their relative

FIG. 5. Radioactivity profile of a CsCl density gradient of lysate produced by infecting *E. coli* C cells in light cold medium with heavy ($5'$ -bromodeoxyuridine) radioactive (^3H -leucine) ϕX174 . A small amount of ^{14}C -labeled light reference phage was added to the lysate before centrifugation. Conditions of centrifugation are described in the text. Symbols: solid line, ^3H -leucine counts per minute; dashed line, ^{14}C -leucine counts per minute.

amounts of ^{14}C and ^3H radioactivity. The density profile of this gradient (Fig. 5) shows the ^{14}C reference phage to band around fraction 20 with some radioactivity at the top of the gradient. This tail may be due to capsomer components of some of the reference virions released by osmotic shock. The parental ^3H label was found in four distinct regions of the gradient: (i) close to the bottom of the tube probably representing unaltered parental phage; (ii) in the same region as the light reference phage, indicating the transfer of radioactivity from originally heavy virions into light progeny virions; (iii) in a peak at a density lighter than the reference phage [this may represent a conversion of some of the 114S parental phages into 70S components through interaction with host cell walls as described by Eigner et al. (8)]; and (iv) at the air interface probably representing empty parental phage capsid fragments.

About 7% of the total input viral protein label which was originally associated with a densely banding component was transferred during the infectious cycle to a component which cobands with light reference ϕX174 particles, indicating that a small percentage of the parental viral protein is utilized in the formation of progeny phage. This observation is consistent with the idea that some viral protein may enter the host cell during the penetration process. To investigate whether reutilization of this label in progeny virus formation occurs as a result of its decomposition into amino acids or if the intact parental protein frag-

ment is incorporated, the cells infected with ^3H -labeled ϕX174 were superinfected with T4. Under these conditions, the host produces only T4 (Zarling and Brown, *in preparation*). No ^3H label could be detected in the purified T4 produced from such doubly infected cells. It thus appears that, if the ϕX174 parental protein is degraded before incorporation into progeny virion, this degradation does not result in free amino acids, as such free amino acids should be incorporated into T4 proteins.

DISCUSSION

ϕX174 is an icosahedral virus which attaches directly to the surface of the host cell without the aid of a complex tail structure. It can be demonstrated by electron microscopy that this attachment occurs by positioning of the phage particle in such a way that the axis through two opposite vertices of the capsid is normal to the cell surface as though the attachment is facilitated by such an orientation; this view would be consistent with the suggestion of Edgell, Hutchison, and Sinsheimer (7) that the vertices of ϕX174 represent the organelles of attachment. Our findings seem to indicate that ϕX174 may not be capable of multiple attachment to cell walls. Two explanations can be offered for this: (i) one of the 12 vertices is specialized for attachment or (ii) after the initial contact of one vertex with the cell surface the remaining vertices lose their binding properties. Such a loss might be due to a configurational change initiated by attachment. The negative outcome of experiments testing the ability of phage to clump susceptible cells at low temperature (15 C) or to bind lysozyme-treated cell wall fragments to more than one vertex (both conditions which do not allow for release of DNA) indicates that a configurational change would require binding only, without ensuing eclipse. The lack of cell agglutination by bridge formation of virus particles could also be caused by a microscopically "invisible" layer or by the action of an electric charge surrounding the cell, which, although permeable to the virus, would keep at the same time two neighboring cells apart from each other at a distance too wide to be bridged by one virus capsid. The existence of such "layers" is known for noncapsulated *E. coli* strains (18); its thickness, however, has not been established in cells without fixation or dehydration, or both.

The results of our experiments with isolated cell wall fragments confirm the findings of Fujimura and Kaesburg (10) that the interaction of ϕX174 with wall fragments results in irreversible adsorption of the phage and the release of DNA.

We have used this experimental system to obtain further information on the nature of the phage-cell wall interaction. Control experiments with fragments of cells not possessing ϕ X174 receptor sites indicate that the interaction of the virus with isolated wall fragments is cell strain-specific and the host range relationship is maintained. Isolated cell wall fragments therefore seem to provide an adequate subcellular model for study of the infective process.

The initial binding of the virion to the host cell appears to involve the interaction of one of the vertices of the icosahedral phage with the lipid-containing layers of the cell wall. Treatment of intact cells or isolated cell walls with an organic solvent (chloroform) eliminates the ability of these structures to bind virions.

Lysozyme has an effect on cell wall fragments more subtle than that of chloroform in that it does not interfere with the binding of phage to the wall fragments but prevents DNA release. Newbold and Sinsheimer have shown that at a low temperature (15 C) the attachment of ϕ X174 to intact cells is not followed by DNA release and penetration. It may be that phage bound to lysozyme-treated cell wall fragments are also unable to proceed to the DNA release step of the three-stage infective process. Release of the DNA from the viral capsid seems, therefore, to require interaction of the virion with some lysozyme-sensitive structure in the host cell wall. Since lysozyme is a β -1-4-glucosaminidase, it is likely that virus DNA release is associated with the muramic acid layer since this is the only structure in the *E. coli* cell wall subject to attack by this enzyme. Because the rigid layer lies beneath the outer lipid layers of the host cell wall, the infecting virus must penetrate these outer layers to interact with the structures responsible for DNA release. The adsorbed virus appears (by freeze-etching) buried to about one-half of its diameter into the host cell envelope with one of its 12 fivefold axis of symmetry normal to the cell surface. This orientation results in the farthest penetration of capsid protein into the cell wall reaching a depth of about 12.5 to 14.0 nm. The thickness of the cross-sectioned *E. coli* cell wall has been measured to be about 16.0 nm in ultrathin sections after fixation, dehydration, and embedding (3). This degree of penetration would therefore allow the phage to interact with the deeper portions of the cell wall.

Lysozyme treatment also leads to the release of cell wall elements with ϕ X174-binding capacity. These fragments vary in length from 22.0 to 30.0 nm but have a relatively constant thickness of 6.5 nm. ϕ X174 can be seen to have attached to both sides of these small residues. The electron density of the virus confirms that this attachment

does not result in the release of the viral DNA. These lysozyme-produced fragments are reminiscent of the T5 receptors released from *E. coli* cells by mild alkali (24).

The second step of infection (DNA release), which is prevented by low temperature (20), can also be arrested by treating the phage with formaldehyde. This fixation does not prevent attachment of the phage but does prevent the subsequent release of the DNA. It appears that a formaldehyde-sensitive structure in the phage vertex must interact with a lysozyme-sensitive structure in the cell wall for eclipse to take place.

The ϕ X174 DNA may escape from the phage capsid by one of two mechanisms: either the interaction of the phage vertex with the muramic acid layer of the host cell causes a configurational change in the capsid which opens a passageway through which the viral DNA passes alone into the host cell or an opening in the capsid results from the removal of capsid material from the phage vertex. This latter possibility is supported by the observation that attempts to remove eclipsed particles from cells by lysozyme-EDTA treatment resulted in some phage protein label remaining associated with the resulting spheroplasts as low molecular material, perhaps as capsomers. Experiments carried out in a similar fashion with formaldehyde-fixed phage do not leave low-molecular-weight material associated with the spheroplasts, even though these virions are capable of attachment. It could also be demonstrated that a small portion of the parental phage protein could be recovered in the new progeny particles. Attempts to show, by disc electrophoresis, that only a few of the four structural proteins of ϕ X174 were being released from the capsid during eclipse failed. All four protein components were found present in the spheroplast fraction in the same relative proportions as in the intact parent particles. Freeze-etchings and negatively stained preparations indicate that the ϕ X174 virion is composed of 12 subunits, the smallest number that can be used to construct an icosahedron. If these 12 subunits are of identical composition (four proteins each in equal ratios), our observations by electrophoresis of the spheroplast-associated component may be explained by proposing that this component is one of the 12 subunits, this intact vertex being released from the capsid providing the passageway for the penetrating DNA. That ϕ X174 protein label could not be incorporated into the progeny of super-infecting T4 suggests that this subunit may not be degraded after entering the cell.

It is also possible that one of the 12 subunits possesses a fifth structural protein too small to be detected by methods employed in this study and

that this subunit facilitates attachment to virus. Eight cistrons have presently been identified by complementation in the ϕ X174 genome; however, there may well be room for information to code for this attachment protein.

It is not unreasonable to expect the icosahedral viruses to have a special point of attachment. Morphogenetic events leading to the production of a special vertex in an icosahedron are not without precedent. The "tailed" phages (e.g., lambda, T4, and P22) apparently have their icosahedral heads synthesized and separately packed with DNA before the complex tail assembly is attached (6, 13, 25). These icosahedral head structures are assembled with one of the 12 vertices especially adapted to receive the tail structure. ϕ X174 may then represent an example of a virus which has never developed a complex tail structure, although it possesses a unique point through which its genome will pass during host cell infection.

ACKNOWLEDGMENTS

This investigation was supported by grants from the Brown-Hazen fund administered by the Research Corporation (New York) and the Maryland Division of the American Cancer Society.

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