Adsorption of Phage P22 to *Salmonella typhimurium*

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**SUMMARY**

Adsorption of phage P22 to its receptor in the lipopolysaccharide (LPS) of the envelope of *Salmonella typhimurium* is accompanied by a hydrolytic cleavage of the O polysaccharide chain. The enzyme, an endorhamnosidase, is found in the phage tail. Propagation of a mutant of phage P22, containing two amber mutations, under restrictive conditions permitted isolation of phage tail parts with endorhamnosidase activity. The tail parts, purified by ion exchange chromatography, were shown to be homogeneous by polyacrylamide gradient gel electrophoresis, isoelectric focusing in polyacrylamide gel electrophoresis and crossed immunoelectrophoresis. The mol. wt. was estimated to 240,000. The optimal pH range for glycosidase activity was 5 to 7 and optimal temperature 37°C. Hydrolysis of the O polysaccharide chain, when estimated with whole bacteria as the substrate, did not seem to be influenced by the cation concentration. Eclipse of P22 phage particles to whole bacteria was likewise uninfluenced by the cation concentration in the reaction mixture, but eclipse by isolated receptor containing LPS required cations. The optimal concentration for divalent cations was $2 \times 10^{-3}$ M, for trivalent cations $1 \times 10^{-3}$ M.

**INTRODUCTION**

It has repeatedly been shown that phages active on encapsulated Gram-negative bacteria, like *Escherichia coli* and *Klebsiella* have a phage-associated endoglycosidase or acetylase activity (Lindberg, 1976). The phage bound capsulolytic enzymes appear to assist in the penetration of the capsule in the search for a proper site for phage DNA injection. This must probably be considered as a necessary mechanism for the infectious process of these phages since a capsule may extend up to 400 nm from the cell wall proper.

It was recently found that phages having their receptor in the O antigen of the lipopolysaccharide (LPS) of *Salmonella* and *E. coli* also have phage associated endolytic activity. Thus, the phage tail of phage $\phi^{18}$ has endorhamnosidase activity hydrolysing the Rha 1 $\rightarrow$ 3 Gal linkage in the O polysaccharide chain of *Salmonella anatum* (Kanegasaki & Wright, 1973; Takeda & Uetake, 1973). At the same time it was found that phage P22, or isolated tail parts from it, destroyed the O antigen of *S. typhimurium* (Israel, Rosen & Levine, 1972), also hydrolysing the Rha 1 $\rightarrow$ 3 Gal linkage of the O polysaccharide chain (Iwashita & Kanegasaki, 1973; Lindberg, 1973). In *E. coli*, phage $\phi^{8}$ was found to hydrolyse the Man 1 $\rightarrow$ 3 Man linkage between successive repeating units in the O polysaccharide chain (Wallenfels & Jann, 1974).

The molecular events involved in binding of the phage to its receptor are, however, incompletely known. For a closer study of the binding step it is advantageous to have the
phage organelle with hydrolytic activity in soluble form. In this paper we describe a method for isolation and purification of isolated P22 tail parts with endoglycosidase activity, and a simple assay system for demonstration of the enzymatic activity.

We furthermore report on the influence of varying the ionic, pH, temperature and time condition on the hydrolytic activity of phage P22 and isolated tail parts.

METHODS

**Bacteria.** *Salmonella typhimurium* LT2, *S. typhimurium* SL 696 (a subline of strain LT2; Wilkinson *et al.* 1972), *S. typhimurium* LT2-M1 (a UDP-galactose-4-epimeraseless mutant), *S. typhimurium* SL 4049 (a subline of strain LT2; *tris* C 527am, *trp* A, *wh2*, *HI*-enx, *str*, *gal*), *S. typhimurium* SL 4051 (a subline of strain LT2 which is *fla B*-36, *am sup W*/*am*) were from the collection of Dr B. A. D. Stocker, Department of Medical Microbiology, Stanford, Calif., U.S.A. *S. typhimurium* CV2 (a *S. typhimurium* LT2 derivative which carries P22 *ts* 29 *am N9* and which is non-permissive for phage carrying an amber mutation) was obtained from Dr A. Wright, Department of Molecular Biology and Microbiology, Tufts Medical School, Boston, Mass., U.S.A.

**Bacteriophage.** Phage P22c2 was from the collection of Dr B. A. D. Stocker, phage P22c1 *am N114, am H 101* was obtained from Dr A. Wright.

**Preparation of bacteriophage P22c2.** Phage P22c2 in high yield was obtained by infecting log phase cells (3 to 5 × 10⁸ bacteria/ml) of *S. typhimurium* SL 696 in nutrient broth (NB) (Difco) at 37°C with a m.o.i. of 2 to 4. The culture was harvested after 90 to 120 min of incubation; Sarkosyl, EDTA and NaCl were added to final concentrations of 0.5%, 5 mM and 500 mM, respectively. Whole cells and debris were removed by centrifuging at 3000 g at 4°C. Phage P22c2 was concentrated by adding polyethylene glycol (PEG 6000) to a final concentration of 10%, according to Yamamoto & Alberts (1970). The precipitate was collected and dissolved in 0.05 M-tris-HCl buffer, pH 7.5. The phage was purified by density equilibrium centrifugation: solid CsCl was dissolved in the precipitate to a density of 1.50 g/ml and spun to equilibrium in an SW 40 rotor at 100,000 g for 20 h. The gradient was harvested by punching the bottom of the tube and collecting 1.0 ml fractions.

**Preparation of phage P22 heads.** Phage heads were prepared according to a procedure given by Dr A. Wright. The P22 lysogen *S. typhimurium* CV2 was grown in NB on a rotary shaker at 30°C for 2 h. The cells were spun down and the pellet resuspended in NB at 40°C and incubated until lysis occurred. Cellular debris was pelleted by centrifuging at 3000 g for 15 min at 4°C. The supernatant fluid contained less than 10⁸ p.f.u./ml. Phage heads were purified either by sedimenting them at 35,000 g for 60 min at 4°C in an angle rotor or by density equilibrium sedimentation in a CsCl gradient as described below. The head titre was assayed in tail-head association experiments.

**Preparation of phage P22 tails.** Phage P22c1 *am N114, H 101* with amber mutations in head protein and lysis gene functions was grown on the non-permissive strain *S. typhimurium* SL 4049. Log phase bacteria (3 to 5 × 10⁸/ml) grown in NB on a rotary shaker, were infected with the phage at m.o.i. 10 and incubated at 35°C for 40 min. The culture was rapidly cooled to 0°C and the cells pelleted by centrifuging in a refrigerated centrifuge at 3000 g. The pellet was suspended in 0.05 M-imidazole buffer, pH 6.5, in 1/20 of the original volume. The bacteria were broken by freeze-thawing three times using a dry ice-ethanol mixture. Whole bacteria and cellular debris were removed by centrifuging in a refrigerated centrifuge at 5000 g for 20 min and whole phage by centrifuging at 35,000 g for 60 min. The supernatant fluid was reduced by pervaporation and then dialysed against the imidazole buffer in
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the cold. The tails were purified by ion exchange chromatography using a column packed with Whatman cellulose DE 32 equilibrated with the imidazole buffer. Tails were eluted with 0.05 M-KCl in the imidazole buffer at pH 6.5, whereas phage was eluted with 0.15 M-KCl in the same buffer. Fractions were assayed for endoglycosidase activity and p.f.u. Tail-containing fractions were pooled and concentrated by ultrafiltration (Collodion-Bags SM 13200, Sartorius Membranfilter, Göttingen, West Germany). The concentrate was dialysed against the imidazole buffer in the cold. The tail preparation was assayed for (i) titre in association experiments with isolated P22 heads, and (ii) endoglycosidase activity using 14C-labelled S. typhimurium LT2-M1 bacteria. Tail-head-assembly experiments were carried out in the Phage-80 buffer of Wright & Barzilai (1971), but bovine serum albumin was used instead of gelatin (Pentex crystallized bovine albumin 81-001, lot 22, Miles Laboratories Inc., Kanakee, Ill., U.S.A.). Pre-warmed dilutions of tails and heads were mixed and buffer was added to 1.0 ml final volume. After 3 h incubation at 37 °C on a rotary shaker the phage titre was assayed by the spot-on-lawn method using the permissive strain S. typhimurium SL 4001.

Phage P22 and tail endoglycosidase activity assays. S. typhimurium LT2-M1, deficient in UDP-galactose-4-epimerase was grown in a tryptone-yeast extract medium with 1% glucose (Hellerqvist et al. 1968) vol. 1000 ml, on a rotary shaker under aeration at 37 °C to mid-log phase (approx. 5 x 10^8 cells/ml). The bacteria were cooled and pelleted by centrifugation at 3000 g for 15 min at 4 °C. For labelling the pellet was resuspended in 500 ml fresh TY-medium with 1% glucose, 0.2% galactose and 250 μCi 1-14C-D-galactose (NEC-302X, New England Nuclear Chemicals), and incubated on a rotary shaker at 37 °C for 3 h. The bacteria were pelleted by centrifugation as above, and resuspended in 500 ml of fresh TY-medium without glucose and galactose to utilize the intracellular lactose pool for 60 min as above. Formaldehyde was added to a final concentration of 0.5% (w/v) to kill the bacteria, but also to make them resistant to autolysis. The cells were stored at 4 °C and found to be stable for at least 12 months. Under these conditions 5 to 10% of the specific label was found in the bacterial LPS. Before each experiment the bacteria were pelleted at 3000 g, resuspended in the buffer to be used in the experiment and stored at 4 °C for 40 min, and then pelleted again. This procedure was repeated twice. Approx. 5 x 10^8 bacteria, equivalent to 4000 to 5000 ct/min, were used in each experiment. For estimation of phage or tail associated endoglycosidase activity, phage or tails were mixed with the bacteria in the appropriate buffer and incubated at 37 °C for 60 min. The reaction was stopped by pelleting the bacteria at 3000 g for 15 min in a refrigerated centrifuge. The supernatant fluid was assayed for 14C activity using Insta-Gel (Packard Instrument International S.A.) 1 ml waterphase and 10 ml scintillation fluid. The samples were counted in an Intertechnique Liquid Scintillation Spectrometer SL31.

Preparation of LPS. Phenol-water extraction of LPS was performed as described earlier (Lindberg & Holme, 1972).

Phage inactivation by LPS. Inactivation experiments were carried out in 0.05 M-tris-HCl, pH 7.4, containing 0.01% bovine albumin (Pentex) supplemented with various concentrations of metal chlorides. LPS of S. typhimurium LT2 and P22c2 were diluted and pre-incubated separately for 1 h at 37 °C and 50 μl LPS of various concentrations and 50 μl P22c2 (10^4 p.f.u.) were mixed and incubated at 37 °C for 18 h on a rotary shaker. From each tube 2 x 10 μl were spotted on a lawn of log phase bacteria (SL 696) and the numbers of plaques were counted after 4 h incubation at 37 °C.

Analytical procedures. Polyacrylamide gradient gel electrophoresis was performed on Pharmacia gradient gel PAA 4/30 (Pharmacia Fine Chemicals, Uppsala, Sweden), batch no.
Bo27. The buffer was 0.09 M tris-HCl, 0.08 M boric acid, 0.003 M EDTA, pH 8.4. The gel was pre-run for approx. 2 h at 120 V before a sample was applied. The electrophoresis was run at 120 V for 17 h and the temperature in the system was kept constant at 5 °C by a refrigerated thermostat bath. Gels were stained in a solution of Coomassie blue R (0.1%, w/v) acetic acid (8.5%, v/v), ethanol (27%, v/v) and destained in a solution of acetic acid (8.5%, v/v) and ethanol (27%, v/v).

Isoelectric focusing was done according to the method of Söderholm & Lidström (1975) and Söderholm & Wadström (1975). Polyacrylamide plates (115 × 250 mm) were prepared with acrylamide (48.5%, w/v), bisacrylamide (0.15%, w/v) and riboflavin 500 ng/ml (T = 5%, w/v, C = 3%, w/w). The total ampholyte concentration was 2.0% (v/v) in the pH range 3.5 to 10. The sample applied on a filter paper square (8 × 6 mm) was placed directly on the gel surface. The electrophoresis was run at a constant 50 W for 1 h at 4°C. At the end of the electrophoresis pH was measured with a surface electrode every 5 mm on a line from anode to cathode. The gel was placed in a fixation bath (2%, w/v, sulphosalicylic acid, 11%, w/v, trichloroacetic acid and 27%, v/v, methanol in distilled water) at 56 °C for 30 min.

The staining procedure was the same as used for gradient gel electrophoresis. The gel was scanned at 600 nm, in a Zeiss PMQ3 spectrophotometer supplied with scanner ZK3.

Crossed immunoelectrophoresis (CIE) was performed as described by Holmberg, Nord & Wadström (1975), using a rabbit anti-phage serum, prepared by immunization with P22c2 (purified on CsCl-gradient) and Freund’s complete adjuvant (Difco). The staining procedure was the same as used for gradient gel electrophoresis.

Sugar analyses: total carbohydrate was estimated by the phenol-sulphuric acid method (Nowotny, 1969) with glucose as the standard. Reducing endgroups were determined according to the Park-Johnson method (Park & Johnson, 1949) with rhamnose as the standard.

RESULTS

Preparation of phage P22 tails

The procedure which was developed for the preparation of large quantities of P22 tails is a modification of the procedure followed by Israel, Anderson & Levine (1967). The major difference is that the purification is done under mild conditions in order not to denature the protein(s). The procedure thus involves propagation of the phage P22 mutant under non-permissive conditions, rupturing of the cells by freeze-thawing and differential centrifugation to remove bacterial cell debris and whole phage particles. The subsequent purification step involved ion exchange chromatography (Fig. 1).

The mixture contained approx. $1 \times 10^8$ p.f.u./ml before it was applied on to the ion exchange column; the pooled and concentrated tail preparation contained approx. $1 \times 10^2$ to $10^4$ p.f.u./ml. The procedure is well suited for large scale preparation. A batch of 1000 ml yields 5 to 10 ml of purified tail protein containing approx. $0.5$ to $0.8$ mg protein/ml and $10^{10}$ to $10^{11}$ tail equivalents/ml. The preparation contained approx. 4% of carbohydrates. No aminosugars were detected.

Characterization of the tail protein

The homogeneity in terms of the mol. wt. of the tail proteins was studied by polyacrylamide gradient gel electrophoresis. One band only was found. The tail protein migrated, as compared to internal standards, close to catalase which has a mol. wt. of 240000 (Fig. 2). The homogeneity in terms of charge of the tail protein was estimated by isoelectric focusing in polyacrylamide gel electrophoresis (Fig. 3). The tail protein focused at pH 4.9, and one
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Fig. 1. Ion exchange chromatography of phage P22 tail preparation. The sample was applied on to a Whatman cellulose DE32 column (1 × 2 cm) equilibrated in 0.05 M-imidazole-HCl, pH 6.5. Arrows indicate stepwise elution by addition of 0.05, 0.10 and 0.15 M-KCl to the elution buffer. ●—●. Endoglucosidase activity tested on ¹⁴C-labelled bacteria. ×—×, p.f.u. tested on the phage P22 ct am N154, am H101 permissive strain SL4051.

Fig. 2. Polyacrylamide gradient gel electrophoresis of purified phage P22 tail preparation. Samples were applied to the gel (Pharmacia gradient gel PAA 4/30) after 2 h pre-running (120 V). Buffer: 0.09 M-tris-HCl, 0.08 M-boric acid, 0.003 M-EDTA, pH 8.4. The electrophoresis was run at 120 V for 17 h at 5°C. The proteins were stained with Coomassie blue. A, 48 µg and B, 24 µg phage P22 tail preparation, C, catalase, D, bovine serum albumin, E, transferrine, F, ceruloplasmine, G, ferritin, H, thyroglobulin.
Fig. 3. Isoelectric focusing of purified P22 tail preparation. Polyacrylamide plates (115 × 250 mm, T = 5%, w/v, C = 3%, w/w) with a total ampholyte concentration of 2%, w/v (pH 3.5 to 10) were run at constant wattage (50 W) for 1 h at 4°C. pH was measured every 5 mm at the end of the run. After fixation, staining was performed with Coomassie blue. The dried gel (thickness 1.5 mm) was scanned at 600 nm. ○—○, pH; — E_{600 nm}

Fig. 4. Crossed immunoelectrophoresis of purified P22 tail preparation. 5 μl protein (1 mg/ml) was run in 1% (w/v) agarose (Miles Seravac, Berks, England) layer size 1 × 50 × 50 mm. The first dimension electrophoretic run was done at 9 V/cm for 60 min and the second dimension (gel containing 5 μl/cm² of anti-P22 serum) at 9 V/cm² for 60 min. Staining procedure with Coomassie blue was the same as used for gradient gel electrophoresis.
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Table 1. Oligosaccharide released by P22 tail parts from S. typhimurium LT2 bacteria pre-treated in various ways

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Total carbohydrate released: μg/mg (dry wt.)</th>
<th>Reducing endgroups released: μg/mg (dry wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>24</td>
<td>1.9</td>
</tr>
<tr>
<td>Heat inactivated</td>
<td>15</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>KCN-poisoned</td>
<td>22</td>
<td>1.6</td>
</tr>
<tr>
<td>Formaldehyde inactivated</td>
<td>45</td>
<td>4.2</td>
</tr>
</tbody>
</table>

band only was seen. The whole P22 phage particle focused at pH 4.7 (data not shown). In crossed immunoelectrophoresis with anti-P22c2 serum one precipitate was seen (Fig. 4).

**Phage P22 mediated hydrolysis of S. typhimurium O antigen**

For investigation of the phage P22 associated endorhamnosidase activity, a simple assay system had to be set up. This was most easily done by using whole bacteria rather than isolated LPS (or polysaccharide fractions of it) as the substrate. Log phase cells of *S. typhimurium* LT2 were used as substrate after washing and resuspending in imidazole buffer: living bacteria, bacteria poisoned by 0.005 M-KCN, bacteria killed by heating to 100°C for 1 h, and bacteria killed by addition of formaldehyde (0.4%, final concentration) were mixed with purified P22 tails (1 phage equivalent/100 bacteria). Bacteria were separated from liberated oligosaccharides by centrifugation (3000 g for 15 min at 4°C) and the supernatant fluid assayed for total carbohydrates by the phenol-sulphuric acid, and reducing end-groups by the method of Park & Johnson (1949).

The amount of oligosaccharides released, expressed as μg/mg bacteria (dry weight), was highest for formaldehyde treated bacteria and was approx. twice the amount from living, cyanide poisoned or heat killed bacteria (Table 1). This conclusion was strengthened by the observation that the amount of reducing end-groups was proportionally the same for all preparations except boiled bacteria. Since the formaldehyde treated bacteria were stable for up to twelve months, they were subsequently used as substrate for demonstration and measurement of phage P22 endorhamnosidase activity.

The method was made even more sensitive by specifically labelling the O polysaccharide chain. This was attained by growing a UDP-galactose-4-epimeraseless mutant of *S. typhimurium* in the presence of 1-14C-D-galactose. These bacteria, unless supplied with exogenous galactose, cannot synthesize the core beyond the Glc residue (Fig. 5) and are also unable to synthesize the O repeating unit.

Labelled bacteria diluted to a concentration of about 5×10^9 bacteria were used in subsequent experiments; approx. 5000 ct/min/experiment.

**Ionic requirement for phage P22 inactivation**

In the bacteria LPS is a structural component of the outer membrane where it is thought to be bound primarily by ionic and/or physical forces, an ionic environment different from that seen with extracted LPS. Therefore, we first studied the phage inactivation with whole bacteria. The phage P22 inactivation experiments were performed in buffer solutions supplemented with different concentrations of mono-, di-, and trivalent cations. No significant ion dependence, either in phage inactivation or O antigen hydrolysis, was observed when the concentration of Na⁺, K⁺, Mn⁡±, Ca²⁺, Mg²⁺, Ni⁡± and Co⁢± was varied within the range 5×10⁻⁵ M to 2×10⁻⁸ M (data not shown). However, with phenol-
extracted LPS as receptor instead of whole bacteria, a marked salt dependence was observed (Fig. 6). A concentration of LPS which caused about 50% inactivation of phage P22 was chosen. The salt dependence of phage inactivation was best observed with divalent cations. For MnCl₂ the optimal concentration was $2 \times 10^{-3} \text{M}$ (Fig. 6). Other divalent cations like Ca²⁺ and Mg²⁺ did not differ from Mn²⁺ in their ability to promote phage P22 inactivation. The effect of one trivalent cation, Co³⁺, was also tested. The optimal concentration was the
Fig. 7. Phage P22 tail-endoglucosidase activity in presence of MnCl₂. Five × 10⁸ ³⁴C-galactose labelled bacteria were mixed with different tail concentrations to a total vol. of 1·0 ml in 0·05 M-tris-HCl, pH 7·4, containing varying amounts of MnCl₂. After 1 h on a rotary shaker at 37 °C, 2·0 ml cold buffer was added and the suspensions were centrifuged at 4 °C, 3000 g, for 15 min. Samples of 2 × 1×0 ml were withdrawn from each supernatant fluid for scintillation counting. ×—×, 1 × 10⁸; ○—○, 1 × 10⁷ and △—△, 2 × 10⁶ tail equivalents/ml.

same as for the divalent cations. With monovalent cations no marked influence on phage inactivation was observed. One must stress, however, that the effect of di- and trivalent cations was on P22 phage inactivation only. The extent of enzymatic hydrolysis of the O chain was unaffected within the concentrations of cations tested.

The influence, if any, of cations on the hydrolytic process of isolated tail parts as opposed to phage particles was also studied. With tail titres equivalent to 1 × 10⁶/ml and bacterial concentrations of 5 × 10⁹ cells/ml, i.e. receptor excess, no salt dependence was observed. With lower tail titres, a slight reduction of the enzymatic activity was observed when the cation concentration was increased. This was most pronounced for di- (Fig. 7) and trivalent cations.

Thus, the hydrolytic process is relatively slightly influenced by the cation concentration. This is true both for phage particles and isolated tails. Phage inactivation by whole bacteria also proceeds without addition of cations, probably as a consequence of the cation content of the cell envelope. Phage P22 inactivation by isolated LPS does not occur, however, unless the reaction mixture is supplemented by di- or trivalent cations. Thus, for inactivation of phage P22, whole bacteria provide the ionic conditions required, whereas isolated LPS has to be supplemented with approx. 10⁻³ M divalent cations for phage inactivation to occur.

Influence of temperature and pH on phage P22 and tail mediated hydrolysis

The optimum temperature for phage P22 and tail mediated hydrolysis was estimated with whole bacteria as the substrate. As expected for an enzymatic reaction the optimum temperature was around 37 °C (Fig. 8). The experiments were done in the tris-BSA buffer at pH 7·4. Addition of 2 × 10⁻³ M-MnCl₂ did not affect the outcome of the experiments.

The optimal pH for hydrolysis with both phage and tails was assayed with the same system using an acetate buffer in the pH region 4·0 to 5·5, an imidazole buffer at pH 6·5, the tris-HCl buffer in the pH region 7·4 to 8·5 and the glycine-NaOH buffer at pH 9·5. The formalin
Fig. 8. Effect of temperature on phage P22 tail endorhamnosidase activity. Assays were performed in 0.05 M-tris-HCl, pH 7.4, and each suspension was separately pre-incubated at the different temperatures. $1 \times 10^8$ p.f.u. or $8 \times 10^7$ tail equivalents were incubated with $5 \times 10^9$ $^{14}$C-galactose labelled bacteria in 1.0 ml total vol. for 60 min. Two ml of cold buffer was then added and followed by immediate centrifugation at 4°C, 3000 g, for 15 min. Samples of $2 \times 10^{-3}$ ml from each supernatant fluid were counted by liquid scintillation. $\Delta - \Delta$, Radioactivity released by tails; $\bullet - \bullet$, radioactivity released by phages.

Fig. 9. Effect of pH on phage P22 endorhamnosidase activity. Buffers used: pH 4.0, 4.7 and 5.5, 0.05 M-acetate; pH 6.5, 0.05 M-imidazole-HCl; pH 7.4, 8.5 M-tris-HCl; pH 9.5, 0.05 M-glycin-NaOH. Washings and dilutions of bacteria, phages and tail preparations were performed in the separate buffers. Experimental conditions were the same as in Fig. 8 and the assays were run at 37°C. Plaque forming units were tested from the supernatant fluid by spotting $2 \times 10^{-3}$ µl on a lawn of log phase bacteria $S. typhimurium$ (SL 696). $\Delta - \Delta$, Radioactivity released by tails; $\bullet - \bullet$, radioactivity released by phages; $\bigcirc - \bigcirc$, p.f.u.
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Fig. 10. Effect on time of phage P22 endorhamnosidase activity. $7 \times 10^{10}$ p.f.u. or $8 \times 10^{7}$ tail equivalents were incubated with $5 \times 10^{8}$ 14C-galactose labelled bacteria in 0.05 M-tris-HCl, pH 7.0 at 37°C. Samples were withdrawn at different times and radioactivity and p.f.u. were measured as in Fig. 9. △—△, Radioactivity released by tails; ●—●, radioactivity released by phages; ○—○, p.f.u.

Maximal hydrolysis was observed around pH 6.5 for both phage particles and isolated tails (Fig. 9).

Influence of time and enzyme concentration of phage P22 and P22 tail mediated hydrolysis

The time course of the cleavage of the O antigen was also studied. In the experiment using P22 phage particles and formaldehyde treated bacteria there was about one phage per bacterium, i.e. the substrate was in considerable excess. As can be seen in Fig. 10, about 50% of the hydrolysis occurred within the first 5 min and had reached 80% within 15 min. Incubation for periods greater than 60 min did not increase the yield of oligosaccharides. With whole bacteria as the substrate, phage P22 is irreversibly inactivated. The data also show that after 15 min only 3% of input phage retained its infectivity.

The experiment with isolated tails instead of phage particles was done under the same conditions. The tail titre, expressed as phage equivalents when determined in head-tail association experiments, was approx. $10^8$. The velocity of the reaction with isolated tails was approx. the same as with whole P22 phage particles (Fig. 10). The total amount of oligosaccharides released by free tails was higher than that released by an equal concentration of P22 phage particles. This is easily explained by the fact that the binding of phage P22 results in an irreversible inactivation of the phage whereas the tails are not inactivated but remain on the bacterial surface releasing oligosaccharides.

One effect of the inactivation of P22 could also be seen in the difference in equivalent of tails and phages required to give maximal release of oligosaccharides, $2 \times 10^{10}$ phage particles compared to only $2 \times 10^7$ tail equivalents (Fig. 11).
Fig. 11. Effect of phage and tail concentration on amount of *S. typhimurium* O-polysaccharide release. Various concentrations of phage and tail parts were incubated with 5 × 10⁶ ¹⁴C-galactose labelled bacteria at 37°C for 1 h. The experimental conditions were as described in legend to Fig. 8. △—△, Radioactivity released by tails; ⋄—●, radioactivity released by phages.

**DISCUSSION**

The details of the initial events in phage adsorption are still incompletely known. It is our intent to study binding and eclipse of phage P22 to *S. typhimurium* in more detail. This system has evident advantages since both the phage and the bacterium are well characterized. There are several P22 mutants available (Levine, 1972), and the structure of the receptor in the LPS is well known (Lindberg, 1973). This study involved the isolation, purification and characterization of the phage tail parts with hydrolytic properties. The activity of the soluble tail parts were also compared with the activity of the intact P22 phage particle.

The main aim in the isolation and purification procedure of P22 tails was to establish a mild procedure suitable for large scale production. Since several P22 phage mutants exist it is not necessary to rely on dissociation of the phage with chemical reagents, a procedure which often damages the structural proteins (Rudolph, Freund-Mölbert & Stirm, 1975). Tail parts were instead produced by propagation of mutant phage P22 *cl am N114, H101*, which has two amber mutations, under non-permissive conditions. The bacteria, after pelleting by centrifugation, were ruptured by freeze-thawing to release the tail parts. The purification procedure then involved only differential centrifugations, dialysis and ion exchange chromatography with stepwise elution. The resulting preparation, about 1 ml from a 11 batch, contained approx. 10¹¹ tail units/ml and 10⁶ to 10⁸ p.f.u./ml. The procedure is simple and has been repeated several times with similar yields.

The tail part preparation contained protein, and less than 4% of carbohydrates and nucleic acid. This indicates a pure preparation suitable for analytical studies. Determination of N-terminal amino acids revealed only one amino acid, which is a further indication of the purity of the preparation (data not shown).

Polyacrylamide gradient gel electrophoresis, which estimates the homogeneity in terms of mol. wt., and isoelectric focusing in polyacrylamide gel electrophoresis, which estimates the homogeneity in terms of charge, revealed one band only. In crossed immunoelectrophoresis also, with antiserum directed against the whole phage particle, only one precipitate was seen.
This procedure would have detected contamination by phage head proteins, but none was seen. The amount of protein had to be about 1 µg for detection. Electron microscopic examination with a negative staining technique, failed to reveal the presence of structures other than those identical to the ones reported by Israel et al. (1967).

The ability of the purified tails to associate with isolated phage P22 heads resulting in infectious phage particles was also routinely tested. Israel et al. (1967) empirically deduced a formula for estimating the number of tail parts per head required to produce one infectious phage particle. The number in our preparation varied between 1.5 and 2.0, whereas Israel et al. (1967) reported a value of 3.3. Assuming that the head preparations were more or less similar, this indicates that our tail preparations have as great an ability to associate with heads forming infectious phages as those preparations used by Israel et al. (1967).

Thus, it seems safe to conclude that the following assays were performed with a pure, and homogeneous, preparation of phage P22 tails.

Phage P22 hydrolyses the Rha 1→3 Gal linkage in the repeating unit of the O polysaccharide chain of S. typhimurium (Fig. 5). This was determined using gas liquid chromatography–mass-spectrometry of permethylated alditol acetates of LPS preparations before and after P22 hydrolysis (A. A. Lindberg, unpublished observations).

A simple assay system had to be set up for estimation of the phage tail endorhamnosidase activity. This was necessary in order to be able to follow both the purification of phage tail parts, and the influence of variation of pH, temperature and ionic concentration on enzyme activity. S. typhimurium bacteria killed by 0.5% formaldehyde proved to be superior to living cells and cells killed by heat or poisoned by KCN. Incubation of phage tails or phage particles with bacteria resulted in enzymatic hydrolysis of the O polysaccharide chain. Released oligosaccharides were then separated from whole cells. The amount of released oligosaccharides in the supernatant fluid could be assayed either colorimetrically (phenol-sulphuric assay of total amount of carbohydrates or reducing end-group assay according to Park & Johnson) or radiometrically (labelling the galactose residue of the O chain by growing a UDP-galactose-4-epimeraseless mutant of S. typhimurium with 14C-galactose as carbon source). The latter method proved to be simple, sensitive and reproducible and was used unless otherwise indicated.

Adsorption of phage P22 apparently involves at least two different steps – binding and eclipse. The enzymatic hydrolysis of the O polysaccharide chain of S. typhimurium is probably equivalent to, or part of, the binding step. Addition of cations to a reaction mixture containing either phage P22 particles or isolated tail parts as the enzyme source, or whole bacteria or isolated LPS as substrate did not alter the amount of oligosaccharides released. This does not imply that the ionic conditions are of no importance for the enzymatic hydrolysis. It may well be that, although extensively purified, the reactants contained sufficient amounts of cations to permit the enzymatic process to take place. Phage particles and isolated tail parts did not differ when other environmental effects on the hydrolytic process were studied: the temperature optimum was around 37°C and the pH optimum between 5 and 7. The rate of hydrolysis was approximately the same; the hydrolytic ability of the tail parts did, however, seem to be higher than that of the phage particles (Fig. 11). This can partly be explained by the fact that the phage particles are rapidly eclipsed by the bacteria. After only 3 min of incubation at 37°C more than 90% of the phages were eclipsed. It is also possible that the head-tail assembly assay underestimates the titre of tail parts, since tail parts with endorhamnosidase activity but an inability to assemble may exist in the preparation.

Phage P22 is eclipsed by S. typhimurium whole bacteria and isolated LPS. Takeda & Uetake (1973) reported that phage e15, which is similar in morphology to P22 and which
hydrolyses the O polysaccharide chain of \textit{S. anatum}, was not eclipsed by LPS from \textit{S. anatum} unless divalent cations were present. This was true also for phage P22, di- and trivalent cations being equally effective. Takeda & Uetake (1973) found that for \(e^{15}\), \(\text{Mn}^{2+}\) was more effective than other divalent cations tested. No such difference was observed for P22. When phage P22 eclipse to whole bacteria was studied, addition of mono-, di- or trivalent cations to the tris-HCl buffer in which the reaction took place did not increase the extent or rate of eclipse. Apparently the outer membrane contains sufficient cations, or the correct charge, for P22 phage eclipse to occur.

The observation that phage binding was associated with hydrolysis of the receptor might have been surprising at first. Taking into account the fact that binding may occur far out from the cell membrane (up to 400 nm for phages having their receptor in the capsule, up to 150 nm for phages binding to O antigens) makes the hydrolytic process more plausible. Triggering of nucleic acid ejection several hundred nm from the membrane would inevitably lead to a high percentage of abortive infections. This is not observed, instead, most phage particles adsorbed give rise to infection. The hydrolytic process is advantageous to the phage, since it makes possible a search for a proper receptor site for nucleic acid injection. This site, the second receptor, would most likely be in association with the cytoplasmic membrane to ensure a high frequency of infection. The points of fusion of membrane and cell wall proposed by Bayer (1968), Bayer & Starkey (1972) and Bayer & DeBlois (1974) for containing phage receptors are the most likely for this event.

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Phage P22 adsorption


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