Adsorption of Bacteriophage $\phi$X174 to Isolated Bacterial Cell Walls

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SUMMARY

Incubation of bacteriophage $\phi$X174 with isolated bacterial cell walls in tris HCl, MgCl$_2$ solution results in disruption of the phage with partial release of virus DNA. After incubation for 5 min., particles which sedimented heterogeneously at about 50S were formed. These particles were infectious for spheroplasts, but not for whole bacteria. Incubation for 40 min. produced 70S particles which contained less DNA than intact phage. Similar results were obtained when $\phi$X174 virus particles were incubated with starved bacteria.

INTRODUCTION

The tails of certain bacterial viruses such as the T-even phage or T5 are known to act as a means of attachment and for penetration of the host cell wall by the virus nucleic acid (Anderson, 1948; Lanni & Lanni, 1953; Simon & Anderson, 1967). In some instances contact with isolated bacterial cell walls or phage receptor sites has been shown to result in partial release of virus nucleic acid, suggesting that this event may trigger the natural injection process (Hershey & Chase, 1952; Weidel & Kellenberger, 1955; Garen & Kozloff, 1959). Less has been reported of the mechanism of injection by the 'tail-less' icosahedral phages such as $\phi$X174 which attach directly to the cell surface. However, recently Newbold & Sinsheimer (1970) demonstrated the formation of modified particles after the attachment of $\phi$X174 to starved cells of Escherichia coli C, while Fujimura & Kaesberg (1962) showed that $\phi$X174 can attach to isolated cell walls under conditions which permit attachment to intact bacteria. Although experimental details were not given, they also stated that attachment to cell walls followed by DNase treatment resulted in the release of material with the u.v. extinction properties of virus nucleic acid. We decided to investigate this process further in order to decide to what extent isolated cell walls can induce release of $\phi$X-DNA, and to determine the nature of any other virus products formed.

METHODS

Organisms. Wild-type bacteriophage $\phi$X174 ($\phi$X-wt) and $\phi$X174 am3 ($\phi$X am3), and E. coli strain C, CR34/c416, and K12w6 were kindly provided by Professor R. L. Sinsheimer, California Institute of Technology, Pasadena. $\phi$X am3 contains an amber mutation in the lysis gene, cistron G (Gelfand & Hayashi, 1969) and gives high yields when grown on the
restrictive host *E. coli* c; the permissive host is strain CR34/C416. *E. coli* K12W6 is resistant to all strains of φX.

*Preparation and purification of φX*. Wild-type phage were grown on *E. coli* c according to Sinsheimer (1959), and purified by the method of Tromans & Horne (1961). Stocks of φX am3 were prepared according to Sedat & Sinsheimer (1964) and purified by density gradient centrifugation. Viable phage were assayed by the agar layer technique (Adams, 1959; Sinsheimer, 1959).

*Preparation of radioactive phage*. [32P]-labelled φX am3 were prepared by addition of 20 mc of carrier-free [32P]-phosphate to 20 ml. cultures of *E. coli* c in low-phosphate medium (TPG 3A, Lindqvist & Sinsheimer, 1967, with 2-7 g./l. of casein hydrolysate, not an amino acid mixture) immediately before infection. Doubly labelled φX am3 were obtained by the addition of 2 mc [35S]-sulphate and 2 mc [32P]-phosphate to 20 ml. cultures of *E. coli* c in low-phosphate medium just before infection. The radioactive phage were purified by CsCl equilibrium density gradient sedimentation followed by dialysis against saturated solutions of Na2B4O7. The first procedure gave phage with a specific activity of 1 × 10⁻⁴ counts/min. of [32P]/viable particle; the second procedure gave preparations with 4 × 10⁻⁶ counts/min. of [35S] and 1 × 10⁻⁶ counts/min. of [32P]/viable particle.

*Preparation of Escherichia coli c cell walls*. Cultures of bacteria in 15 l. batches of glycerol medium (Fraser & Jerrell, 1953) were aerated at 37° until a cell density of 1 × 10⁸/ml. was reached. The bacteria were collected by centrifuging and resuspended in 200 ml. of water. The bacteria were broken with glass beads (Ballotini; Grade 12) in a Braun disintegrator (B. Braun, Apparatebau, Melsungen), and the beads and unbroken cells removed by filtering and centrifuging. The cell walls were collected by centrifuging the first supernatant fluid at 34,000 × g for 15 min. and then suspended in 100 ml. of water. The suspension was stored at 0° over CHCl₃. Colorimetric tests for DNA and RNA showed that there was no detectable contamination with cytoplasmic material. About 1 mg. of cell walls was obtained from 5 × 10¹⁰ bacteria.

*Bacteria for adsorption experiments*. Bacteria were grown in glycerol medium to a cell density of 5 × 10⁸/ml., when they were chilled, harvested, and resuspended in distilled water. The bacteria were again sedimented and resuspended in distilled water. The suspension was kept on ice until used (Fujimura & Kaesberg, 1962).

*Bacterial spheroplasts*. These were obtained from *E. coli* K12W6 according to Guthrie & Sinsheimer (1963).

*Sucrose density gradients*. Samples (0·1 to 0·2 ml.) which contained φX and derived particles were layered on to 5 ml. linear gradients of 5 to 30 % (w/v) sucrose in 0·05 M-tris-Cl, 0·003 M-EDTA, pH 8·1. The gradients were centrifuged at 4° for 2 hr at 100,000 g in the SW39 rotor of a Spinco Model L centrifuge. Fractions were collected through a hole made in the bottom of the centrifuge tube. Sedimentation coefficients of particles derived from φX were estimated by comparison with those of known markers. These were intact φX, which sediment with a coefficient of 114S (Sinsheimer, 1959), and [3H]-labelled ribosomal particles from *E. coli* and which have coefficients of 30, 50, and 70S. Particles with the properties of the 70S component of φX preparations (Sinsheimer, 1959) sedimented to the same position as 70S ribosomes. The [3H]-labelled ribosomal markers were kindly provided by Dr M. R. Blundell.

*Caesium chloride gradients*. Solid CsCl was added to suspensions of φX and derived particles to give a density of 1·44 and the mixtures centrifuged for 44 hr at 90,000 g in the SW39 or 50Ti rotor of the Spinco centrifuge. For each gradient, samples were collected from a hole in the bottom of the tube.
Adsorption of CXr74 to cell walls

Radioactive materials and their measurement. Carrier-free $[^{32}P]$-phosphate and $[^{35}S]$-sulphate were obtained from the Radiochemical Centre, Amersham, Bucks. In experiments to determine proportions of acid-soluble material, samples of solutions which contained $[^{32}P]$ were neutralized, dried down on aluminium planchettes and counted in a Lobetamat counter (Isotope Developments Ltd). For volumes of 0.1 to 0.3 ml. the counts recorded were proportional to volume; at least 1000 counts were recorded for all samples. Samples from gradients were collected on to glass-fibre papers (Whatman Chromedia GF82) which were dried and immersed in scintillation fluid which contained 7 g./l. of Butyl PBD (Ciba, Duxford, Cambs.). The $[^{3}H]$, $[^{35}S]$ and $[^{32}P]$ were measured in a Beckman scintillation counter using isosets for $[^{3}H]$, $[^{14}C]$, $[^{32}P]$ with $[^{14}C]$, and $[^{32}P]$. External standard values showed there was no significant quenching of the $[^{32}P]$ and $[^{35}S]$ counts throughout the gradients. All counts were corrected for background and overlap between channels; samples in the peaks on gradients normally contained 15,000 to 20,000 counts/min. The ratio $[^{35}S]/[^{32}P]$ was used to estimate the proportion of protein to DNA in particles derived from $\phi X$; for comparison of different gradients the $[^{35}S]/[^{32}P]$ ratio of the 114S particles on each gradient was taken as 1.

Enzymes. DNase (Grade I) was from Worthington Biochemical Corporation, RNase (Grade I) and lysozyme (Grade A) from Sigma Chemical Co.

RESULTS

Conditions for adsorption of $\phi X$ to bacteria or cell walls

Fujimura & Kaesberg (1962) reported that there was maximum, irreversible adsorption of $\phi X$ to $E. coli$ or to cell walls in unbuffered solutions of 0.1 M-CaCl$_2$ at pH 7.5 and 36°. We found that under these conditions, but in the absence of cell walls or bacteria half the viable phage were inactivated in 1 min. An adsorption medium in which no inactivation occurred contained tris-Cl (0.1 M, pH 7.2 to 9.0) or $\beta\beta'$-dimethyl glutaric acid (0.1 M, pH 4.0 to 7.0) 0.01 M-KCl, and either 0.01 M-CaCl$_2$ or 0.01 M-MgCl$_2$; this medium at pH 8.1 was routinely used for the experiments described here.

We found that maximum adsorption rates occurred at pH 7.2 to 9.0 and at 35 to 45°; Ca$^{2+}$ or Mg$^{2+}$ ions were essential. Adsorption occurred with $E. coli$ C or with cell walls from this strain, but not with bacteria or cell walls of $E. coli$ B which is not a host for $\phi X$. The rate constant $k$ was determined from the equation

$$k = \frac{2.3}{B_t} \log \frac{P_0}{P_t},$$

where $B$ = bacterial concentration, $t$ = time, $P_0$ = initial concentration of $\phi X$, and $P_t$ = concentration of $\phi X$ after time $t$. Under our conditions $k = 3.6 \times 10^{-9}$ ml. sec.$^{-1}$ bacteria$^{-1}$. Fujimura & Kaesberg (1962) obtained $k = 1 \times 10^{-10}$ ml. sec.$^{-1}$ bacteria$^{-1}$ in 0.1 M-CaCl$_2$ at 36°.

Release of DNA from $\phi X$ after adsorption to bacterial cell walls

Fujimura & Kaesberg (1962) concluded that DNA was released from $\phi X$ particles following their adsorption to isolated bacterial cell walls. We tested this in the following way. $[^{32}P]$-labelled $\phi X$ am3 (1 x 10$^8$/ml.) and 0.1 mg. of cell walls were incubated in a total volume of 10 ml. adsorption medium at 37° for 30 min. After addition of DNase (1 µg./ml.) and MgSO$_4$ (0.015 M final concentration) the mixture was incubated for a further 30 min. Samples of 1 ml. were removed and added to chilled tubes which contained 0.5 mg. of bovine serum albumin and HClO$_4$ (0.25 M final concentration). After 15 to 30 min. the tubes
Table 1. Release of acid-soluble $[^{32}P]$-labelled material by DNase treatment of $[^{32}P]$-labelled φX before and after adsorption to cell walls

<table>
<thead>
<tr>
<th>Treatment of sample of $[^{32}P]$ φX</th>
<th>Acid-soluble</th>
<th>Acid-insoluble</th>
</tr>
</thead>
<tbody>
<tr>
<td>No incubation, no DNase</td>
<td>11</td>
<td>91</td>
</tr>
<tr>
<td>Incubated, no DNase</td>
<td>21</td>
<td>75</td>
</tr>
<tr>
<td>Lysozyme, DNase</td>
<td>23</td>
<td>72</td>
</tr>
<tr>
<td>Cell walls, DNase</td>
<td>37</td>
<td>61</td>
</tr>
<tr>
<td>Cell walls; lysozyme and DNase</td>
<td>50</td>
<td>44</td>
</tr>
</tbody>
</table>

Each incubation mixture contained a total of 20,000 counts/min. of $[^{32}P]$.

Table 2. Analysis of components formed after incubating $[^{32}P]$-$[^{35}S]$-labelled φX with cell walls or whole bacteria

(a) φX + cell walls

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>5 min.</th>
<th>40 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total $[^{35}S]$ counts/min. in original 2 ml. incubation mixture</td>
<td>96,500</td>
<td>94,700</td>
</tr>
<tr>
<td>Fraction of total $[^{35}S]$ recovered on gradients</td>
<td>0.900</td>
<td>0.890</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Supernatant fluid fractions:</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Low mol. wt material</td>
<td></td>
<td></td>
</tr>
<tr>
<td>φX particles, 114S</td>
<td>0.004</td>
<td>0.006</td>
</tr>
<tr>
<td>50S particles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>70S particles</td>
<td>0.005</td>
<td>0.014</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pellet fraction:</th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Low mol. wt material</td>
<td></td>
<td></td>
</tr>
<tr>
<td>φX particles</td>
<td>0.320</td>
<td>0.080</td>
</tr>
<tr>
<td>50S particles</td>
<td>0.120</td>
<td></td>
</tr>
<tr>
<td>70S particles</td>
<td>0.330</td>
<td>0.660</td>
</tr>
</tbody>
</table>

(b) φX + washed Escherichia coli c

| Total $[^{35}S]$ counts/min. in 2 ml. incubation mixture | 79,300 | 75,600 |
| Fraction of total $[^{35}S]$ recovered on gradients | 0.660 | 0.760 |

<table>
<thead>
<tr>
<th>Pellet Fraction:</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Low mol. wt material</td>
<td></td>
<td></td>
</tr>
<tr>
<td>φX particles</td>
<td>0.180</td>
<td>0.060</td>
</tr>
<tr>
<td>50S particles</td>
<td>0.110</td>
<td></td>
</tr>
<tr>
<td>70S particles</td>
<td>0.390</td>
<td>0.54</td>
</tr>
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</table>

were centrifuged, the supernatant fluids removed for acid-soluble counts, and each pellet dissolved in 1 ml. of 1 M-NaOH for measurement of acid-insoluble $[^{32}P]$. Samples of the original cell-wall-φX mixture were also treated with lysozyme (0.1 mg./ml. in 0.25 M-tris-Cl, pH 8.1, 0.01 M-EDTA) at room temperature for 30 min. before incubation with DNase. In addition suspensions of φX were incubated with lysozyme and DNase in the absence of cell walls. In each case after acid precipitation, the $[^{32}P]$ in the supernatant fluid and pellet fractions was measured.

The results (Table 1) showed that after adsorption to cell walls there was an increase in the DNase-sensitive $[^{32}P]$-labelled material of the φX preparation. The increase was larger if the virus–cell wall complex was treated with lysozyme before the DNase. It was concluded that some DNA was released from the virus particles following their attachment to cell walls. The effect of the lysozyme is not clear: it may remove cell wall fragments which either protect the DNA from DNase action, or which bind the acid-soluble products formed.
Adsorption of \( \phi X174 \) to cell walls

Fig. 1. Distribution of radioactivity in samples from sucrose density gradient analysis of eluates from bacterial cell walls following adsorption of \([^{32}P]-[^{35}S]\)-labelled \( \phi X \). Upper section: cell wall-virus mixture incubated for 40 min. Lower section: same mixture incubated for 5 min. -\( \Delta \)-\( \Delta \), \([^{32}P]\); -O--O--O, \([^{35}S]\); -\( \square \)--\( \square \), ratio \([^{35}S]/[^{32}P]\).

Identification of particles formed after attachment of \( \phi X \) to bacterial cell walls or starved bacteria

Samples which contained \( 2 \times 10^8 \) p.f.u. of \([^{32}P]-[^{35}S]\)-labelled \( \phi X \) am3 were incubated with 0.02 mg. of cell walls in 2 ml. of adsorption medium pH 8.0 at 37° for 5 min. or for 40 min. The mixtures were then chilled, centrifuged at 0° for 30 min. at 32,000 g and the supernatant fluid removed. The pellets which contained 74 % of the initial [\( {^{35}S} \)] were suspended in 2.25 ml. of 0.05 M-Na\( _2 \)BO\( _4 \), 0.003 M-EDTA and left overnight at 4° to elute virus and other components from the cell walls. Samples (0.1 to 0.2 ml.) of the eluates and of the original supernatant fluid were then analysed by sedimentation through sucrose density gradients.

Exactly similar experiments were done with suspensions of whole bacteria. The results are summarized in Table 2. They show that after 5 min. incubation with cell walls, most of the virus [\( {^{35}S} \)] is associated with the pellet fraction from which it is recovered as a mixture of intact particles and 50S and 70S particles. After 40 min. incubation no 50S particles were recovered, and most of the pellet fraction [\( {^{35}S} \)] was present as 70S particles. Similar results were obtained when intact bacteria were incubated with \( \phi X \).

Characterization of components from cell wall-\( \phi X \) complexes

(a) [\( {^{35}S}/^{32}P \)]-ratio

The relative amounts of [\( {^{35}S} \)] and [\( {^{32}P} \)] indicate the proportions of protein and DNA respectively associated with \( \phi X \) and particles derived from it. The results shown in Fig. 1
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indicate that the 50s component contains the same proportions of DNA and protein as intact φX. The $^{[35S]/[32P]}$-ratio of the 70s component is higher than that of φX and its density (see (c)) is less.

(b) Infectivity of 50s particles

A sample which contained $2 \times 10^9$ p.f.u. of $^{[32P]}$-$^{[35S]}$-labelled φX and $4 \times 10^3$ unlabelled φX was incubated with 0.02 mg. of cell walls in 2 ml. of adsorption fluid at 37° for 2 min., and the borate-EDTA eluate of the pellet fraction obtained as before. The eluate of radioactive components was fractionated by sedimentation through a sucrose density gradient. Alternate drops were collected for scintillation counting and the others each diluted into 0.6 ml. of phage dilution fluid. The infectivity of these samples towards *E. coli* cr34/c416 and towards spheroplasts of strain K12.w6 was measured. The result shown in Fig. 2 indicated that the 45 to 50s component was infectious towards spheroplasts but not towards whole bacteria.

(c) Buoyant density and action of DNase

Following procedures described in (b) above, radioactive material corresponding to the 50s and 70s components was isolated from sucrose density gradients. Samples of the separately pooled components were treated with pancreatic DNase (1 µg./ml.) in the presence of 0.01 M-MgSO₄ at 37° for 30 min. Wild-type φX were then added as a reference marker and the contents of the mixtures analysed by sucrose density gradient centrifugation (Fig. 3), or by centrifuging to equilibrium in CsCl density gradients (Fig. 4).

The results show that 50s particles have the same buoyant density as intact φX particles, and that treatment with DNase converts 50s particles to material with the same sedimenta-
Adsorption of φX174 to cell walls

Fig. 3. Sucrose density gradient sedimentation analysis of isolated components eluted from bacterial cell walls after incubation with [32P]-[35S]-φX am3. (i) 70s component. (ii) 50s component after DNase treatment. 5 × 10⁶ p.f.u. of wild-type φX were included in each gradient as a sedimentation marker. –△—△–, [32P]; –○—○–, [35S]; ■—■–, plaques of wild-type φX assayed on Escherichia coli C.

tion velocity and the same density as 70s particles. The 70s component was unaffected by DNase treatment.

The high [35S/32P]-ratio, reduced density and sedimentation velocity relative to intact φX show that the 70s material has a higher protein/DNA ratio than whole particles. These properties together with the value of the sedimentation coefficient indicate that the 70s material is probably identical with the ‘top component’ of partially filled phage particles obtained from lysates by Sinsheimer (1959) and others (Maclean & Hall, 1962; Daems et al. 1962; Bleichrodt & Knijnenburg, 1969).

The 50s component’s buoyant density and [35S/32P]-ratio show that it has the same proportions of protein and DNA as intact φX. This, its infectivity towards spheroplasts, and the changes in density and sedimentation velocity caused by DNase suggest that the 50s component consists of partially disrupted particles in which some virus DNA is trailing from the protein coat. They therefore resemble the 57s particles obtained from cell lysates by Dann-Markert, Deutsch & Zillig (1966), and the 47s particles produced by heating φX particles to 70° (Guthrie & Sinsheimer, 1960).
Fig. 4. Caesium chloride equilibrium density gradients of the same components described in Fig. 3. (i) 50s component incubated without DNase. (ii) 50s component after DNase treatment. (iii) 70s component after DNase treatment. —Δ—Δ—, [32P]; —O—O—, [35S]; ■ ■, plaques of wild-type φX density marker.

DISCUSSION

These results show that attachment to bacterial cell walls can cause disruption of φX particles with the partial release of virus DNA from its protein coat. One product is a 50s particle which is infectious to spheroplasts, while incubation for extended times results in the appearance of 70s particles which have a diminished DNA content.

The 50s particles may be intermediates in natural infections, but our experiments cannot provide direct evidence on this point since we used cell walls or starved bacteria.
Adsorption of φX174 to cell walls

Knippers et al. (1969) have shown that the transfer of φX DNA from virus particles to an intracellular site requires metabolizing bacteria. Newbold & Sinsheimer (1970) showed that at 37°, particles previously attached to starved bacteria in a tris-Cl-salts-starvation buffer were converted to particles which sediment at about 84s. When nutrients were added a fraction of the 94s particles attached to bacteria disappeared and equivalent amounts of empty protein coats and virus DNA (as intracellular replicating intermediate) were formed. The 94s or ‘eclipsed phage’ particles were infectious for spheroplasts but not for intact bacteria. Nuclease treatment converted them to 70s particles. Newbold & Sinsheimer (1970) concluded that the 84s particles were intermediates, or were derived from intermediates, of infection and consisted of modified phage with a portion of virus DNA protruding from the protein coat. The particles were reported to be unstable in Mg²⁺-tris buffer, and to show a slow decrease in sedimentation coefficient on storing at 0° in borate-EDTA solution.

It is therefore not surprising that we did not detect 84s particles because even if formed they would be unstable in the adsorption buffer used. However, even in our experiments 84s particles may have been formed initially and then converted to those of 50s which they resemble except for the difference in sedimentation coefficients, and this could depend only on differences in the amounts of DNA trailing from the protein coats. The absence of 50s particles in the systems studied by Newbold & Sinsheimer (1970) could be accounted for by the stability of 84s particles in their conditions; in addition, rapid uptake by bacteria of virus DNA from 84s particles may not permit isolation of a 50s type of particle.

In conclusion it appears that contact with bacterial cell walls can initiate disruption of φX virus particles, the 50s and 70s particles representing intermediate and final products when normal infection does not occur. The appearance of these components in lysates of bacteria infected with φX may be accounted for in this way.

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REFERENCES


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