ON SPECIFIC INHIBITION OF BACTERIOPHAGE ACTION BY BACTERIAL EXTRACTS*

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In a brief note dealing with the specificity of bacteriophage on organisms of the typhoid group, Hadley (3) mentioned the hypothesis that the action of phage on the sensitive bacillus is determined by the constitution of the heat-stable agglutinogens. The theme has been considered by Burnet in investigations covering the several recognized groups of the Salmonella organisms (4–6). It is generally agreed that lysis by phage requires a preliminary absorption of the active agent onto the bacterial surface, and the assumption is that the specificity of the absorption is attributable to the carbohydrate haptens which in the case of the Salmonella group of organisms, as shown by Furth and Landsteiner (7, 8), are endowed with the same specificity as the heat-stable agglutinogens. The hypothesis of Hadley and Burnet requires that the agglutinin-absorbing property of an organism and its phage-absorbing capacity run parallel.

The evidence for the hypothesis is based in the main part on direct tests of phages on representative cultures of the Salmonella group. Thus, in the experiments of Burnet made on agar plates a certain phage which lysed *B. enteritidis*, lysed also several organisms which share with the former strain the same heat-stable agglutinogens (heat-stable factors), namely *B. pullorum*, *B. sanguinarium*, *B. typhosus*, but did not act on cultures of *B. aertrycke*, *B. derby*, *B. paratyphosus* A, and *B. newport*. That the heat-labile flagellar antigens played no rôle in the specificity, was shown by the action of this *enteritidis* phage on the non-motile organisms *B. pullorum* and *B. sanguinarium*. Other phages active for *B. enteritidis* were not entirely specific but merely gave a greater incidence of reactions on the typhoid-*enteritidis* group of bacilli than on those of *paratyphosus* A or *paratyphosus* B.

Further support for the hypothesis that the specificity of phage depends upon

* See preliminary papers (1, 2).

the same antigenic components that have affinity to antibodies, was found by Burnet in the specific behavior of rough and smooth cultures to a series of phages. In accordance with a striking change in antigenic specificity, as evidenced by both agglutinin absorption experiments and also distinct bacterial carbohydrate extracts, accompanying the change of phase from smooth to rough, there was a more or less sharp difference in the behavior of the two sorts of cultures to phages. Thus Burnet described phages which acted mainly on a smooth phase, others which lysed mainly rough derivatives of the same bacilli, and a third variety which acted on both (5).[1] There were indications also that phages sensitive for the rough organisms generally had a more extensive scope of action covering several species of the Salmonella, an observation which is in harmony with the loss of specific antigens in such strains and the presence of an antigen common to the entire group (4)[2] (6).[3]

Burnet's experiments with both the Flexner group of dysentery organisms and varieties of staphylococci are also in harmony with the view expressed above (9, 10). Of particular interest in this connection is an early observation of Marcuse (11) who described a phage active for *B. dysenteriae* Flexner Type Y, and also for certain coliform strains; only the sensitive coliform organisms, and not the resistant strains, absorbed the agglutinins for the Type Y organisms.

Burnet recognized that a number of his own observations as well as those of others were not compatible with the hypothesis under consideration.[4] In the first place, as already mentioned, some of the phages, although apparently selective for a certain group, reacted also with cultures outside of the group. Also the organisms obtained from the overgrowth after the action of phage on sensitive bacilli and in the same phase as the parent strain are resistant to lysis, although it is not possible to demonstrate a difference in the heat-stable agglutinogens in the two strains by cross-absorption of the two sorts of sera (13, 14). The lack of action of phage on such resistant cultures can be understood if these organisms fail to absorb the phage particle, presumably by virtue of a corresponding change in the specific carbohydrate complex not serologically detectable (6). While the failure of phage absorption by resistant organisms has been reported by Burnet (13), Prausnitz and Firle (15), Lepper (16), Kimura (17), and others,

[1] Burnet (5), Table 1, p. 19.

[2] Burnet (4), Table 5, p. 127.

[3] Burnet (6), Table 1, p. 654.

[4] More recently Burnet (12) has published on other aspects of the specificity of bacteriophage, using the antiphage reaction together with the various resistance groups after the method of Bail.

positive results have been obtained by some workers—Flu (18), Applemans (19), Gohs and Jacobson (20).

Should the hypothesis be correct, it ought to be possible to demonstrate specific binding of phage or inhibition of its action in suitably prepared mixtures of phage and sterile solutions of the specific soluble carbohydrate-containing extracts. Actually such experiments have been carried out by several workers with negative results (6, 15). Since a final explanation for the specificity involved in bacteriophage action is not available, a reinvestigation has seemed warranted.

Our first tests were made according to the general principle employed by Burnet; namely, by direct experimentation on a large number of smooth and a smaller number of rough Salmonella cultures by using suitable dilutions of active phages derived against representative smooth organisms of the several groups of White (22) and Kauffmann (23).[5] In contrast to the work of Burnet, our experiments were made in test-tubes. The results of these investigations with phages active against *B. paratyphosus* B (factors I and II),[5] *B. enteritidis* (factor III), *B. paratyphosus* A (factor VI) soon revealed that a clear-cut group specificity sufficient to establish the identity of cultures within any group was not readily demonstrable. The difficulties encountered were in part similar to those already mentioned; namely, a small number of cultures were totally resistant to phage action, while a larger number in our collection, especially in the typhoid-*enteriditis* group, were lysogenic and cleared spontaneously in broth. Only one of these phages (*B. paratyphosus* B) showed a tendency towards a group specificity, giving a relatively greater incidence of lytic reactions on members of the *paratyphosus* B group than on organisms of the *paratyphosus* A, hog cholera, and *newport* groups. The phage for a smooth *B. enteritidis* showed action on numerous Salmonella cultures irrespective of serological grouping, except for a few strains which resisted the action of several phages employed. Another phage active for a rough strain of *enteritidis* gave a considerable number of reactions with rough strains of many Salmonella organisms and also with numerous smooth cultures irrespective of antigenic composition.

While direct tests of phages on numerous bacilli did not yield clear-cut results in our hands, nevertheless they gave indications of some rough parallelism between phage action and antigenic composition of heat-stable agglutinogens. Tests by means of another method not yet extensively employed for the study of phage specificity, namely absorption, a procedure found to be so useful for investigations on the

[5] In this paper White's numberings of the somatic factors (heat-stable agglutinogens) are given.

specificity of antigen-antibody reactions, have thus far yielded conflicting results. Consequently for the further investigation of the question, the important experiments of Prausnitz (15) and Burnet (6), designed to demonstrate a reaction between phage and specific soluble substance, were repeated. We employed lysis in test-tubes instead of placque counts since it appears from the literature (21) that the former may be a more delicate criterion of phage activity, allowing for continuous observations on the progress of lysis. Under such conditions, as the following data show, clear-cut specific inhibition of phage action by bacterial extracts was obtained.

Description of Material

Phages.—All the phages employed in these experiments were obtained from chicken stool filtrates. The titer of the phage was increased by several successive passages in beef extract broth (pH 7.4–7.6) against the homologous organism.

Cultures.—The cultures were the same employed by Furth and Landsteiner (8) in their studies on precipitable substances of the Salmonella group and were originally obtained by them from the National Collection of Type Cultures, Lister Institute, London.

Bacterial Extracts.—The carbohydrate-containing extracts were prepared, on the whole, according to the method of Furth and Landsteiner (8). Organisms were grown in Blake bottles on beef extract agar for 48 hours at 37°C. The bacilli were taken up in 0.85 per cent sodium chloride solution, centrifuged, and the sedimented bacteria were suspended and washed in 95 per cent alcohol. The bacterial mass was extracted in boiling 95 per cent alcohol (10 cc. per bottle) and then in absolute alcohol (5 cc. per bottle). After centrifugation the alcohol-extracted bacteria were heated twice for 1 to 2 hours in a steam bath in 0.85 per cent sodium chloride solution (5 cc. per bottle). The combined saline extracts were precipitated with 3 to 4 volumes of 95 per cent alcohol. (The precipitation of proteins by acid from the saline extracts and the addition of alkali prior to flocculation by alcohol were omitted from our procedure.) The precipitates were then washed in absolute alcohol, ether and dried.

EXPERIMENTAL

The experiments were made in the following manner: the crude bacillary saline extracts of *B. dysenteriae* Shiga and *B. paratyphosus* B were dissolved in saline in sufficient amounts to make a 1:200 solution and filtered through a Seitz filter. Equal volumes (0.25 cc.) of the extracts and dilutions of phage were mixed and incubated at 37° overnight. The test to detect inhibition was made the following day by adding 4.5 cc. beef extract broth to each of the tubes together with 3 to 4 drops of a young suspension of the homologous test organisms (1 loopful of a

TABLE I, *a*

Test with B. dysenteriae Shiga

Phage dilution incubated with 1:200 extract of	Dilutions of anti-*B. dysenteriae* Shiga phage							
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
B. dysenteriae Shiga	+	+±	++	++	++	++	++	++
	cl	±	++	++	++++	++++	++++	++++
	cl	cl	cl	cl	++++	++++	++++	++++
B. paratyphosus B	cl	±	+	+±	+±	++	++	++
	cl	cl	cl	±	+±	+±	++++	++++
	cl	cl	cl	cl	cl	cl	++	++++
Saline solution	cl	cl	±	+±	++	++	++	++
	cl	cl	cl	cl	+±	+±	++++	++++
	cl	cl	cl	cl	cl	cl	++	++++

TABLE I, *b*

Test with B. paratyphosus B

Phage dilution incubated with 1:200 extract of	Dilutions of anti-*B. paratyphosus* B phage							
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
B. dysenteriae Shiga	cl	+	+±	++	++±	+++	+++	+++
	cl	cl	cl	cl	++	++±	++++	++++
	cl	cl	cl	cl	cl	+	++++	++++
B. paratyphosus B	+±	+±	++±	++±	+++	+++	+++	+++
	cl	+±	++++	++++	++++	++++	++++	++++
	cl	+	+	+±	++	++++	++++	++++
Saline solution	cl	+	+±	++	++±	++±	+++	+++
	cl	cl	cl	cl	++	++±	++++	++++
	cl	cl	cl	cl	cl	+	++++	++++

Readings were made after incubation at 37° for 3, 5, and 7 hours following the addition of the test organism.

In these and in the following protocols, cl indicates clearing, tr, trace, and the signs ±, +, +±, ++, ++±, etc. indicate increasing degrees of turbid'ty.

young agar slant to 50 cc. broth). The mixtures were incubated and turbidity readings were made at frequent intervals in order to follow the course of the lysis. Tests were made at the same time with anti-*B. paratyphosus* B phage and with

anti-*B. dysenteriae* Shiga phage in order to make observations on the specificity of the reaction.

The results in Table I showed a distinct specific inhibition of phage action by the homologous carbohydrate-containing extract. Thus, the course of lysis was specifically delayed only for a short interval in those tubes containing the larger quantity of phage and the effect was seen only in the early readings; whereas in the tubes containing small quantities of phage, the more striking effects were observed in the later readings.

In the experiments tabulated there was little or no inhibition resulting from contact with the heterologous extract, but in several cases a slight non-specific inhibition was at times evident in the tubes containing the smaller quantities of phage. In a few instances in which this effect was observed it did not increase much on prolonged incubation and did not obscure the specificity.

The specific effect in the case of the Shiga phage was still present on the following day but in the phage for *B. paratyphosus* B overgrowth occurred rapidly in those tubes which had cleared, so that the inhibition was more or less obscured. However, the effect could still be verified even in the set of tubes which were equally turbid (for instance those containing phage 10^{-5} and 10^{-6}), by the observation that where heterologous substance was present the turbidity was due to organisms resistant to phage; in the tubes containing the homologous extract, however, the union of phage and extract completely inhibited lysis so that the bacteria still remained sensitive to fresh addition of the lytic agent.

For routine experiments, observations were made at frequent intervals until lysis in the control progressed practically to its endpoint.

At this point it may be mentioned that in several experiments the specific inhibition was evident when placque counts were used as a criterion of phage action. In these tests the incubated mixtures were plated together with a few drops of a young suspension of homologous organisms.

The Effects of Time and Temperature

For further progress it seemed desirable to gather information relative to the conditions most suitable for the demonstration of the specific inhibition.

For this purpose, mixtures containing 4 volumes (1 cc.) of varying dilutions of anti-*B. dysenteriae* Shiga phage and 4 volumes (1 cc.) of crude saline extracts, were prepared in a manner similar to those of the previous experiment. These mixtures were divided into four equal parts, each tube of the series containing 0.25 cc. of phage and 0.25 cc. of the crude extract. One part was allowed to incubate overnight at 37°C., another kept overnight at room temperature, a third set for the same period in the ice chest, and the fourth was tested without previous incubation. The tests were made by adding 4.5 cc. of beef extract broth to each of the tubes followed by 3 drops of a 1 hour old broth suspension of *B. dysenteriae* Shiga. The

TABLE II

*Test with B. dysenteriae Shiga**

Phage dilution incubated with 1:200 extract of	Dilutions of anti-*B. dysenteriae* Shiga phage						Incubation at
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
							°C.
B. dysenteriae Shiga.....	±	+±	+++	++++	++++	++++	37
B. paratyphosus B.....	cl	cl	cl	+	++	++++	
B. dysenteriae Shiga.....	+	+±	++±	+++	++++	++++	22
B. paratyphosus B.....	cl	+	+±	++±	++++	++++	
B. dysenteriae Shiga.....	cl	cl	cl	+±	++++	++++	5
B. paratyphosus B.....	cl	cl	cl	+	+++	++++	
B. dysenteriae Shiga.....	cl	cl	+	++	++	++++	Unincubated
B. paratyphosus B.....	cl	cl	cl	±	++	++++	mixtures

The reading recorded was made after incubation at 37° for 7 hours following the addition of the test organism.

* Since the degree of non-specific inhibition was negligible, the saline controls were omitted from the table.

tests were incubated at 37° and turbidity readings made at frequent intervals to observe the course of lysis.

Due to lack of space, one representative turbidity reading is given, save in Table I where several are presented to illustrate the general course of events. While the results are definite as shown in Table II, and in all others containing a single reading, the effects, in all cases, were clearer when the progress of lysis was observed at frequent intervals.

These tests showed that the most pronounced inhibition occurred in those mixtures incubated overnight at 37°. Practically no effect could be seen in the mixtures kept in the refrigerator. In the other two sets intermediate results were obtained, the inhibition being more distinct in the mixtures held overnight at room temperature. It is noteworthy that the inhibition was better in the unincubated mixtures (tubes with phage 10^{-4}) than in those incubated overnight in the ice box.

In similar experiments using anti-*B. paratyphosus* B phage, the results, on the whole, corresponded to those obtained with the anti-*B. dysenteriae* Shiga phage, in that the best effect was observed in the set incubated overnight at 37° and the least in the set kept at the lowest temperature. The results differed in that the tests with anti-*B. paratyphosus* B phage kept in the ice box and the series without incubation showed inhibition of lysis which was more distinct than the corresponding tests with anti-*B. dysenteriae* Shiga phage. From these observations it follows that the specific union of the anti-*B. paratyphosus* B phage and bacillary extract may be greater than that of the anti-*B. dysenteriae* Shiga phage and Shiga bacillary extract. An alternative view, however, is that the affinity between the anti-*B. paratyphosus* B phage and the homologous bacillus is weaker than the union of the anti-*B. dysenteriae* Shiga phage and the Shiga bacillus.

Another series of experiments was made with the two phages in which temperatures of 37°, 45°, and 55°C. were employed and intervals of incubation varying from 3 to 14 hours. These indicated that incubation of the mixtures at the higher temperatures did not yield a specific inhibition which was markedly better than that obtained after incubation for the same length of time at 37°. Consequently for all further experiments the routine procedure of incubation at 37° overnight was adopted. In addition these tests showed an increase in the specific inhibition with the time increment at any one temperature employed. Again the two phages behaved differently in that the degree of inhibition, especially after the shorter period of incubation, was distinctly better in the case of the anti-*B. paratyphosus* B phage.

It is still to be determined whether these conditions of time and temperature obtain, also, for other phages and extracts derived from other organisms. That the state of affairs may be somewhat different for other cases seems not unlikely in view of the differences in the

properties of the anti-*B. dysenteriae* Shiga and anti-*B. paratyphosus* B phages just discussed.

Specificity of Phage Inhibition within the Salmonella Group

It has been demonstrated in the first part of this paper that the action of the anti-*B. paratyphosus* B phage and the anti-*B. dysenteriae* Shiga phage is specifically inhibited by extracts of the homologous bacilli. These organisms, however, belong to entirely different and unrelated species. A more severe test of the specificity of the reaction could be applied to extracts of organisms and phages within the Salmonella group which, although possessing a number of substances in common (those connected with flagellar antigens, the substance G in the terminology of White (22), and some proteins studied by Furth and Landsteiner (7) and also White (24)) nevertheless fall into distinct groupings which are defined by the distribution of the various soluble specific substances of the heat-stable or somatic factors.

One series of experiments was made with extracts and phages for *B. paratyphosus* B (somatic factors I and II), and for *B. suipestifer* (somatic factor V). Both phages were specific in their reactions on the two cultures when the tests were put up in tubes. The tests were made in the usual manner, save that a trace of alkali was employed to aid solution of the extracts.

The results, Tables III, *a*, and III, *b*, show a striking specific inhibition of the two phages since the course of the lysis of the anti-*B. suipestifer* phage was distinctly retarded by the homologous extract but only slightly by an extract of *B. paratyphosus* B. On the other hand the extract of *B. suipestifer* but weakly influenced the action of anti-*B. paratyphosus* B phage which was inhibited to a considerable degree by its own extract. It is not without significance that the degree of non-specific inhibition by the extracts of the Salmonella organisms was not much greater than that by the more distantly related *B. dysenteriae* Shiga (compare with Tables I and II).

In the same set of experiments the two phages were tested also with extracts of *B. dysenteriae* Shiga, *B. paratyphosus* A, (somatic factor VI), *B. stanley* and *B. aertrycke*, the latter two organisms sharing with *B. paratyphosus* B the same somatic factors I and II, but differing from each other by virtue of specific flagellar antigens. None of these extracts influenced the action of the anti-*B. suipestifer* phage, an

observation which is apparently in agreement with the failure of this phage to lyse any of the organisms mentioned. However, extracts of *B. stanley* and *B. aertrycke* distinctly inhibited the action of anti-*B. paratyphosus* B phage. On testing this phage directly on these two organisms it was observed that while it lysed *B. aertrycke* to a titer of 10^{-5}, it had very weak action on *B. stanley*, 10^{-1}. Consequently other experiments were made to investigate the relationship of phage

TABLE III, *a*

Test with B. paratyphosus B

Phage dilution incubated with 1:200 extract of	Dilutions of anti-*B. paratyphosus* B phage						
	1×10^{-4}	1×10^{-5}	1×10^{-6}	3×10^{-6}	1×10^{-7}	3×10^{-7}	1×10^{-8}
B. suipestifer	cl	cl	±	++	++	++++	++++
B. paratyphosus B	+±	+++	++++	++++	++++	++++	++++
B. dysenteriae Shiga	cl	cl	cl	+	+++	++++	++++
Saline solution	cl	cl	cl	cl	++±	++±	++++

TABLE III, *b*

Test with B. suipestifer

Phage dilution incubated with 1:200 extract of	Dilutions of anti-*B. suipestifer* phage						
	3×10^{-2}	1×10^{-3}	3×10^{-3}	1×10^{-4}	3×10^{-4}	1×10^{-5}	3×10^{-5}
B. suipestifer	±	++	+++	+++	++++	++++	++++
B. paratyphosus B	cl	cl	±	+	++	++	++++
B. dysenteriae Shiga	cl	cl	cl	+	++	+++	++++
Saline solution	cl	cl	cl	+	+±	+++	++++

The reading recorded was made after incubation at 37° for 5 hours following the addition of the test organism.

titer on the living bacillus to inhibition of phage by extracts of these organisms. For this purpose two other organisms were included; namely, *B. tidy* of the same antigenic composition and a smooth resistant strain of *B. paratyphosus* B, isolated from the overgrowth following phage action on the sensitive smooth parent culture employed throughout these experiments. This organism, still resistant to phage following numerous transplants on agar over a period of 10 months after its isolation, could not be differentiated morphologically

or biochemically from the parent culture. Also cross-absorption of the two sorts of immune sera with the two organisms failed in our hands to reveal a serological difference (13, 14).

The titer of the anti-*B. paratyphosus* B phage on the homologous organism was about 10^{-8}, on *B. tidy*, 10^{-7}, *B. aertrycke* and *B. stanley*, as already mentioned, 10^{-5} and 10^{-1} respectively, and of course no lytic action at all on the resistant strain.

Crude saline carbohydrate-containing extracts of all these organisms were prepared and tested for their capacity to react with the anti-*B. paratyphosus* B phage, using the homologous culture as a test

TABLE IV

Test with B. paratyphosus B

Phage dilution incubated with 1:200 extract of	Dilutions of anti-*B. paratyphosus* B phage						
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
B. paratyphosus B (sensitive)	cl	+±	+++±	++++	++++	++++	++++
B. paratyphosus B (resistant)	cl	+	++	++++	++++	++++	++++
B. aertrycke	cl	+	+±	++±	++±	++++	++++
B. stanley	+	++±	++++	++++	++++	++++	++++
B. tidy	cl	+	+±	++++	++++	++++	++++
B. dysenteriae Shiga	cl	cl	cl	cl	+	++±	++++
Saline solution	cl	cl	cl	cl	+	++±	++++

The reading recorded was made after incubation at 37° for 5 hours following the addition of the test organism.

organism. With regard to the collection of resistant organisms for the preparations of the extracts, preliminary tests made on each individual Blake bottle, showed that the bacilli grown under such conditions retained their characteristic property.

The results of the tests (Table IV) demonstrate a specific inhibition by extracts of all organisms containing the factors I and II.[6] The most intense effect was exhibited by an extract of *B. stanley*. The extracts of the four other organisms gave effects which did not differ

[6] In these and other experiments with the anti-*B. paratyphosus* B phage, specific inhibition was evident also when *B. tidy* and *B. aertrycke* were employed as test organisms.

much from each other, the *aertrycke* extract giving the weakest inhibition.[7] Certainly the order of activity of the extracts does not run parallel to the titer of the phage against the living bacillus since the *stanley* strain was acted on only to a titer of 10^{-1}. Additional support for this view is furnished by the behavior of the resistant strain, the extract of which gave a pronounced inhibition. The failure to establish a relationship between phage inhibition by extracts and the phage titer against the living bacillus suggested other ideas, for instance the phage-absorbing capacity of the whole bacillus, the precipitating titer of the bacillary extracts, or both. Decisive evidence to form the basis for definite conclusions as to any correlation of other functions with inhibition is still wanting and experiments are under way to supply information on these issues. However, it may be stated that the *stanley* strain which gave the most intense inhibition also gave the highest precipitin titer; extracts of the resistant organism which gave distinct but somewhat weaker precipitin reactions than that of the sensitive strain, also gave weaker inhibition reactions.

In the course of these experiments the anti-*B. dysenteriae* Shiga phage was tested for inhibition after contact with extracts of eight different organisms belonging to the Salmonella group and also with its homologous extract and only the latter gave inhibition.

That the phage-absorbing capacity of the whole bacillus may play a rôle is indicated in a number of preliminary experiments, which show a distinct absorption of phage by resistant organisms obtained by fishing a single smooth colony derived from the overgrowth after action of anti-*B. paratyphosus* B phage on several strains having factors I and II (*paratyphosus* B, *stanley*, *aertrycke*, and others). These tests, carried out with the same technique which proved effective for the demonstration of inhibition of phage by bacillary extracts, were made in the following manner.

[7] No final attempts have been made as yet to determine how sensitive the reaction is, but in a preliminary way it can be stated that in one of the earliest experiments as little as 1:100,000 dilution of the crude *aertrycke* extract—the weakest inhibitor—showed a distinct specific inhibition of the anti-*B. paratyphosus* B phage. Probably the reaction has about the same order of sensitivity as that of the precipitin antibodies.

The 24 hours' growth of bacilli from one agar slant was suspended in 5 cc. saline and killed by heating at 70° for 1 hour. The suspension was diluted ten times in saline and 0.25 cc. was allowed to incubate overnight at 37° with an equal volume of varying concentrations of phage. The mixture caused no turbidity on dilution with 4.5 cc. broth the following day when the test was made.

A typical protocol showing the absorption of phage by heat-killed sensitive and resistant *paratyphosus* B organisms is presented in Table V.

While in these instances there was undoubtedly a fixation of phage by resistant organisms, the objection may be raised that the absorption was due to the splitting off of a small number of sensitive bacteria.

TABLE V

Absorption of Anti-B. paratyphosus B Phage by Heat-Killed Bacilli Test with B. paratyphosus B

Phage dilution incubated with a suspension of heat-killed	Dilutions of anti-*B. paratyphosus* B phage					
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
B. paratyphosus B (sensitive)....	+	++++	++++	++++	++++	++++
B. paratyphosus B (resistant)....	±	+	++	++++	++++	++++
B. dysenteriae Shiga.....	cl	cl	cl	±	±	++++
Saline solution.....	cl	cl	cl	tr	tr	++++

The reading recorded was made after incubation at 37° for 5 hours following the addition of the test organism.

However, the several resistant strains when tested with undiluted phage both on plates and in test-tubes showed no evidence of lysis.

However, organisms resistant to other anti-Salmonella phages derived from the overgrowth by isolation of a single smooth colony, absorbed little or no phage. This controversial question requires investigations taking into account all known criteria for resistance.

DISCUSSION

In these experiments evidence is presented that extracts of organisms containing carbohydrate material unite specifically with bacteriophage as indicated by inhibition of the lytic effect on young susceptible growing organisms. Apparently the affinity of the phage for the extract is weak and probably weaker than that of the phage for the

test organism. This fact may perhaps serve to explain the previous negative reports in such experiments. Indeed, it is difficult to determine the relative affinity of phage for a solution as compared to the affinity for a formed element.

As for the nature of the reaction, very little can be said at present except that no turbidities or precipitates have been observed in mixtures giving specific inhibition. Also such mixtures in a few experiments failed to fix complement. More progress in this direction could be made if some suitable material were found with no affinity to either the phage or extract, to be used as an indicator for the supposed specific reaction product.

The experiments suggest that the specificity of the reaction may be connected with those specific soluble substances which define the heat-stable agglutinogens. By means of this reaction, extracts derived from organisms related to *B. paratyphosus* B have been differentiated from the extracts of *B. suipestifer*. Reasoning by analogy from the work of Furth and Landsteiner, the substances involved are probably carbohydrate in nature. Indeed in a few experiments the specific phage inhibition (anti-*B. paratyphosus* B phages and anti-*B. dysenteriae* Shiga phages) was obtained also with the same substances boiled in alkali and subsequently neutralized. Certainly in these instances neither the substances connected with flagellar antigens nor the proteins played a rôle in the reaction. These experiments should be extended to other phages and substances to determine whether or not the reaction will define the remaining somatic factors of the Salmonella group and other species of bacteria in general. Perhaps investigations along this line may throw considerable light on the question of polyvalent phages (25).

In any event the demonstration of a reaction between phage and solutions of bacillary products makes it possible to study the chemistry of these products as disclosed by the effects of purification, fractionation, and hydrolysis, in terms of bacteriophage reaction and to investigate whether the findings will parallel those recently obtained on the chemistry of antigens in relation to the precipitating antibodies.

In view of our experience with phages and extracts of *B. paratyphosus* A and *B. typhosus*, it seems possible that the reaction may not be readily demonstrable in all instances. In the case of the

phage for *B. typhosus* specific inhibition has so far not been obtained and only a weak specific reaction could be demonstrated with the anti-*paratyphosus* A phage. Perhaps the most suitable conditions of time and temperature have not been as yet determined for these cases. That various phages may not be influenced in entirely the same way by time and temperature has already been indicated (see page 220). Another possibility to be considered is that the specific soluble substances mentioned above are not the only ones having an affinity for the phage. In these instances in which marked specific inhibition has not yet been demonstrated, and perhaps also in cases of those phages that have a wide scope of action covering organisms with various somatic factors, possibly the proteins described by Furth and Landsteiner and White (24) or other carbohydrates are involved (see Bronfenbrenner (25)).

The inhibition of phage by soluble bacillary products recalls some serological reactions dealing with inhibition of antibody action. Several such instances recently studied are: first, that of the haptens, thoroughly investigated by Landsteiner and van der Scheer (the inhibition of the precipitin for the coupled protein antigen by the hapten (26)); second, the inhibition of the precipitin reaction by fractions of low molecular weight derived from the specific soluble substance of Pneumococcus Type III (Heidelberger and Kendall (27)); and finally a group of cases in which the action of isoagglutinins is specifically inhibited by soluble body fluids, such as urine or saliva.

SUMMARY

1. Experiments are presented demonstrating specific inhibition of phage by soluble products of bacteria.

2. The inhibition proceeds more rapidly when the phage and bacterial extracts are incubated at 37° than at ice box temperature.

3. The specificity of the reaction in the instances studied is probably connected with the presence of specific soluble carbohydrates.

4. A reaction is available for the study of the chemistry of bacillary antigens in terms of bacteriophage.

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