The RNA Injection Step of Bacteriophage f2 Infection

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(Accepted 5 July 1968)

SUMMARY

In this communication we describe the events occurring during the injection step in the infection of *Escherichia coli* Hfr cells with the RNA bacteriophage f2. The phage RNA is partially injected into the F-pilus of the Hfr cell. This step was found to be necessary for f2 RNA to block the penetration of the DNA phage fI. A cold sensitive mutant of f2 is described. It is able to inject its RNA at 41° but not at 31°.

After contact is made between the phage RNA and the F-pilus, the coat protein of the phage desorbs from the F-pilus as an empty shell, leaving the RNA free to enter the cell. In the absence of divalent metals, however, the phage RNA is unable to leave the shell. When the shell desorbs from the F-pilus, the RNA remains inside in a ribonuclease sensitive state. A small fraction of the RNA in cultures depleted of divalent metal ions remains bound to the cell. It is sensitive to ribonuclease and is removed from the cell by treatments which remove F-pili.

INTRODUCTION

The first stage of the infective process of the male-specific RNA phage f2 is the penetration of the viral RNA into the cell. This stage has been divided into several steps (Valentine & Wedel, 1965) (Fig. 1). We believe that the F-pilus of the F+ or Hfr cell is crucial throughout this process. Valentine, Wedel & Ippen (1965) showed that the F-pilus is necessary for infection of *Escherichia coli* by male-specific phage. Male-specific phages, including f2, can be seen to adsorb to F-pili in the electron microscope (Crawford & Gesteland, 1964; Caro & Schnös, 1966). Free pili, removed from the cell, also adsorb f2 (Ippen & Valentine, 1965). However, the details of the involvement of F-pili in stages of infection subsequent to adsorption are less clear. Ippen & Valentine (1966) showed that, although the male-specific DNA phage fI does not compete with f2 for adsorption sites, (fI adsorbing to the tip, f2 to the sides of the F-pilus (Caro & Schnös, 1966)), these phages do compete at some later stage for a common injection pathway. These results are consistent with the model proposed by Brinton (1965), that the F-pilus serves as a common duct for nucleic acid transport during male-specific phage infection.

Cell mutants have been isolated which are resistant to f2 (Silverman, Mobach & Valentine, 1967 a, b; Silverman et al. 1968). One such mutant is unable to adsorb the RNA phages f2 or Qβ, although the DNA-containing, male-specific phage infects these strains normally (Silverman et al. 1968). A second mutant is unable to support

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the growth of f2 while Qβ and f1 grow normally (Silverman et al. 1967a). This mutant is able to adsorb f2 but subsequent steps are blocked (cf. Fig. 1) since the adsorption is reversible (Silverman, Rosenthal & Valentine, 1967b). These mutants tend to substantiate the scheme shown in Fig. 1. They are most easily explained as F-pili mutants blocked in one or another step in the extracellular events of f2 infection.

In this report we describe the biochemical events involved in the second step in f2 infection, which we term the injection step (Fig. 1). We define this step as that step during which the plaque-forming ability of the virus becomes sensitive to CHCl₃ and the viral RNA becomes sensitive to ribonuclease. An important guide in this investigation has been the observation by Paranchych (1966) that infection by another RNA phage, R17, is blocked after the injection step by depriving cultures of divalent metal ions. We have confirmed these findings for f2 and shown that (1) competition for the penetration pathway between f1 and f2 depends on the occurrence of the RNA phage injection step, (2) conditional mutants of f2 can be isolated which are blocked in the injection step, (3) a small fraction of the cell-bound RNA from cultures treated with EDTA is sensitive to ribonuclease and that this fraction is quantitatively removed by blending, a treatment which also removes F-pili, and (4) the coat protein of f2 is desorbed after the injection step whether or not RNA penetration occurs. These findings provide considerable insight into the extracellular steps of infection by f2.

**METHODS**

**Bacterial strains.** *Escherichia coli* W1895 is an Hfr₁ male strain auxotrophic for methionine. M27 and G26 are nitrosoguanidine-induced mutants of W1895 selected for resistance to phage f2. Silverman *et al.* (1967a) have described the isolation procedure for these mutants. A19 is an RNase I deficient strain of *E. coli* isolated by Gesteland (1965).

**Bacteriophages.** (1) Standard phage stocks. Lysates of f2, Qβ and f1 were made by infecting a young (∼ 10⁶ cells/ml) culture of *Escherichia coli* with 20 to 40 p.f.u./cell and incubating until lysis occurred (f2 and Qβ) or for 2 to 2½ hr (f1). The cells were centrifuged and the lysates used without further purification. Final titres were 10¹¹ to 10¹² p.f.u./ml.

(2) Cold-sensitive f2 mutants. CS10 is a cold-sensitive nitrous acid-induced mutant of f2. A stock of f2 was treated with nitrous acid (1 M-NaNO₂) in 0.5 M acetate buffer,
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Phage f2 infection at pH 4.3, 25° to 99.9% killing (about 30 min.). Survivors were plated at 42° and plaques picked with sterile toothpicks into 1 to 2 ml. sterile broth. A loopful of each suspension was plated on each of two plates, one incubated at 32–33° and the other at 42°. After 5 to 6 hr the plates were inspected and suspensions containing phage growing at 42° but not at 32 to 33° were further analysed. The yield of cold sensitive mutants was about 1%. The mutants were analysed by temperature shift experiments for early and late function. Both types were found (Silverman, unpublished). One early mutant, cs10, was analysed further since preliminary experiments indicated that it might be an injection mutant.

(3) Radioactive phage. For 32P phage, male cells (K37) were grown to a concentration of 10⁸ cells/ml. in 20 ml. of low phosphate medium containing 4 µc of 32P as sodium phosphate (Mallinckrodt Chemical Works). Phage f2 was added at a multiplicity of about 20 p.f.u./cell and CaCl₂ to a final concentration of 1 mM. The infected culture was incubated for 3 hr at 37°. 32P cs10 was prepared the same way.

For ³⁵S phage, lysates were obtained according to the method of Edgell & Ginoza (1965) except that 0.5 ml. (5 µc) of Na₂[³⁵S]O₄ (New England Nuclear) was added to 25 ml. of cells.

After the incubation period the cells were removed by centrifugation. (NH₄)₂SO₄ was then added to the supernatant fluid to a final concentration of 50%, along with about 20 mg. of serum albumin to aid in the precipitation. The mixture was allowed to precipitate at 0° for at least 1 hr. The precipitate was collected by centrifugation, suspended in 2 ml. of broth, and dialysed for 4 hr at 0° against 16 l. of distilled water. After dialysis, the total volume was brought to 4 ml. by addition of broth, and CsCl added to the solution; (2.5 g. CsCl/4 ml. when f2 was being purified, 1.70 g./4 ml. for f1). The solution was then centrifuged at 37,000 rev./min. on a Spinco Model L ultracentrifuge in the SW-39 rotor for 24 hr at 5°. Fractions of 3 drops were collected from the centrifuge tube, after piercing the bottom, and those fractions containing phage (measured by radioactivity and titre) are pooled. CsCl and broth were again added and the centrifugation step repeated. The main phage-containing fractions were pooled and dialysed against 16 l. of H₂O to remove the CsCl. The purified phage was stored in the refrigerator in vials containing 1 drop of chloroform. The phage yield was generally about 10¹² p.f.u./ml. and 10⁷ counts/min./ml. for the [³²P]-phage and 10¹¹ p.f.u./ml. and 10⁶ counts/min./ml. for the [³⁵S]-phage.

Media. TYE broth was described by Loeb & Zinder (1961), low phosphate medium by Davis & Sinsheimer (1963), the TBrC medium for f2 phage by Edgell & Ginoza (1965) and tris-salts buffer by Denhardt & Sinsheimer (1965).

Assays. The ³²P phage binding assay using cellulose nitrate filters was described by Ippen & Valentine (1965). Duplicate 0.5 ml. samples were taken and the filters washed with 10 ml. of TYE broth. The penetration assay was performed by diluting 1.0 ml. samples into 2 ml. cold saline and blending at half speed for 2 min. in the cold in a Sorvall Omnimix fitted with a micro-attachment unit. The cells were centrifuged and the supernatant fluid discarded. The cell pellet was resuspended in cold 5% (w/v) trichloracetic acid and filtered over glass membrane filters (Whatman GF/C 2.4 cm.).

Two binding assays were used with ³⁵S phage. In the first, 1 ml. samples of the phage cell mixture were diluted into 20 ml. of cold TYE. Five ml. of female carrier cells were added and the cells sedimented by centrifugation, resuspended in cold 5% (w/v) trichloracetic acid, filtered and counted. In the second, 1 ml. samples were placed in cold centrifuge tubes and allowed to stand for 30 to 45 min. When all the remaining
phage capable of adsorbing had done so. 1 ml. of female cells was added to each tube as carrier. The cells were sedimented, precipitated with 5% (w/v) trichloracetic acid, plated and counted.

Phage injection was measured by the procedure of Denhardt & Sinsheimer (1965) except that gentle shaking with a few drops of CHCl₃ was substituted for freezing and thawing.

Sucrose gradients. Thirty ml. 5 to 20% (w/v) linear sucrose gradients were prepared in TKM buffer (0.05M-tris, pH 7.2; 0.001M-MgCl₂; 0.1M-KCl). Two ml. of the sample to be analysed were layered on top of the gradient and centrifuged in the SW25 rotor in Spinco model L centrifuge for 5½ hr at 23,500 rev./min. After centrifugation a hole was punched in the bottom of the tube and 60 to 80 four-drop fractions collected. A background of 10 counts/min. was subtracted from each fraction.

Radioactive counting. ³²P was counted on filters in a Nuclear Chicago gas flow counter. ³⁵S was counted in a Packard Tri-Carb Liquid Scintillation spectrometer at a gain setting of 20 and a window setting of 50 to 1000. The counting vials contained, in addition to 0.4–0.5 ml. aqueous sample, 10 ml. of scintillation fluid (Liquifluor, New England Nuclear) diluted as directed into sulphur-free toluene and 6 ml. of ethylene glycol monomethyl ether. For the ³⁵S protein adsorption assays, filters were dried at 60o for 60 min. and counted directly in 10 ml. scintillation fluid.

RESULTS

Ippen & Valentine (1966) showed that f₂ and the male specific DNA phage f₁ compete for some stage in the penetration pathway subsequent to adsorption. The addition of unlabelled f₂ to a culture containing cells and ³²P-labelled f₁ inhibited the penetration of f₁ DNA into the cell but not the adsorption of f₁, presumably to the tip of the pilus (Caro & Schnöbs, 1966). They further showed that the addition of RNase to the culture reversed the inhibitory effect of f₂. RNase is known to prevent the penetration of f₂ RNA (Valentine & Wedel, 1965). With the isolation of a bacterial mutant preventing the injection stage of f₂ infection but allowing normal growth of Qβ and f₁ (Silverman et al. 1967 a, b), it became possible to extend this type of experiment. If the results of Ippen & Valentine (1966) were due to the physical blockage of the pilus lumen by the entering RNA, thus preventing the entry of f₁ DNA, then f₂ should not inhibit f₁ penetration into the mutant bacterium. On the other hand, Qβ should still inhibit the entry of f₁ DNA. At high multiplicities of infection non-infectious RNA does penetrate the mutant cell (Silverman et al. 1967 b). Therefore, as the multiplicity of f₂ is increased, it should inhibit the entry f₁ DNA (Table 1). At a low multiplicity of infection Qβ inhibited f₁ penetration approximately 50% in both the mutant and the wild type strains while f₂ inhibited significantly only in the wild type strain. As the multiplicity of the RNA phage was raised, f₁ penetration became sensitive to f₂ even in the mutant strain. These results support the explanation proposed by Ippen & Valentine (1966) that the lumen of the pilus is used by both f₁ and f₂ for nucleic acid transport; it also rules out the possibility of an indirect effect mediated by the simple adsorption of the RNA phage, since the mutant bacterial strain adsorbs f₂ normally (Silverman et al. 1967 a).
An f2 injection mutant

Assuming that the initial stages in the injection step involve interactions between phage and sites on the F-pilus which render the plaque-forming ability of the phage sensitive to CHCl₃ (P. M. Silverman, unpublished experiments), we wondered if it would be possible to isolate conditional mutants of f2 blocked in this step. Such mutants have been isolated for φX174 by Dowell (1967) using a selection for cold-sensitive phage. We have isolated a series of mutants of f2 which form plaques at 41° but not at 32° (see Methods). The latter temperature is at the lower limit of permissive

<table>
<thead>
<tr>
<th>Competing RNA phage</th>
<th>Multiplicity of infection of competing RNA phage</th>
<th>Bacterial strain</th>
<th>f1 DNA penetrated (counts/min./5 min./5 ml. cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>f2</td>
<td>0</td>
<td>Wild type</td>
<td>306</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td></td>
<td>188</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td></td>
<td>71</td>
</tr>
<tr>
<td>f2</td>
<td>0</td>
<td>Injection</td>
<td>356</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Mutant</td>
<td>318</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td></td>
<td>101</td>
</tr>
<tr>
<td>Qβ</td>
<td>0</td>
<td>Wild type</td>
<td>393</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td></td>
<td>185</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td></td>
<td>65</td>
</tr>
<tr>
<td>Qβ</td>
<td>0</td>
<td>Injection</td>
<td>328</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Mutant</td>
<td>171</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td></td>
<td>58</td>
</tr>
</tbody>
</table>

Both f1 (m.o.i. = 4) labelled with 32p in its DNA and the unlabelled RNA phage at the desired multiplicity were preadsorbed for 10 min. at 0° to cells prechilled for 30 min. At 0 time the cultures were shifted to 37° and a sample removed and blended as described by Valentine & Wedel (1965). The process was repeated 5 min. later. The 0 time control was subtracted from the 5 min. value to give counts/min. penetrated/5 min.

Fig. 2. Properties of the cold sensitive f2 mutant cso. (a) Adsorption of cso to free F-pili prepared according to Ippen & Valentine (1965a). ○, 41°; △, 31°. (b). Injection of cso on Hfr cells. ○, cso, 41°; △, cso, 31°; ○, wild type f2, 41°; △, wild type f2, 31°. (c). Penetration of cso RNA into Hfr cells. ○, cso, 41°; △, cso, 31°; ○, wild type f2, 41°; △ wild type f2, 31°.
temperatures for plaque formation by f2. The e.o.p. of wild type f2 at 32° is about 80% relative to 37°. Preliminary experiments showed that one of the mutants we isolated, cs10, failed to enter an eclipse period in liquid cultures at 31° but did so normally at 41°. The plaque forming ability of the virus remained resistant to CHCl₃ at 31° but not at 41°. Experiments with aP-cs10 (Fig. 2) demonstrated that at the low temperature cs10 adsorbed normally to free pili (Fig. 2a). However, injection and penetration assays (Fig. 2b, c) showed that relative to cultures at 41° both these events were reduced by about 90% at 31°. Control experiments with wild type f2 (Fig 2b, c) showed that reducing the temperature from 41° to 31° caused a two or threefold reduction in both penetration and injection. Either one of the shell proteins (the coat or the maturation protein (Steitz, 1968)) or the RNA itself was probably affected by a mutation in cs10 so that the phage failed to inject its RNA at 31° as measured by sensitivity to CHCl₃. Other experiments with cs10 showed that when infection of cells was allowed to occur at 41° and the infected cells shifted to 32°, a normal yield of phage resulted. We argue that this fact points to the RNA as being normal and to one of the protein components as being responsible for the failure to inject at 31°.

Table 2. Effect of EDTA on f2 infection

<table>
<thead>
<tr>
<th>EDTA (mm)</th>
<th>p.f.u./ml. injected (×10⁻⁷) after 20 min.</th>
<th>Phage penetrated (counts/min./ml.) after 10 min.</th>
<th>Phage/infective centre after 60 min.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1·9</td>
<td>275</td>
<td>1000</td>
</tr>
<tr>
<td>2·5</td>
<td>1·4</td>
<td>0</td>
<td>1500</td>
</tr>
<tr>
<td>7·5</td>
<td>1·1</td>
<td>—</td>
<td>160</td>
</tr>
<tr>
<td>20</td>
<td>2·3</td>
<td>—</td>
<td>&lt; 8</td>
</tr>
</tbody>
</table>

* Phage yield is determined by allowing infection to occur for 10 min. in TYE broth. An aliquot is removed and treated with anti-f2 antibody after which it is plated for infective centres. At 10 min. after infection EDTA is added to the desired final concentration and 50 min. later a second aliquot is removed and plated. Results are expressed as phage/infective centres.

The effect of divalent metal depletion on f2 infection

Loeb & Zinder (1961) observed that divalent metals were essential in the medium used for f2 infection, and Paranchych (1966) showed that for the f2-related phage R17 depletion of divalent metals prevented penetration of RNA into the cell, but did not prevent initial steps in infection which rendered the phage RNA sensitive to RNase.

In order to eliminate centrifugation we used EDTA to deplete the supply of divalent ions in a normal TYE broth culture. The addition of 2·5 mM EDTA to a culture about 1 min. before phage prevented penetration of aP-phage RNA, while the amount of phage RNA injected was unchanged relative to an untreated control culture (Table 2). The ability of the cell to support an f2 infection once the phage RNA had entered the cell was also unchanged (col. 3, Table 2). In all subsequent experiments using EDTA it was added to a concentration of 2·5 mM about 1 min. before the phage.

A cell-bound RNase sensitive intermediate

Paranchych (1966) observed that when RNase is present from the beginning of R17 infection the same amount of RNA is degraded in metal-deprived cultures as in cultures supplied with Mg²⁺ ions. As reported in the previous section, the addition of
EDTA mimics the effect of Mg²⁺ deprivation: injection step occurs but subsequent steps are blocked so that no RNA enters the cell.

We wondered if we could isolate a cell-bound, RNase-sensitive intermediate from cultures depleted of divalent metal ions. If so, it should then be possible to determine the nature of this intermediate, especially whether or not it is bound to F-pili. We detected such an intermediate and made several experiments which indicated it was bound to F-pili. We reasoned that if the injected intermediate were stable it should be possible to add the RNase to the cell fraction some time after infection in the presence of EDTA rather than before infection and still detect the RNase-sensitive RNA fraction. To minimise breakage of the RNA due to cellular nucleases we employed Escherichia coli \( \lambda \) 19, an RNase I deficient strain (Gesteland, 1965) for these experiments. \( \lambda \) 19 was grown to a concentration of about \( 2 \times 10^8 \) bacteria/ml. and divided into two samples of 25 or 50 ml. each; one contained 2.5 mM EDTA and both were exposed for 30 min. to \( ^{32} \)P-f2 at a multiplicity of 2. After 30 min. the cells were collected by centrifugation and resuspended in 5 ml. of cold tris-salts buffer (Denhardt & Sinsheimer, 1965). The suspension was then assayed for trichloracetic acid precipitable radioactivity and radioactivity adsorbable to cellulose nitrate filters (Ippen & Valentine, 1965) before and after treatment with RNase (Table 3). All the radioactivity in both fractions was trichloracetic acid precipitation although about 50 to 75% of the RNase-sensitive fraction could not bind to filters. The RNase-sensitive fraction was not observed in cultures of the bacterial injection mutant (Silverman et al. 1967a, b).

When the cell fraction of an EDTA-treated culture was blended to remove F-pili (Valentine & Wedel, 1965), the RNase-sensitive fraction was quantitatively removed from the cells (Table 4), although this treatment also eliminated about 90% of the radioactivity adsorbable on the filters. The RNase fraction could also be removed

### Table 3. Accumulation of an RNase-sensitive intermediate of f2 infection during divalent metal ion deprivation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total cell bound phage RNA (counts/min./ml.)</th>
<th>RNA remaining TCA precipitable (counts/min./ml.)</th>
<th>RNA remaining adsorbable to filters (counts/min./ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ RNase*</td>
<td>- RNase</td>
<td>+ RNase*</td>
</tr>
<tr>
<td>EDTA 2.5 mM</td>
<td>865</td>
<td>432</td>
<td>850</td>
</tr>
<tr>
<td>Control</td>
<td>1230</td>
<td>1056</td>
<td>1164</td>
</tr>
</tbody>
</table>

* RNase treatment is 4 μg/ml.; 10 min.; 37°C.

### Table 4. Extracellular localization of RNase-sensitive intermediate

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total phage RNA (counts/min./ml.)</th>
<th>Intermediate* (counts/min./ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cells</td>
<td>356</td>
<td>102</td>
</tr>
<tr>
<td>Blended cell pellet</td>
<td>19</td>
<td>---</td>
</tr>
<tr>
<td>Blended cell supernatant</td>
<td>316</td>
<td>100</td>
</tr>
</tbody>
</table>

* Intermediate is difference in 5% TCA precipitable counts/min./ml. between RNase-treated samples and untreated samples. RNase treatment is 5 μg./ml.; 5 min.; 37°C.
from EDTA-treated cultures by three cycles of gentle (3000 g; 10 min.) centrifugation and resuspension, which also removed F-pili (Valentine et al. 1965).

Thus, under conditions where penetration of the RNA into the cell was prevented but injection occurred normally, a cell-bound RNase-sensitive fraction of RNA appeared. It was outside the cell and could be removed by treatments which also removed F-pili. Experiments to confirm its association with F-pili are in progress.

The fate of the phage coat during the injection step

The results of the previous section led us to ponder the role of the coat protein of the virus in infection. In particular, we were interested in knowing whether the coat protein of the phage remained with the cell through the entire infection or whether it was desorbed. Edgell & Ginoza (1965) showed that the coat protein remained outside the cell during normal infection of *Escherichia coli* with R17.

Initial experiments with $^{35}$S-labelled f2 using the filter adsorption assay (Ippen & Valentine, 1965) gave ambiguous results and further experiments were conducted using centrifugation assays (see Methods). At 10° a normal adsorption curve was obtained when f2 labelled with $^{35}$S was mixed with a male strain of *Escherichia coli*. However, when the temperature was increased to 37°, a slight increase was followed by a decrease in adsorbed $^{35}$S radioactivity (Fig. 3a). This might have been due to the coat desorbing (from the F-pilus?) as soon as the RNA left it. We may, therefore, have been observing two competing reactions, adsorption and desorption, which tended to cancel each other. To measure only the latter process, samples were removed from the phage + cell mixture and chilled on ice for 30 to 45 min. This is sufficient time for all the phage that can still adsorb to do so, while at 0° subsequent steps are blocked. Phage coat that had been desorbed would not readсорb during this interval and a decrease in adsorbed radioactivity should be observed (Fig. 3b). Cells infected at 10°
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still carried about as much phage after 12 min. at 10° as they did at the beginning of the experiment. When the bacteria were held at 37°, however, a rapid decline in adsorbed radioactivity was observed, indicating that sometime during normal infection the coat was irreversibly desorbed. To demonstrate that this event is directly related to

RNA injection the experiment was repeated with the bacterial mutant in which this phase can not occur. There was little loss in adsorbed radioactivity (Fig. 3 b).

As it desorbs, the phage protein could exist either as an empty shell or as monomers. In the second case the separation of phage RNA and phage protein could be thought of

![Graphical representation of Sucrose gradient analysis of coat protein desorbed during normal infection.](image)

Fig. 4. Sucrose gradient analysis of coat protein desorbed during normal infection. ○, radioactivity; △, infectivity. (a). Free phage. (b). Supernatant fluid from infection of wild type cells. (c). Supernatant fluid from infection of bacterial injection mutant.
as the reverse of assembly (Hohn, 1967; Roberts & Steitz, 1967). These alternatives were tested by sucrose gradient sedimentation (Fig. 4). Cells (2 × 10^8 bacteria/ml.) and phage were mixed at 37° and incubated for 15 min. The cells were removed by centrifugation and the supernatant fluid analysed on sucrose gradients. Free phage sedimented as a single peak and the radioactivity profile corresponded to the viable phage profile (Fig. 4a). The supernatant of an infected culture, however, contained a second peak (Fig. 4b) sedimenting more slowly than the first. The total radioactivity on the two gradients was about the same. The supernatant fluid contained only a small proportion of the viable phage used to infect the culture. The second peak sedimented about where one would expect the empty shell (Hohn, 1967), and there was no radioactivity seen at the top of the gradient where one would expect phage coat monomer.

When this experiment was repeated with the bacterial mutant in which the eclipse phase could not occur (Fig. 4c), most of the radioactivity was missing from the supernatant fluid and none was seen in the segment of the gradient corresponding to where empty shells should have been. This supports the results from the adsorption experiments in which it was shown that the phage remained adsorbed to the bacterial mutant. Thus, the coat protein is desorbed from the bacterial cell as a hollow shell rather than as monomer.

When EDTA was added to cultures before infection with f2 labelled with ^35S, the coat was desorbed just as during normal infection (Fig. 5). Penetration of the RNA is therefore not a requirement for desorption of the coat shell. Use of the bacterial injection mutant as a control further shows that the loss of adsorbed phage was not due directly to the presence of the EDTA. No adsorbed phage was lost if EDTA was added to f2 and the bacterial injection mutant. The loss of coat requires eclipse of the phage but is not dependent on the penetration of the RNA into the bacterial cell.

This conclusion was verified by sucrose gradient analysis of supernatants of EDTA-treated cultures infected with f2. Conditions were as in the experiments of Fig. 4,
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except for the presence of EDTA during infection. Under these conditions there was a single broad peak of radioactivity sedimenting at approximately the same rate as whole phage. Viability was reduced to about the same level as seen during normal infection (Fig. 6 a). When the supernatant was first treated with RNase, the radioactivity shifted toward the top of the gradient with the bulk sedimenting where empty shells are found in normal infection (Fig. 6 b). Treating free phage with RNase had little effect on the sedimentation rate (Fig. 6 c), although some radioactivity seemed to sediment more slowly. Divalent metal ion depletion, while not preventing phage RNA injection did prevent the release of the RNA from the shell. The coat protein

![Graphs showing sucrose gradient analysis of coat protein desorbed during abortive infection in the presence of EDTA.](image)

Fig. 6. Sucrose gradient analysis of coat protein desorbed during abortive infection in the presence of EDTA. ●, radioactivity; △, infectivity. (a) Supernatant fluid from culture infected in the presence of EDTA. (b) Supernatant fluid as in (a), treated with RNase 50 μg/ml, 30 min, 37°C. (c) Free phage treated with RNase as in (b).
was still desorbed, but now contained some of the phage RNA in an RNase sensitive state. The remainder of the RNA remained with the cell in an RNase sensitive state, probably attached to F-pili.

**DISCUSSION**

Brinton (1965) proposed a model for the role of the F-pilus in bacterial conjugation and in male specific phage infection in which it acts as a tube through which nucleic acids pass *en route* to or from the male cell. Parts of this model have received substantial experimental support. Male-specific phage bind to F-pili; filamentous DNA phages to the tip, RNA phages to the side (Caro & Schnös, 1966). Although these two types of phage do not compete for binding sites, they do compete for some subsequent step in the infection pathway, presumably transport through the lumen of the pilus (Ippen & Valentine, 1966). Furthermore, blended male cells are unable to transfer DNA to female cells and this ability returns along with the regrowth of F-pili (Ippen & Valentine, 1967). Both f1 and f2 inhibit mating under conditions where no phage infection is allowed (Ippen & Valentine, 1967; Knolle, 1967). While all of this information substantiates the theory that F-pili are necessary in all these processes involving nucleic acid transport, the exact role of the F-pilus remains obscure.

We think that the most direct prediction of the model proposed by Brinton (1965) is that nucleic acid ought to be found in the lumen of the F-pilus and, because of the ease with which assays are performed and the availability of radioactively labelled material, we chose f2 to try to isolate the complex of phage nucleic acid and F-pilus.

Aside from the role of the F-pilus in nucleic acid transport, we were also interested in the dynamics of infection from the standpoint of the phage. The f2 particle is about 200 Å in diameter and contains a single strand of RNA of molecular weight about $1 \times 10^6$ daltons (Zinder, 1965). In order successfully to infect, the phage must transfer its genome intact into the cell. EdgeU & Ginoza (1965) showed that only the RNA of f2 enters the cell, the protein remaining outside. How, then, does the RNA dissociate itself from its coat and enter the host? What proteins in the virus are important in this process? The virus particle contains only two proteins; the coat protein is the major component (Zinder, 1965) and the existence of a second minor component was inferred from genetic evidence for f2 (Zinder & Cooper, 1964; Lodish, Horiuchi & Zinder, 1965) and recently isolated as a structural component of R17 (Steitz, 1968). Particles lacking this minor component, or maturation protein, are unable to adsorb to the male cells (Valentine & Strand, 1965) and it is tempting to speculate that this protein plays a key role in infection. However, these particles also contained defective RNA (Lodish, *et al.* 1965) so conclusions on this point cannot yet be drawn.

We have now observed a fraction of cell-bound phage RNA in divalent metal-depleted cultures infected with f2. This fraction is RNase-sensitive and is removed from cells under conditions which also remove F-pili. We have also shown, however, that under these conditions, some of the phage RNA leaves with the viral protein and that these 'eclipsed particles' can be isolated on sucrose gradients. The RNA contained in these particles is sensitive to RNase and in this property these particles are similar to 'dead' particles lacking maturation protein (Argetsinger & Gussin, 1966; Heisenberg, 1966). Experiments to determine the exact nature of both the cell bound and desorbed intermediate are now in progress.

It is interesting that complete separation of viral RNA and viral coat protein is not
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...a requirement for desorption of the latter. One might argue that in order to afford maximum protection to the RNA, the coat might cover the particle until the nucleic acid is safely en route to the cell. On the other hand, if the RNA is tightly packed inside the phage head, it might hold back the RNA by the bonds formed between the RNA and the viral protein. One role divalent metals might play is to weaken these forces so that the RNA is left associated with the cell when the coat desorbs by some mechanism independent of the presence of divalent metal ions. In the absence of such ions, however, the phage shell and the pilus would compete for the RNA and either each would get a piece or the entire RNA molecule would remain with the shell or with the pilus. Alternatively, the divalent metal ions might act on the pilus to pull the RNA out of the shell. However, the same struggle would ensue between the cell and the pilus for the phage RNA with either mechanism.

The competition experiments between f1 and f2 or Qβ reported in this paper rule out secondary effects to explain the fact that f2 blocks the penetration of f1 DNA (Ippen & Valentine, 1966), and definitely places the point of inhibition after injection of the phage RNA. Heretofore, it might have been argued that simple adsorption of the RNA phage prevented the penetration of f1 DNA by some indirect means. The competition experiment with the bacterial mutant, which prevents injection but not adsorption of f2, proves that it is necessary for the phage RNA to inject in order to inhibit f1 DNA penetration. Since the coat protein of the RNA phage falls away soon after the phage RNA injects, it is very likely that the RNA itself is preventing f1 DNA penetration, although the possibility cannot be ruled out that a minor protein component of the RNA phage (maturation protein?) blocks phage DNA penetration. In any event, it is easiest to reconcile these results with the model (Brinton, 1965) which proposes that the F-pilus acts as a tube for nucleic acid transport of both DNA and RNA male-specific phages.

Finally, the isolation of conditionally defective virus particles, such as the cold sensitive mutant reported here, permits the determination of the role of the various proteins in the virus shell, the coat protein (Zinder, 1965) and the maturation protein (Hohn, 1967; Roberts & Steitz, 1967). By complementation experiments similar to those reported for f2 amber mutants (Valentine, Engelhardt & Zinder, 1964), we hope to be able to determine which protein in the phage injection mutant cs10 is defective.

The injection step in f2 bacteriophage infection is a complex process involving interactions between the F-pilus, the phage RNA and the phage protein. Essentially, the RNA originally bound in its shell, becomes attached to the pilus and its linkage to its own coat is weakened. The coat falls away leaving the RNA to complete its infection of the cell. In the absence of divalent metal ions the phage RNA is desorbed with the shell or remains bound to F-pili. In either case, it is sensitive to ribonuclease.

We gratefully acknowledge the excellent technical assistance of Mrs Helen Mobach and Miss Mette Strand. This work was supported by USPHS grants TI-GM-31-10 and AM 10109.

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(Received 29 April 1968)