METHODS OF STUDY OF ANTIPHAGE AGENTS PRODUCED BY MICROORGANISMS[1,2]

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Within recent years attempts have been made to determine whether microorganisms have the capacity of producing substances active against animal viruses in a manner comparable to the production of antibiotics or antibacterial agents (Jones *et al.*, 1945; Robinson, 1943). In this work the lack of techniques analogous to those commonly used for the study of antibiotics necessitated a more or less random choice of cultures. Needless to say, this indiscriminate testing would appear to be relatively inefficient as contrasted with the use of selective procedures such as those which have proved helpful in antibiotic work. In view of the increasing interest in the possibilities of an approach to the virus problem through studies of microbial antagonisms and the production of specific antiviral agents, attempts have been made to modify certain methods for investigating agents active against bacterial viruses or bacteriophages.

The practicability of utilizing bacteriophages as test agents in the search for chemotherapeutic substances active upon true viruses may be questionable in view of the marked differences between the bacteriophages and viruses, and even among members of each group with regard to their susceptibility to the direct action of chemical agents (Klein *et al.*, 1945). Nevertheless, any information concerning substances inhibitive to bacteriophages may contribute generally to knowledge of the intimate relationship between obligate intracellular parasites and their hosts. Moreover, experiments with bacterial viruses can be conducted with particular facility because of the ease and rapidity with which antiphage action can be detected and measured. In addition, the relative simplicity of the phage phenomenon, as compared with animal and plant virus systems, allows greater control of conditions.

The work reported here had as its objective the isolation of agents active against viruses, and is essentially quite distinct from studies on phage inhibition by bacterial extracts, which aimed at the elucidation of antigenic structure (Burnet, 1934) or the relationships and classification of bacteria (Levine and Frisch, 1933).

MATERIALS AND METHODS

Cultures of *Staphylococcus aureus* and *Escherichia coli* together with three bacteriophages employed in previous investigations (Jones, 1945) were used in

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these studies. Unless otherwise stated, 1.0 per cent nutrient agar was employed throughout. In the course of investigating various actinomycetes and fungi for antiphage action, an agar plate method for isolating microorganisms antagonistic to bacteriophages, agar streak and cup techniques to test such antagonists, and agar dilution and diffusion procedures for assaying cell-free preparations were developed. In view of the doubtful value of the soil enrichment procedure for the development of organisms antagonistic to bacteria (Waksman and Schatz, 1946), no studies were made concerning changes in the microbial population following the addition of bacteriophage to soil.

EXPERIMENTAL RESULTS

Several methods have been developed for the isolation of microorganisms antagonistic to bacteriophages and for the assay of antiphage activity. The first is similar to the bacterial agar method of Foster and Woodruff (1946) for the isolation of microorganisms antagonistic to bacteria. An agar medium seeded with the phage against which an antagonist is desired is employed for plating out a heterogeneous microbial flora, such as soil, compost, or manure, in sufficiently high dilutions to obtain well-isolated colonies. After a few days' incubation, the plates are coated with a thin layer of agar seeded with phage-susceptible bacteria, care being taken not to cause bacterial colonies to "run." For this purpose, an agar concentration of 0.5 per cent has been found satisfactory. If any of the organisms on the plates have produced diffusible antiphage agents, such colonies become surrounded by a zone of growth of the host cells; whereas at a distance from antagonistic organisms, as well as in the immediate vicinity of inactive colonies, the host cells will be lysed. Thus, with this method a zone of bacterial growth surrounding an antagonistic colony indicates antiphage activity, whereas in the corresponding technique involving bacteria-washed agar a clear zone of bacterial inhibition surrounding the antagonist is the criterion of antibacterial action (Waksman and Schatz, 1946).

The medium employed in the agar plate method affects greatly the practicability of the procedure. For example, nutrient agar, inoculated with suspensions of different soils and manures, allowed such rapid growth of spreading sporeformers that the host-cell-seeded agar, subsequently used to flood the plates, became contaminated. Acidified peptone glucose agar, which allows primarily the development of fungi, caused a rapid destruction of the phage. In general, neutral glucose asparagine 1 per cent agar has been found to be the most satisfactory medium so far tried.

In regard to the significance of phage antagonism, the fact must be appreciated that the elaboration of an antiphage agent is but one of the several possible mechanisms whereby the lytic action of phage can be inhibited. Consequently, the presence of a zone of host-cell growth is not absolute proof that the particular organism involved produces an antiphage substance. This has been shown to be the case with the corresponding plating procedure for isolating microorganisms antagonistic to bacteria. Actually, it has been found that many organisms, the original colonies of which showed good antiphage zones, produced no demon-

strable antiphage action in the filtrates of different liquid media. It has also been observed that some organisms which were isolated from phage agar plates because of antibacterial zones surrounding the original colonies yielded broth filtrates active against phage but lacking demonstrable activity against the host cells (table 1). Some microorganisms isolated at random likewise produced liquid culture filtrates which inhibited bacteriophages but were without ap-

TABLE 1

Action of certain soil actinomycetes, isolated by the agar plate method, against Staphylococcus aureus phage K

CULTURE NO.	ORIGINAL COLONY ON GLUCOSE ASPARAGINE PHAGE AGAR	CULTURE MEDIUM*	AGAR TUBE TEST, FILTRATE DILUTION†		
			1:2	1:20	1:100
69	Antiphage	1	++++		
		2	++++		
		3	+++		
79	Antiphage	1	++++		
		2	+	++	
		3	+++		
87	Antiphage	1	+++		
		2	++	+++	++++
		3	+	++	++++
19	Antibacterial	1	+	++	
		2	0	+	+++
		3	0	+	++
56	Antibacterial	1	+	++	+++
		2	0	0	+
		3	0	0	+
74	Antibacterial	1	+++	++++	
		2	++	++++	
		3	++	++++	

* 1 = meat extract peptone; 2 = glucose tryptone; 3 = glycerol yeast extract. Each medium contained 0.2 per cent agar.

† Activity of filtrates of stationary cultures grown for 8 days at 28 C. ++++ = normal number of plaques; ++ = 10-fold reduction; + = at least a 100-fold reduction; 0 = no plaques.

parent effect upon host-cell growth. It would appear, therefore, that certain antibiotic agents in subinhibitive concentrations may prevent lysis by bacteriophages. This would indicate that the antiphage action of such substances was due to an effect on the host cells rather than directly upon the phages.

Although it is possible to isolate microorganisms which produce antiphage filtrates in liquid cultures by the use of the phage-seeded agar medium, the limited data available (table 2) do not indicate any significant advantage for this

procedure as contrasted with a random selection of organisms for testing. The relatively high percentage of active fungi, as compared with the actinomycetes (table 2), is very likely due to a pH effect in some cases, since the mold filtrates varied from pH 2.7 to 8.4. The tests with the two groups of organisms are not directly comparable for a number of other reasons, such as treatment of filtrates and phage exposure periods.

The agar streak method was employed for determining activity against different phages in a manner comparable to its use in the study of the antibacterial properties of microorganisms. For this purpose, the culture to be

TABLE 2

Antiphage activity of fungi and actinomycetes isolated at random and from glucose asparagine agar seeded with S. aureus phage K

SOIL SOURCE	ORIGINAL COLONIES	CULTURES TESTED	ACTIVE ORGANISMS, AGAR DILUTION TUBE TEST†
	Fungi*		
Enriched with fowl pox virus	Selected at random	8	7
Untreated	Selected at random	38	16
Untreated	Antiphage on phage agar	5	4
Untreated	Antibacterial on phage agar	9	7
	Actinomycetes‡		
Untreated	Selected at random	100	27
Untreated	Antiphage on phage agar	17	3
Untreated	Antibacterial on phage agar	10	2

* Grown in Czapek-Dox medium, glycerol nutrient broth, fungus broth, and corn steep liquor media for 10 days. Filtrates tested against a laboratory strain of *E. coli* phage and *S. aureus* phage K.

† Active organisms produced at least one filtrate active against one or more of the phages.

‡ Grown in media listed in footnote of table 1. Filtrates tested against laboratory strain of *E. coli* phage, *E. coli* PC phage, and *S. aureus* phage K.

tested is streaked diametrically across a plate of agar seeded with phage. As in antibacterial studies, several different media are used. After appropriate incubation, cell-seeded agar is placed immediately adjacent to the growth of the antagonist, and the plate is then tipped so that the agar runs perpendicularly away to form a thin layer. After several hours' incubation, growth of the host cells occurs up to varying distances from the antagonist, depending on the degree to which the bacteriophage has been inhibited. Often, as shown in figure 1, there is an antibacterial zone within an antiphage zone. By the use of different phages, a spectrum of activity may be obtained. Some organisms are inactive by the streak test, although in the corresponding

agar-free medium they produce filtrates capable of inhibiting bacteriophages (table 3).

It is also possible to determine antiphage action by growing organisms in liquid medium in cylinders set on phage-seeded nutrient agar plates. After the desired period of incubation, the surface is carefully flooded with cell-seeded 0.5 per cent agar. If diffusible antiphage agents have been produced, the cup will be surrounded by a zone of host-cell growth. With this technique several

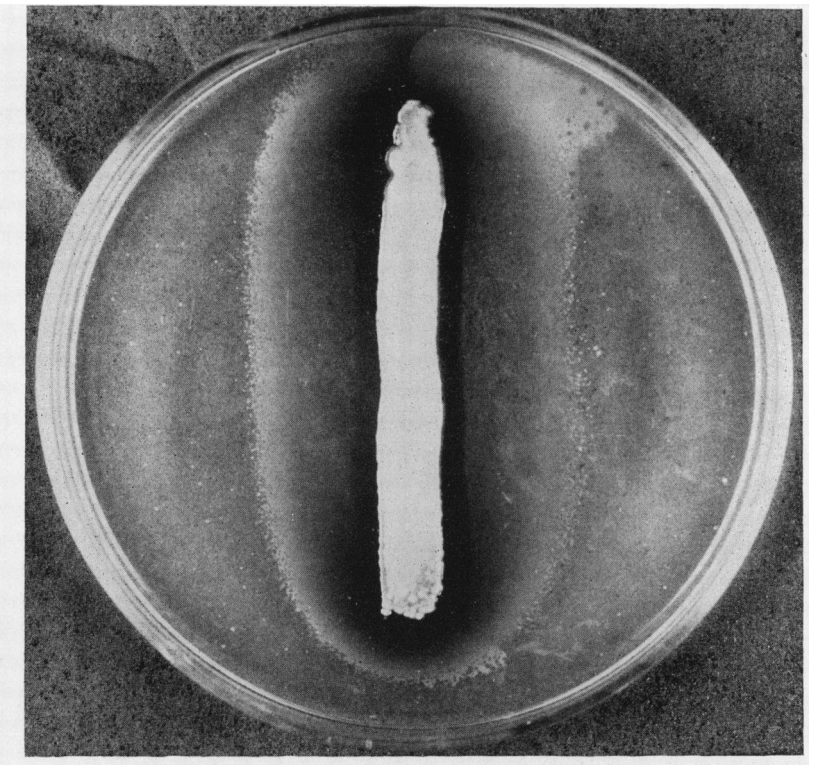

FIG. 1. STREAK TEST OF STREPTOMYCES NO. 193 AGAINST S. AUREUS PHAGE K ON TRYPTONE GLUCOSE AGAR
Zone of antiphage action surrounds inner zone of antibacterial action

organisms or different media can be tested on a single plate. Though the method works satisfactorily for actinomycetes, it frequently fails with certain bacteria which grow under the cup and spread out over the surface of the plate.

In the agar dilution method, various dilutions of the preparations to be tested are placed in petri dishes and 10-ml portions of agar seeded with one or more phages are added. When culture fluids are tested, they may be sterilized by filtration or by heat treatment before use. After suitable incubation, individual areas of each plate are flooded with separate lots of agar, each seeded with a different bacterial host. The relative number of plaques is compared with the

plaques on a control plate, and a spectrum of activity against different phages can thus be obtained.

A modification of this dilution procedure with only one phage can be conducted in test tubes. For this purpose, 1-ml portions of the phage suspensions and of the preparation being tested are mixed and incubated as desired. Subsequently, 3-ml portions of 1.7 per cent agar seeded with the bacterial host are added to each tube, which is then shaken and slanted; the higher agar concentration is used to compensate for the 2 ml of agar-free solution originally present. As in the fore-

TABLE 3

*Action of some soil actinomycetes against S. aureus phage K**

CULTURE NO.	MEDIUM	AGAR STREAK ZONE	AGAR TUBE TEST, CULTURE FILTRATE DILUTION		
			1:2	1:20	1:100
		mm			
56	1	31†	++++		
	2	16	+	++++	
	3	7	AB‡	++++	
79	1	0	0	0	++++
	2	2	0	0	++++
	3	3	++++		
87	1	0	++++		
	2	0	0	++++	
	3	2	0	++++	
97	1	0	+++		
	2	4	+	+	
	3	3	+	+	+
193B	1	0	+	++	
	2	16†	0	0	++
	3	15	0	+	++++

* See footnotes of table 1.

† Inner antibacterial zone present.

‡ AB = antibacterial action.

going method, the number of plaques per square centimeter is compared with the concentrations of plaques in the controls. In the present work, the potency of different filtrates was compared by taking as the end point that dilution which, when mixed with the phage overnight at 37 C, produced at least a 100-fold reduction in plaque titer.

In the agar diffusion or cup test, the usual procedure is used for pouring plates of phage-seeded agar and for placing and filling the cylinders with the preparations to be tested. After incubation overnight, the plates are flooded with a thin layer of host-seeded agar. Where diffusible antiphage agents are present, the cups are surrounded with zones of bacterial growth, the host cells being lysed at a distance from the cups. With inactive preparations, complete lysis occurs

right up to the cylinders. Figure 2 illustrates this technique. As has been found for the previous tests, increasing phage concentration yields progressively smaller zones of phage inhibition. The antibacterial zone in figure 2 indicates either that the antiphage agent is antibacterial in greater concentrations or that there is also present an antibiotic in addition to the antiphage agent.

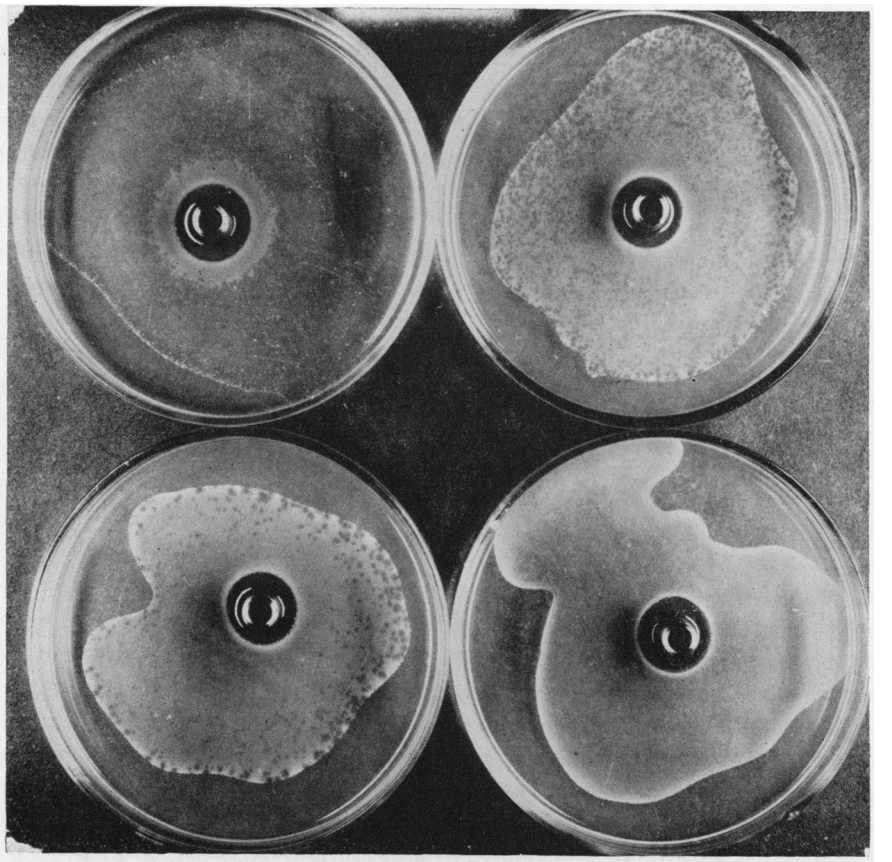

FIG. 2. AGAR DIFFUSION OR CUP TEST WITH THE ISOLATED AGENT OF STREPTOMYCES NO. 193 AGAINST THREE DIFFERENT CONCENTRATIONS OF S. AUREUS PHAGE K AND A PHAGE-FREE CONTROL

DISCUSSION

The various methods described have been developed for the study of agents active against bacteriophages. It is essential that the phages used be stable in the media over that period of time during which they are subjected to the metabolic products of the antagonists or to other preparations tested. In this respect, different phages, and possibly even different lots of the same phage, may vary considerably. It should be remembered that factors such as acid production may cause phage inactivation by the streak test, and that concentration of solvents, such as alcohol, must be considered in the agar cup tests.

In general, the techniques for the study of antiphage agents appear to possess

inherent limitations which make their standardization more difficult than is the case with the analogous methods employed in antibiotic investigations. However, this may be expected in view of the greater complexity of the bacteriophage system. The antimicrobial tests involve (1) an antagonistic organism or an antibiotic substance, (2) a single medium, and (3) microorganisms whose growth is antagonized or inhibited. On the other hand, the system in which antiphage action is tested consists of (1) the antagonist or antiphage agent, (2) frequently two different media (one for plating and another for flooding the original agar layer with host cells), (3) a phage, and (4) the bacterial host of the phage. In addition, the time factor as well as other conditions of culture differ in the two systems. For example, the cup test for antiphage action requires a preincubation period to allow diffusion before the host cells are placed upon the surface of the phage-seeded agar. On the other hand, both cells and active agent are usually added simultaneously in the agar diffusion method for determining antibiotic action, the bacteria being present throughout the agar.

Any method for demonstrating and measuring the inhibition of phage action is considerably more complex in regard to the number and kinds of possible mechanisms which may be involved. Whereas an antibiotic must, of necessity, act directly upon the microbial cell, an antiphage agent may inhibit phage multiplication by acting directly upon the phage particle or by affecting in some manner the medium or host cell. With bacteriophage, the latter type of action may be due to the prevention of phage adsorption on, or entrance into, the cell; an alteration of host metabolism which renders impossible phage multiplication; or some other mechanism. It is, therefore, quite logical to anticipate difficulties in modifying for phage studies the procedures used for the isolation and study of antibiotic agents.

The limited data available indicate no particular advantage to the use of the agar plate method for isolating microorganisms antagonistic to phage. However, this very same comment has been made with respect to the bacteria-washed agar medium for the isolation of antibiotic-producing microorganisms, in which the criterion of activity has been the formation of lytic zones (Waksman and Schatz, 1946). Nevertheless, the selective streak and cup techniques for detecting and measuring antagonism and the agar dilution and diffusion method for the study of the agents themselves can be applied to phage investigations. Although some substances do not diffuse and as a result appear inactive by the cup procedure, such preparations can be satisfactorily handled by the agar dilution technique.

It is suggested that similar methods may possibly find application in the study of plant and animal viruses. It may be possible, for example, to test the infectivity of virus-seeded agar taken at various distances from a colony or a streaked organism, or from a cup containing a diffusible preparation.

SUMMARY

Methods useful in the screening of antagonistic organisms and studies of the antibiotic substances can also be applied to the isolation of microorganisms pos-

sessing antiphage properties and the study of the antiphage agents themselves. The resulting techniques, however, involve more variables than do the original and simpler procedures used in antibiotics.

The production of antiphage agents active against bacterial phages was investigated by the agar plate, agar streak, cup culture, agar dilution, and agar diffusion methods. The principle of all these methods is the ability of the virus host to grow on the medium in which virus action is inhibited. To what extent these methods or modifications of them can also be utilized in the study of substances active against plant and animal viruses remains to be determined.

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