A SMALL RNA CONTAINING PSEUDOMONAS AERUGINOSA BACTERIOPHAGE[1]

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The single-stranded deoxyribonucleic acid (DNA) bacteriophage ØX174 (Sinsheimer, 1959) and the ribonucleic acid (RNA) phage f2 (Loeb and Zinder, 1961) have recently stimulated much interest with respect to biosynthetic processes occurring in virus-infected cells (Sinsheimer, et al., 1962; Rueckert and Zillig, 1962; Cooper and Zinder, 1962; Doi and Spiegelman, 1962). Kay (1962) reports another bacteriophage which contains a single-stranded chain of DNA, while Bradley (1962) presents further evidence that small, spherical bacterial viruses containing single-stranded nucleic acid molecules may be more common than previously suspected. The isolation of additional spherical phages of either the single-stranded DNA or RNA types should prove useful for comparative purposes.

Phage 7s is one of three bacteriophages isolated in this laboratory from a single multilysogenic strain of Pseudomonas aeruginosa. This virus forms large clear plaques ranging from 3 to 6 mm in diameter on several strains of P. aeruginosa, but is incapable of plaque formation on several strains of Escherichia coli (including E. coli, B, C and K-12)

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tested in preliminary host range studies. Among the several strains of P. aeruginosa found sensitive to this virus, phage 7s plated with highest efficiency on strain 1 of Holloway (1955), but formed plaques which were more regular in size on strain Ps-1 (one of a collection of P. aeruginosa strains isolated in this laboratory). Examinations of crude 7s phage lysates by electron microscopy using the neutral phosphotungstic acid method (Brenner and Horne, 1959) revealed small spherical particles with an average diameter of around 25 mu (photographs to be published elsewhere). The small viral size suggested that phage 7s might contain only a single-stranded nucleic acid chain which in turn raised the question regarding its DNA or RNA content. The following data indicate that phage 7s is an RNA virus.

Methods. Crude lysates of phage 7s were prepared by elution of virus obtained through agar plate confluent lysis techniques. The basal medium consisted of nutrient agar (Difco) with 0.5% yeast extract (BBL) and 0.5% NaCl; the soft agar contained 0.75% Difco agar with 0.5% NaCl and 0.08% NH_4NO_3. To facilitate rapid inoculation of a large number of plates, soft agar was made up in 500 ml batches, autoclaved, and cooled to 45°. Sufficient phage 7s and host bacteria were then added to produce confluent lysis; this mixture was dispensed by an automatic Cornwall syringe in 6.5 ml volumes to pre-poured plates containing basal medium. Following 8 to 10 hours' incubation at 37° the soft agar layers were scraped into 0.005 M phosphate buffer pH 7.2, held at 4°. This mixture was homogenized in 300 ml portions with 5 ml chloroform for 90 seconds in the cold in a Waring blendor, then transferred to a beaker and agitated continuously in the cold by a magnetic stirrer for 3 to 4 hours to permit elution of virus from the soft agar. The homogenate was next centrifuged, first for 45 minutes at 8,000 X g to sediment the agar, then for 30 minutes at 17,000 X g to sediment bacterial

debris. In this manner it was possible to obtain approximately one liter of crude lysate containing 8 X 10^{11} to 2 X 10^{12} plaque forming units (pfu) per ml per 100 inoculated agar plates.

Partial purification of phage 7s from crude lysates was obtained by treatment with RNAse and DNAse followed by differential centrifugation. Crude lysates were first treated with 4 μg/ml of RNAse (Sigma) for 60 minutes at room temperature. $MgSO_4$ was then added to a final concentration of 0.002M followed by the addition of DNAse (Sigma) to a final concentration of 4 μg/ml. Following a 60-minute incubation interval at room temperature, the enzyme treated lysates were centrifuged 120 minutes at 70,000 X g in a 30 rotor of the Spinco Model L centrifuge. Supernatant fluids were carefully decanted and phosphate buffer was added to the pellets. After standing in the cold for 24 hours without agitation, the resuspended pellets were pooled and centrifuged 30 minutes at 17,000 X g; the resulting supernatant fluids were brought to one-tenth the volume of the crude lysate with phosphate buffer. The preparation was again centrifuged 120 minutes at 17,000 X g as above. Pellets obtained from this second high-speed centrifugation were resuspended to one-fiftieth the volume of the crude lysate and subjected to a final 30-minute centrifugation at 17,000 X g. Pellets obtained from the 17,000 X g centrifugations contained insignificant amounts of phage, so were discarded.

DNA was measured by the modified diphenylamine reaction as described by Burton (1956). Highly polymerized calf thymus DNA (Mann) was used as a standard. RNA was measured by the orcinol reaction as standardized against yeast RNA (Pabst).

The base composition of phage 7s nucleic acid was determined from formic acid hydrolysates of whole virus (Smith and Wyatt, 1951) at concen-

trations of 2×10^{13} to 2×10^{14} pfu/ml. To determine viral base compositions, 0.5 ml aliquots of various virus preparations were taken to dryness, resuspended in 0.5 ml 90% formic acid and hydrolyzed 30 minutes in sealed Pyrex tubes at 175°. Hydrolysates were evaporated to dryness, dissolved in 25 to 50 µl 1 N HCl, spotted on Whatman No. 1 filter paper and the bases separated by descending chromatography using an isopropanol-HCl-water (4:1:6) solvent (Wyatt, 1951). Four spots containing the bases were located by ultraviolet light and each was eluted in 10 ml 0.01 N HCl.

Phenol extracted phage 7s nucleic acid (Sinsheimer, 1959) was hydrolyzed 60 minutes at 100° in sealed Pyrex tubes containing 1 N HCl in a total volume of 1 ml. The sugar liberated by this hydrolysis was identified by descending chromatography using the solvent system of Hall (1962). Yeast RNA and ribose were treated appropriately and these served as standards for the comparison of Rf values.

Results and Discussion. Tables 1 and 2 show plaque titers as determined by the agar layer method using Pseudomonas Ps-1 as the plating bacteria. DNA and RNA content of the various fractions obtained by differential centrifugation are also recorded. When crude lysates were treated with RNAse, but not DNAse (Table 1), essentially all of the infectivity and approximately 40% of the RNA remaining after RNAse treatment were sedimented in 120 minutes at 70,000 X g, while most of the DNA remained in the supernatant. On the other hand, when crude lysates were treated with DNAse as well as RNAse (Table 2), most of the DNA was rendered acid soluble. In both experiments, approximately 90% of the virus titer was recovered in the final high speed pellet.

Table 1. Virus titer, DNA and RNA content of lysate fractions obtained by differential centrifugation. Experiment 1.

Fraction	Volume	pfu/ml	μg DNA/ml	μg RNA/ml
Crude lysate	2500 ml	8.9×10^{11}	130	325
Enzyme treated lysate*	2500 ml	9.0×10^{11}	135	225
1st 70,000 X g supernatant	2500 ml	4.6×10^{10}	112	86
1st 70,000 X g pellet	250 ml	8.8×10^{12}	**	**
2nd 70,000 X g supernatant	250 ml	**	2.2	83
2nd 70,000 X g pellet	50 ml	3.9×10^{13}	<0.5	4612

* Crude lysate treated with 4μg RNAse/ml.
** Not tested.

Table 2. Virus titer, DNA and RNA content of lysate fractions obtained by differential centrifugation. Experiment 2.

Fraction	Volume	pfu/ml	μg DNA/ml	μg RNA/ml
Crude lysate	3500 ml	1.0×10^{12}	129	323
Enzyme treated lysate*	3500 ml	8.8×10^{11}	<0.2	206
1st 70,000 X g supernatant	3500 ml	6.5×10^{10}	<0.2	103
1st 70,000 X g pellet	350 ml	9.8×10^{12}	<0.07	710
2nd 70,000 X g supernatant	350 ml	4.4×10^{11}	<0.02	134
2nd 70,000 X g pellet	70 ml	5.0×10^{13}	<0.2	3130

* Crude lysate treated with 4 μg DNAse and 4 μg RNAse/ml.

When the final 70,000 X g pellet from either experiment 1 or 2 was diluted with 0.005M phosphate buffer, pH 7.2 and centrifuged at 63,000 X g in the Spinco Model E analytical rotor, two peaks were observed, one with a sedimentation coefficient of 53S, the other, faster moving peak with a sedimentation coefficient of 88S. When these preparations were diluted in phosphate buffer containing 0.005M ethylenediaminetetracetic acid (EDTA) at pH 7.2, only a single peak with a coefficient of 88S was observed. The two pellets were pooled; the virus titer was 5.2×10^{13} pfu/ml when tested on strain Ps-1 and 1.1×10^{14} pfu/ml on strain 1, while the RNA content was 3.72 mg/ml. A 30-ml sample of this pool was mixed with 30 ml phosphate buffer containing 100 μg RNAse/ml and 0.005M EDTA, then incubated 45

minutes at 37°C. The RNAse-treated pool was centrifuged 150 minutes at 70,000 X g. Pellets were covered with phosphate buffer and held 24 hours in the cold without agitation. The resuspended pellets were diluted to 15 ml with phosphate buffer and subjected to a final 30-minute centrifugation at 17,000 X g. This preparation contained 1.18 mg RNA/ml, while the plaque titer was found to be 7.2×10^{12} pfu/ml when tested on strain Ps-1 and 1.8×10^{13} pfu/ml when grown on strain 1.

Base compositions of the 2 high-speed pellet nucleic acid fractions, as well as that of the RNAse-treated pooled pellet preparation, are shown in Table 3. No thymine was detected in any of the chromatograms. The base ratios obtained in all of the preparations show close agreement and differ markedly from those values published for P. aeruginosa RNA (Belozersky and Spirin, 1960) and also differ from the base ratio values reported for P. aeruginosa ribosomal RNA (Hayashi and Spiegelman, 1961, and Midgley, 1962).

Table 3. Base ratios of phage 7s pellets before and after treatment with RNAse.

Virus Preparation	mg RNA/ml	Moles per cent			
		Adenine	Guanine	Cytosine	Uracil
Experiment 1	4.62	23.8	25.5	24.9	25.8
Experiment 2	3.13	22.8	24.8	26.6	25.8
RNAse-treated pool	1.18	24.7	23.4	25.8	25.8

The sugar liberated by acid hydrolysis of phenol extracted nucleic acid of various phage 7s pellets had an Rf characteristic of ribose.

In summary, the evidence indicates that phage 7s contains RNA and not DNA as its nucleic acid component. This conclusion is based on the distribution of the two types of nucleic acid in different lysate fractions fol-

lowing differential centrifugation, the absence of thymine in partially purified virus hydrolysates, the absence of deoxyribose as tested by the diphenylamine reaction and the identification of ribose in acid hydrolysates of phenol extracted phage 7s nucleic acid. Further studies on the biology and chemistry of phage 7s are in progress.

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