OBSERVATIONS ON THE CHEMICAL INHIBITION OF STREPTOMYCES GRISEUS BACTERIOPHAGE MULTIPLICATION

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The practical significance of Bennett's (1946) discovery of a bacteriophage for streptomycin-producing strains of *Streptomyces griseus*, which was quickly confirmed in other laboratories (Saudek and Colingsworth, 1947; Woodruff, Nunheimer, and Lee, 1947; Smith, Kuhn, and Miesel, 1947; Koerber, Greenspan, and Langlykke, 1950), spurred efforts to circumvent this threat to streptomycin manufacture. While these early reports and that by Reilly, Harris, and Waksman (1947) indicated that bacteriophage-resistant cultures could be isolated which produced as much streptomycin as the bacteriophage-susceptible parent culture, the studies by Koerber, Greenspan, and Langlykke (1950), which showed that a second type of bacteriophage existed to which resistant cultures could be obtained only with difficulty, suggested that chemical means of preventing or limiting bacteriophage infections should be investigated.

Survey of the literature indicated that several hundred compounds have been tested for ability to prevent multiplication of many different bacteriophages. Very few of those tested appeared to be able to prevent the multiplication of the bacteriophage without affecting the metabolism of the host cell and, as noted by Woodruff, Nunheimer, and Lee (1947), the most effective of these, the acridine series, unfortunately affect the growth and metabolism of the *S. griseus* cultures when added to media in concentrations effective in inhibiting the multiplication of the bacteriophage.

Consideration of this problem indicated that a chemical inhibiting agent must meet the following requirements if it was to be useful: (1) It should be effective in preventing the multiplication of all types of bacteriophages attacking *S. griseus*. (2) Addition of this substance to the medium should not affect the production of streptomycin by the actinomycete. (3) Addition of this substance to the medium should not affect the recovery and isolation of the streptomycin and other desired products from the fermented medium. These requirements restrict the number of compounds considerably and practically eliminate the use of basic compounds that would interfere with the recovery of streptomycin.

Only a few of the compounds mentioned in the literature appeared to have possibilities, and the one that looked most promising was citrate. This substance had been demonstrated to inhibit the multiplication of different bacteriophages by Stassano and de Beaufort (1925) and has been studied more recently by Burnet (1934), who noted that the quantities of citrate necessary to inhibit the multiplication of *Salmonella* bacteriophages varied with different bacteriophage types. Presumably the citrate interferes with the normal course of bacteriophage

infection by sequestering the calcium ions, which appear either to be required as adsorption cofactors (Delbrück, 1948; Cohen, 1949; Anderson, 1949) or to be involved in the release of the bacteriophage from the infected cells (Cherry and Watson, 1949). Following preliminary experiments which demonstrated that citrate was suitable for our purposes and suggested that other calcium-sequestering agents might be of use, several studies were started on compounds affecting the chemical inhibition of the bacteriophages infecting streptomycin-producing strains of *S. griseus*.

EXPERIMENTAL METHODS

The methods used in the following experiments resembled in a general way those used by other investigators (Reilly *et al.*, 1947; Koerber *et al.*, 1950). Bacteriophage counts were made by adding quantities of the bacteriophage-containing solutions (diluted in peptone solution—Koerber *et al.*, 1950) to a 15-cm petri dish together with 0.1 to 0.3 ml of spores obtained from 5- to 7-day submerged culture of a bacteriophage-susceptible culture of *S. griseus* and mixing well with 10 ml of sterile liquefied Difco yeast beef agar (a yeast extract, meat extract, glucose agar). Plaque counts were made after 4 to 5 days' incubation of the petri dishes at 25 C, unless otherwise indicated. Two to four replicate plates at several dilutions were made of each bacteriophage preparation.

When inhibitors were tested for activity by the plating technique, the volume of the bacteriophage dilution added to each petri dish did not exceed 0.5 ml and was usually of the order of 0.1 ml, and it was assumed that the amount of calcium added from this source was negligible. Experimental observation suggested that approximately 1×10^7 to 1×10^9 viable spores were present. Substances tested as bacteriophage inhibitors in the agar plate test were added to the petri dish as neutral aqueous solutions and mixed with the liquid-agar, spore-bacteriophage mixture.

The studies on the effect of bacteriophage and bacteriophage inhibitors on the production of streptomycin in submerged culture were conducted in shaken flask fermentations. Cotton-plugged 500-ml Erlenmeyer flasks containing 100 ml of a medium composed of 15 g of soybean meal and 20 g of glucose per liter (Rake and Donovick, 1946), with supplements added as indicated, were sterilized by autoclaving at 126 to 128 C for 30 to 40 minutes. When cool, these flasks were inoculated with the 48-hour-old vegetative growth of a bacteriophage-sensitive, streptomycin-producing culture of *S. griseus* grown on a similar soybean glucose medium sometimes modified by the addition of bacteriophage or other solutions as shown in the tables. An inoculum volume equal to 5 per cent of the fermentation volume was used. After inoculation the flasks were incubated on a reciprocating shaker (120 four-inch-stroke cycles per minute) in a 25 C constant temperature room. Samples were removed periodically for analysis for streptomycin potency and bacteriophage content. The samples were prepared for streptomycin assay (Donovick *et al.*, 1945) by acid extraction at pH 2.0 to 2.5 (Rake, Koerber, and Donovick, 1949). The samples used for bacteriophage counts were first clarified by centrifugation in a low-speed angle head type of

centrifuge to remove mycelium and residual soybean meal, and bacteriophage counts were made on the supernatant solution without further treatment. The fermentations were arranged in triplicate, and pooled samples were used for analysis.

The type II bacteriophage (Koerber *et al.*, 1950) was used in all the following experiments. Several other experiments indicated that similar results could be obtained with type I bacteriophage (Koerber *et al.*, 1950).

EXPERIMENTAL RESULTS

Inhibition of bacteriophage multiplication by organic acids. Preliminary studies showed that the addition of sodium citrate to agar plates containing bacteriophage and susceptible host cells resulted in complete inhibition of the bacteriophage as measured by the formation of plaques. In one of these experiments summarized in table 1, as little as 0.001 M citrate resulted in marked reduction in bacteriophage multiplication. Many actinomycetes are able to utilize citrate,

TABLE 1

Effect of sodium citrate on bacteriophage multiplication

(Agar plate test)

SODIUM CITRATE ADDED	PLAQUES PER PLATE AFTER INDICATED INCUBATION PERIOD			
Molarity	3 days	7 days	10 days	17 days
0	C.P.*	C.P.	C.P.	C.P.
0.001	10	75	>3,000	C.P.
0.005	1	100	500	*ca.* 750
0.01	0	10	150	200
0.1	0	0	0	10

* C.P.: Clear plate, e.g., greater than 5,000 plaques per plate.

and this perhaps accounts for the increase in plaque counts with time, as the growing host cells presumably degraded the citrate and then were infected with the bacteriophage.

To follow this lead, sodium citrate was added to the fermentation medium, and the effects on the multiplication of the bacteriophage and on the production of streptomycin were noted. Some of the results of these experiments are summarized in table 2. For comparative purposes the streptomycin yields obtained in the uninfected control fermentations have been assigned a value of 100, and those obtained when bacteriophage or organic acid salts have been added to the fermentation have been expressed in terms of percentage of those obtained with this control. In most experiments these streptomycin yields were of the order of 300 to 350 units per ml after a 6-day fermentation period. The data summarized in table 2 show that the addition of sodium citrate at a concentration of 0.01 M did not adversely affect the streptomycin yields and may, in fact, have resulted in significant increases. When bacteriophage was added to the fermentations at the time of inoculation, the streptomycin yields were markedly reduced, and

when it was added to the inoculum flask, the reduction was even more marked. Addition of sodium citrate to the fermentation medium at the time of bacteriophage infection resulted in reduced multiplication of the bacteriophage and practically normal streptomycin production. When the inoculum had been infected with bacteriophage and then transferred to the fermentation medium that contained a quantity of sodium citrate, no multiplication of bacteriophage

TABLE 2

Effect of sodium citrate on bacteriophage multiplication and streptomycin production

ADDITION OF SODIUM CITRATE (0.01 M)	BACTERIOPHAGE ADDED, PARTICLES PER ML	FERMENTATION DATA			
		4 DAYS		6 DAYS	
		Streptomycin*	Bacteriophage particles per ml	Streptomycin*	Bacteriophage particles per ml
		%		%	
None	None	100	—	100	—
At time of inoculation	None	115	—	127	—
None	2×10^4 at time of inoculation	73	7×10^9	71	1×10^{10}
At time of inoculation	2×10^4 at time of inoculation	97	1×10^4	111	3×10^3
At time of inoculation	2×10^4 after 1 day's fermentation	103	7×10^3	100	3×10^4
None	2×10^4 after 1 day's fermentation	71	3×10^8	67	8×10^8
At time of inoculation	2×10^4 added to inoculum medium 6×10^7 present at inoculation	41	7×10^8	38	6×10^8

* For convenience in comparison, streptomycin yields are expressed as percentages of those obtained in uninfected control fermentations.

occurred; but as the infection was already so heavy, it is not surprising that very low streptomycin yields were obtained.

A number of sodium salts of other organic acids were also studied. As shown by the data in table 3, sodium oxalate, sodium phytate, sodium hydroxyisobutyrate, and perhaps sodium tartrate inhibited the phage in the agar plate test. Of this group, sodium oxalate and sodium phytate were also found useful in the fermentation test. Sodium gluconate and sodium tartrate, which appeared effective in the agar plate test, were unsatisfactory in the fermentation test, presumably because they were utilized by the actinomycete; and sodium hy-

TABLE 3

Effect of organic acids in preventing bacteriophage multiplication

A. Agar plate test

SUBSTANCE ADDED	CONCENTRATION ADDED, M	PLAQUE COUNT PER PLATE AFTER INDICATED INCUBATION INTERVAL		
		2 days	3 days	4 days
Sodium citrate	0.003	50	325	104
	0.01	0	0	0
	0.03	0	0	0
Sodium gluconate	0.003	23	226	234
	0.01	37	166	169
	0.03	0	91	146
Sodium oxalate	0.003	0	0	0
	0.01	0	0	0
	0.03	0	0	0
Sodium tartrate	0.003	0	175	244
	0.01	0	199	226
	0.03	0	0	0
Sodium phytate	0.003	10	34	47
	0.01	0	0	0
	0.03	0	0	0
Sodium hydroxyisobutyrate	0.003	10	27	56
	0.01	0	0	0
	0.03	(Growth of actinomycete inhibited by this level of sodium hydroxyisobutyrate)		
None		>5,000		

B. Fermentation test

SUBSTANCE ADDED (0.01 M)	BACTERIOPHAGE ADDED, PARTICLES/ML	FERMENTATION DATA 5 days	
		Streptomycin*	Bacteriophage particles/ml
		%	
None	—	100	—
	2×10^6	81	6×10^9
Sodium citrate	—	116	—
	2×10^6	111	1×10^5
Sodium gluconate	—	93	—
	2×10^6	81	1×10^{10}
Sodium oxalate	—	115	—
	2×10^6	114	6×10^4
Sodium tartrate	—	81	—
	2×10^6	74	4×10^9
Sodium phytate	—	96	—
	2×10^6	115	3×10^5

* For convenience in comparison, streptomycin yields are expressed as percentages of yields in control fermentations.

droxyisobutyrate, which also showed promise in the agar plate test, was rather unsatisfactory in the fermentation test as it seemed to inhibit the growth of the actinomycete. A number of other organic acids were tested by these procedures; among them were ethylenediamine tetraacetic acid, amino acids, including glutamic and aspartic, and straight chain and branched chain fatty acids. Many of this group were effective in the agar plate test but either failed to inhibit bacteri-

TABLE 4

Effect of inorganic salts on inhibition of bacteriophage multiplication caused by sodium citrate

A. Agar plate test

SALT ADDED*	PLAQUES PER PLATE WHEN INDICATED SALT CONCENTRATION WAS ADDED TO AGAR MEDIUM				
	0.001 M	0.003 M	0.01 M	0.03 M	0.1 M
None (sodium citrate, 0.01 M, control)	50				
$CaCl_2$	*ca.* 3,000	*ca.* 3,000	>5,000	>5,000	>5,000
$BaCl_2$	254	1000	700	0	0
$ZnSO_4$	100	†	†	†	†
$FeSO_4$	0	0	†	†	†
$MnSO_4$	100	100	1,500	1,000	†

* When no sodium citrate was added, more than 5,000 plaques were noted.

† No growth in control plates indicating that there was a bacteriostatic effect of salt on the growth of actinomycete.

B. Fermentation test

$CaCl_2$ ADDED 0.01 M	SODIUM CITRATE ADDED 0.01 M	BACTERIOPHAGE ADDED, PARTICLES PER ML	FERMENTATION DATA			
			4 days		6 days	
			Streptomycin*	Bacteriophage particles per ml	Streptomycin*	Bacteriophage particles per ml
			%		%	
−	−	—	100	—	100	—
−	−	6×10^4	92	2×10^{10}	77	2×10^9
−	+	6×10^4	102	5×10^3	96	1×10^5
+	+	6×10^4	90	2×10^{10}	79	1×10^{10}
+	−	6×10^4	106	2×10^{10}	81	5×10^{10}

* For convenience in comparison, streptomycin yields are expressed as percentages of yields obtained in control fermentations.

ophage multiplication or limited streptomycin production in the fermentation test.

Mechanism of action of bacteriophage inhibitors. Consideration of the properties of those organic acids found to be useful indicated that they all had the ability to sequestrate calcium. Other studies (Koerber *et al.*, 1950) may be interpreted to demonstrate that calcium is a cofactor for adsorption during some stage of the bacteriophage-host-cell interaction, and it was not surprising that substances

that are able to interfere with the ability of calcium to act as a cofactor were useful. These organic acids also form complexes with other metallic ions; and as indicated in table 4, metallic ions forming citrate complexes were able to reverse the effect on bacteriophage multiplication when studied in the agar plate test or the fermentation test. This limits the use of sodium citrate or sodium oxalate as bacteriophage inhibitors to media that do not contain large amounts of these metallic ions; and of the media used in the production of streptomycin, the soybean meal glucose medium is singularly low in its metallic ion content. Such media components as cornsteep water, hydrolyzed rabbit fur, or meat hydrolyzates and extracts (Bennett, 1946; Woodruff *et al.*, 1947; Reilly *et al.*, 1947) are heavily contaminated with metallic ions, and the application of this method of inhibiting bacteriophage multiplication to fermentations in which they are included would require prohibitively large amounts of sodium citrate.

Preliminary experiments (summarized in part in Table 2) suggested that addition of the organic acids that were found effective in inhibiting the multipli-

TABLE 5

Effectiveness of oxalate in preventing bacteriophage multiplication

TIME OF ADDITION OF OXALATE (0.02 M)	BACTERIOPHAGE PARTICLES PRESENT IN MEDIUM AFTER INDICATED FERMENTATION INTERVAL (PARTICLES/ML)						
	0 min	1 min	10 min	60 min	180 min	540 min	18 hr
(Not added)	6×10^3	2×10^2	1×10^1	6×10^1	2×10^2	9×10^5	1×10^7
−1 min		1×10^2	1×10^1	3×10^1	7×10^1	6×10^1	1×10^3
+1 min		3×10^2	7×10^1	1×10^2	4×10^2	4×10^3	3×10^4
+10 min		7×10^2	1×10^2	1×10^2	3×10^2	5×10^4	9×10^3
+60 min		9×10^1	1×10^1	1×10^1	3×10^3	4×10^4	8×10^7

Host: 18-hr vegetative growth of *S. griseus* growing on soybean meal glucose medium. Original cell count was about 10^7 per ml of flask contents.

cation of the bacteriophage to infected fermentations sometime after infection had occurred would not stem the spread of the infection. This hypothesis was strengthened by the experiments summarized in table 5, where it may be seen that addition of the sodium oxalate even 10 minutes after infection had no real effect on the multiplication of the bacteriophage. These experiments also suggest that the latent period for the bacteriophage under these conditions was less than 9 hours, and probably about 6 hours, as determined graphically.

DISCUSSION

The foregoing experiments suggest that the multiplication of bacteriophages may be controlled in infected *S. griseus* cultures if substances that are able to sequester calcium are added to the media. Although the studies reported in the literature (Saunders and Sylvester, 1947; Dulaney, 1948; Thornberry and Anderson, 1948) have not indicated whether calcium is required for the growth of this actinomycete in synthetic media, it has been included in many media and found essential for streptomycin production by certain cultures (Perlman, 1949).

However, the amounts required are extremely small, usually ranging from 1 to 10 ppm; most synthetic and natural media would contain sufficient calcium as impurities in the other ingredients. Calculation of the quantity of citrate or oxalate necessary to inhibit the multiplication of the bacteriophage indicates that about 30 M of the sequestering agents are required per mole of calcium present. Of course, the sequestering agents are taken up by elements other than calcium, and they are probably needed in excess because they are slowly metabolized by the actinomycete. From a practical viewpoint this would appear to indicate that the use of these sequestering agents to inhibit the multiplication of the bacteriophage is limited to those media in which calcium compounds, e.g. calcium carbonate, and other metallic ions are not present as major ingredients.

The experiments summarized in table 5 suggest that the bacteriophages infecting the actinomycetes resemble those infecting other bacteria. The "free" bacteriophage count decreased rapidly after infection of the fermentations, possibly due to adsorption by the sensitive host cells. Cherry and Watson (1949) noted that addition of citrate to the medium did not allow multiplication of the bacteriophage, although the bacteriophage was adsorbed on the host cells; and perhaps the same explanation may be applied to the effect of oxalate noted in table 5. This is of considerable importance as it suggests that addition of the sequestering agents, e.g., oxalate, to heavily infected cultures is impractical as a means of controlling or destroying the infection. However, where occasional or sporadic infection is encountered, the routine addition of sequestering agents to the media may prevent the disastrous results of free multiplication of the phage. Though the use of these inhibitors may offer a temporary solution to the practical problem of phage infection, the ultimate goal should, nevertheless, be to secure resistant cultures.

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SUMMARY

Multiplication of the bacteriophages infecting *Streptomyces griseus* may be limited by the addition of substances capable of sequestering calcium in the media before infection of the host cells occurs. Among the sequestering agents found effective in inhibiting bacteriophage multiplication in submerged culture fermentations without adversely affecting streptomycin production are citrate, oxalate, and phytate. The effectiveness of the inhibition of bacteriophage multiplication may be reversed by the addition of metallic ions forming complexes with these sequestering agents.

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