The Nucleic Acid Composition of Bacteriophage φR

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SUMMARY

Bacteriophage φR was prepared and purified in sufficient quantity to determine the composition and base ratios of its nucleic acid. The ratios are such that the complementary double-stranded structure usually found in deoxyribonucleic acid (DNA) seems unlikely to be present. This observation and the accessibility of the DNA of phage φR to the action of formaldehyde support the view that phage φR, like phage φX-174 which it resembles, carries its nucleic acid in a single strand.

INTRODUCTION

Bacteriophage φR has been in our collection for some years. From electron micrographs taken in 1954 it was known to be very small in size and appeared to be tail-less. It was studied by Fildes (1954) and by Tucker (1961), when it was referred to as phage anti-R. Sinsheimer (1959), working with the very small bacteriophage φX-174, found that certain unusual properties of its nucleic acid could be accounted for by assuming a single-stranded instead of a double-stranded structure. This discovery prompted an investigation of the nucleic acid composition of phage φR to determine whether it, too, was of the single strand type.

Bacteriophage φX-174 resembles another coliphage, S13, in several ways; a comprehensive comparison between the two was made by Zahler (1958). A specimen of coliphage S13 was kindly made available by Dr K. Burton (Department of Biochemistry, University of Oxford) and this was compared with phage φR. Although the two phages have an entirely different history it has nevertheless proved difficult to differentiate them on grounds of host range alone. Bacterial strains showing well-marked differential sensitivity to these phages are rare and the results depend somewhat on the divalent metal ion content of the test media. Zahler (1958) found similar difficulties in distinguishing between φX-174 and S13. Phages φR and S13 can however be shown to differ immunologically even more markedly than do phages φX-174 and S13. It is therefore unlikely that φR is merely another isolate of φX-174 or S13 and it must be regarded as a different species of bacteriophage. The fine structure of phage φR is of interest. Purified specimens of the phage made as described in this paper have been examined in the electron microscope. The results are described in a subsequent paper (Kay & Bradley, 1962).
METHODS

Bacteriophages and host bacteria. Bacteriophage φ5R was received from the late Dr A. Felix in 1953. He described it as an anti-rough phage, meaning that it lysed only the 'rough' stains of Salmonella typhi that he was studying. It was carried by a strain of S. paratyphi A isolated in Egypt in 1948 and had been maintained at first on S. paratyphi strain A59R and later on S. paratyphi strain 6SR. For the purpose of the present work it was found more convenient to grow the phage on Escherichia coli C (referred to as coli C) which was kindly supplied by Dr K. Burton (Department of Biochemistry, University of Oxford), who also supplied a specimen of phage S18.

Assay of phage φ5R. The phage was assayed by the two-layer method described by Adams (1950). The Petri dishes were kept at 37° for only 3 hr., by which time the plaques were countable. Further incubation allowed the plaques to grow to a large size, fuse and become uncountable.

Growth and purification of phage φ5R. Some difficulty was found in growing this phage to sufficiently high titre for purification. The rate of lysis of an infected culture depended on the divalent metal ion concentration, but unfortunately rapid lysis always resulted in low phage titres. A medium was devised containing peptone (Evans, 1% (w/v)) and NaCl (0.5% (w/v)). It was decalcified by boiling and filtering at pH 9.1 and was then readjusted to pH 7.6. Cultures of coli C were grown in 1 l. batches in 5 l. flasks rotated at 37° (Kay, 1959). At a population density of $4 \times 10^8$ bacteria/ml. phage was added to give a ratio of 1:1. Lysis took place slowly over 5 hr., giving phage titres of about $10^{11}$ plaque-forming units (pfu)/ml. When maximum clearing had occurred the phage was purified by a procedure modified from that of Sinsheimer (1959). The phage was precipitated with ammonium sulphate (400 g/l.) in the presence of ethylenediaminetetra-acetic acid (EDTA; 0.0025 M) at 4° overnight. The precipitate was collected by centrifugation at 15000 g for 1 hr. The sediment was extracted in borate + EDTA buffer (pH 9.7) for 3 hr. at 4°, centrifuged and re-extracted with the same buffer. The extracts were pooled, dialysed against borate + EDTA buffer (pH 9.1) at 4° and again precipitated with ammonium sulphate (280 g/l.) in the cold. The precipitate was extracted twice in borate + EDTA buffer (pH 9.1) and centrifuged for 2 hr. at 5000 g to remove inactive material. The phage was then sedimented at 140,000 g for 1 hr., the supernatant fluid discarded and the phage pellet resuspended in 0.033 M-phosphate buffer (pH 7.6) containing 0.01% (w/v) human serum albumin. The suspension was then treated with deoxyribonuclease (1 µg./ml.) and MgSO₄ (0.004 M) for 1 hr. at room temperature and then at 4° overnight. The phage was then sedimented at 140,000 g for 1 hr. and resuspended in 0.033 M-phosphate buffer (pH 7.6).

Thirty litres of lysate containing about $2 \times 10^{15}$ phage particles were treated in this way and yielded $2 \times 10^{14}$ active phage particles.

Column chromatography of phage φ5R. The product from the large-scale phage preparation described above was not subjected to further purification but was used entirely for the nucleic acid composition studies described later. Other preparations of phage on a smaller scale were made for different purposes and were purified by a combination of the above procedure and chromatography on a column of DEAE (diethylaminoethyl)-cellulose.
The nucleic acid composition of bacteriophage φR

The DEAE-cellulose was prepared by washing, successively, in N-HCl, water, N-NaOH and again water. The pH of the DEAE-cellulose was adjusted to 7.4 with HCl and washing with water continued. The column (2 cm. diameter) was packed with the DEAE-cellulose suspended in 0.2 M-ammonium acetate (pH 7.4) to a height of 7.5 cm. The column was washed with ammonium acetate until the effluent was at pH 7.4 and then washed with 200 ml. 0.01 M-ammonium acetate (pH 7.4).

Phage for chromatography was prepared as described above, up to the first centrifugation at 140,000 g. The pellet was resuspended in 0.01 M-ammonium acetate (pH 7.4), centrifuged at 4,000 g to remove insoluble matter and applied to the column. Stepwise elution was carried out with ammonium acetate buffer (pH 7.4) at 0.04 M, 0.1 M, 0.2 M, 0.5 M and M. Six fractions of 6 ml. were collected at each concentration and assayed for phage. The light absorption at 260 μm was also measured. The phage was eluted as a single band in 0.2 M-ammonium acetate. It was spread over two 6 ml. fractions and also contained most of the 260 μm absorbing material. Recoveries of phage were about 90%. Eluates containing 5 × 10¹¹ pfu/ml. possessed no visible turbidity and were used for the electron microscopy described in a subsequent paper (Kay & Bradley, 1961).

Antiphage sera. Antisera to phages φR and S13 were prepared by intravenous injection of 0.5 ml. phage suspension at weekly intervals. The phage suspensions used were an ammonium acetate eluate containing 10¹¹ pfu/ml. in the case of φR and a centrifuged lysate containing 2 × 10¹¹ pfu/ml. in the case of S13. The animals were bled one week after the last injection.

RESULTS

Host range of phages φR and S13

The two phages were tested by the cross-streak method against several coli and typhoid bacteria from our collection. In no case was it possible to demonstrate a difference in the host range of these phages. The cross-streak test is not a quantitative method, so the plaque-forming ability of the two phages was determined by counting plaques on several strains of bacteria. The titres found, expressed as % of those given on coli C, were: coli strain 518, 85 and 35, typhoid strain O901 R, 50 and < 1, typhoid strain R4, 1 and < 1 for φR and S13, respectively. These figures show that the two phages are definitely different but that the differences are only quantitative since all the bacteria were sensitive to both the phages at some concentration. A more clear-cut distinction was given by the reaction of the phages to antiserum.

Serological difference between phages φR and S13

The antiphage activity of the sera prepared against the two phages was determined by mixing the phages, at 10⁸ pfu/ml., with ten-fold serial dilutions of the homologous serum, incubating at 37° for 30 min. and comparing the number of plaque-forming units remaining with those in the controls without serum. All dilutions were made in a solution of 1% (w/v) peptone + 0.5% (w/v) NaCl. Each antiserum inactivated about 50% of the homologous phage at a dilution of 1/50,000. Both phages were then tested against both sera at a dilution of 1/20,000. The phage
remaining active was assayed at intervals of 5 min. for a period of 30 min. (Fig. 1). The phages were inactivated by the homologous antisera only and were therefore clearly different from one another.

Univalency of phage φR

Electron micrographs of phage φR (Kay & Bradley, 1961) show that the particle is an icosahedron and that it probably carries a small tail. This protuberance is so small that it could be regarded as merely a specialized area of the phage protein envelope. Nevertheless this phage must function in much the same way as other phages in that it must first absorb to its host cell and then transfer its DNA to the host through a perforation in the cell wall. These phage particles would therefore be expected to carry an organ concerned with absorption to the host and an enzyme which can digest the cell wall material at that site. If there were not a unique 'tail' on the phage in the accepted sense, and any part of the particle could act as an absorption site with an affinity for the bacterial receptor, then it might follow that a mixture of a great excess of phage particles with bacteria would lead to agglutination of the bacteria in the same way as certain animal viruses can give rise to haemagglutination. On the other hand, if under these circumstances no bac-
The nucleic acid composition of bacteriophage \( \phi R \)  

If bacterial agglutination occurred, then it would appear that there was only one adsorption site on the particle and that the phage must have a single area capable of combining with the host cell. That is, it must have a single ‘tail’.

In an attempt to resolve this matter a mixture of logarithmic-phase bacteria at \( 2 \times 10^8 \text{/ml.} \) and phage at \( 3 \times 10^{11} \text{pfu/ml.} \) was made in a solution containing 1\% (w/v) peptone + 0.5\% (w/v) NaCl + 0.0025M-CaCl\(_2\). After 5 min. at 37\(^\circ\) a portion of the mixture was concentrated and the free phage titre determined. It was found that 9\% of the phage was still free and that there must have been an average of

![Fig. 2. Effect of formaldehyde (2\%, w/v) on the ultraviolet absorption of phage \( \phi R \) nucleic acid. A, immediately after mixing; B, after 4.5 hr.; C, after 24 hr.](image URL)

1450 absorbed phage particles/bacterium. Part of the mixture was kept at 37\(^\circ\) and part at room temperature. No gross agglutination was observed in either case, nor did the cells appear to be clumped together in a stained smear. Although this evidence is of a negative kind it can reasonably be taken to mean that phage \( \phi R \) possesses only one absorption site or ‘tail’.
The purine and pyrimidine bases of phage φR nucleic acid

The nucleic acid was extracted by the procedure of Sinsheimer (1959) from a suspension of phage containing $2 \times 10^4$ pfu/ml, prepared from 30 l. lysate as described in Methods. It was extracted into 2-methoxy-ethanol, precipitated with ethanol (60%, v/v, in water) in the presence of a little sodium chloride, and washed successively in 60%, 90% (v/v) ethanol in water, absolute ethanol, then ether, and dried over phosphorus pentoxide. The bases were liberated from the nucleic acid by hydrolysis with 6 N-HC1 at 100° for 3 hr. in a sealed Pyrex tube. The hydrolysate was evaporated to dryness in vacuo over KOH, taken up in water and chromatographed in propan-2-01 + HCl (Wyatt, 1951). Four spots containing the bases were located with ultraviolet irradiation and extracted into 0.1 N-HCl. The bases were identified as adenine, guanine, thymine and 'cytosine' from their ultra-violet absorption curves and their positions on the chromatogram. The 'cytosine' spot, which runs to the same place as hydroxymethylcytosine in propan-2-01 + HCl, was again chromatographed in propan-2-01 + ammonia (Hershey, Dixon & Chase, 1953) and found to run to the same place as authentic cytosine.

The molar ratios of the bases found were: adenine, 1.00; thymine, 1.41; guanine, 1.03; cytosine, 0.85. The comparable ratios for phage φX-174 nucleic acid (Sinsheimer, 1959) were 1.00; 1.31; 1.06; 0.82.

Effect of formaldehyde on phage φR nucleic acid

It was shown by Sinsheimer (1959) that the ultraviolet absorption of phage φX-174 nucleic acid was increased and the peak moved to a slightly longer wavelength by treatment with formaldehyde. This change, which does not take place with native deoxyribonucleic acid (DNA) from other sources, was explained by assuming a single-stranded structure for the phage φX-174 DNA, in which the amino groups of the bases would not be involved in hydrogen bonding to the bases in the complementary strand. The phage φR nucleic acid dissolved in 0.2M-NaCl was treated with formaldehyde (2%, w/v) at 37° in a glass-stoppered tube. The ultraviolet absorption curve was determined at the beginning, after 4.5 hr. and after 24 hr. (Fig. 2). The nucleic acid from phage φR showed the same hyperchromicity as does that from phage φX-174. This observation and the lack of complementarity in the bases support the conclusion that the nucleic acid of phage φR is of the single-stranded variety.

DISCUSSION

Bacteriophage φR clearly belongs to the group of phages, now three in number, with phages φX-174 and S13. The distinguishing features of this group are the small size of the particle (245–300 Å diameter compared, for example, with 1000 × 700 Å diameter for coliphage T2) and the unusual single-stranded form of the DNA. In general, all the bacteriophages that have been examined, with the exception of the one containing ribonucleic acid (Loeb & Zinder, 1961) possess the 1:1 molar ratios of adenine:thymine and guanine:cytosine (or hydroxymethylcytosine) that are found in native DNA from all other sources (Evans, 1959). The new group of small bacteriophages does not show complementarity between its bases which, since they are not involved in hydrogen bonding, are accessible to formaldehyde.
The nucleic acid composition of bacteriophage $\phi R$

In phage $\phi R$, as in phage $\phi X$-174, formaldehyde causes an increase in the ultraviolet absorption of the nucleic acid, an effect which is demonstrable with whole phage (Tucker, 1961), as well as with isolated nucleic acid, and which could form the basis of a screening test for bacteriophages possessing single-stranded nucleic acid.

Some of the electron micrographs of phage $\phi R$ have shown what appears to be a very small ‘tail’ (Kay & Bradley, 1961) but the evidence is not conclusive. The behaviour of phage $\phi R$ when absorbed in large numbers to bacteria supports the contention that there is only one absorptive site on the particle since bacterial agglutination does not take place, as it might be expected to do by analogy with haemagglutination by animal viruses. The evidence is of a negative kind and it would therefore be desirable to obtain further information about the absorption to and penetration of, the bacterial cell by phage $\phi R$ in order to determine conclusively whether a ‘tail’ is present. Work is in progress with this end in view.

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REFERENCES