THE EFFECT OF ANTIBIOTIC SUBSTANCES UPON BACTERIOPHAGE[1,2]

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The problem of finding effective agents that might be utilized for controlling virus infections of animal tissues still remains unsolved (Jones, Beaudette, Geiger, and Waksman, 1945). Methods used in studying the effects of such agents upon animal viruses are complicated by the necessity of employing tissue cultures, chick embryos, or animal inoculation techniques. In view of certain similarities between bacteriophages and animal viruses and because of the relative simplicity of culturing such phages, an investigation was undertaken to determine the effects of known antibiotic substances upon them in the hope of uncovering clues leading to a method of attack upon the animal virus problem.

Levine and Frisch (1933) first reported a specific inactivation of phage by the polysaccharide components of susceptible host cells; Williams *et al.* (1940) later demonstrated a nonspecific inactivation of phage by phospholipids contained in bacterial cells. Cultures of *Bacillus subtilis* were found to have an inactivating effect upon a *Staphylococcus aureus* phage; this was believed to be caused by the adsorption of the phage particles upon the *B. subtilis* cells (Rakieten, Rakieten, and Doff, 1936).

The effect of antibiotic substances upon phage is still little understood. Tyrothricin and actinomycin A were reported to be inactive against certain bacteriophages (Neter, 1942); and penicillin was found to cause no destruction of a strain of staphylococcus phage (Neter and Clark, 1944). Penatin, or penicillin B, was shown to be active, however, against *Escherichia coli* phage, 0.1 mg per ml reducing the lytic activity of the phage to one one-thousandth of its original titer, in 18 hours, at room temperature (Anderson, 1943).

EXPERIMENTAL

Several antibiotic substances known to be active against gram-positive and gram-negative bacteria and three phages were selected for this study. The effect of each substance upon the phage free from the host and upon the phage added to the host cells was determined. Although no sweeping conclusions can be drawn from these experiments, certain facts concerning the action of the antibiotics upon bacteriophage have been elucidated.

Bacterial cultures, phages, and antibiotic substances. The following phages and their corresponding hosts were selected for this study: (1) *Escherichia coli*

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phage, laboratory strain, isolated from sewage in 1943. (2) *E. coli* PC phage, obtained from Dr. J. J. Bronfenbrenner; its host is *E. coli* PK3AR. (3) *S. aureus* K phage and its host *S. aureus* S_3K, obtained from Capt. Albert P. Krueger of the University of California.

The antibiotic agents used in this work were (1) streptomycin containing 125 *E. coli* units per 1 mg; (2) streptothricin, 100 *E. coli* units per 1 mg; (3) crystalline actinomycin, approximately 10 *E. coli* units per 1 mg; (4) crystalline clavacin, 200 *E. coli* units per 1 mg; and (5) penicillin, 200 Oxford units per 1 mg.

Methods. Plaque counts of *E. coli* phage, laboratory strain, were made on 1 per cent nutrient agar adjusted to pH 7.6; the *E. coli* PC phage was counted on a glucose tryptose agar at pH 7.2; the *S. aureus* phage, on nutrient agar at pH 7.0. Cell counts of the bacteria were made on 1.5 per cent nutrient agar adjusted to pH 7.0 to 7.2.

Effects of antibiotics on phage filtrates. Certain of the antibiotic substances were found capable of causing inactivation of bacterial phages, whereas others had no measurable effect. A mixture of a given concentration of the antibiotic and of the bacteria-free phage filtrate was incubated at 37 C for a desired length of time, usually overnight. The residual phage particles were determined by a simplified plaque count. This consisted in diluting 1 ml of the mixtures in distilled water, placing samples in sterile petri dishes, and thoroughly mixing with 10 ml of agar seeded with host cells and kept at 45 C. The plates were incubated at 37 C.

Of the five agents tested, streptomycin, streptothricin, and clavacin were found to cause varying degrees of inactivation of the phages, whereas penicillin and actinomycin were without effect.

The laboratory strain of *E. coli* phage was effectively reduced by as little as 10 to 20 *E. coli* dilution units per ml of streptomycin, streptothricin, and clavacin. At higher concentrations, the inactivation was progressively greater, as shown in table 1. Plaque counts in all cases were made at dilutions which eliminated the possibility of any inactivation of the host cells by the antibiotics.

In the case of the *E. coli* phage PC, a progressive inactivation was demonstrated with clavacin, streptothricin, and streptomycin after 6 hours' incubation (table 2); repeated tests with this strain indicated, however, that its sensitivity to different preparations of streptomycin varied, whereas the sensitivity of samples of the same phage to clavacin remained constant. No attempt is made at this time to explain these differences (table 3). The phage host, *E. coli* PK3AR, was tested by the streak method against clavacin and streptomycin and was found to be of the same degree of sensitivity as the laboratory strain of *E. coli*.

The *S. aureus* phage was inactivated by streptomycin but not by penicillin and actinomycin. Concentrations of streptomycin as low as 0.01 mg per ml, or one unit, caused some destruction, as indicated in table 4. Actinomycin showed no inactivation beyond the probable action of the solvent.

These results indicate that certain antibiotics exert a specific activity against phage. The mere fact that a substance is active against the bacterial host does

not insure action upon the corresponding phage as well. Thus penicillin, highly active against the *S. aureus* host, causes no destruction of its phage; the resistance of the *E. coli* phage PC varied, whereas the bacterial host remained susceptible to the action of streptomycin. It is interesting to note that those agents that have a strong effect upon gram-negative bacteria have also shown activity against phage. Those antibiotics that affect both phage and host must attack some metabolic system or substance common to the two, whereas those affecting the host alone must attack another system not possessed by or vitally necessary to the phage.

TABLE 1

Effects of five antibiotics upon E. coli phage, laboratory strain

ANTIBIOTIC			PHAGE PARTICLES SURVIVING PER ML OVERNIGHT AT 37 C	PER CENT SURVIVAL
Nature	mg/ml	*E. coli* units/ml		
Broth (1 ml)	0	0	$2,210 \times 10^3$	
Clavacin	10	2,000	$<0.1 \times 10^3$	<0.004
Clavacin	1.0	200	1.5×10^3	0.1
Clavacin	0.1	20	96×10^3	4.0
Clavacin	0.01	2	$1,080 \times 10^3$	49.0
Streptothricin	10	1,000	$<0.1 \times 10^3$	<0.004
Streptothricin	1.0	100	8.6×10^3	0.4
Streptothricin	0.1	10	770×10^3	35.0
Streptothricin	0.01	1	$1,600 \times 10^3$	72.0
Streptomycin	10	1,250	$<0.1 \times 10^3$	<0.004
Streptomycin	1.0	125	177×10^3	8.0
Streptomycin	0.1	12.5	325×10^3	15.0
Streptomycin	0.01	1.25	$1,905 \times 10^3$	86.0
Actinomycin*	0.1	1	620×10^3	28.0
Actinomycin	0.01	0.1	$1,725 \times 10^3$	78
Penicillin	0.1	1,000†	$8,650 \times 10^3$‡	79

* Actinomycin was prepared in a 10 per cent alcoholic solution containing 0.1 mg per ml. An alcoholic control which was run to test the survival of phage indicated that the apparent reduction of phage particles was due only to the action of the alcohol.

† *S. aureus* dilution units.

‡ Control for penicillin was $11,000 \times 10^3$.

Effects of antibiotics on phages and their hosts. An attempt was made to study the effect of antibiotic substances upon phage multiplication in the presence of treated host cells, i.e., host cells previously exposed to the action of the antibiotic agents before the addition of the phage.

Eighteen-ml portions of 12- to 18-hour broth cultures, diluted 1:20, were exposed to concentrations of the antibiotics, ranging from 2 *E. coli* units per ml to 20 units per ml, for 2 to 3 hours at 37 C. After that, 2 ml of phage filtrate were added, bringing the total volume to 21 ml. Controls included samples of untreated cells, cells treated only with the antibiotics, phage with untreated cells, phage in broth, and phage in the presence of the antibiotics. Counts of

the bacterial cells and of the surviving phage particles were made at the beginning and after 2 to 3, 8, and 24 hours.

TABLE 2

Effects of clavacin, streptothricin, and streptomycin on E. coli phage PC

Results × 10^5

TREATMENT OF PHAGE	MG/ML	E. COLI	PHAGE PARTICLES/ML AFTER HOURS		
			2	6	30
		units/ml			
Control	0	0	>1,700*	>1,200*	296
Clavacin	1	200	450	>150*	<0.01
Streptomycin	1	125	500	46.4	<0.01
Streptothricin	1	100	335	>150*	0.32

* Plaques estimated.

TABLE 3

Effects of clavacin and streptomycin on E. coli phage PC

TREATMENT	MG/ML	E. COLI	PHAGE PARTICLES PER ML AFTER 24 HOURS
		units/ml	
Control	0	0	128 × 10^5
Streptomycin	10	1,000	300 × 10^5
Streptomycin	1	100	240 × 10^5
Clavacin	2.5	500	0.045 × 10^5

TABLE 4

Effect of penicillin, actinomycin, and streptomycin on S. aureus phage

SAMPLE	MG/ML	S. AUREUS	PLAQUES/ML AFTER 24 HOURS AT 37 C
		units/ml	
Water	0	0	77.0 × 10^5
Acetone, 2%	0	0	40.5 × 10^5
Penicillin	0.1	1,000	49.5 × 10^5*
Penicillin	0.01	100	52.0 × 10^5
Actinomycin, 2% acetone solution	1.0	50,000	59.5 × 10^5
Actinomycin, 0.2% acetone solution	0.1	5,000	77.0 × 10^5
Actinomycin, 0.2% acetone solution	0.01	500	68.5 × 10^5
Streptomycin	10	1,250	<0.01 × 10^5
Streptomycin	1.0	125	<0.01 × 10^5
Streptomycin	0.1	12.5	0.19 × 10^5
Streptomycin	0.01	1.25	0.21 × 10^5

* Control for penicillin was 50.5 × 10^5.

The effects of streptothricin upon *E. coli* phage and host are presented in table 5. Tests with clavacin and streptomycin indicated that, although there were

differences due to individual rates and the nature of activity of the antibiotics upon the *E. coli* cells, there was a suggestion of a rather close correlation of effects upon the phage in the presence of the treated cells. These results may be summarized as follows:

(1) Concentrations of 20 units per ml of clavacin, streptothricin, and streptomycin plus *E. coli* cells, followed by the addition of phage after 2 to 3 hours' exposure of the cells to the action of the antibiotic at 37 C, gave a decrease of phage after 8 hours and a further decrease after 24 hours. Thus, phage was not only unable to multiply in the presence of the treated cells, but it was actually decreased. That this was not the result of the action of the antibiotics alone was shown by the controls; possibly it was due to the adsorption of the phage on the inactive cells, or to its inactivation by the lysed cells.

TABLE 5

Effect of streptothricin on E. coli phage, laboratory strain, and host

Results × 10^5

TREATMENT	E. COLI	BACTERIAL CELLS/ML AFTER HOURS AT 37 C				PLAQUES/ML AFTER HOURS AT 37 C		
		0	3	8	24	3	8	24
	units/ml							
E. coli control	0	27	2,200	4,900	9,300			
Cells + streptothricin	20		<0.001	<0.001	<0.001			
Cells + streptothricin	2		0.1	<0.01	>90			
				<0.01				
Cells + phage	0		2,200	117	1,500		17,000	2,050
Phage + broth	0					38	40	19
Phage + streptothricin	20						49	22
Phage + streptothricin	2						48	26
Cells + phage + streptothricin	20		<0.01	<0.001	<0.001		0.12	0.01
Cells + phage + streptothricin	2		<0.01	<0.01	6.4		4	109

(2) At the 2-unit-per-ml level of treated cells, the phage appeared to be able to multiply only in those mixtures in which there were viable cells. Multiplication of the phage occurred only when the cells were increasing.

The effect of prolonged action of streptomycin on *E. coli* cells and phage is brought out in table 6. Plate and plaque counts were made after 5 days of incubation, the cultures being diluted either with 20 volumes of broth, in order to determine the ability of the cells to multiply, or with a fresh 24-hour broth culture of *E. coli*, in order to see whether the phage could multiply when removed from the action of the antibiotic. The results show a definite reduction of *E. coli* cells to $<0.01 \times 10^4$ in both the *E. coli* plus antibiotic control and in the phage-treated flasks. On dilution of the antibiotic, no growth of bacteria resulted in either flask. This proved that there were no viable cells after 5 days of exposure to 10 units per ml of the antibiotic.

The phage count of the mixture of phage and bacterial cells was reduced 100-fold in 5 days by 10 units of streptomycin per ml. Redilution of this sample with broth (1:20) further lowered the plaque count after 24 hours, indicating again that no cells remained in the original culture on which the phage could multiply. On dilution with the *E. coli* culture, there resulted an increase of the

TABLE 6

Effect of dilution on streptomycin-treated E. coli cells and phage

Results × 10^4

TREATMENT	E. COLI	E. COLI			PHAGE		
		Undiluted 5 days	Diluted with broth + 24 hr	Diluted with *E. coli* culture + 24 hr	Undiluted 5 days	Diluted 1:20 with broth + 24 hr	Diluted 1:20 with 24-hr *E. coli* culture + 24 hr
	units/ml						
E. coli control	0	12,000	180,000				
E. coli + streptomycin	10	<0.01	<0.01				
Broth phage control	0			18,850	65	4.3	22,700
E. coli + phage + streptomycin	10	<0.01	<0.01	37,500	0.63	0.001	210

TABLE 7

Effects of penicillin and streptomycin on S. aureus phage and its host

Results × 10^5

TREATMENT	UNITS/ML	BACTERIAL CELLS/ML AFTER HOURS AT 37 C					PLAQUES/ML AFTER HOURS AT 37 C			
		0	3	8	24	48	3	8	24	48
Culture control	0	114	320	750	3,300	570				
Cells + streptomycin	2		<0.01	<0.01	4.25	4,200				
Cells + penicillin	10		0.15	<0.01	0.273	55				
Cells + phage	0				<0.01				4,700	
Phage + broth	0						75	2.1	3.7	<0.001
Phage + streptomycin	2								120	0.04
Phage + penicillin	10								100	0.98
Cells + phage + streptomycin	2		<0.01	<0.001	<0.001	0.294		0.012	0.41	0.2
Cells + phage + penicillin	10		0.09	<0.01	<0.001	0.025		19	309	231

phage from 0.001 × 10^4 to 210 × 10^4 in 24 hours. The rate of increase corresponded to that occurring in the similarly treated phage-broth control; however, the lower count in the test sample, as compared to the control, suggests that the phage particles that were rendered inactive by the streptomycin were not reactivated when the antibiotic was removed.

A study was finally made of the effects of penicillin and streptomycin upon

S. aureus phage and its host, *S. aureus* S_3K. Broth cultures of the cells, diluted 1:20, were first exposed for 3 hours at 37 C to 2 *E. coli* units per ml of streptomycin and 10 dilution units per ml of penicillin, after which phage filtrate was added. The fates of the cells and of the phage were followed for 48 hours. As shown in table 7, these concentrations of the antibiotics had no destructive effect on the phage alone. Cells exposed to the streptomycin were first decreased from 10^5 per ml to $<10^3$ per ml in 8 hours, but thereafter began to multiply until at 48 hours a count of 10^7 was reached. Penicillin had a similar effect upon the cells. When phage was added to the treated cells, the results of bacterial and phage counts brought to light two distinct effects. The phage added to the penicillin-treated cells remained at a high count and then increased as the cells multiplied. On the other hand, the phage added to the streptomycin-treated cells was first reduced from 10^5 per ml to 10^3 per ml and finally began to multiply as the bacterial cells entered a phase of rapid growth.

These effects suggest differences in the mode of action of the two antibiotic agents. Streptomycin appears to inactivate the bacterial cells in such a way that phage may still become adsorbed on the inactive cells, or be inactivated by products of cell lysis, the residual particles multiplying as the effect of the streptomycin is reduced. Penicillin, however, seems to affect the cell in such a manner that it no longer adsorbs phage particles. It is suggested, therefore, that penicillin and streptomycin attack two different mechanisms of the bacterial cell, the former having no relation to phage activity and the latter being directly concerned in some way with the growth of the bacteriophage.

SUMMARY

Streptothricin, streptomycin, and clavacin were found to cause inactivation of various phages in bacteria-free filtrates, whereas penicillin and actinomycin were without effect. There appeared to be no correlation between the susceptibility of the host cells and that of the phage to an antibiotic agent.

The action of different concentrations of antibiotic substances on *Escherichia coli* hosts and phage was also determined. When the concentration of the substance was great enough to inactivate all of the viable cells, the phage added to such mixtures was decreased progressively in 24 hours. With lower concentrations of the antibiotic, the phage multiplied only when the cells were increasing. Phage in suspensions of streptomycin-treated cells was not reactivated by dilution after prolonged incubation. Tests with penicillin and streptomycin on *Staphylococcus aureus* phage and its host, at concentrations of the substances which had no destructive effect on the phage alone, indicated that no reduction of the phage occurred when it was placed in the presence of penicillin-treated cells, whereas a definite decrease took place when streptomycin was used. In both cases, multiplication of the phage later paralleled an increase of host cells.

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