THE ADSORPTION OF BACTERIOPHAGE ϕX174 TO ITS HOST

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ABSTRACT The adsorption of purified ϕX174 to *E. coli* C and to *E. coli* C cell walls was investigated. Adsorption was measured by assaying for unadsorbed plaque formers. The amount of irreversible and reversible adsorption depends upon pH and divalent ion concentration. Maximum irreversible adsorption occurs in 0.1 M $CaCl_2$ at 36°C. There is no detectable reversible adsorption at conditions of pH and $CaCl_2$ concentration optimum for irreversible adsorption. Under these optimum conditions, diffusion is not the rate-limiting factor, and the encounter efficiency appears to be low. The rate constant is 1.0×10^{-10} ml/sec. Phages adsorbed irreversibly to live cells cause infection and to the isolated cell walls apparently cause release of DNA. There is a specific ϕX174 receptor site on the mucocomplex portion of the cell wall.

INTRODUCTION

It is known that the bacterial virus T2 invades its host by diffusion to a bacterium, adsorption to a receptor site on the bacterium by its tail, release of DNA from the phage, and penetration of DNA into the bacterium (Adams, 1959; Kozloff, 1960).

However, little is known concerning the mechanism of invasion by viruses lacking tails. Presumably ϕX174 is such a virus (Tromans and Horne, 1961). Many of its physical and chemical properties are known. It has a molecular weight of 6.3×10^6 and contains 25 per cent by weight of DNA (Sinsheimer, 1959*a*). Its DNA is single-stranded (Sinsheimer, 1959*b*).

In this paper we report a study of the adsorption between ϕX174 and its host, *E. coli* C, in a medium containing only the ingredients essential for adsorption. We investigated reversibility of adsorption, the effect of various salts, temperature, and viscosity on the rate of adsorption, and we also investigated the nature of the bacterial receptor site.

MATERIALS AND METHODS

(*a*) *Assay of Phages and Bacteria*

Phage concentration was determined by the agar layer method as described by Adams (1950). *E. coli* C cells were grown in glucose tryptone medium consisting of 4.08 gm

KH_2PO_4, 5 gm glucose, 10 gm tryptone, 0.03 gm $CaCl_2$, 0.12 gm $MgSO_4$, and 1.0 gm NaOH per liter of distilled water, pH approximately 7.4. Bottom agar, as used by us, consists of 10 gm agar, 10 gm tryptone, 2.5 gm KCl, 6 ml of 1 N NaOH and 2 ml of 0.1 M $CaCl_2$ per liter of distilled water. Top agar is made by diluting two volumes of bottom agar with one volume of distilled water.

Bacterial concentration was determined by measuring optical density at 650 mμ. Optical density was calibrated to the number of bacterial cells by comparing it with the number of colonies formed on agar plates after the proper dilution. The dilutions were such that about 100 colonies were counted.

(b) Growth of Phages

The original cultures of ϕX174 and *E. coli* C were obtained from Professor R. L. Sinsheimer.

When it was desired to obtain 5 liters or less of lysate an appropriate number of 2-liter shakers were used, each containing 500 ml of the casamino acid–glycerol medium of Fraser and Jerrel (1953). Bacteria were grown to a titer slightly above 10^8/ml with vigorous stirring and then phages were introduced with a multiplicity of about three and the resultant suspension was incubated further for about 1.5 hours. The resulting lysates were stored in a cold room. The titer of phage obtained was about 2×10^{11}/ml.

For volumes of 10 to 15 liters a 30-liter stirred-jar made of stainless steel was used. Concentrations and multiplicity were as above. The titer of phage in the lysate from the stirred-jar was usually about 1×10^{11}/ml.

(c) Concentration and Purification of Phages

When the quantity of lysate was 2 liters or less the debris was sedimented at 5360 *g* for 20 minutes in the 1-liter capacity swinging bucket head of a Spinco model K centrifuge. The supernatant solution was centrifuged in a Spinco model L No. 30 rotor at 30,000 RPM for 150 minutes. The pellets contain the phage, and these were resuspended in 5×10^{-3} M phosphate buffer, pH 7.2 (hereafter called phosphate buffer). Insoluble materials were removed by centrifugation for 20 minutes at 14,000 RPM. The suspension was dialyzed against phosphate buffer and introduced onto a 2 cm diameter 20 cm long DEAE (diethylaminoethyl)-cellulose column equilibrated against the buffer. Phages were eluted with 0.05 M NaCl in phosphate buffer. The flow rate was about 1 ml/min. The maximum quantity of sample added was 20 cc of 1×10^{14} plaque formers per cc. The sample was concentrated by pervaporation and was then dialyzed against saturated sodium tetraborate at pH 9.1. The yield of phage from these steps is about 50 per cent. If the sample at this stage of purification is run in an analytical centrifuge, two peaks are observable—the so called *top* and *bottom* components of Sinsheimer (1959*a*).

When the volume of lysate was 5 to 15 liters, debris was removed by continuous centrifugation in a Sharples centrifuge operated at maximum speed. The debris was resuspended in 10^{-3} M phosphate buffer containing 0.0025 M EDTA (ethylenedinitrilotetraacetic acid) at pH 7.2 and left standing in a cold room while the supernatant solution was processed further. The latter was concentrated by flash evaporation at 30°C to about 2 liters (the minimum operating volume of the apparatus). The concentrate was dialyzed against distilled water for 1 day to reduce the salt concentration. Insoluble materials were sedimented at low speed and discarded. The resuspension of the debris from the Sharples centrifuge was similarly sedimented. Supernatant solutions were combined and processed as described for the small scale preparative method commencing from the step utilizing a Spinco Model L centrifuge. The yield of phage is about 20 per cent.

Top and bottom components were separated by means of density gradient centrifugation in an SW25 rotor. About 4 ml of phage solution consisting of about 1×10^{14}/ml of plaque formers were layered on top of a constant gradient formed from 35 per cent and 40 per cent RbCl solutions. Centrifugation was carried out for at least 12 hours at 25,000 RPM. The top component forms an opalescent band about one-third of the distance down from the meniscus and the bottom component forms a band about two-thirds of the distance down from the meniscus. The components were separated by puncturing a hole in the bottom of each tube and collecting the fractions. Those fractions that contained top or bottom components were dialyzed first against saturated sodium tetraborate solution containing 0.0025 M EDTA and then against saturated sodium tetraborate solution. The recovery of phage from the density gradient separation is nearly 100 per cent. The phage preparation was stored in saturated sodium tetraborate at 4°C.

(d) Preparation of E. coli C to be used as the Absorbing Agent

Bacteria were grown in 100 ml of casamino acid-glycerol medium in a 500-ml shaker flask to a titer of about 1×10^9/ml. They were immediately chilled in an ice bath, sedimented in a Spinco model K centrifuge at 5360 *g* for 10 minutes and then resuspended in distilled water. The centrifugation was repeated, and the resuspension was stored in an ice bath. The bacteria were prepared 30 hours or less before an experiment.

(e) Preparation of Cell Walls

Cell walls were prepared by a slight modification of a method developed by Wylie and Johnson (1962).

Bacteria were grown to a concentration of about 1×10^9/ml in eight 2-liter shaker flasks each containing 500 ml of casamino acid–glycerol medium. The bacteria were centrifuged down and resuspended in 100 ml of distilled water. The suspension, 500 ml at a time, was subjected to sonic oscillation for 3 minutes in a Raytheon sonic oscillator. Unbroken cells were centrifuged down. The pellets were resuspended in 50 ml of distilled water and subjected to sonic oscillation again. This process was repeated until only a small pellet remained after centrifugation. The supernatant fractions were subjected to sonic oscillation once more, centrifuged at 4,000 RPM for 10 minutes, and the pellet was discarded. The supernatant fractions were combined and centrifuged at 12,000 RPM for 10 minutes. The pellets, which are the cell walls, were resuspended in 50 ml of distilled water, and the centrifugation step was repeated two more times. The final cell wall fraction was resuspended in 100 ml of distilled water and stored in a refrigerator over a few ml of chloroform. The cell wall preparation was checked by electron microscopy to insure that no unbroken cells were present.

Phenol Treatment. A cell wall preparation was treated with phenol, according to a modification of the method of Weidel, Koch, and Lohss (1954).

A sample of cell wall preparation was centrifuged down at 13,000 RPM for 10 minutes in a No. 40 rotor. The precipitate was resuspended to its original volume in 90 per cent phenol solution. It was centrifuged at 13,000 RPM for 10 minutes. The phenol-insoluble fraction, *i.e.* the pellet, was resuspended in 0.05 M sodium acetate to the original volume. Then both the phenol-soluble and the insoluble fractions were dialyzed against 0.05 M sodium acetate for 2 days with three changes of the dialysis medium. After the dialysis, the remaining phenol was removed from the phenol-soluble fraction by extraction with chloroform. The phenol-soluble material precipitates out of the aqueous layer. This was redissolved in 0.1 M sodium borate, pH 9.1, to the original volume. The aqueous layer and the chloroform layer were discarded. The phenol-insoluble fraction was further

dialyzed against distilled water. Both fractions were saturated with chloroform and stored at 4°C.

Trypsin Treatment. The method is based on that of Weidel (1951).

Trypsin solution was made by dissolving 0.24 mg of trypsin (Worthington Corp., Harrison, New Jersey) in the phosphate buffer, and bringing the volume of the solution to 5 ml. 5 ml of a cell wall preparation were sedimented at 13,000 RPM for 10 minutes, resuspended in the trypsin solution, and left at 37°C for 48 hours with occasional shaking. A small crystal of merthiolate was added to prevent bacterial growth. Then the cell walls were centrifuged at 15,000 RPM for 15 minutes in a No. 40 rotor. The sediment was slightly brown and transparent. It was resuspended in distilled water to the original volume. The solution was stored over chloroform.

Lysozyme Treatment. The method is based on that of Guthrie and Sinsheimer (1960).

Lysozyme solution was made by adding 0.4 mg lysozyme (Worthington) to 1 ml of water. 3 ml of cell walls were centrifuged at 13,000 RPM for 10 minutes in a No. 40 rotor, and resuspended in 4 ml of 0.05 M tris buffer pH 8.1. The sample was then transferred to 1 ml of a lysozyme solution. 0.1 ml of 0.2 M EDTA was added. The mixture was left at room temperature for 15 minutes. It was then centrifuged at 13,000 RPM for 10 minutes, and the pellet was resuspended in 3 ml of distilled water and stored at 4°C.

Fluorodinitrobenzene Treatment. 70 ml of a cell wall preparation were centrifuged at 12,000 RPM in a No. 30 rotor, and the pellets were resuspended in 10 ml of 0.2 M carbonate-hydrogen carbonate buffer, pH 8.5, treated with fluorodinitrobenzene (Koch and Weidel, 1956), and centrifuged again. The final pellet was resuspended in 9 ml of water and stored at 4°C.

C^{14}-Labeled Cell Walls. Cell walls were prepared from bacteria grown to a titer of about 4×10^8/ml in Hershey's medium (Hershey, 1955) containing 0.08 mc/liter of invert sugar. Cell walls prepared from 4 liters of a bacterial suspension and resuspended in 50 ml of distilled water had an activity of 4000 CPM/ml.

(*f*) *Method of Measuring Adsorption of Phages to Bacteria*

Procedure. The procedure consists essentially of three steps: (*a*) A quantity of adsorbing agent is added to a reaction medium which has already been equilibrated to a desired temperature. 5 minutes later phages are added to a concentration of 5×10^4/ml. (*b*) Adsorption is stopped at a specific time by diluting the reaction mixture 50-fold by transferring 0.1 ml of reaction mixture to 5 ml of dilution medium. Dilution medium is either the reaction medium or 0.1 M NaCl solution. If the adsorbing agents are live bacteria the dilution medium is saturated with chloroform (Adams, 1959) in order to kill the bacteria at the time of dilution. (*c*) From 5 to 20 minutes after dilution, unadsorbed phages are assayed. The control suspension for the above procedure will give about 100 plaques when assayed at step (*c*).

In the subsequent sections, *standard conditions* are defined as 1.5×10^7/ml of bacteria and 5×10^4/ml of phages in a reaction mixture at 36°C.

Analysis of Data. The *number* of phages remaining unadsorbed is the number of plaques obtained by the above procedure. The *fraction* of phages remaining unadsorbed is the number of plaques formed divided by the number of plaques formed in a corresponding control experiment.

Deviations of experimental values from the mean are expressed as average deviations.

When a final value is obtained by addition, subtraction, or division of uncertain values the uncertainty is expressed as the probable error. In assessing the significance of a value, standard statistical criteria were used (Bauer, 1960).

Validity of Method of Measuring Adsorption. It was observed in preliminary studies that Ca^{++} or Mg^{++} at least in the amount of 10^{-3} M is necessary for irreversible adsorption. Na^{+} is not necessary and cannot be substituted for these divalent ions. Near neutral pH both phosphate buffer and veronal acetate buffer, both at 5×10^{-3} M, inhibit adsorption even in the presence of Ca^{++} or Mg^{++}. Thus in the studies to be reported the only inorganic salts used were $CaCl_2$ or $MgSO_4$.

In order to insure that the decrease in phage titer in the presence of the host is due to adsorption, controls were run concurrently under identical conditions except that the absorbing agent was absent. Thus, inactivation caused by constituents of the medium other than adsorbing agent is taken into account. Most experiments were done on 0.1 M $CaCl_2$ or 0.01 M $MgSO_4$, in which there was no significant inactivation for the duration of an experiment. However, about 50 per cent of the phages become inactivated in 2 hours at room temperature in these solutions.

Several experiments in which various dilution factors were used were in agreement that 50-fold dilution is sufficient to stop adsorption, and that the dilution mixture saturated with chloroform does indeed kill the infected bacteria. In one experiment bearing on these points, an adsorption mixture was allowed to interact for 10 minutes and then was diluted 50-fold into a chloroform-saturated solution. The resulting solution was assayed at various times. It was found that for assay times from 5 minutes to at least 20 minutes the phage titer remained constant, indicating that the infected bacteria had been killed, that unadsorbed phages were not being inactivated, and that 50-fold dilution was sufficient to stop further adsorption. The results were the same when the bacteria were removed by centrifugation instead of being killed by chloroform. Since under our conditions no appreciable adsorption occurs when the bacterial concentration is less than 2×10^8/ml, the 50-fold dilution effectively stops adsorption from a medium containing as many as 10^9 bacteria per ml.

RESULTS

(a) *Adsorption of φX174 to E. coli C*

The adsorption behavior was studied at a phage concentration of 5×10^4/ml with bacterial concentration, divalent ion concentration, temperature, and viscosity of the medium as variables. It is convenient to use $-d \log n_p/dt$ as a measure of the adsorption rate k'. Here n_p is the per cent of phages remaining unadsorbed. If adsorption is first order with respect to phage concentration, k' will be the apparent rate constant.

Effect of Bacterial Concentration. It was found (Fig. 1) that the adsorption rate is linearly dependent on bacterial concentration up to about 2×10^7/ml, and then levels off. Below a bacterial concentration of 2×10^6/ml, the adsorption rate is too small to be determined by our method. Thus the standard bacterial concentration of 1.5×10^7/ml used in subsequent experiments results is close to the maximum rate of adsorption, but still the rate is dependent on bacterial concentration.

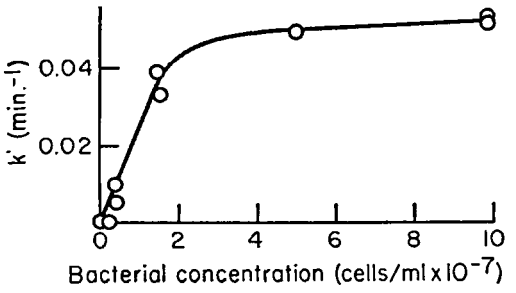

FIGURE 1 Apparent rate constant k' *versus* bacterial concentration at zero time at 36°C in 0.01 M $MgSO_4$, pH 6.3 ± 0.2. The phage concentration at zero time was about 5×10^4/ml in each determination.

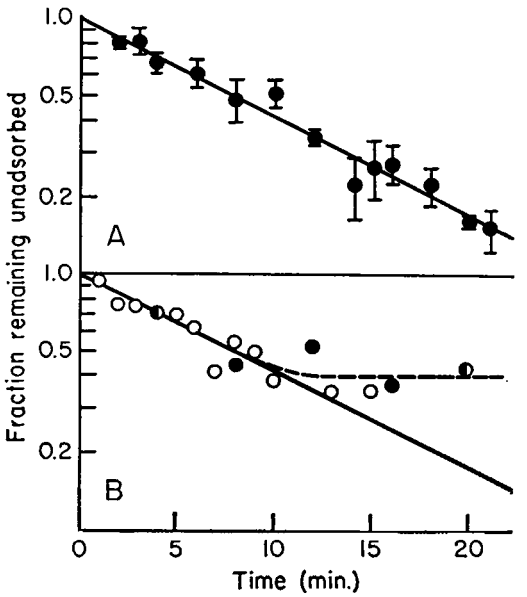

FIGURE 2 The rate of adsorption under standard conditions at various concentrations of divalent ions. The graphs show semi-long plots of the fraction of phages remaining unadsorbed as a function of time.

A. In 0.1 M $CaCl_2$, pH 6.8, $k' = 0.039$/min. The uncertainty shown at each point is the average deviation among several sets of data. The slope, k', is the average value obtained from seven sets of data. Its 95 per cent confidence limits are ± 0.004.

B. The solid line is drawn so as to have the same slope as the line shown in Fig. 2A.

● 0.01 M $CaCl_2$ + 0.1 M NaCl, pH 7.1.

○ 0.01 M $MgSO_4$, pH = 6.2

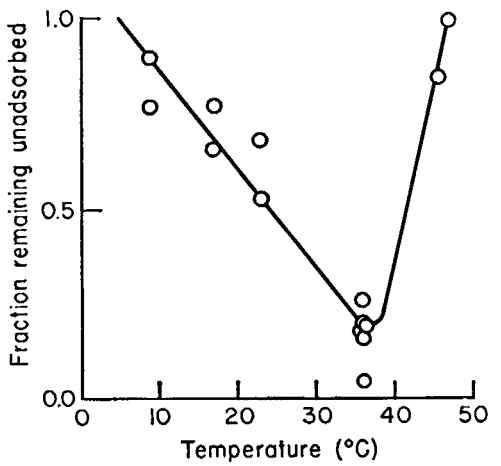

FIGURE 3 Dependence of adsorption of the phage on temperature in 0.1 M $CaCl_2$. The measurements were made under standard conditions except for temperature.

Effect of Divalent Ion Concentration. The rate dependence of adsorption was determined in 0.0001, 0.001, 0.01, 0.1 and 1 M $CaCl_2$ and $MgSO_4$. Appreciable adsorption occurs only in the 0.01 M and 0.1 M solutions. Representative results are shown in Figs. 2A and 2B. They show a semi-log plot of the fraction of phages remaining unadsorbed as a function of time. The ordinate is thus a measure of n_p while the slope is a measure of k'.

It may be seen in Fig. 2A that the experimental points fall on a straight line indicating that adsorption is first order with respect to phage concentration in 0.1 M $CaCl_2$, pH 6.8 at 36°C. The average k' from several determinations with different preparations of bacteria and phage was 0.039/minute with 95 per cent confidence limits of ± 0.004.

In 0.1 M and 0.01 M $MgSO_4$ the adsorption rate for approximately the first 10 minutes is not significantly different from that in 0.1 M $CaCl_2$—the average k' is 0.036. However, after this time it is apparent that the adsorption rate is decreasing with time and at the end of 20 minutes it is nearly zero (Fig. 2B).

In 0.01 M $CaCl_2$ there is considerable loss of titer due to the solvent. However, if 0.1 M NaCl is present in addition to 0.01 M $CaCl_2$, the loss of titer due to the solvent is prevented and the adsorption curve appears to be similar to that in 0.01 M $MgSO_4$ (Fig. 2B). Adsorption does not occur in 0.1 M NaCl alone nor does the addition of 0.1 M NaCl affect the adsorption behavior in the presence of 0.1 M $CaCl_2$.

Effect of Temperature. Adsorption was studied at several temperatures between 0° and 45°C in 0.1 M $CaCl_2$. Fig. 3 shows the fraction remaining unadsorbed after 20 minutes of interaction. The fraction adsorbed was practically nil at 0°C, but it increased with temperature and reached a maximum at about 36°C. Above 40°C most of the plaque formers become inactivated, but there was no additional reduction in number of plaques in the presence of bacteria.

Effect of Viscosity. The apparent rate constant was determined in 0.1 M $CaCl_2$ in the presence of 0, 20, and 40 per cent sucrose, otherwise at the conditions where maximum adsorption occurs. The results (Table I) show that increase in

TABLE I

EFFECT OF VISCOUS MEDIUM ON THE RATE OF ADSORPTION

Sucrose concentration by weight	Viscosity (36°C)	k' (observed) per min.	k'* (expected) per min.
per cent	*centipoises*		
0	0.71	0.043	
20	1.30	0.031	0.024
40	3.66	0.036	0.008

The effect of viscosity was determined by introducing sucrose into the 0.1 M $CaCl_2$ solution. The rates were determined at the standard conditions. All the determinations were made concurrently. Final reaction mixtures all had a pH of 6.7.

* Calculated by using 0.043 as the value for k' in 0 per cent sucrose.

viscosity by about fivefold has not much effect on the rate of adsorption indicating that diffusion is not rate-limiting.

Reversibility of Adsorption. Whether the adsorbed phages can be freed and remain active was checked by means of the following procedures. With our usual dilution method only the irreversibly adsorbed phages are measured. If instead, the adsorption mixture is centrifuged without dilution, both the reversibly and irreversibly adsorbed phages are removed as the bacteria are sedimented. Thus the difference of the titers should indicate the phages adsorbed reversibly. Reversibly adsorbed phages can also be counted by resuspending the sedimented cells in 0.1 M NaCl solution in which medium they are presumably eluted. Table II shows the results at 11°, 36°, and 47°C. Column A shows the reduction in titer caused by irreversible adsorption. Column B shows the reduction in titer caused by reversible plus irreversible adsorption. Column C, which gives the difference between Columns A and B indicates that there are practically no reversibly adsorbed plaques under these conditions. Column D shows the titers of reversibly adsorbed phages as obtained by elution from the sedimented bacteria. Again hardly any reversibly adsorbed phages are indicated. At 47°C there was considerable inactivation, but no

TABLE II

REVERSIBILITY OF ADSORPTION

Temperature	A	B	C	D	E
°C					
11	66 ± 12	69 ± 3	−3 ± 12	8, 4	0.65
11	63 ± 5	72 ± 4	−9 ± 6	0, 0	0.62
36	42 ± 5	35 ± 1	8 ± 5	1, 2	0.42
36	37 ± 7	29 ± 6	8 ± 9	2, 0	0.37
47	13 ± 2	23 ± 3	−10 ± 4	0, 0	0.9
47	15 ± 6	8 ± 4	7 ± 7	0, 0	1.1
11 and 36 (controls)	101 ± 7	103 ± 9	−2 ± 11	0, 1	1.0
47 (controls)	14 ± 5	14 ± 1	0 ± 5	1, 0	1.0

The adsorptions were allowed to proceed for 15 minutes in 0.1 M $CaCl_2$, pH 7.2 at the standard conditions except for the temperature. Duplicate experiments were done at the same time, and duplicate assays were made of each fraction.

A. Number of plaques (indicating the unadsorbed phages) obtained by diluting into chloroform-saturated 0.1 M $CaCl_2$ solution.

B. Number of plaques (indicating the unadsorbed phages) obtained after the dilution of the supernatant following the centrifugation of infected bacteria.

C. The difference between column A and column B.

D. Each of the sediments from B was resuspended in 0.1 M NaCl solution, and the resulting suspension was centrifuged. Each of the supernatants was diluted and assayed. The numbers are plaque counts.

E. Values in column A divided by the values determined for the controls.
Controls for 11° and 36° were incubated for 15 minutes at 36°C, since previous studies have indicated that there was no inactivation of phages at either of these temperatures.

additional inactivation was observed in the presence of bacteria (as shown by Column E which gives the fraction of the phages remaining unadsorbed).

It should be emphasized that the above results apply only under conditions of optimum adsorption. For various technical reasons it is more feasible to do extensive studies of reversible adsorption with cell walls rather than with live cells. In a later section it will be shown that with cell walls there is no detectable reversible adsorption under optimum adsorption conditions, but under other conditions reversible adsorption occurs. Under the latter conditions the rate of irreversible adsorption declines with time as a consequence of the effective removal of phage from the adsorption medium by the reversible adsorption process.

Biological Significance of Adsorption. We investigated whether phages adsorbed under our conditions lead to infection. Some results are shown in Fig. 4.

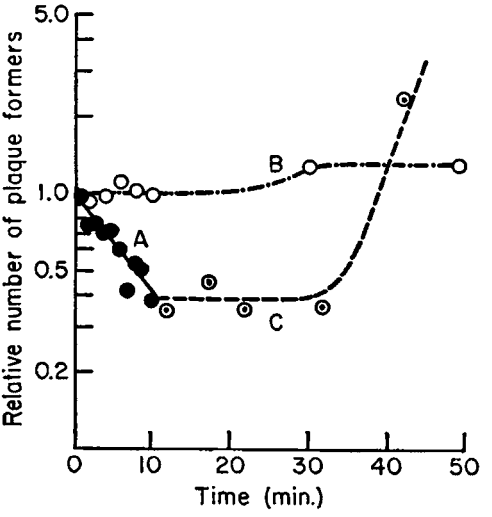

FIGURE 4 Infectivity of phages adsorbed in 0.01 M $MgSO_4$, pH 6.2 at the standard conditions.
A. Fractions taken out of the reaction mixture at the times indicated and assayed after 50-fold dilution into chloroform-saturated 0.01 M $MgSO_4$ solutions.
B. Fractions taken out of the reaction mixture at the times indicated and assayed after 50-fold dilution into 0.01 M $MgSO_4$ solutions.
C. Fractions were diluted into growth medium after 12 minutes of interaction. They were further incubated for the various times indicated, and infected bacteria were then killed by chloroform. Samples were then assayed.

Curve A is from a routine adsorption experiment in which a fraction of the reaction mixture in 0.01 M $MgSO_4$ was transferred at various times into a chloroform-saturated $MgSO_4$ solution. The data give the fraction of phages remaining unadsorbed in the reaction mixture as a function of time and thus show that adsorption is occurring. For curve B, the data were obtained by transferring an aliquot of the reaction mixture at various times into a $MgSO_4$ solution, lacking chloroform so that the infected bacteria were not killed. The data show that the *sum* of the unadsorbed phages and the adsorbed phages which caused plaques upon assaying is a constant. If desorption had occurred accompanied by inactivation, the titer (curve B) should have decreased with time. For curve C several fractions of the reaction mixture were transferred to growth medium after 12 minutes' interaction so that the infected bacteria should start synthesizing phages. The constant titer (for the next 20 minutes) after the transfer indicates that there was no further adsorption and that the

adsorbed phages remained adsorbed. Had there been desorption with the phage remaining active the titer (curve C, 10 minutes to 30 minutes) should have increased with time. The sudden increase in the titer 20 minutes after the transfer indicates that newly synthesized phage had been released into the medium. (The phages that were in the $MgSO_4$ solution throughout also seem to have produced a few progeny as indicated by the slight rise in the right hand portion of curve B.) Thus the results of curves B and C are in accord that in these experiments *every adsorption of plaque formers leads to infection.*

(*b*) *Attachment Site on the Host*

Adsorption to the Cell Walls of E. coli C. Typical adsorption curves of ϕX174 to the cell walls in 0.01 M $MgSO_4$ are shown in Fig. 5. They are similar to

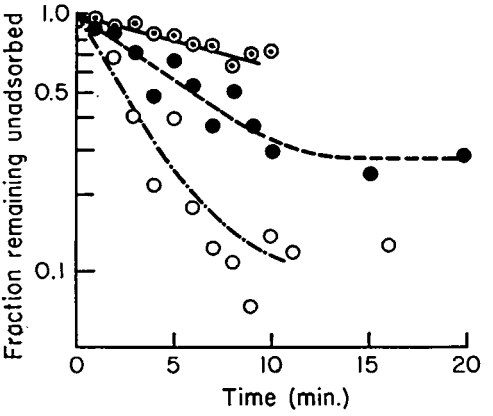

FIGURE 5 Adsorption to the cell wall of *E. coli* C. The curves were obtained at several concentrations of the same preparation of cell wall in 0.01 M $MgSO_4$, pH 6.2 at 36° C with initial phage concentration of 5×10^4/ml. Relative concentrations are 1, 4, and 10 in order of increasing slope. The unit of concentration is 0.1 ml of stock suspension per 100 ml of adsorption mixture.

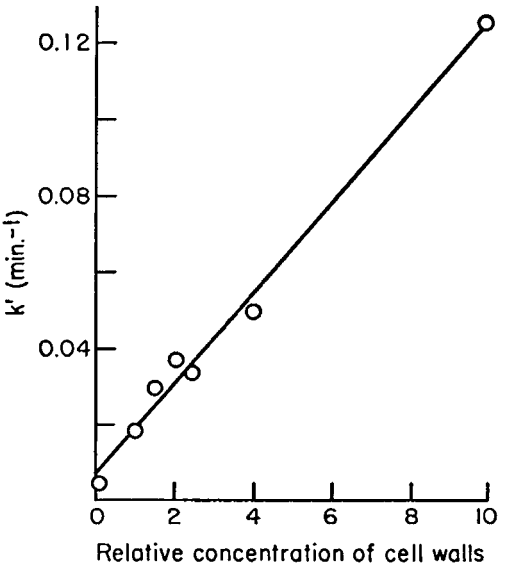

FIGURE 6 Apparent rate constant *k' versus* the relative concentration of cell walls. The conditions were the same as given for Fig. 1.

the adsorption curves of bacteria under similar conditions in that in both cases after a few minutes of interaction it is apparent that the adsorption is ceasing. Evidently the fraction adsorbed at the limit of adsorption increased with cell wall concentration, although it should be noted that the precision in this portion of the curve is not high and not enough data have been obtained to establish this point unequivocally.

The apparent rate constant of adsorption was determined with several concentrations of cell walls using data obtained within about the first 10 minutes of interaction. The plot of k' *versus* cell wall concentration was found to be linear (Fig. 6). When compared with the similar plot for live bacteria (Fig. 1) it may be seen that the apparent rate constant increased with cell wall concentration to a much higher value of k' without reaching a limit.

We have found that all of our cell wall stocks had about the same k' provided they were similarly prepared and were analyzed under comparable conditions. Nevertheless, in the subsequent sections comparisons of the effect of various factors on adsorption to cell walls were made with the same amount of a single stock of cell wall suspension.

The effect of salts on adsorption to cell walls and to live bacteria is very similar. Again, for about the first 10 minutes of interaction the adsorption rate is similar in 0.1 M and 0.01 M $CaCl_2$ and $MgSO_4$, but thereafter the adsorption rate decreases in 0.01 M $CaCl_2$ and in 0.1 M and 0.01 M $MgSO_4$ but remains constant in 0.1 M $CaCl_2$. This is illustrated in Table III which gives the per cent of phages

TABLE III

PER CENT ADSORPTION OF THE PHAGES TO THE CELL WALLS IN PRESENCE OF VARIOUS SALTS

A. Effect of divalent ion concentration

Concentration	$MgSO_4$	$CaCl_2$	$CaCl_2$ in 0.1 M NaCl
M			
10^{-1}	37 ± 8	3 ± 2	3 ± 1
10^{-2}	35 ± 5	39 ± 4	30 ± 3
10^{-3}	120 ± 8	130 ± 25	66 ± 8
10^{-4}	120 ± 14	78 ± 14	109 ± 8

B. Effect of NaCl

Concentration	NaCl
M	
10^{-1}	85 ± 16

The per cent of phages remaining unadsorbed was measured after 20 minutes' interaction at 36°C with initial phage concentration = 5×10^8/ml and relative cell wall concentration = 5. The pH was 6.8 ± 0.3.

remaining unadsorbed after 20 minutes of interaction. It may be seen that practically complete adsorption has occurred in 0.1 M $CaCl_2$, and only about two-thirds of the phages have been adsorbed in 0.01 M $CaCl_2$ and in 0.01 M $MgSO_4$. In 10^{-3} M and 10^{-4} M divalent salts there was higher titer of free phages remaining in the presence of cell walls after 20 minutes' incubation than in the corresponding control suggesting that at low ionic strength the phage may be more stable in the presence of cell walls.

In 0.1 M NaCl alone there is no appreciable adsorption. Also, when 0.1 M NaCl was added in the presence of various concentrations of the divalent salts there was no effect at the higher concentrations. However, at divalent ion concentration lower than 10^{-3} M the inactivation which ordinarily occurs at low ionic strength is prevented, and there occurs some adsorption at 10^{-3} M but not at 10^{-4} M. Thus, while high ionic strength is essential for phage stability, it is not a sufficient condition for adsorption. The requirement for Ca^{++} or Mg^{++} is specific for some other reason.

Reversibility of Adsorption to Cell Walls. The reversibility of adsorption as a function of pH was studied in $CaCl_2$ solutions. Since in preliminary studies it was found that several buffers, among them phosphate and veronal-acetate, inhibit adsorption, pH was varied by the addition of dilute NaOH or HCl. Initial phage concentration was 5×10^4/ml and 0.5 ml of the standard cell wall stock was used per 100 ml of the adsorption mixture. The incubation temperature was 36°C. The incubation time was 15 minutes. Irreversible and reversible adsorption were measured as has been described previously in the case of live cells.

As shown in Fig. 7, the irreversible adsorption in 10^{-1} M $CaCl_2$ depends markedly upon pH; there is no appreciable reversible adsorption at any pH. Maximum irrever-

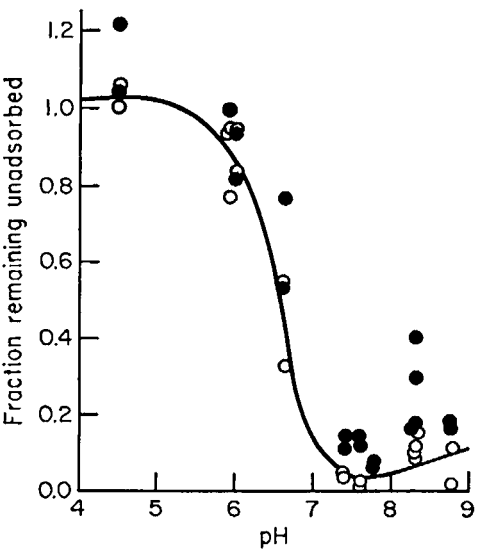

FIGURE 7 Effect of pH on adsorption in 10^{-1} M $CaCl_2$. The fraction unadsorbed was measured after 15 minutes' interaction under the conditions stated in the text.

○ Fraction remaining after *both* reversibly and irreversibly adsorbed phages were removed;

● Fraction remaining after irreversibly adsorbed phages were removed.

sible adsorption occurs at about pH 7.5. There is no adsorption below pH 6. All the data shown were obtained from experiments in which the phage sample was stored in saturated sodium tetraborate at pH 9.1 and diluted into appropriate $CaCl_2$ solutions. When corresponding experiments were performed by dialysis against the appropriate 10^{-1} M $CaCl_2$ solutions, there was considerable inactivation due to aggregation of the phages but otherwise the results were the same.

In 10^{-3} M $CaCl_2$ no appreciable irreversible adsorption occurred at any pH during the 15 minute incubation period. (At neutral pH there is some irreversible adsorption which is detectable by extending the incubation period to 1 hour.) However, practically the whole population of phage adsorbed reversibly at acid pH as shown in Fig. 8. Most experiments were done with phage dialyzed against the appropriate

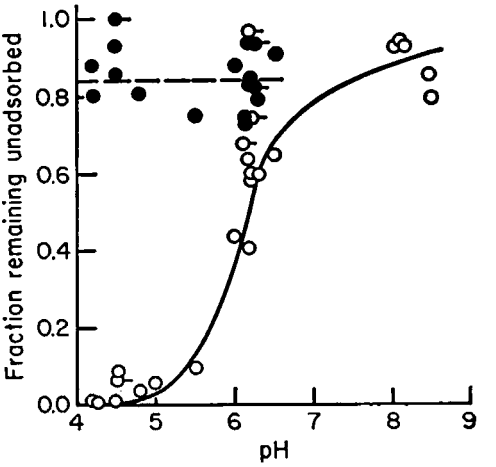

FIGURE 8 Effect of pH on adsorption in 10^{-4} M $CaCl_2$. The fraction unadsorbed was measured after 15 minutes' interaction under the conditions stated in the text.

○ Fraction remaining after *both* reversibly and irreversibly adsorbed phages were removed. In this experiment the phage was dialyzed against 10^{-4} M $CaCl_2$.

○– same as above except that the phages were diluted into 10^{-4} M $CaCl_2$.

● fraction remaining after irreversibly adsorbed phages were removed. In this experiment the phages were dialyzed against 10^{-4} M $CaCl_2$.

●– same as above except that the phages were diluted into 10^{-4} M $CaCl_2$.

10^{-3} M $CaCl_2$ solutions. When the phage was diluted directly from stock (as was done at 10^{-1} M $CaCl_2$) there was considerable inactivation particularly at the more acidic pH's which made it difficult to obtain reproducible data.

Adsorption to Treated Cell Walls. Cell walls treated separately with phenol, trypsin, and lysozyme were kept at comparable concentration by keeping the volume of the treated cell wall solution the same as before the treatment. Adsorption experiments were done at several concentrations.

Fig. 9 shows that phages adsorbed to the phenol-insoluble fraction, and to the trypsin treated cell walls, but not to the lysozyme treated cell walls. Control experiments showed that the media for the treated cell walls were not causing phage inactivation.

The adsorption to the phenol insoluble fraction and the trypsin treated cell walls indicates that the receptor site for ϕX174 is the lipopolysacchairide layer of the cell wall. The loss of capacity to adsorb after the treatment with lysozyme further

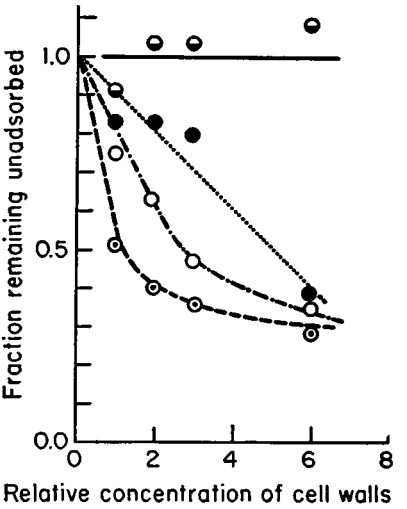

FIGURE 9 Adsorption to the treated cell walls. Fraction remaining unadsorbed after 20 minutes' interaction with various concentrations of cell wall in 0.01 M $MgSO_4$, pH 6.6 at 36°C with 5×10^4/ml of phages.

- O cell walls untreated.
- ⊙ cell walls treated with trypsin.
- ◒ cell walls treated with lysozyme.
- ● phenol insoluble fraction of cell wall.

indicates that the receptor site must be in the same area as that attacked by lysozyme. According to Salton's review (Salton, 1960), lysozyme attacks the mucocomplex which consists of D-alanine, D-glutamic acid, muramic acid, glucosamine, and diaminopimelic acid.

Adsorption on the K12 Strain of E. coli. Although ϕX174 has several hosts among other genera (Sertic and Boulgakov 1935; Zahler, 1958) it is known to attack only strain C of *E. coli*. This specificity may be due to the failure to adsorb or the failure to be synthesized in the cell. Adsorption was studied in the case of K-12 under standard conditions in 0.01 M $MgSO_4$, pH 6.3. The result is shown in Table IV along with results that would be expected with *E. coli* strain C.

TABLE IV

ADSORPTION OF PHAGES TO *E. COLI* K12

Time	Fraction remaining unadsorbed with *K*12	Expected result with C
min.		
10	1.0 ± 0.1	0.4
20	1.0 ± 0.3	0.4

It is seen that no significant amount of adsorption has occurred. Thus the adsorption is specific to some structure in the cell wall of strain C which is inaccessible or absent in strain K12.

(c) *DNA Penetration*

Search for an Enzyme in φX174. Several investigators have found that soluble materials are released from the cell wall of *E. coli* due to the action of T2 phage enzyme (Koch and Weidel, 1956; Barrington and Kozloff, 1956; Brown and Kozloff, 1957). Thus we sought degradation products of cell walls after interaction with φX174.

A solution consisting of 4.4×10^{12}/ml of plaque formers was dialyzed against 10^{-2} M $MgSO_4$ for 24 hr. One ml aliquots of the stock of dinitrophenylated cell walls were centrifuged and each sediment was resuspended in 2 ml of 0.01 M $MgSO_4$ having appropriate concentrations of phages. Up to 4.8×10^{12} particles of plaque formers per 2 ml of reaction mixture were used. The reaction mixtures and a control containing only dinitrophenylated cell walls in 0.01 M $MgSO_4$ were incubated at 36°C for 3 hours. The mixtures were then centrifuged at 13,000 RPM for 10 minutes. The supernatants were measured for the release of yellow substances at 336 mμ. No significant amount of yellow material was released above that obtained in the control. Under comparable conditions Koch and Weidel (1956) have obtained with T2 phage a significant release of color with as little as 1.2×10^{11} phages.

Various concentrations of C^{14}-labeled cell walls giving as many as 29,000 CPM were centrifuged down from the stock suspension, and resuspended in a solution of 5×10^{12}/ml phage particles in 0.01 M $MgSO_4$. The mixtures were incubated at 36°C for 3 hr. Cell walls were sedimented at 13,000 RPM for 10 minutes. There was no significant release of non-sedimentable C^{14} materials above that obtained in the control which contained no phages. Brown and Kozloff (1957) had cell walls with a radioactivity 14,000 CPM mixed with as little as 1.7×10^{10} T2 phages in about 0.5 ml of solution, and after the reaction obtained from 0.1 ml of the supernatant about 160 CPM.

Thus we could find no evidence of a cell wall degrading enzyme on the phage. However, there is evidence that DNA may be released from phage particles adsorbed to the cell walls under these conditions. When infected cell walls freed of unadsorbed phages were treated with DNase, materials having an absorption spectrum like that of φX174 DNA were released into the solution in large excess over similarly treated uninfected cell walls. Evidently DNA was released upon phage adsorption, but remained entangled with cell wall materials until it was degraded by DNase.

DISCUSSION

For some of the T-phages of *E. coli* it is known that infection involves at least two steps before the penetration of DNA into a host bacterium occurs. The first step

involves a reversible adsorption. The second step is irreversible and involves bond formation between the phage tail fibrils and the bacterial receptor site. Diffusion is the rate-limiting step with an encounter efficiency of close to unity. The high efficiency of encounter is believed to be due both to the electrostatic nature of the adsorption and to the structure of the tail fibrils (Tolmach, 1957, 1960).

Maximum irreversible adsorption of ϕX174 to *E. coli* C and its isolated cell walls occurs in 0.1 M $CaCl_2$ at 36°C. Under these optimum adsorption conditions, there is not an obligatory reversible adsorption and diffusion is not rate-limiting. Furthermore it was shown by Koch (1960) that in the range where the rate constant is close to maximum, the rate constant is strongly dependent upon encounter efficiency. Absence of appreciable dependence indicates that the efficiency is low. This must be the case under our adsorption conditions since increased viscosity of the medium, which causes decrease in collision frequency, did not appreciably affect the rate constant.

When the rate of adsorption is first order with respect to phage concentration and the rate is dependent linearly upon bacterial concentration the rate constant may be expressed by the equation $-d n_p/d t = k n_p n_b$, where n_p *is* phage concentration, n_b is bacterial concentration, t is time, and k is the rate constant (Delbruck, 1940; Schlesinger, 1932). Then $k' = k n_b/2.303$. In 0.1 M $CaCl_2$ at our standard conditions $k = 1.0 \times 10^{-10}$ ml/sec.

The optimum pH and divalent ion concentration for reversible and irreversible adsorption are different. Reversible adsorption occurs under conditions where there is no irreversible adsorption. On the other hand in 0.1 M $CaCl_2$, a medium favorable for irreversible adsorption, there is no reversible adsorption at any pH. Thus reversible adsorption is not an essential step for the infection process and is likely not of great biological significance.

In our conditions for irreversible adsorption it was shown that phage adsorbed to live bacteria leads to infection of its host, and phage adsorbed to isolated cell walls leads to release of DNA (or at least leads to its accessibility to nuclease). Yet under these conditions no cell wall–degrading enzyme was found. This suggests that the mucocomplex part of the cell walls to which ϕX174 becomes attached may be permeable to DNA under proper pH and salt concentration without the aid of an enzyme. Apparently no phage enzyme is necessary for the penetration of the cell membrane, since it has been shown by several laboratories that it is possible to infect *E. coli* protoplasts with ϕX174 DNA (Guthrie and Sinsheimer, 1960; Hofschneider, 1960; Sekiguchi *et al.*, 1960).

Without the presentation of considerable additional experimental evidence it is not practical to discuss the adsorption experiments reported in this paper in terms of the physical characteristics of ϕX174. However it may be pointed out that it has been reported elsewhere (Fujimura, 1961) that the sedimentation rate of ϕX174, in media optimum for irreversible adsorption, is about 15 per cent lower than it is

in the usual tetraborate buffer. On the other hand, the sedimentation rate of top component of ϕX174 is unchanged. Furthermore it was shown that top component does not adsorb under conditions in which the phage itself is adsorbed irreversibly. These facts suggest that in favorable adsorption media the phage may undergo a shape change which makes adsorption feasible. Since top component is presumed to differ from infectious phage only in that its DNA content is small it may be assumed that the phage DNA is in some way involved in the shape change and in the adsorption process.

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