Properties of a Small Bacteriophage and the Action of some Compounds on it

By R. F. N. MILLS

*Virology Section, Bacteriology Division, Research Department,
Boots Pure Drug Co. Ltd., Nottingham*

SUMMARY: Phage S13, a small phage active against a strain of *Escherichia coli*, had a latent period of 20 min. on this host. Intracellular phage multiplication was detected only toward the end of the latent period. Photo-reactivation of ultraviolet irradiated phage occurred, but the inactivated phage did not kill its host. More than 1600 compounds were tested for ability to prevent growth of phage S13 on *E. coli* at concentrations allowing growth of the bacterium, but only thirteen were active. Seven of these compounds were examined further; none affected free S13 phage or the adsorption of S13 phage to its host. The only compound investigated in detail, 4 : 4-bis-(2-dihydroglyoxalanyl) stilbene dihydrochloride, inhibited both early and late, but probably not intermediate, stages in the intracellular development of S13 phage. This was different from the action of this compound on T1 phage. No relationship was found between structure and action against phage, nor between compounds active against S13 phage and those active against larger phages.

As part of an investigation into virus chemotherapy, S13 phage of *Shigella paradysenteriae* (*S. flexneri*) Y6R was used as a representative of the smallest viruses. Elford & Andrewes (1932) by ultrafiltration estimated its diameter to be 8-12 m μ ., while Elford (1936) by a centrifugation method found it to be 15-17 m μ . i.e. it was of the same order of size as the viruses of poliomyelitis and foot and mouth disease. Because of its small size S13 phage might differ from larger phages in some of its properties. This paper reports studies on these properties, including the action of certain compounds on this phage, and a comparison of the activity of one of these compounds on S13 with its activity on T1 phage of *Escherichia coli*.

EXPERIMENTAL

S13 phage, and a strain of *Escherichia coli* against which S13 phage was active, were obtained through the courtesy of Dr C. H. Andrewes, F.R.S. In this paper the bacterium will be termed *E. coli* CHA.

Strains. Stock S13 phage was prepared by the method of Swanstrom & Adams (1951). Best results were obtained by concentrating a 24 hr. broth culture of *Escherichia coli* CHA tenfold by centrifugation and using the concentrate for sowing the plates. The lysate was filtered through a Gradocol membrane of porosity 0.9 μ . The final titre was 4×10^{10} particles/ml.

T1 phage was prepared by sowing a 2 hr. Lab-Lemco broth culture of *Escherichia coli* CHA with 10^4 particles of T1 phage previously obtained by lysis of *E. coli* B, incubating at 37° for 6 hr., then filtering through a Gradocol membrane of porosity 0.9 μ . The final titre was 3×10^9 particles/ml. T1 phage

prepared from *E. coli* B gave 100 times more plaques when plated on *E. coli* B than when plated on *E. coli* CHA. The phage obtained by lysis of *E. coli* CHA gave the same titre on both bacterial strains. The variation appears to be a phenotypic one similar to that described by Bertani & Weigle (1953).

Escherichia coli CHA grew (slowly) on a defined medium which contained lactic acid and ammonia as sole sources of carbon and nitrogen. It was sensitive to T1, T3 and T7 but resistant to T2, T4, T5 and T6 phages of *E. coli* B.

Media. Lab-Lemco broth was used as liquid medium and nutrient (tryptic digest) agar as solid medium.

Titration of phage. For one-step growth experiments: to 1 ml. phage suspension in a test tube, 1 ml. of a 24 hr. culture of *Escherichia coli* CHA and 3 ml. nutrient agar were added. After the contents had been mixed, the tubes were sloped and incubated at 37° for 4 hr. before reading. In all other experiments the mixture was poured on 15 ml. agar bases instead of sloping, and the plates incubated for 24 hr. at 37° before reading. The shorter period of incubation was necessary after sloping, as after 24 hr. plaques were too large for convenient numbers to be counted in tubes.

RESULTS

Stability of S13 phage. S13 phage was stable in Ringer's solution at 37° for at least 30 min. and at 5° for at least 6 months; at 60° the titre fell from 3×10^6 particles/ml. to 4×10^3 particles/ml. in 30 min. In M/15 phosphate + citrate buffer at 37°, S13 phage was stable at pH 8 for at least 1 hr.; at pH 5 the titre fell from 1×10^6 /ml. to 1×10^5 particles/ml. in 1 hr. pH values outside the range 5–8 were not investigated.

Latent period (Fig. 1). The method of Dickinson & Codd (1952) was used. To obtain good absorption a 2 hr. culture of *Escherichia coli* CHA was suspended in Ringer's solution, incubated at 37° for 5 min. only, and S13 phage added to give 2×10^8 host cells and 10^8 phage particles/ml. The mixture was incubated 10 min. before dilution into broth. The latent period was about 20 min.

No increase in the titre of S13 phage was found when KCN (final concentration 0.02 M; Rountree, 1951) was added at any time during the first half of the latent period (Fig. 1). Attempts to break open the bacterial cell at earlier times by the method of Rountree (1951) were unsuccessful.

Effect of ultraviolet radiation on S13 phage. S13 phage (4×10^9 particles/ml.) in broth was irradiated with a 15 W. 18 in. British Thomson-Houston germicidal lamp (which gave 99 % of its energy at a wavelength of 2537 Å.) at a distance of 37.5 cm. An exposure of 60 min. (the dose usually given) was required to decrease the titre one hundredfold. The irradiated phage had a significantly higher titre when absorbed on a 2 hr. culture of *Escherichia coli* CHA for 5 min. in daylight before counting than when counted immediately (Table 1). The effect increased with decreasing phage concentration over a fourfold range, so that it was not due to multiplicity reactivation. No increase occurred on dilution in absence of *E. coli* CHA. No work was done in absence of light but it seems reasonable to attribute the observed effect to photo-reactivation.

Fig. 1.

Fig. 2.

Fig. 1. Premature liberation of intracellular S13 phage by addition of 0.02 M-KCN at various times. x—x, latent period in absence of cyanide; ●—●, phage yield when cyanide added at various times.

Fig. 2. The action of compound no. 347 (20 µg./ml.) on the growth of *Escherichia coli* CHA. x—x, compound no. 347 absent; ●—●, compound no. 347 present.

Fig. 3. The effect of compound no. 347 (20 µg./ml.) on the latent periods on broth of (a) S13 phage, (b) T1 phage on *Escherichia coli* CHA. x—x, compound no. 347 absent; ▲—▲, compound no. 347 present throughout; ■—■, compound no. 347 added at 8 min.; ●—●, compound no. 347 added at 20 min.

Table 1. Reactivation of ultraviolet-inactivated S13 phage in the presence of *Escherichia coli* CHA

Treatment	Host presence	Phage count (particles/ml.)
Unirradiated	—	1.6×10^9
Irradiated	—	1.6×10^7
Irradiated, diluted* 1:2	+	1.2×10^7
Irradiated, diluted 1:4	+	7.3×10^6
Irradiated, diluted 1:8	+	4.4×10^6
		Host count (no./ml.)
Host + broth	.	1.4×10^7
Host + unirradiated phage	.	8×10^4

* Dilutions were in broth. The first dilution was due to mixing equal volumes of phage and host. Phage and host were incubated together for 5 min. before counting.

In the course of this work it was noted that though infection of *Escherichia coli* CHA with normal S13 phage decreased the viable bacterial count, infection with ultraviolet (u.v.)-inactivated S13 phage failed to do so (Table 2). Studies on antiserum neutralization by u.v.-inactivated S13 phage failed to

Table 2. *Effect of ultraviolet-inactivated S13 and T1 phages on the viability of Escherichia coli CHA*

Equal volumes of host and phage were incubated together for 5 min. before host counts were made.

	S13 phage	T1 phage
	Phage count (particles/ml.)	
Before irradiation	4×10^9	2.5×10^9
After irradiation	5.6×10^6	5.8×10^6
	Host count (no./ml.)	
Host + broth	1.6×10^8	9.0×10^6
Host + unirradiated phage	3.6×10^7	1.4×10^4
Host + irradiated phage	1.7×10^8	1.5×10^4

Fig. 4.

Fig. 4. Effect of compound no. 347 (20 $\mu\text{g./ml.}$) on the yields of S13 and T1 phages from *Escherichia coli* CHA when added at different times during their latent periods. $\bullet$ — $\bullet$, T1 phage; $\times$ — $\times$, S13 phage. Tubes were 'sloped' at 60 min.

Fig. 5.

Fig. 5. The effect of diluting out compound no. 347 (20 $\mu\text{g./ml.}$) on the latent period of S13 phage on *Escherichia coli* CHA. $\times$ — $\times$, compound no. 347 absent; $\blacktriangle$ — $\blacktriangle$, compound no. 347 initially present, diluted out at 15 min.; $\bullet$ — $\bullet$, compound no. 347 added at 5 min., diluted out at 15 min.

show whether it was adsorbed on *E. coli* CHA, but as reactivation occurred only in presence of *E. coli* CHA it appears probable that adsorption did take place. It was confirmed (Table 2) that u.v.-inactivated T1 phage killed *E. coli* CHA.

Action of various compounds on S13 phage

A preliminary test of each compound against S13 phage was made by a cup-plate diffusion method using a 1% (w/v) neutral aqueous solution or suspension of the compound in a single cup. Those compounds which gave an appreciable zone of phage inhibition were retested by the serial dilution method of Chantrill, Coulthard, Dickinson, Inkley, Morris & Pyle (1952), using five-fold

serial dilutions. Some of the compounds found active in this test were examined in greater detail by the method of Mills (1953).

Of more than 1600 compounds screened against S13 phage only thirteen were active by the serial dilution method (Table 3), though many showed appreciable activity in the cup-plate test. No reason was found for this

Table 3. *Compounds which prevented the growth of S13 phage on Escherichia coli CHA*

Ref. no.	Compound	Activity*	Action on absorption	Action on intracellular phage
347	4:4-bis-(2-dihydroglyoxalanyl) stilbene dihydrochloride, monohydrate	5·1/4·4 _p	0	+
348	2-hydroxy-4:4'-diamidinostilbene dihydrochloride, dihydrate	5·1/4·4 _p	Not tested	
690	phenyl guanidine	2·7/2·1	0	+
774	1:2-bis-(4'-benzamidinylphenoxy) ethane di-HCl	3·4/2·7	Not tested	
776	1:4-bis-(4'-benzamidinylphenoxy) butane dihydrochloride	3·4/2·7	0	+
1105	bis-(<i>p</i> -N-2-pyridylamidophenyl) ether	4·8/4·1	0	+
1202	di-(<i>p</i> -N- <i>p</i> '-methoxyphenylamidinophenyl) ether	4·8/4·1	Not tested	
1148	9-(<i>p</i> -aminoanilino) phenanthridine	5·1/4·4	0	+
1207	2:7-diamino-9-anilinophenanthridine	4·4/3·7	Not tested	
1367	2:7-bis-(2-dihydroglyoxalanyl) 9-phenylphenanthridine, trihydrochloride, dihydrate	5·1/4·4	0	+
2177	2:7-diamino-10-methyl-9- <i>p</i> -tolylphenanthridinium bromide	4·8/4·1	Not tested	
2202	2:7-diamino-9:10-dimethylphenanthridinium bromide	5·5/4·8	Not tested	
1475	2, 4, 5, 6-tetra-aminopyrimidine sulphate, monohydrate	3·4/2·7	0	+

* Activity is expressed as

$$\frac{-\log (\text{minimum concentration (g./ml.) preventing phage growth)}}{-\log (\text{minimum concentration (g./ml.) preventing host growth})}.$$

difference in response. No compound affected the titre of S13 phage at its active concentration, and none of seven compounds tested prevented adsorption of S13 phage on *Escherichia coli* CHA. All compounds tested except one, no. 347, 4:4-bis-(2-dihydroglyoxalanyl) stilbene dihydrochloride, decreased host growth too much at the active concentration for more detailed work to be of value.

Action of compound 347

In a one-step growth experiment compound 347 prevented phage multiplication at 20 μ g./ml. and caused little phage loss (Fig. 3*a*). This concentration was used for the rest of the work reported here. Compound 347 did not diminish the titre of free S13 phage (Table 4), nor did it prevent a burst when present during absorption if, after absorption, it was diluted well below the limit of activity (Table 5). It slightly retarded the growth of *Escherichia coli* CHA as measured by plate colony counts (Fig. 2).

Action on intracellular S13 phage. Compound 347 was added at various times

during the latent period to samples of the phage + host system, and left till the burst had ended. There was no phage multiplication when compound 347 was added during the first 15 min. When added after 15 min., phage yield increased the more the later compound 347 was added (Fig. 4). When added at 20 min. compound 347 did not increase the latent period (Fig. 3a).

Table 4. *Action of compound 347 on free S13 and T1 phages in broth*

Time (hr.)	Concentration of compound 347			
	0		20 µg./ml.	
	S13 phage (phage count $\times 10^{-4}$)		T1 phage (phage count 1.25×10^{-5})	
0	47, 40		22, 17	
1	28, 22		23, 21	

Table 5. *Action of compound 347 on the adsorption of S13 and T1 phages on Escherichia coli CHA*

Concentration of compound 347 was 20 µg./ml.

Presence (+) or absence (-) of compound 347		S13 phage, plaques/tube at		T1 phage*, plaques observed	
				Diluted	
				1/10 at 7 min.	1/100 at 60 min.
During adsorption	After dilution	10 min.	60 min.		
-	-	30, 29, 38	133, 183, 158	26, 23, 23	80, 79, 93
-	+	28, 25, 15	15, 6, 6	27, 26, 29	39, 36, 29
+	-	29, 25, 31	135, 155, 180	29, 23, 22	71, 77, 84
+	+	23, 19, 27	15, 6, 15	23, 28, 29	3, 5, 2

* In the experiment on T1 phage, the phage was diluted ten-fold immediately before counting at 7 min. and 100-fold immediately before counting at 60 min. The figures given are the counts observed.

Effect of diluting out. When compound 347 was present initially and was diluted out at 15 min., the latent period was lengthened by about 10 min. (Fig. 5). The results of this and the preceding experiments suggested that compound 347 was acting at the beginning and at the end of the latent period. To determine whether it was also acting at intermediate stages it was added at 5 min., diluted out at 15 min., and the latent period measured. The results (Fig. 5) were equivocal but suggested that any action it had on intermediate stages could only be slight.

Effect of the active compounds on T1 coliphage

For comparison with the results reported above, some experiments were made on T1 coliphage grown on *Escherichia coli* CHA, using the same methods of testing. Eleven of the thirteen compounds active against S13 phage were tested by the serial dilution method against T1 phage; only three were active, including compound 347, and a few experiments on its mode of action were

made. It prevented multiplication of T1 phage on *E. coli* CHA at 0.3 $\mu\text{g./ml.}$, compared with 8 $\mu\text{g./ml.}$ for S13 phage. At 20 $\mu\text{g./ml.}$ it had no action on free T1 phage (Table 5), nor on the absorption of T1 phage on to *E. coli* CHA. It had little effect on the yield of T1 phage when added at 20 $\mu\text{g./ml.}$ at 3 min. or more after the start of the latent period (Figs. 3*b* and 4).

DISCUSSION

The only unusual property observed of the S13 phage + *Escherichia coli* CHA system was the failure of S13 phage after inactivation by ultraviolet irradiation to kill its host. In this it differed from T2 (Dulbecco, 1952) and T1 coliphages. On the other hand, S13 phage, in its response to the compounds tested for inhibitory activity against it, differed considerably from certain larger phages. Thus proflavine was inactive against S13 phage though active against T2 phage (Foster, 1948), Pb phage of *Pseudomonas aeruginosa* (Dickinson, 1948) and staphylococcus phage K (Hotchin, 1951): several phenanthridines active against Pb phage (Dickinson, Chantrill, Inkley & Thompson, 1953) were inactive against S13 phage, while two (nos. 1148 and 1207) were active against S13 phage but not Pb phage. Of the eleven compounds active against S13 phage and tested against T1 phage, only three (nos. 347, 1367 and 2202) were active against the latter. While there was no relationship between activity against S13 phage and against larger phages, equally it was clear from consideration of the active compounds (Table 3) and certain inactive ones (not listed here) that there was no relationship between the structure of a compound and its activity against S13 phage.

The only compound (no. 347) whose action on S13 phage was studied in detail had previously been reported to be active against Pb phage of *Pseudomonas aeruginosa* (Dickinson & Codd, 1952). Its action on T1 phage resembled that of the phenanthridine no. 1367 on Pb phage (Mills, 1953; Table 3). Its action on S13 phage appeared to combine such an action with one similar to that of proflavine on T2 phage (Foster, 1948).

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