BACTERIOPHAGE INHIBITION BY EXTRACTS FROM PHAGE-INSENSITIVE BACTERIA OF THE GENUS *PSEUDOMONAS*

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INTRODUCTION

The correlation among related bacteria between phage susceptibility and possession of a common antigen noticed first by Hadley (1926) and repeatedly confirmed, led Burnet (1927, 1929, 1930) to suggest that attachment of phages to bacteria takes place through the specific surface antigen. Support for this suggestion came first from the many instances in which bacteria lose their susceptibility to one or more phages when undergoing the S→R transformation, and secondly from the discovery by Levine & Frisch (1933), soon extended by other workers, that extracts from susceptible bacteria could in many cases protect these organisms from certain types of phage (Burnet, 1934; Gough & Burnet, 1934; Bruce White, 1936; Rakieten, Rakieten & Doff, 1936; Beumer, 1947; Miller & Goebel, 1949; Goebel, 1950). Andrewes & Elford (1933) postulated that the bacteriophage surface consists of a mosaic of antigenic components and bacteriotropic components. The former combine with antibody in antiphage serum which then prevents or delays attachment of the phage to bacteria through neighbouring bacteriotropic components. Intact receptors present in suitably prepared extracts of susceptible bacteria combine with bacteriotropic constituents of phage and hinder penetration into bacteria probably by mechanical obstruction (Burnet & Freeman, 1937).

Although these theories have been supported by a host of observations on the relation between phage susceptibility and antigenic structure and by many examples of phage inhibition by extracts from sensitive bacteria, homogeneous phage inhibitors have been isolated in only a few instances. Indeed, it has been common experience that attempts to purify crude bacterial extracts cause progressive loss of their phage-inhibiting properties. As bacteria sometimes undergo a change from phage sensitivity to phage resistance without detectable effect on their immunological properties, it has been inferred that phage sensitivity must be determined by minute details in the architecture of the surface antigen. If this is the case, it is hardly surprising that the most active phage-inhibitory extracts have been obtained by the mildest methods (which, however, yield very complex extracts) and that attempts at purification may cause changes in molecular pattern detectable by phages but not by routine immunological techniques.

Of the pure substances extracted from susceptible bacteria and proved to inhibit phages, one, isolated by Gough & Burnet (1934) from *Bacillus sanguinarum* appeared to be a pure polysaccharide haptene but showed activity against only one phage. The pure haptene from *Salmonella enteritidis*, likewise effective

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against this phage, provides the second example. The only other example of pure phage inhibitors of which we are aware are the polysaccharide lipoprotein complexes of phases I and II *Shigella sonnei*, used by Miller & Goebel (1949) and Goebel (1950).

Inhibition of phages by extracts from non-susceptible bacteria is a phenomenon less easy to interpret in terms of Burnet's phage-receptor theory. Examples have been noted by Levine & Frisch (1933), by Burnet (1934), and by Beumer (1947), but no attempts to investigate the nature of the phenomenon or to identify the inhibitory substances appear to have been made. While investigating the action of a group of phages on strains of *Pseudomonas aeruginosa* and their variants, we have discovered several examples of this effect. One strain yields on treatment with cold 0·25 N-trichloroacetic acid (TCA) (Boivin & Mesrobèanu, 1933) an apparently homogeneous antigenic extract which not only inhibits the action of several phages to which it is sensitive but is also effective against two phages to which it is resistant. Three other apparently unrelated strains resistant to these two phages also yield extracts which inhibit them. It would appear, therefore, that in *Ps. aeruginosa* somatic antigens may determine phage resistance as well as phage susceptibility.

Another interesting property of the extract from one strain is that while diminishing or abolishing the activity of at least two phages (as judged by plaque counts on a sensitive variant) it increases the activity of a third phage. The preparation of the inhibitory extracts will be described in a separate paper (Mead).

MATERIALS AND METHODS

Bacteria

The origin, characteristics and serological reactions of the strains AII, BII, GII, LII and LIII–3bi of *Ps. aeruginosa* have been described elsewhere (Don & van den Ende, 1950; van den Ende, 1952). The strains were stored in the dry state (Stamp, 1947) and a fresh subculture was invariably used for each day's experiments.

Bacteriophages

The origin, characteristics and serological grouping of *Ps. aeruginosa* phages numbered 1–17 have been described (van den Ende & Mead, 1952). Phages 1 and 3 are active against *Ps. aeruginosa* strain LIII–2; phages 3–17 against strain LIII–3bi. Phages O, W, X(L) and AD are active on strain LII and very slightly active on LIII–3bi (see Tables 2 and 3).

The phage 5 used for this work was propagated on strain LIII–3bi and concentrated by differential centrifugation to a titre of 2×10^{12} plaque-forming particles/ml. The other phages were broth 'lysates'* of infected cultures of LIII-3bi clarified by centrifugation and sterilized at 56° C. for 30 min. For the inhibition experiments the preparations were diluted in Difco nutrient broth containing 0·5 % NaCl to a concentration of approximately 2×10^3 particles/ml.

* Cultures of LIII–3bi are not in fact visibly lysed by several of these phages but the term 'lysate' has its usual significance.

Media

Water, except that used for cooling, was distilled water condensed in Pyrex glass. The solid medium consisted of Hartley's digest broth containing 1·2 % of Bacto agar. It was poured in Petri dishes (6 in. diam.) to form a layer 6–8 mm. thick. One batch of medium was used for most of the experiments described in this paper. Difco nutrient broth (0·8 %) containing sodium chloride (0·5 %) adjusted to pH 7·2–7·4 and sterilized by autoclaving was used for dilutions and for all cultures except when the use of the following special media is specifically mentioned.

Dialysed Difco-medium

Difco (dried) nutrient broth (100 g.) in water (400 ml.) was dialysed with stirring against water (2·5 l.) at 60° C. After 1·5 hr. the diffusate was cooled rapidly in ice and replaced by water (2·5 l.) which was maintained at 80° C. Two hours later this was cooled in ice and replaced by water (2·5 l.) at 90° C. Diffusion was allowed to proceed at this temperature during 4½ hr. and in the refrigerator (without stirring) overnight. Next day the diffusate was replaced by hot water (2·5 l.) and diffusion continued at 90–95° C. for a final period of 4½ hr. The diffusates were mixed, sodium chloride (50 g.) added, pH adjusted to 7·2, water added to 10 l. and the medium autoclaved in 1 l. bottles.

Casein hydrolysate medium H.I.D.

(*a*) *Dialysed casein hydrolysate.* Casein (200 g.) mixed with hydrochloric acid (170 ml. of sp.gr. 1·18) and water (110 ml.) was autoclaved for 45 min. at 15 lb. pressure (Gladstone & Fildes, 1940). The hydrolysate was neutralized, filtered through paper pulp and concentrated to about half the original volume. The solution was dialysed with stirring against water (3 l.) at 80° C. for 2–3 hr. The diffusate was cooled, treated with chloroform and replaced by fresh water into which diffusion continued in the refrigerator overnight. Diffusion at 80° C. was continued during the next day. In the evening the diffusate was cooled, treated with chloroform and replaced by fresh water. The apparatus was put into the refrigerator overnight and diffusion at 80° C. continued during the third day. These conditions were chosen on the basis of amino-nitrogen determinations (Pope & Stevens, 1939) carried out on the successive diffusates. The three lots of diffusate were combined (volume, 8·5 l.) and preserved with chloroform.

(*b*) *Dialysed yeast extract.* Brewer's yeast (500 g.) in two equal lots was added to boiling water (1 l. per 250 g. yeast), and boiling was continued for about 5 min. (Gladstone & Fildes, 1940). The insoluble debris settled out in the refrigerator overnight. The supernatant was concentrated under reduced pressure to about 200 ml. and dialysed during 2 days against two successive portions of water. The bulked diffusate (volume 2·5 l.) was preserved with chloroform. To make up the medium casein hydrolysate diffusate (6 l.) and the diffusate from 500 g. yeast were boiled briefly in order to remove dissolved chloroform, and mixed with an aqueous solution containing diammonium hydrogen phosphate (40 g.), potassium dihydrogen phosphate (30 g.), sodium chloride (90 g.) and sodium lactate (200 ml. of 70 %).

A solution of magnesium chloride hexahydrate (2·0 g.) was added to the mixture followed by water to 20 l. After adjustment of pH to 7·5–7·8 the mixture was autoclaved in 2 l. Pyrex flasks, filtered, and re-autoclaved.

Bacteriological techniques

(*a*) *Pipettes.* Pipettes delivering 0·02 ml. drops of broth were made as described by Donald (1915) and Miles & Misra (1938). The volume delivered by each pipette was checked by weighing at least three drops of broth. The volume delivered from a '50 dropper' pipette depends on the surface tension of the fluid used, and since inhibitor preparations might influence this property, tests were made by weighing drops of phage suspensions with and without inhibitor extracts delivered from several pipettes. No significant differences were found.

(*b*) *Bacterial cultures on solid medium for plaque counts.* The following details are given because phage counts appeared susceptible to small variations in technique. Ten ml. of broth to which a pellet of the dried phage-sensitive strain LIII–3bi had been added were aerated overnight at 37° C. Next day a flooding culture was prepared by adding 0·06 ml. of the overnight culture to 35 ml. of broth and incubating with aeration at 37° C. until the turbidity measured in a 0·5 cm. cell of the Hilger absorptiometer with neutral grey filters (Hilger H. 508) against sterile medium corresponded to an optical density of about 0·08. This usually required 3–3½ hr. About 5·0 ml. of the culture were poured on to the surface of a plate which had been dried for 1½–2 hr. at 37° C. The plate was rapidly tilted to ensure that the whole surface was wetted by the culture, the excess of which was then immediately poured off. The flooded plates were re-dried at 37° C. for 25 min. and used at once.

(*c*) *Determination of phage susceptibility.* Plates seeded with the different strains of *Ps. aeruginosa*, as described in the preceding section, were spotted with at least two 0·02 ml. drops of each of two dilutions of the phages. The dilutions employed were calculated to give respectively about 200 and about 20 plaques per drop on the sensitive organism (LIII–3bi for phages 3–17; LII for phages AD, X(L), W and O).

Variation in the number of plaques given by the same suspension of phage 5 spotted with the same pipette on different plates may be due to the plating efficiency of this phage with our technique falling short of 100 %. This phage was therefore chosen for a comparison of our method with the agar overlayer technique recommended by Gratia (1936). Sixteen 0·02 ml. drops of a given suspension plated on one plate gave a total count of 992 plaques or $3{\cdot}1 \times 10^3$/ml. Four plates, each overlayered with 1 ml. of a mixture of the same phage suspension (0·5 ml.) with a culture of the sensitive organism LIII–3bi (3·5 ml.) and agar in broth (1·0 ml. of 2·5 %), gave counts of 349, 299, 207 and 333 plaques respectively. The average count indicated a titre of $2{\cdot}97 \times 10^3$/ml.

(*d*) *Measurement of phage inhibition.* For reasons discussed later, it was found essential to compare control and inhibited phage suspensions under conditions as nearly identical as possible. For most of our work the following standard procedure was adopted. Inhibitor solutions were normally prepared by diluting extracts (of

which the solid content had been determined by drying an aliquot from the frozen state) with 0·0023 M ($I = 0·005$) phosphate buffer of pH 7·0–7·2 to a concentration of 4 mg./ml. Alternatively, the freeze-dried solid sterilized, if necessary, by being moistened with 70 % ethanol (Miller & Goebel, 1949) was dissolved directly in buffer to a concentration of 4 mg./ml. The initial solution was diluted with an equal volume of broth and subsequent dilutions were made with a 1:1 mixture of broth and buffer. To determine the inhibitory power of a preparation, 0·5 ml. of the phage (2×10^3/ml.) was added to each of a series of tubes containing 0·5 ml. amounts of serial dilutions of inhibitor and to a control tube containing 0·5 ml. of diluent. The tubes were placed in a water-bath at 37° C. for 2 hr. then cooled in tap water and plated at once. Eight 0·02 ml. drops from each tube and eight drops from the control were placed on one or more plates seeded with LIII–3bi and re-dried as described in § (*b*). The spots of experimental and control suspensions were arranged in alternate rows of four spots each. The plates were incubated 16–18 hr. at 37° C. If incubation is prolonged, the plaque count of the inhibitor-containing suspension may increase—especially with phage 5. The plaques, however, remain smaller than those from the control suspension. Plaques were counted with the aid of a hand-lens. A direct comparison was made in every case between the number of plaques given on the same plate by (i) a phage in contact with inhibitor, and (ii) the control phage suspension. If the control suspension gave n plaques and the experimental suspension m plaques, percentage inhibition was calculated as $(n-m)\,100/n$. If m exceeded n, $(m-n)100/n$ was recorded as percentage exhibition. At an early stage it was found that phage 13 is not inhibited by extracts of *Ps. aeruginosa* LII active against phages 5 and 14. Tests on phage 13 were frequently made in parallel with those on the other phages as a safeguard against errors in technique.

In some experiments the diameters of 10 (occasionally 5) control, and the same number of experimental plaques chosen at random were measured with a Hilger microcomparator capable of being read to 1μ. Comparisons were always made between plaques formed on the same plate, and the percentage reduction (if any) in the average diameter of the experimental plaques was calculated.

(*e*) *Phage-resistant mutants LIII–3bi/5 and LIII–3bi/14 of* Pseudomonas aeruginosa. Bacteria scraped from plaques or areas of confluent lysis produced by phages 5 and 14 on surface growth of LIII–3bi were grown in broth. The cultures when faintly turbid were used to flood plates which after re-drying were treated with excess of the corresponding phage. Bacteria scraped from these plates were grown in broth. The cultures were diluted and plated. Single colonies were picked for inoculation of agar slopes which after incubation at 37° C. overnight were kept in the refrigerator and used for the preparation of dried pellets (Stamp, 1947). The phage susceptibilities of these mutants are given in Table 5. Culture filtrates from strain *Ps.* LIII–3bi/14 contain free phage. This property is retained when the parent strain has been subcultured in an effective concentration of antiphage 14 serum. A culture after induction (Lwoff, Siminovitch & Kjeldgaard, 1950) by exposure (in buffer) to an amount of ultraviolet radiation, comparable to that required by a lysogenic strain of *Escherichia coli* (Weigle & Delbrück, 1951), and re-incubation in broth is lysed with liberation of free phage 14 at high titre. This

phage, though neutralized by antiphage 14 serum, appears to be less sensitive than the original phage 14 to inhibition by purified TCA extracts from *Ps.* LII. No lysogenic properties were detected in strain *Ps.* LIII–3bi/5.

Inhibitors

The preparation and properties of extracts from *Ps. aeruginosa* strains AII, BII, GII and LII will be described in a separate paper (Mead) and need only be summarized here.

Bacteria grown in liquid culture and in the case of strain LII on media from which large molecular components had been removed by dialysis, were extracted with cold 0·25 N-trichloracetic acid (TCA) essentially as described by Boivin & Mesrobèanu (1933, 1937). The neutralized extracts after dialysis against water were fractionally precipitated by acetone at 0° C. after the addition of $\frac{1}{10}$th volume of a 20% w/v solution of sodium acetate trihydrate. In the case of the *Ps.* LII extract, for example, the precipitate obtained between the levels of 60% and 70% acetone was redissolved in water, one-tenth volume of sodium acetate solution added and the inhibitor precipitated by addition of acetone to 64%. The precipitate was dissolved in water, the solution dialysed and then centrifuged at 18,000 r.p.m. for 2 hours. The supernatant fluid was removed and the deposit suspended in water. The suspension, clarified by centrifuging at 5,000 r.p.m. for $\frac{1}{2}$ hour, was spun at 20,000 r.p.m. for 2 hours. The final supernatant fluid was added to that from the first high speed run and the mixture termed Fraction A (Table 1). This solution was kept at 4° C. Material prepared from acetone-dried organisms of strain *Ps.* LII by extraction with ethylene glycol (Morgan, 1937), was purified in the same way and behaved in inhibition experiments similarly to extracts made with TCA.

An acetone-precipitated TCA extract from strain *Ps.* LII used for many of the inhibition experiments appeared homogeneous when submitted to electrophoresis at pH 7·5 and 4·0. Centrifugation experiments, however, indicated that the preparation was polydisperse.

The preparation of a TCA extract from strain *Ps.* LIII–3bi will be described in a separate paper. In addition, a lysate of this strain was made in the following manner. The organisms were grown in H.I.D. broth (8 l.) to which a few drops of tributyl phosphate were added as antifoam. The culture was incubated at 37° C. in two 5 l. flasks fitted with sintered glass disks through which oxygen was passed. After 18 hr. the bacteria were collected in a refrigerated Sharples centrifuge. The yield was 47 g. (moist weight). To prepare the lysate, moist organisms (17 g.) were suspended by vigorous mechanical stirring in buffered saline (30 ml., pH 7·0). Chloroform (5 ml.) was added and the mixture stirred at 37° C. during 3·5 hr. Centrifuging for 30 min. at 6000 r.p.m. yielded a slimy deposit, a brown, slightly turbid fluid and a supernatant (fatty?) scum. The fluid was separated as completely as possible from the other phases, clarified by centrifugation at 12,000 r.p.m. for 30 min. and dialysed. The product (100 ml.) was a faintly brown solution of marked viscosity containing 3·2 mg. of solids per ml. It was slightly turbid but lacked the blue-grey opalescence characteristic of the TCA extracts.

RESULTS

A. *Inhibitor preparations*

The derivation and some properties of the inhibitor preparations are given in Table 1.

B. *The phage susceptibility of strains of* Pseudomonas aeruginosa

Tables 2 and 3 give the results of tests for the sensitivity of various *Ps. aeruginosa* strains to the phages used in this investigation.

The significant results are: (i) the susceptibility of strain LIII–3bi, which is a variant from strain LII, to phages 3, 5, 11, 13, 14 and 17; (ii) the susceptibility of strain LII to phages O, AD, X(L) and W; (iii) the resistance of strain LII and of the antigenically distinct and unrelated strains AII, BII and GII to phages 5, 13 and 14 with which we are now principally concerned. The complementary relation between the resistance patterns of strains LIII–3bi/5 and LIII–3bi/14 is also of interest.

In accordance with these results strain *Ps.* LIII–3bi has been used as indicator for phages 3, 5, 11, 14 and 17 and strain LII as indicator for phages O, AD, X(L) and W.

C. *The inhibition of phages* 5 *and* 14, *by extracts from* Pseudomonas aeruginosa *strain LII*

These results are of special interest because the strain yielding the inhibitor is insensitive to these phages while the sensitive strain when extracted in the same manner gives no inhibitor.

(1) *Summarized results of inhibition experiments with phage* 5

Forty inhibition tests were made with a variety of *Ps.* LII extracts at different stages of purification and at concentrations ranging from 0·125 to 2·0 mg./ml. Complete inhibition was recorded on three occasions, while on five there was less than 20 %. The overall average inhibition for all extracts and concentrations was 46 %.

(2) *Summarized results of inhibition experiments with phage* 14

Thirty-seven inhibition tests were made with various extracts at concentrations between 0·025 and 1·0 mg./ml. Complete inhibition was recorded only once. The overall average inhibition was 81 %.

(3) *The reproducibility and significance of results*

A difficulty in working with the phages to which strain LIII–3bi is sensitive is that their plating efficiency is not uniformly high. This applies especially to phage 5. We have tested the following media solidified with 1–1·2 % Bacto agar; Difco nutrient broth, Hartley's digest medium, MacConkey broth (prepared without indicator) and H.I.D. medium. The best results, both as regards plating efficiency and demonstration of inhibitor effect, were obtained with Hartley's digest agar poured in thick (6–8 mm.) layers. Even on this medium the phage count (of

Table 1. *Preparation and properties of inhibitor preparations*

Ps. aeruginosa strain	Method of extraction	Fraction and treatment	Yield (% of moist bacteria)	Precipitin titre*	Percentage inhibition of phages (concentration in mg./ml. given in brackets)			
					3	5	13	14
LII	TCA	(*a*) TCA extract neutralized and dialysed	0·8	1/200,000	—	—	—	—
LII	TCA	(*b*) Fraction A. Extract (*a*) twice precipitated by acetone (70 %) and freed from largest particles by centrifugation at 20,000 r.p.m.	0·4	1/200,000	—	75 (1)	—	—
LII	TCA	(*c*) Fraction A 3. Fraction A purified by electrophoresis at pH 7·5 and 4·0‡	0·32†	1/200,000	Nil (1)	75 (1)	Nil (1)	80 (1)
LII	TCA	(*d*) Fraction C. Recovered from centre section of electrophoresis cell‡	—	1/100,000	—	—	—	68 (1)
LII	Glycol	(*e*) Acetone-dried organisms extracted with glycol. Solvent removed by dialysis	6§	—	—	—	—	—
LII	Glycol	(*f*) Crude extract (*e*) twice precipitated by acetone‖	3§	1/250,000	—	100 (2)	—	—
LII	Glycol	(*g*) Crude extract (*e*) shaken with chloroform and butanol.¶ Substance in aqueous layer precipitated by acetone‖	2·4§	1/200,000	—	100 (2)	—	—
AII	TCA	Extract neutralized and dialysed. Fraction precipitated by acetone‡	0·7	1/200,000	82 (1)	65 (1)	Nil (1)	54 (1)
BII	TCA	Extract neutralized and dialysed. Fraction precipitated by acetone‡	1·0	1/200,000	20 (0·5)	20 (0·5)	10 (0·5)	35 (0·5)
GII	TCA	Extract neutralized and dialysed. Fraction precipitated by acetone‡	1·1	1/200,000	93 (1)	66 (1)	Nil (1)	81 (1)
LIII–3bi	TCA	Extract neutralized and dialysed only	0·1	1/10	—	17 (0·7)	9 (0·7)	3 (0·7)

* Precipitin titre (ring test) against undiluted serum prepared against whole homologous organisms.
† Estimated recovery of homogeneous substance.
‡ Fractions so marked act as complete antigens.
§ Yields based on weight of dried organisms.
‖ For gradocol filtration experiments on these fractions see Mead.
¶ Sevag, Lackman & Smolens (1938).

a given suspension plated with one pipette) and percentage inhibition (by a single preparation at one concentration) varied somewhat from plate to plate. Thus in a typical experiment an inhibitor solution gave 81, 67 and 60 % inhibition on three plates prepared at the same time.

Table 2. *The susceptibility of strains of* Pseudomonas aeruginosa *to phages active on strain* Ps. *LIII–3bi*

Phages			Strains of *Ps. aeruginosa*						
No.	Serological group	Concentration	LIII–3bi	LII	AII	BII	GII	LIII–3bi/5*	LIII–3bi/14†
3	II	Dilute	24	0	0	0	0	0	16
		Concentrated	∞	N.T.	0	0	0	—	—
5	II	Dilute	23	0	0	0	0	0	56
		Concentrated	∞	0	N.T.	N.T.	N.T.	—	—
11	III	Dilute	22	N.T.	0	0	0	0	38
		Concentrated	N.T.	N.T.	0	0	0	—	—
13	IV	Dilute	46	0	0	0	0	0	37
		Concentrated	∞	0	0	0	0	—	—
14	II	Dilute	72	0	0	0	0	29	0
		Concentrated	∞	0	0	0	0	—	—
17	V	Dilute	30	0	0	0	—	0	33
		Concentrated	∞	N.T.	0	0	—	—	—

The figures are the average plaque counts per 0·02 ml. spot. The 'concentrated' suspensions were diluted $1/10^2$ to give the 'dilute' suspensions.

∞, confluent lysis or number too large for counting.

N.T., not tested.

* Strain LIII–3bi/5 was also resistant to phages 9 and 15 (serological group II).

† Strain LIII–3bi/14 was also sensitive to phages 9 and 15.

Table 3. *The susceptibility of* Pseudomonas aeruginosa *strains LIII–3bi and LII to phages active on strain LII*

(The notation is the same as that in Table 2.)

Phages		Strains of *Ps. aeruginosa*	
		LIII–3bi	LII
AD	Dilute	0	28
	Concentrated	23	—
X(L)	Dilute	0·75	39
	Concentrated	44	—
O	Dilute	0	23
	Concentrated	6	—
W	Dilute	0	8
	Concentrated	0	—

Apparent percentage inhibitions of less than 20 % are probably not significant, especially with phage 5, but good evidence for the reality and specificity of the inhibition effect is provided by the close agreement between plaque counts of control and experimental solutions in cases where inactive extracts or inhibitor-resistant phages have been tested.

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(4) *The rate of reaction between phage and inhibitor*

Many inhibitors derived from phage-sensitive bacteria require prolonged incubation (at temperatures up to 50° C.) with phage in order to exert their maximum effect (see, for example, Burnet, 1934). The rates of interaction of phages 5 and 14 with extracts of the resistant strain LII have not been investigated, but an experiment in which an ice-cold suspension of phage 5 was completely inhibited when mixed with ice-cold LII inhibitor (2 mg./ml.) and plated at once indicated that at high concentrations the inhibitor must act very rapidly. This conclusion was supported by experiments in which phages 5 and 14 were plated on agar treated with inhibitor either before or after being seeded with the indicator organism.

(5) *The effect of inhibitor on plaque size*

As plaques formed by phages 5 and 14 in the presence of inhibitor are smaller than control plaques, whereas the size of plaques formed by the uninhibited phage 13 is not affected, we hoped that this phenomenon would provide a quantitative basis for assessment of inhibition. The average diameters of control and inhibited plaques were estimated in twenty-seven inhibition tests, but the results showed that no simple quantitative test for inhibition could be based on such measurements. In fact, it appeared that reduction in the size of plaques in the presence of an inhibitor is a more frequent occurrence than inhibition defined in terms of reduction in plaque count.

(6) *The influence of inhibitor concentration and period of incubation of plates*

On the basis of early results obtained with phage 5 and relatively crude extracts of *Ps.* LII we adopted 1 mg./ml. as the standard concentration for inhibition tests. Later it became apparent, first that phage 14 is sensitive to much smaller con-

Table 4. *The effect of inhibitor concentration and of delayed reading of plates on the inhibition of phages 5 and 14*

Phage	Inhibitor preparation	Concentration of inhibitor (mg./ml.) ...	2	1	0·5	0·25	0·125	0·05	0·01	0·002
			Percentage inhibition							
5	C		0	28	35	35	10	—	—	—
5	A		—	—	—	68 (−7)	—	35 (−20)	12 (−25)	−30* (−50)
14	A		—	—	—	79 (76)	—	76 (80)	56 (61)	44* (38)

* These solutions and their controls were replated after 48 hr. at 4° C. with the following results: phage 5, −45 %; phage 14, +85 %.

The figures in brackets are percentage inhibitions obtained when the corresponding plates were re-counted after standing for 48 hr. at room temperature. A figure preceded by a minus sign denotes 'exhibition'.

centrations than phage 5, and secondly that for the latter phage there is an optimum concentration above and below which inhibition is less complete. Instances of similar 'pro-zone' effects have been noted previously, e.g. by Gough &

Burnet (1934). The influence of inhibitor concentration is shown in Table 4, which also indicates the pro-zone phenomenon with phage 5 and the effect on the inhibition of this phage (but not phage 14) of delay in counting the plates. The exhibition effect with phage 5 at the low inhibitor concentration and after the plates had stood 2 days at room temperature is noteworthy.

(7) *The neutralization of inhibitory effect by homologous antiserum*

Portions of an inhibitor solution (1·2 mg./ml.) were treated respectively with an equal volume of homologous precipitating serum from a rabbit and with an equal volume of normal rabbit serum. A precipitate formed in the mixture containing the immune serum. After centrifugation, the mixture to which normal serum had been added retained the faint opalescence associated with the inhibitor, whereas that containing the immune serum was clear.

The results in Table 5 show that treatment with antiserum diminished the inhibitory power of the solution against phages 5 and 14 and its activating power on phage 13. In addition, the results show that the inhibition of phage 14 (but not phage 5) was diminished by normal rabbit serum. This latter phenomenon has been demonstrated repeatedly in other experiments, although not so strikingly as in the one recorded in Table 5.

Table 5. *The effect of* Ps. *LII extract after treatment with* (*i*) *homologous antiserum and* (*ii*) *normal serum on phages* 5, 13 *and* 14

	Inhibition (%)	
Phage	By LII extract treated with antiserum	By LII extract treated with normal serum
5	22	72
13	18	−32
14 (1st expt.)	−7	21
14 (2nd expt.)	4·5	−18

Inhibitor concentration 0·25 mg./ml.

D. *The inhibition of phages active on* Pseudomonas aeruginosa *strain LIII–3bi by extracts from* Pseudomonas *strains AII, BII and GII*

Strains AII, BII and GII were obtained from sources differing from each other and from that of strain LII. Acetone-precipitated fractions of TCA extracts of all four strains were antigenic in rabbits, and precipitin tests with extracts and antisera revealed no cross-reactions, although the sera precipitated homologous extracts diluted as much as 1:200,000.

Pseudomonas strains AII, BII and GII are, like LII, resistant to phages 3, 5, 13 and 14 (see Table 2). Nevertheless, their extracts, like that of LII, were inhibitory to phages in this group as shown by the results in Table 1.

The extracts of strains AII, BII and GII inhibit phage 3, whereas tests of the effect of extracts of strain LII on this phage gave inconsistent results. Strains AII and GII yielded the most potent inhibitors, but BII is of interest in that its extract alone inhibited phage 13.

E. *Experiments on the inhibition of phages active against* Pseudomonas aeruginosa LIII–3bi by extracts of this organism

Trichloracetic acid extracts of *Ps.* LIII–3bi organisms failed to inhibit phages 5 and 14 (Table 1); a chloroform lysate, however, proved to be an efficient inhibitor of many phages acting on the homologous strain. The following phages were inhibited by the lysate at a concentration of approximately 0·75 mg./ml. The percentage inhibitions are given in brackets: 3 (93 %), 5 (48 %), 9 (40 %), 10 (68 %), 11 (68 %), 13 (15 %), 14 (38 %), 17 (36 %). The lysate appeared to possess very weak immunological properties. A 1/10 dilution failed to react with homologous antiserum (ring test) but gave traces of precipitate with sera prepared against *Ps.* LII organisms and the TCA extracted *Ps.* LII antigen. The 10^{-2} dilution gave a very faint reaction and only with the serum prepared against the whole LII organism.

F. *The inhibition of phages active on* Pseudomonas aeruginosa *strain LII by the extract from this strain*

The results in Table 6 show that the acetone-precipitated fraction from TCA extracts of *Ps.* LII inhibits phages active against this strain (in addition to phages 5 and 14 to which the strain is resistant). This finding accords with the phage-inhibition theory of Burnet. The activity of the extract at a concentration of 0·5 mg./ml. against phages AD and X(L) is about equal to its activity against phages 5 and 14, but the slope of the activity/concentration curve is greater for the phages active on *Ps.* LII. Phages O and W, which are less sensitive, are also less active against *Ps.* LIII–3bi (Table 3).

Table 6. *The inhibition of phages active on* Ps. *LII by homologous extract*

Concentration of inhibitor (mg./ml.)	Inhibition (% of phages)			
	AD	X(L)	O	W
0·5	90	87	57	47
0·05	22	17	28	18

The inhibitor was an acetone-precipitated fraction from TCA extract of *Ps.* LII. The figures are averaged results from several experiments.

G. *Experiments on the mechanism of inhibition*

(1) *The effect of dilution on mixtures of phage and inhibitor*

To demonstrate indirectly that phage and inhibitor combine, suspensions of phages 5 and 14 at concentrations between 10^7 and 10^3 particles/ml. were incubated for 2 hr. at 37° C. with a fixed concentration of inhibitor and diluted to 10^3 particles/ml. before plating for measurement of inhibition. Controls consisting of corresponding dilutions of inhibitor mixed with phage (2×10^3/ml.) immediately before plating proved that the inhibition observed in the experimental series was not due to residual inhibitor in the dilutions as plated. The fact that relatively

Table 7. *The effect of dilution on mixtures of inhibitor (purified* Ps *LII extract) with phages* 5 *or* 14

Phage	Initial concentration of phage	Inhibitor concentration (mg./ml.) In initial mixture	Inhibitor concentration (mg./ml.) As plated	Dilution of mixture before plating	Inhibition (%)	Concentration of inhibitor in control	Inhibition in control (%)
5	$1{\cdot}5 \times 10^{7}$	0·25	$0{\cdot}25 \times 10^{-4}$	10^{-4}	−5	$0{\cdot}25 \times 10^{-4}$	Nil
	$1{\cdot}5 \times 10^{6}$	0·25	$0{\cdot}25 \times 10^{-3}$	10^{-3}	−9	$0{\cdot}25 \times 10^{-3}$	Nil
	$1{\cdot}5 \times 10^{5}$	0·25	$0{\cdot}25 \times 10^{-2}$	10^{-2}	45	$0{\cdot}25 \times 10^{-2}$	Nil
	$1{\cdot}5 \times 10^{4}$	0·25	$0{\cdot}25 \times 10^{-1}$	10^{-1}	72	$0{\cdot}25 \times 10^{-1}$	26
	$1{\cdot}5 \times 10^{3}$	0·25	0·25	0	80	0·25	54
14	$0{\cdot}95 \times 10^{7}$	0·025	$0{\cdot}025 \times 10^{-4}$	10^{-4}	80	$0{\cdot}025 \times 10^{-4}$	Nil
	$0{\cdot}95 \times 10^{6}$	0·025	$0{\cdot}025 \times 10^{-3}$	10^{-3}	77	$0{\cdot}025 \times 10^{-3}$	Nil
	$0{\cdot}95 \times 10^{5}$	0·025	$0{\cdot}025 \times 10^{-2}$	10^{-2}	86	$0{\cdot}025 \times 10^{-2}$	Nil
	$0{\cdot}95 \times 10^{4}$	0·025	$0{\cdot}025 \times 10^{-1}$	10^{-1}	74	$0{\cdot}025 \times 10^{-1}$	31
	$0{\cdot}95 \times 10^{3}$	0·025	0·025	0	75	0·025	47

small inhibitions were noted in the controls is presumably due to the short period of contact between phage and inhibitor.

The results (Table 7) indicate that phage 14 combines with the inhibitor, and that the combination is not dissociated during dilution by a factor of 10^4. The lower sensitivity of phage 5 is reflected in the greater tendency of the phage-inhibitor complex to dissociate during dilution.

We have been unable to demonstrate combination between phage and inhibitor in any more direct manner. We have also failed to detect significant adsorption of phage 14 to heat (56° C.) killed *Ps.* LIII–3bi or *Ps.* LII organisms even at bacteria/phage ratios of $10^4/1$.

(2) *The inhibition of phage* 5 *by cultures of* Pseudomonas *LII*

The results of a typical experiment given in Fig. 1 show the inhibition of phage 5 added to an actively growing culture of *Ps.* LII which is itself lysogenic. The phage was titrated on *Ps.* LIII–3bi on which the typically regular plaques of the phage normally carried by *Ps.* LII can be differentiated from plaques of phage 5 propagated on *Ps.* LIII–3bi. The concentration of phage producing the regular plaques did not appear to be reduced even during incubation for 24 hr.

H. *The 'Exhibition' phenomenon*

It was realized at an early stage that phage 13 (which is serologically distinct from the inhibitor-sensitive phages 5 and 14) is not inhibited by extracts of *Ps.* LII active against phages 5 and 14. Suspensions of phage 13 with and without inhibitor were therefore plated in parallel with suspensions of the other phages as a negative control to prove that the inhibition observed with these latter phages was not caused by faulty technique. On tabulating the results of several experiments it was obvious that phage 13 is not merely uninhibited but actually gives an increased number of plaques when plated with at least the crude extracts of *Ps.* LII. In nine tests with a variety of such extracts at concentrations between 0·065 and

1 mg./ml. percentage 'exhibitions' of between 12 and 71 % were recorded, the average being 43 %. Exhibition of phage 13 was also obtained when the phage suspension was spotted on to plates which had been seeded with *Ps.* LIII–3bi, re-dried and treated on one-half with a solution containing 0·4 mg. of *Ps.* LII extract in buffer, and on the other with buffer only. This indicates that the exhibition effect, like inhibition, does not necessarily depend on prolonged contact between phage and inhibitor. The phenomenon also resembles the inhibition of phage 5 in that it is subject to a pro-zone effect. A crude TCA extract of *Ps.* LII, for example,

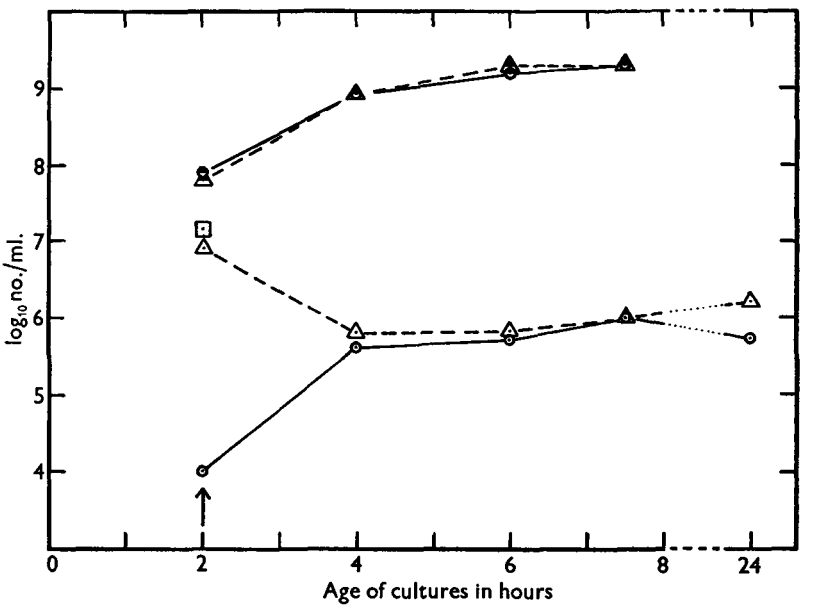

Fig. 1. Changes in the concentration of bacteria and total phage during the incubation of (i) a culture of *Ps.* LII, (ii) an identical culture to which phage 5 was added at the time indicated by the arrow. ◓—◓, bacteria in control culture; △—△, bacteria in culture infected with phage; ⊙—⊙, phage in control culture; △---△, total phage in culture to which phage 5 was added; ⊡, calculated concentration due to added phage.

gave 14 % exhibition when tested at 1 mg./ml., 33 % at 0·5 mg./ml., 57 % at 0·25 mg./ml., 71 % at 0·125 mg./ml. and 45 % at 0·062 mg./ml. Fractionation of the extract by high-speed centrifugation and fractional precipitation by acetone appeared to be accompanied by some loss of exhibitory activity. The exhibition phenomenon has occasionally been observed with phage 5 at low inhibitor concentrations or after prolonged incubation of the plates (see, for example, Table 4).

DISCUSSION

Many of the strains of *Ps. aeruginosa* which have been investigated in this laboratory have been found to be lysogenic. Some of them have produced, on repeated subculture, several variants which differ not only in antigenic structure but also in sensitivity to phages, and in the properties of the phages which they carry. One lysogenic strain (L), in particular, appeared to be of interest in that a rough

variant (LIII–3 bi) was sensitive to many phages, some being derived from other variants of the same parent strain; whilst 3bi itself appeared to be non-lysogenic. This observation led us into a detailed investigation on the mode of action of the phages on this indicator strain.

It appeared to be of particular interest that apparently homogeneous antigenic extracts of the lysogenic parent strain LII inactivated two phages active on the indicator variant 3bi, whilst similarly prepared extracts of the indicator strain itself had no phage-inactivatory powers. The phage-inhibitory extracts, which were prepared by treatment of bacteria with TCA or glycol, appeared to contain the type-specific somatic antigen (van den Ende, 1952).

Whilst it is well known that relatively crude antigenic extracts of bacteria have inhibitory powers against phages active on these strains, little has previously been done on the purification of such phage-inhibitory substances. Apart from the work of Miller & Goebel (1949) and of Goebel (1950), who obtained inhibition with very low concentrations of apparently homogeneous antigens derived from susceptible strains of *Shigella sonnei*, the most direct and convincing evidence for the identity of some of the inhibitors prepared by earlier workers with undegraded somatic antigens or haptens is the neutralization of inhibitory power *pari passu* with precipitinogens by homologous antisera. The possibility, however, that an inhibitor might be neutralized by normal serum, as with phage 14 in our experiments, may have been overlooked.

The inhibitor extracted from the variant *Ps.* LII by TCA is effective at a low concentration (0·002 mg./ml. for phage 14), appears on electrophoresis at two widely different pH's to contain but one major component, is a sensitive precipitinogen, is antigenic, and, at least as far as its action on phage 5 is concerned, appears to be neutralized by homologous antiserum. Although these facts suggest that the inhibitor may be relatively homogeneous, we wish to take advantage of the more refined physico-chemical methods now available for fractionating macro-molecules before accepting the hypothesis that the inhibitor and antigen or a portion of the antigen are identical.

It is generally accepted that phage inhibitors extracted from susceptible bacteria act by combining with specific bacteriotropic receptors of bacteriophage, thereby preventing their penetration into otherwise susceptible bacteria. Crude chloroform lysates of our indicator strain 3bi do, in fact, inhibit many phages normally active upon it, probably by preventing penetration. More difficult to explain is the inhibition of phages normally active upon a rough variant by an extract from the apparently resistant smooth parent of the indicator strain. Not only has the isolated somatic antigen this inhibitory power, but an actively growing culture of the smooth parent will inactivate phage added to it even in high concentration.

We have not obtained direct evidence of combination between phages and the inhibitor. However, the result of our experiments on the effect of dilution on mixtures of phage 5 or 14, and the inhibitory extract afford indirect proof that such combination does occur. Phages 5 and 14 differ in their affinity for the sensitive organism *Ps.* 3bi, and this difference is reflected in their behaviour towards the inhibitor. Phage 5 is very poorly adsorbed by the bacterium, and its combination

with the inhibitor is dissociated by dilution beyond 1:100. Phage 14 is more strongly adsorbed by its host and its combination with inhibitor resists a dilution of $1:10^4$.

To explain the susceptibility of *Ps.* LIII–3bi and inhibition by the somatic antigen of the smooth parent of 3bi we are therefore forced to conclude that the same phage receptors may be present in the surface components of several organisms. In the case that we have studied the phage receptor in the smooth somatic antigen must then be so situated that its combination with phage prevents penetration and therefore further multiplication of phage, whilst in the rough variant adsorption can be followed by a complete phage multiplication cycle. It is possible that the receptors for phages 5 and 14 may be so insecurely attached to the surface of LII that union of phage and receptor is followed by rupture of the receptor-to-surface link. This would explain the neutralization of phage 5 by LII cultures. The phages active on the smooth parent organism LII (phages AD, W, O and X (L)), which are also inhibited by the LII extract, can presumably penetrate the organism either because their receptors are more firmly bound to the LII surface or are better situated than the phages 5 and 14 receptors for further action by the phage.

Rupture of inhibitor particles, rather than deproteinization, is provisionally considered to be the more likely explanation of the finding that an inhibitor preparation which was retained by a gradocol filter of average pore diameter 500 mμ, passed readily through a similar filter after being shaken with chloroform and butanol (Mead).

As an alternative to the concept of interference with penetration, it is necessary to consider the possibility of interference at a later stage of the phage multiplication cycle. We have, in fact, considered the possibility that the inhibitor may act by converting lytic phage to symbiotic phage (Boyd, 1951). The behaviour of phage 5 from LIII–3bi lysates when plated on *Ps.* LIII–3bi in the presence of inhibitor resembles that of phage in Boyd's (1952) α-lysate. Thus both *Ps.* LII and some or all of its variants, including 3bi, may be sensitive to phages 5 and 14 but in different degrees. In some, e.g. *Ps.* LII, the tendency is towards symbiosis, so that this strain which is normally lysogenic appears resistant when tested in the usual way and yields culture filtrates of low titre. Furthermore, plaques formed on 3bi by phage from LII cultures resemble those of Boyd's β-particles. The smooth somatic antigen, when added to the susceptible rough variant, may have a symbiosis promoting effect. We have, however, at present no experimental proof of such an hypothesis.

The 'exhibition' phenomenon which has been observed regularly with phage 13, as well as with phage 5 in the presence of low inhibitor concentrations, may indicate that the antigen or part of it can under certain conditions act as an adsorption co-factor.

Whatever the explanation of the phenomenon we have studied, it appears that the mechanism of phage inhibition by bacterial extracts is not always as simple as earlier work has led us to believe.

SUMMARY

1. An electrophoretically homogeneous antigen preparation extracted from a strain (LII) of *Pseudomonas aeruginosa* by trichloracetic acid inhibits four phages active against this strain, and two phages (5 and 14) to which it is resistant but which attack a mutant strain LIII–3bi.

2. An extract prepared in the same way from strain LIII–3bi has no antigenic or inhibitory properties. Lysis of LIII–3bi organisms by chloroform, however, yields a potent inhibitor active against several phages.

3. TCA extracts from immunologically distinct *Ps.* strains AII, BII and GII also inhibit phages 5 and 14 to which these strains are resistant.

4. The effect of a third phage (13) on *Ps.* LIII–3bi is activated by the extract from strain LII which normally inhibits phages 5 and 14. Prolonged contact with very dilute solutions of the extract also causes activation of phage 5. Activation of phage 14 has not been observed.

5. The above findings are discussed in relation to current theories of phage action and phage inhibition.

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