The Action of Ultrasonic Vibrations on Actinophages

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SUMMARY

High-frequency sonic oscillations destroyed the plaque-forming ability of actinophages. Among the 31 actinophage strains studied, the fraction of viral particles which survived an exposure for 1 min. to vibrations of 20 kcyc./sec. varied from 100 to 0.0002%. Coliphage T2hr+ was more susceptible to ultrasonic treatment than were some actinophages and was more resistant than other actinophages. In general, large actinophage particles were more sensitive to sonic treatment than small particles. A 60 W. 20 kcyc./sec. M.S.E. Ultrasonic Disintegrator destroyed actinophages more rapidly than a 50 W., 9 kcyc./sec. Raytheon Sonic Oscillator. Electron micrographs of actinophage MSP8 treated with sonic vibrations showed progressive disruption of the viral particles. Susceptibility to sonic inactivation gave an additional criterion for grouping actinophages.

INTRODUCTION

Ultrasonic oscillations produced by vibrating crystals or diaphragms have been shown to inactivate various types of bacteriophages suspended in liquid media (Anderson, Boggs & Winters, 1948; Beumer & Beumer-Jochmans, 1950; Fadeeva, Rautenshtein & El'piner, 1959). Anderson and co-workers reasoned that if bacteriophages were more resistant to sonic vibration than were their hosts, early stages of phage multiplication could be studied by sonically-disrupting bacteria at intervals after infection. Anderson & Doermann (1952) used this method in studies on the intracellular development of coliphage T3. Beumer & Beumer-Jochmans (1950) found large differences in the sensitivities of staphylococcus phage 'Twort' and coliphages PF and pfi, to sonic vibrations of 1000 kcyc./sec. Fadeeva *et al.* (1959) found a phage of *Bacillus megaterium* to be much more sensitive to oscillations at a frequency of about 650 kcyc./sec. than were phages virulent for *Actinomyces olivaceus* (*Streptomyces olivaceus*) and *Actinomyces streptomycini* (*Streptomyces griseus*). Hydrogen and argon atmospheres had no protective effect against the inactivation of the *B. megaterium* phage.

We wished to determine whether ultrasonic treatment could be used to prepare actinophage 'ghosts', to separate phage heads from tails, and to determine whether actinophages with similar host ranges had similar sensitivities to sonic treatment. Susceptibility to ultrasonic vibrations has been generally correlated with other criteria for classifying actinophages. Electron microscopic examination of treated actinophage suspensions showed that mechanical disruption was correlated with loss of plaque-forming ability.

METHODS

Actinophages. The actinophages used and their hosts are given in Table 1. The origins of these actinophages were recorded previously (Anderson, 1961).

Actinophages were grown on the appropriate host in a medium composed of: 5 g. Difco peptone; 3 g. Difco yeast extract; 0.5 g. $\text{Ca}(\text{NO}_3)_2$; per litre of de-ionized water. The methods used for propagating and assaying the actinophages were described by Bradley, Anderson & Jones (1961). Phage lysates were filtered through unglazed porcelain filters to remove host debris, and the filtrates stored at 4°.

Ultrasonic treatments. Filtered phage suspensions were subjected to 18–20 kcy./sec. generated by a 60 W. M.S.E. Ultrasonic Disintegrator (Measuring and Scientific Equipment Ltd., London) or to 9 kcy./sec. from the 50 W. Model S102A Raytheon Oscillator (Raytheon Manufacturing Company, Waltham, Massachusetts, U.S.A.). Containers holding 10 ml. of phage suspension were immersed in an ice bath during the 12 min. treatment period. Samples (0.3 ml.) were removed at intervals; the numbers of infectious particles were enumerated on peptone yeast-extract agar by the soft agar overlay method (Jones & Bradley, 1962).

Electron microscopy. Suspensions of actinophage MSP8 to be observed by electron microscopy were purified on columns of G-75 coarse Sephadex (Pharmacia, Uppsala, Sweden) and ECTEOLA-cellulose (Bio-Rad Corp., Richmond, Calif., U.S.A.) or by several cycles of centrifugation (Kolstad & Bradley, 1964). Treated suspensions were dried on Formvar-coated specimen grids, shadowed with uranium, and observed in an RCA EMU-3F electron microscope.

RESULTS

Effects on infectivity

Actinophages markedly lost infectivity as measured by plaque forming ability when subjected to ultrasonic oscillations of 18–20 kcy./sec. or 9 kcy./sec. for various periods (Tables 1 and 2). The actinophage strains differed substantially in sensitivity to ultrasonic treatment. For example, 10 % of the infective units of phages MSP8, WSP1, and WSP3 survived 12 min. ultrasonic treatment, whereas the infective titre of other actinophages (e.g. MSP11) decreased from 10^8 particles/ml. to undetectable values after treatment for 2 min. Coliphage T2hr+ was more sensitive to ultrasonic treatment than were some of the actinophages and was more resistant than others. A comparison of phage inactivation produced by the M.S.E. Disintegrator (Table 1) and by the Raytheon Oscillator (Table 2) suggested that the former instrument was more effective. The M.S.E. apparatus destroyed more than 10 times as many particles of coliphage T2hr+ as did the Raytheon apparatus after treatment for 2 min., about 100 times as many particles after 4 min., and about 1000 times as many particles after 12 min. Similar results were obtained in comparing the susceptibilities of actinophages MNP8 and WSP3 to treatment in these two instruments.

To determine the effect of the initial phage concentration on the inactivation rate, the initial concentration of several phage suspensions was adjusted so that their titres differed by factors of 10 to 1000. In general, the rate of inactivation of separate suspensions of a particular phage was independent of initial phage concentration (Table 3).

For those actinophages whose size had been determined, large phages proved more sensitive to ultrasonic treatment than small phages. For example, actinophages MSP 19, MNP 8, MNP 6, all of which were quickly inactivated by ultrasonic treat-

Table 1. *The effects of ultrasonic treatment at 20 kcyc./sec. on the infectivity of various actinophages and coliphage T2hr +*

Phage	Host	Log. initial titre	Log. $\frac{\text{Initial phage titre}}{\text{Phage titre after ultrasonic treatment for (min.)}}$					
			0.25	1	2	4	7	12
MSP 8	<i>Streptomyces venezuelae</i> S13	8.8	0.1	0.3	0.4	0.5	0.7	1.0
MSP 8	<i>S. griseus</i> S104	4.4	-0.1	0.1	0.1	0.4	0.3	0.9
MSP 1	<i>S. griseus</i> S86	9.0	0.2	0.8	2.6	3.5	3.5	3.6
MSP 2	<i>S. griseus</i> S86	7.7	0.2	0.3	0.4	0.8	1.3	1.7
WSP 1	<i>S. griseus</i> S86	7.8	-0.1	-0.2	0.1	0.2	0.6	1.1
WSP 3	<i>S. griseus</i> S104	6.3	-0.2	-0.1	0.2	0.1	0.5	1.1
MSP 7	<i>S. violaceoruber</i> S199	8.3	0.4	1.3	2.8	4.5	5.7	5.8
MSP 11	<i>S. violaceoruber</i> S199	8.1	2.6	4.3	—	—	—	—
MSP 15	<i>S. violaceoruber</i> S199	7.5	0.7	2.4	4.3	5.1	6.5	6.0
MSP 16	<i>S. violaceoruber</i> S199	7.2	1.5	2.8	3.4	3.6	6.2	—
MSP 17	<i>S. violaceoruber</i> S199	7.7	2.3	3.7	4.2	—	—	—
MSP 18	<i>S. violaceoruber</i> S199	9.4	1.6	3.0	4.1	5.1	6.2	6.4
MSP 19	<i>S. violaceoruber</i> S199	7.0	4.0	5.3	—	—	—	—
MSP 21	<i>S. vinaceus</i> S208	8.0	1.0	2.3	3.9	3.7	4.1	5.9
MSP 22	<i>S. vinaceus</i> S208	7.9	1.3	3.1	4.5	—	—	—
MNP 4	<i>S. griseus</i> S104	9.4	1.0	2.4	3.9	4.7	—	—
MNP 3	<i>S. griseus</i> S104	9.6	0.3	1.1	2.3	2.6	3.5	4.4
MVP 4	<i>S. cinnamomeus</i> 1285	7.7	0.5	1.6	3.2	4.3	4.7	6.7
MNP 1	<i>Nocardia brasiliensis</i> N301	6.8	0.4	0.5	1.3	2.4	4.4	4.6
MNP 2	<i>N. brasiliensis</i> N301	7.3	2.3	3.2	3.9	4.1	—	—
MNP 6	<i>N. brasiliensis</i> N301	7.7	0.2	0.7	2.2	3.7	4.2	6.4
MNP 7	<i>N. brasiliensis</i> N301	8.1	0.6	2.3	3.5	4.9	5.9	6.2
MMP 1	<i>N. brasiliensis</i> N301	6.2	0.4	0.1	2.2	—	—	—
MJP 1	<i>Jensenia canicruria</i> 1574L	8.9	0.8	2.1	3.0	3.7	4.7	5.3
MMP 9	<i>Mycobacterium</i> sp. A405	5.6	0.2	1.7	—	4.2	—	—
MMP 14	<i>M. friburgensis</i> A403	5.5	1.0	2.2	4.8	—	—	—
MMP 13	<i>M. friburgensis</i> A403	5.1	0.6	1.9	4.1	—	—	—
MMP 8	<i>M. rabinowitsch</i> A402	4.1	1.1	3.4	—	—	—	—
MMP 10	<i>M. stercoides</i> A406	6.9	0.9	2.1	4.1	3.7	3.9	—
MMP 7	<i>M. rabinowitsch</i> A402	6.9	4.0	5.7	—	—	—	—
T2hr +	<i>Escherichia coli</i> B	9.0	0.1	0.4	1.4	3.3	4.5	5.4
T2hr +	<i>E. coli</i> B	9.1	0.1	0.4	1.2	3.0	4.9	5.2

Table 2. *The effects of ultrasonic treatment at 9 kcyc./sec. on the infectivity of various actinophages and coliphage T2hr +*

Phage	Host	Log. initial titre	Log. $\frac{\text{Initial phage titre}}{\text{Phage titre after ultrasonic treatment for (min.)}}$						
			0.5	2	4	7	12	27	57
WSP 3	<i>Streptomyces griseus</i> S104	8.2	—	—	—	0.2	0.4	0.5	0.6
MNP 8	<i>Nocardia corallina</i> W5	5.2	-0.3	0.9	2.3	—	4.2	4.2	—
T2hr +	<i>Escherichia coli</i> B	9.1	-0.1	-0.1	0.9	0.7	1.8	3.7	4.4

ment, were longer than 300 m μ and had head dimensions of about 70–80 m μ (Anderson, 1961). Conversely, actinophages MSP2, WSP1, WSP3, which were highly resistant to ultrasonic treatment, were all about 250 m μ in overall length. Actinophages WSP1 and WSP3 had head dimensions of less than 60 m μ .

Table 3. *The effects of initial phage concentration on the ultrasonic inactivation rate*

Ultrasonic treatment by a 20 kcyc./sec. M.S.E. instrument.

Phage	Host	Log. initial titre	Log. $\frac{\text{Initial phage titre}}{\text{Phage titre after ultrasonic treatment for (min.)}}$					
			0.25	1	2	4	7	12
MSP 10	<i>S. violaceoruber</i> S199	6.6	0.2	1.0	3.4	4.3	—	—
MSP 10	<i>S. violaceoruber</i> S199	5.6	0.5	1.3	2.7	4.6	4.6	—
MNP 8	<i>N. corallina</i> W5	7.9	0.9	2.7	5.0	6.4	—	—
MNP 8	<i>N. corallina</i> W5	4.8	1.0	3.1	—	—	—	—

Table 4. *The relationship between mechanical disintegration of actinophage MSP8 and loss of plaque-forming ability*

Min. of treatment	Log. plaque forming units	Total heads (no.)	Whole phage (%)	Phage with broken heads (%)	Whole detached heads (%)	Broken detached heads (%)	Whole free tails (no.)
0	9.9	155	95	3	—	2	6
0.5	9.8	212	83	15	—	1	29
2	9.7	194	64	9	2	25	34
4	9.5	542	51	8	—	41	106
7	Undetermined	162	49	7	1	44	56
12	8.7	294	31	6	—	62	142
22	8.3	227	7	2	8	83	90
42	7.7	121	3	1	3	95	56

Effects on structure

Samples for electron-microscope examination were taken at intervals during ultrasonic treatment of actinophage MSP8, and the frequencies of whole particles, particles with broken heads, broken detached heads, and whole detached heads were determined (Table 4). Almost all the viral particles of the untreated control were found to be intact (Fig. 1). After a short period of treatment, some phage heads were broken and separated from their tails; other particles appeared to be undisturbed (Pl. 1, figs. 2, 3). As the treatment progressed, more particles were disrupted; after 42 min. of ultrasonic treatment no intact particles were found (Pls. 1, 2, figs. 4–8). Electron micrographs at this point showed masses of debris which included short tail fragments.

DISCUSSION

Fadeeva *et al.* (1959) showed that actinophages were inactivated by ultrasonic treatment at a frequency of about 650 kcyc./sec. Treatments of 45 min. to 6 hr. produced large decreases in infective titres. In the present work, exposure to ultrasonic treatment at a frequency of 18–20 kcyc./sec. for 1–12 min. caused a comparable loss of infectivity. Moreover, 20 kcyc./sec. was more effective in destroying the infec-

tivity of the phages studied than a frequency of about 9 keyc./sec. The 650 keyc./sec. treatment used by Fadeeva *et al.* (1959) apparently was not as effective in destroying actinophages as our 20 keyc./sec. treatment; but meaningful comparisons cannot be made because their actinophages may have been quite different from those we studied. Moreover, our results cannot be compared directly with those of Beumer & Beumer-Jochmans (1950), who used a 120 W. instrument with a frequency of 1000 keyc./sec. The intensity and the frequency of oscillation are undoubtedly both important factors in the destructive action of any ultrasonic treatment.

Anderson *et al.* (1948) suggested that small phages such as coliphage T3 probably escape the destructive cavitation which disrupts the larger phages such as coliphages T2, T4 and T6. In the present work, actinophages WSP1, WSP3 and MSP2 were inactivated less rapidly than the smaller coliphage T2hr+ (overall length 190 m μ). The head of coliphage T2hr+, however, is about 95 m μ long which is larger than the actinophage head structure. This suggests that susceptibility to ultrasonic treatment may be primarily dependent upon the dimensions of the head. Consistent with this view are the observations that coliphage T5 is far more susceptible to ultrasonic treatment than is coliphage T1 and that the head of coliphage T5 (65 m μ) is larger than the head of coliphage T1 (50 m μ). The tails of coliphages T5 and T1 are of about the same length (170 m μ and 150 m μ , respectively; Stent, 1963).

Mechanism of ultrasonic inactivation of actinophage MSP8. Electron-microscope examination of samples of an ultrasonically treated suspension of actinophage MSP8 taken at intervals showed that mechanical disruption was important in inactivation (Pls. 1, 2, figs. 1-8). No sharp sequence of destructive events was detected (for example, breaking of heads, separation of heads from tails, fragmentation of tails). Tails, however, did not appear to fragment extensively until the ultrasonic treatment was prolonged from 22 to 42 min. (Pl. 2, figs. 6, 7). Free tails consistently clumped after prolonged treatment (Pl. 2, fig. 8). If the time of irradiation were controlled, it might be possible to separate intact actinophage tails from the phage heads, and then purify each component by appropriate physical techniques.

Taxonomic implications. One of the objects of this work was to determine whether ultrasonic treatment data would provide one more criterion in grouping actinophages. The sensitivity pattern of actinophages to ultrasonic treatment agrees in general with the classification based upon host range (Bradley *et al.* 1961). For example, actinophages MSP15, MSP16, MSP17, MSP18, MSP19, which are closely related in host range, are all markedly susceptible to ultrasonic treatment. Similarly, actinophages WSP1 and WSP3, which are quite resistant to ultrasonic treatment, are indistinguishable in host range. Adams (1959) stated that one of the major objections to the use of inactivation of any type of phage as a taxonomic criterion is that phage strains often differ quantitatively rather than qualitatively; indeed, there is limited diversity in sensitivity among members of a group with similar host ranges. When the sensitivities of different groups are compared, there is overlapping in ranges of variation among the groups. Obviously no one type of inactivation can be used as a sole taxonomic criterion; inactivation characteristics should be correlatable with other criteria, such as morphology and serology.

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EXPLANATION OF PLATES

PLATE 1

- Fig. 1. Untreated particles of actinophage MSP8; $\times 25,000$.
- Fig. 2. Actinophage MSP8 treated with 20 keyc./sec. ultrasonic vibrations for 30 sec.; $\times 25,000$.
- Fig. 3. Actinophage MSP8 after 4 min. treatment; $\times 25,000$.
- Fig. 4. Actinophage MSP8 after 7 min. treatment; $\times 26,818$.

PLATE 2

- Fig. 5. Actinophage MSP8 particles and debris after 12 min. treatment; $\times 25,000$.
- Fig. 6. Actinophage MSP8 particles and debris after 22 min. treatment; $\times 25,000$.
- Fig. 7. Head coats and debris from actinophage MSP8 particles after 42 min. treatment; $\times 25,000$.
- Fig. 8. Large clump of tails apparently held together by debris from actinophage MSP8 particles after 42 min. treatment; $\times 25,000$.

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