THE REACTIONS OF CERTAIN BACTERIOPHAGES WITH THEIR RECEPTORS ISOLATED FROM *SALMONELLA TYPHI* O 901 R.

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Received for publication March 7, 1955.

THE adsorption of a bacteriophage to its host cell is now considered to involve the formation of electrostatic bonds between the phage particles and specific receptor sites on the bacterial surface (Puck, 1953). It has been shown by Burnet (1934) and by Jesaitis and Goebel (1952) that it is possible to extract from bacterial cells material which can inactivate homologous phages and which is considered to contain the specific phage receptors. This assumption is based on the observation that the phage-inactivating power of the extracts is as specific as the phage-adsorbing power of the bacteria. For example a strain of *Shigella sonnei* which is lysed by phages T2, T3, T4, T6 and T7 yields on extraction a material which can inactivate all five of the phages, whereas the extract prepared from a variant which is resistant to T3, T4 and T7 inactivates only phages T2 and T6 (Goebel and Jesaitis, 1952).

The purified material from *Sh. sonnei* was found to be an antigenic complex of phosphorylated lipocarbohydrate and protein, but it could be degraded to give a serologically active protein-free lipocarbohydrate which still retained the ability to inactivate phages T3, T4 and T7. It had lost the power to inactivate phages T2 and T6. The action of the lipocarbohydrate on phage T4 was studied by Jesaitis and Goebel (1953) and it was found to result not only in the inactivation of the phage but also in the release of its nucleic acid into solution. This discovery is of great interest in the study of bacteriophage multiplication because it suggests that the phage receptor can be considered as the agent which causes the release of the phage nucleic acid at the bacterial surface ready for incorporation into the host cell.

The work described in this paper concerns the isolation from *Salmonella typhi* O 901 R of the receptors for some of the phages to which it is susceptible. The reaction of one of these phages with its receptor has been studied and found to result in the almost immediate release of nucleic acid.

MATERIALS AND METHODS.

Organisms.

The phage receptors were prepared from acetone-dried cells of *Salm. typhi* O 901 R, which is a " rough " variant of O 901. This organism is called " rough " because of its colony morphology, instability of suspensions in salt solutions and its susceptibility to an " anti-R " phage obtained from Dr. A. Felix, labelled " A.59/65.R ". O 901 R is lysed by four phages in use in this laboratory, namely phage 1, phage 2, T3 and anti-R.

Stocks of bacteria for extraction purposes were grown in a medium containing acid hydrolysed casein 1 per cent, sodium lactate (syrup) 2 per cent v/v, sodium glycerophosphate

1 per cent, tryptophan 0·0001 M and an aqueous extract of yeast 10 per cent v/v (Gladstone and Fildes, 1940). Batches of 1 l. were sterilised in 5–l. flasks, inoculated, and incubated at 37° in a spinning apparatus similar to that described by Mitchell (1949). After overnight growth the cells were collected and washed in 1 per cent NaCl by sedimentation. Suspensions of O 901 R in culture medium or saline are unstable and the cells settle to the bottom of the vessel in a few minutes. The concentrated cells were then centrifuged, suspended in water and precipitated with 6 vol. acetone. Several changes of acetone were used to dehydrate the cells which were finally filtered off and dried in air. The yield was between 3 and 4 g. of dried cells per l. medium.

Bacteriophages.

Four bacteriophages were used. These were T3, one of seven " T " coliphages, " anti-R " (Felix), a phage which attacks only " rough " strains of *Salm. typhi*, and phages 1 and 2. These latter were derived by single plaque isolations from a phage which attacked *Salm. typhi* N.C.T.C. 3390. Phage 2 is indistinguishable serologically from the " anti-O No. 2 " phage used by Dr. Felix (personal communication). Phage 1 has a different host range from phage 2 and is unrelated serologically. Stocks of phage T3 were prepared on *Bacterium coli* B grown in a defined medium containing 0·05 M phosphate, buffer pH 7·6, 0·02 M–NH_4Cl, 0·00016 M–$MgSO_4$ and 0·02 M glucose. Phages 1 and 2 were prepared by lysis of *Salm. typhi* R4 (derived from N.C.T.C. 3390) grown in the above medium supplemented with 0·00025 M–$CaCl_2$ which is an essential factor for the multiplication of these two phages. Phage anti-R was prepared by the lysis of R4 grown in tryptic meat broth supplemented with 0·002 M–$MgSO_4$. A high magnesium concentration is necessary for the adsorption of this phage to its host (Tucker, personal communication). All the lysates were centrifuged to remove bacterial débris and stored at 4° with the addition of chloroform to prevent bacterial contamination.

Radioactive phage 1 labelled with ^{32}P was prepared as follows: *Salm. typhi* R4 was grown in the defined medium modified by reduction of the phosphate concentration to 0·02 M and the addition of 10 μc./ml. of ^{32}P as orthophosphate.

Seven 10-ml. lots of this medium were rocked at 37° in " L " tubes (Kay, 1952) until the bacteria had grown to 4×10^8/ml. Phage 1 was then added to give a ratio of one phage particle per cell. Lysis was complete in 4 hr. and the phage was then separated and purified by three cycles of low (2000 g.) and high (30,000 g.) speed centrifugation. A concentrate containing $3·7 \times 10^{11}$ active phage particles per ml. with a ^{32}P radioactivity of 120,000 counts/ml./min. was obtained. High speed centrifugation sedimented 88 per cent of the radioactivity and a suspension of O 901 R cells in 0·03 M phosphate buffer was found to adsorb 98·5 per cent of the phage activity and 80 per cent of the radioactivity.

All four phages were assayed by the two-layer method on R4. Phages 1 and 2 were incubated at 37° overnight, but phages T3 and anti-R were ready for counting after 4–6 hr. Further incubation was undesirable because the plaques grew in size and coalesced.

Measurement of radioactivity.

Samples (0·1 ml.) were dried on aluminium dishes (2 cm. diameter) and counted under a thin window Geiger-Müller counter. When samples of larger volume had to be counted a liquid counter tube holding 10 ml. was used. At least 3000 counts were obtained from each sample.

EXPERIMENTAL.

Extraction of Phage Receptors from O 901 R.

A suspension of acetone-dried cells (50 g.) in water (300 ml.) was heated at 60° for 1 hr. with occasional shaking. After centrifugation the supernatant fluid was removed and the sedimented cells again extracted with water at 60° for 1 hr. Six serial extracts made in this way were clarified by centrifugation at 10,000 r.p.m. and assayed for phage-inactivating power by the following method. Serial tenfold dilutions of the extracts (2 ml.) in water were added to 0·067 M phosphate buffer at pH 7·6 (2 ml.) containing 0·004 M magnesium and 0·02 per cent gelatin. A

dilute suspension of the phage (0·1 ml.) was then added to each dilution of the extract and to a control tube containing buffer but no extract. The final phage concentration was about 1×10^3/ml. A sample from the control tube was assayed immediately to determine the initial phage titre. After incubation at 37° for 90 min. the phage titre in each tube was determined and the percentage of the phage particles inactivated was calculated. Phages 1, 2 and anti-R were tested in this way, but in order to test phage T3 the medium had to be modified to contain only 0·0002 M magnesium instead of 0·002 M. The magnesium concentration of 0·002 M had been chosen because it promoted the rapid inactivation of phage anti-R and was effective in preserving phage 1 from non-specific inactivation. Phage T3 however has little affinity either for its host bacteria or for its receptor in solution in the presence of 0·002 M-Mg, but at 0·0002 M-Mg both reactions proceed at a satisfactory rate.

The percentage of phage 1 particles inactivated by different concentrations of each of the six extracts is shown in Table 1. A very approximate estimate of the

TABLE I.—*The Inactivation of Phage* 1 *by Six Serial Extracts of* Salm. typhi *O* 901 *R.*

Extract tested.	Concentration of extract in phage-extract mixture (μg./ml.).* 1000.	100.	10.	1.
1	66	27	22	0
2	99	53	0	0
3	100	79	22	0
4	100	93	43	20
5	100	96	23	0
6	100	93	71	0

* The figures represent per cent of total phage inactivated.

amount of receptor that inactivates 50 per cent of the phage was made by interpolation of a plot of the logarithm of the concentration against the percentage of phage inactivated. For example extract No. 1 inactivates 50 per cent of the phage at 400 μg./ml. but extract No. 4 is equally active at only 15 μg./ml. The " 50 per cent inactivation " concentration was calculated for each extract against all four phages. These figures obtained are shown in Table II, from which it can be seen

TABLE II.—*The Anti-phage Activity of Six Serial Extracts of* Salm. typhi *O* 901 *R against Four Strains of Phage.*

Extract tested.	Concentration of extract (μg./ml.) to inactivate 50 per cent of the phage. Phage 1.	Phage 2.	T 3.	Anti- R.
1	400	+	+	+
2	90	*	*	2,500
3	30	4,000	2,700	180
4	15	1,500	1,500	40
5	20	2,000	1,500	40
6	20	1,500	1,000	40

\+ No activity at highest concentration tested.
* Highest concentration caused less than 50 per cent inactivation.

that material active against phage 1 is found in all the extracts and against all four phages in the last five extracts. However, the activities against phage 1 and phage anti-R are much greater than those against phage 2 and phage T3. The weight of material in the extracts varied from 8·9 g. in the first to 0·66 g. in the sixth, and totalled 17·96 g. or 35·9 per cent of the weight of cells taken. In this series of extractions the sixth extract still contained anti-phage material and in another series the thirteenth was still active. Under the microscope the extracted cells appeared to be of normal bacillary shape and did not seem to have disintegrated to any marked extent. The low yield of phage 2 receptor can be explained by its lability to heat. A washed suspension of O 901 R in 0·033 M phosphate buffer was found to adsorb 50 per cent of phage 1 and 99 per cent of phage 2 in 40 min. In another suspension of bacteria which had been heated at 60° for 1 hr. the adsorption of phage 2 was reduced to less than 10 per cent, whereas that of phage 1 was almost unaffected at 49 per cent.

Purification of Phage Receptors.

Acid precipitation.

Following the procedure described by Jesaitis and Goebel (1952) an attempt was made to precipitate the phage receptors at an acid pH. Extracts 3, 4, 5 and 6 which contained most of the active material were pooled and adjusted to pH 4·2 with acetic acid after cooling to 4°. After 18 hr. the precipitate which formed was separated by centrifugation at 4° and dissolved in water by the addition of sufficient NaOH to bring the pH to 7·6. The supernatant was also adjusted to pH 7·6. Both fractions were dialysed against distilled water and tested for anti-phage activity. The supernatant was found to be inactive against phages 2, T3 and anti-R but slightly active against phage 1 (50 per cent inactivation required 1 mg./ml.). The precipitate was highly active against phage 1 (7 μg./mg.), but the activity against phage anti-R was reduced to one half. There was no activity against phage 2 and very little against phage T3. The lost activity against phages 2 and T3 could not be recovered by mixing the two fractions or adding the dialysate. It appears therefore that acid precipitation is only of value for concentrating the phage 1 receptor, as the receptors for phages anti-R, T3 and 2 are destroyed to a greater or lesser extent. At this stage work was restricted to the phage 1 receptor.

Precipitation with sodium chloride.

It was found that the phage 1 receptor was precipitated by the addition of 10 per cent NaCl. The calculated quantity of solid NaCl was dissolved in the cold solution (pH 7·6) of the acid-precipitated phage 1 receptor and stood at 4° overnight. The precipitate was removed by centrifugation and dissolved in water. After dialysis against distilled water the activity was found to have increased to a " 50 per cent inactivation " concentration of 2 μg./ml., an improvement of 3·5 times over the acid-precipitated fraction. The yield of this material was 230 mg. from 50 g. dried bacteria. It had a nitrogen content of 6·9 per cent.

Dissociation with phenol.

Jesaitis and Goebel (1952), working with the somatic antigen from *Sh. sonnei* which is active against phages T2, T3, T4, T6 and T7, found that treatment with

90 per cent phenol dissociated the antigen into protein and lipocarbohydrate parts. Activity against T3, T4 and T7 was retained in the lipocarbohydrate fraction, but phages T2 and T6 were no longer attacked. A sample of the NaCl-precipitated phage 1 receptor in solution was mixed with solid phenol until a solution containing 85 per cent w/v of phenol was obtained. This was stirred at room temperature for 30 min. and then dialysed until the phenol was removed. The dialysis sac then contained a precipitate which had a nitrogen content of 12·4 per cent and a solution containing material with a nitrogen content of 1·5 per cent. These materials were not active against phage 1 either alone or when mixed together.

A similar result was obtained with an antigen from O 901 kindly supplied by Dr. D. A. L. Davies. This material was produced by extraction of the cells with trichloracetic acid and was subsequently degraded into protein, carbohydrate and lipid fractions. When tested against phage 1 the complete antigen (250 μg./ml.) destroyed 94 per cent of the phage in 1 hr. but the degraded materials were all inactive.

The Inactivation of Phage 1 *by its Receptor.*

In order to study the release of nucleic acid from inactivated phage it was necessary to use an amount of phage which would contain sufficient nucleic acid to be measurable by the available techniques. It was therefore desirable to know the maximum amount of phage which could be inactivated by a given amount of receptor. After some preliminary experiments to decide the approximately equivalent quantities of phage and receptor the following experiment was carried out.

Phage 1 ($1{\cdot}2 \times 10^{11}$ particles) in 0·033 M phosphate buffer pH 7·6 (1·9 ml.) containing 0·002 M magnesium and 0·01 per cent gelatin was added to each of two tubes. Purified phage 1 receptor (0·1 ml. containing 325 μg.) was added to one tube and water (0·1 ml.) to the other (control). Both tubes were incubated at 37°. The phage titre in the control tube was determined at 0 and 20 min. and in the other at 5, 10 and 22 min. At 24 min. phage 1 ($1{\cdot}2 \times 10^{11}$ particles) was again added to each tube. Phage titres in the control were determined at 24 and 105 min. and in the tube containing the receptor at 31, 41, 46 and 110 min. The results in Fig. I show that the first addition of phage is almost completely inactivated (92 per cent), whereas the second was not damaged at all. The phage titre in the control tube remained constant within the experimental error. All the sites on the receptor to which phage 1 could adsorb must have been occupied by the first addition of phage, which appeared to have been in excess, since 8 per cent remained active. Under these conditions therefore 325 μg. of receptor were saturated by 92 per cent of $1{\cdot}2 \times 10^{11}$ particles of phage 1. In another test 325 μg. inactivated 82 per cent of $1{\cdot}5 \times 10^{11}$ particles. From the figures it can be calculated that one particle of phage 1 is inactivated by $2{\cdot}6\text{–}3{\cdot}0 \times 10^{-15}$ g. of receptor. Jesaitis and Goebel (1953) found that their lipocarbohydrate fraction from *Sh. sonnei* required only 2×10^{-16} g. to inactivate one particle of phage T4. However, the phage 1 receptor has received only two stages of purification whereas the *Shigella* material had been purified more rigorously and had been de-proteinised.

Release of nucleic acid from phage 1.

If the reaction between phage and its receptor releases nucleic acid which may then go into solution it should be possible to detect this by removing the phage

bodies by centrifugation and measuring the amount of nucleic acid left in solution. By the use of phage labelled with ^{32}P which is believed to go almost entirely into the nucleic acid it is possible to follow the release of nucleic acid into the medium in non-sedimentable form.

Radioactive phage 1 ($3{\cdot}7 \times 10^{11}$ particles) was incubated at 37° with 11·5 mg. purified receptor in 0·03 M phosphate buffer pH 7·6 (30 ml.) containing 0·002 M magnesium and 0·01 per cent albumin. A control of phage alone was also set up. Samples (5 ml.) were removed at intervals to determine the non-sedimentable ^{32}P. These were stored at 2° until all the samples had been collected and then centrifuged at 15,000 *g.* for 60 min. also at 2°. The supernatants were removed and assayed for ^{32}P. The non-sedimentable ^{32}P in the control was determined

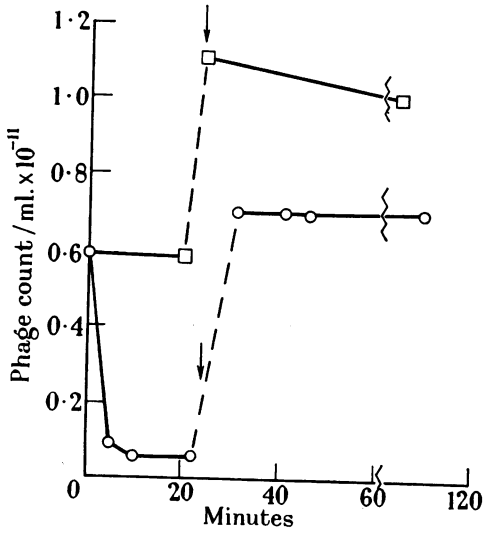

FIG. 1.—The inactivation of phage 1 in high concentration by purified receptor preparation (325 μg.). ⊡——⊡ Phage alone. ⊙——⊙ Phage plus receptor. Arrow indicates time of further addition of phage.

three times during the incubation period and found to be constant. The phage titres in both tubes were also determined. The storage of samples at 2° until a convenient time for centrifugation is justified since it was found that no ^{32}P is released for a period of 60 min. if the mixture of phage and receptor is kept at 2°. After making a correction for the amount of ^{32}P initially non-sedimentable the amount rendered non-sedimentable by the action of the receptor was calculated. Fig. 2 shows the result obtained together with a curve showing the inactivation of the phage. It can be seen that inactivation of the phage proceeds more rapidly than the release of ^{32}P especially in the first minute when 53 per cent of the phage is inactivated but only an insignificant amount of ^{32}P is found in the medium.

DISCUSSION.

The repeated extraction of O 901 R cells with water at 60° provides a satisfactory means of preparing the receptors for phages 1 and anti-R but not those for phages 2 and T3. The phage 2 receptor was found to be heat-labile as judged

by the lowered affinity for phage 2 caused by heating the cells at 60° and this could account for the low yield of anti-phage 2 activity in the extracts. The low yield of T3 receptor is unexplained. The action of the receptor in solution on phage 1 results in the rapid inactivation of the phage and a somewhat slower release of ^{32}P-containing material which may be phage deoxyribonucleic acid (DNA). The results of Jesaitis and Goebel (1953) showed that DNA was released when phage T4 was inactivated. Although there is a lag of about one minute's duration before a significant amount of ^{32}P appears in solution it is clear from the slope of the curve (Fig. 2) that ^{32}P and presumably DNA escapes from the phage

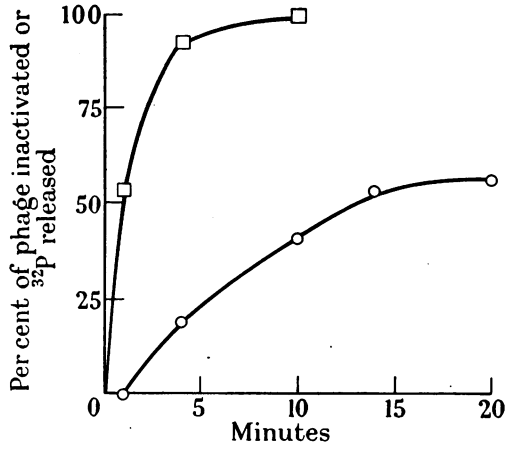

FIG. 2.—The liberation of ^{32}P from labelled phage 1 ($3 \cdot 7 \times 10^{11}$ particles) incubated with purified receptor (11·5 mg.). ⊙——⊙ Percentage of initially sedimentable ^{32}P rendered non-sedimentable. ⊡——⊡ Percentage of phage inactivated.

particles within a few minutes of their combination with the receptor. If the reaction between the phage and its receptor on the bacterial surface proceeds at a comparable rate then the first of the DNA will have left the phage particle and appeared on the bacterial surface in an equally short time. Whether the release of DNA at the bacterial surface is sufficient to constitute an active infection of the cell is unknown. It may be that an energy-requiring step then occurs which transfers the DNA across the cell wall. However the known properties of the phage receptor are sufficient to account for the specific adsorption of the phage particles to the host cell and the transfer of the DNA from the inside of the phage particle to the bacterial surface.

SUMMARY.

The receptors for three of the bacteriophages which attack *Salm. typhi* O 901 R can be isolated from the acetone-dried cells by extraction with water at 60°. The phages are inactivated by treatment with the receptor solution. Purification of the receptor material by precipitation with acid (pH 4·2) and NaCl (10 per cent) gave a product which was highly active against one of the phages (phage 1), one particle of which is destroyed by $2 \cdot 6 \times 10^{-15}$ g. of the receptor.

The inactivation of phage 1 results in the release into solution of material containing phosphorus which is presumably nucleic acid. The rate of release was

followed by the use of phage labelled with ^{32}P and found to be slower than the rate of inactivation.

The author wishes to thank Sir Paul Fildes, F.R.S., the Director of the Unit, for his valuable criticism and advice during the course of this work, and the British Empire Cancer Campaign for a personal grant.

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