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Isolation and Composition of Bacteriophage-Like Particles from Kappa of Killer Paramecia

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Summary. Phage-like particles from kappa of stock 562 of *Paramecium aurelia* have been isolated by CsCl density gradient centrifugation. Analyses show that the particles contain about 1.6×10^{16}g DNA and 2.0×10^{-16}g protein. Their buoyant density is approximately 1.47. DNA from the particles has a buoyant density very close to that of whole kappa DNA. The presence of DNA in the particles has been confirmed by a cytochemical technique. The results support the conclusion that kappa contains a bacteriophage.

Introduction

Kappa represents one class of several kinds of bacterial endosymbionts which are found in the cytoplasm of *Paramecium aurelia* (Beale, Jurand and Preer, 1969). Kappa is distinguished by the fact that generally about 25% of the kappa in each kappa population contains a refractile (R) body. The R body is a roll of tape which may unwind to produce a long twisted protein filament approximately 0.2–0.5 μ wide, 130 Å thick, and up to 20 microns long (Mueller, 1962; Anderson *et al.*, 1964). When kappa develops an R body three things occur. First, a variable number of cytologically visible phage-like particles appear (Preer and Preer, 1967). Second, there is a loss of the capacity of the kappa to reproduce (Sonneborn, 1959; Mueller, 1963). Third, the kappa acquires the ability to kill kappa-free sensitive paramecia when it is ingested (Jurand, Rudman, and Preer, 1971). These facts have been interpreted to mean that kappa harbors a bacteriophage, possibly defective, which is generally borne in a prophage state, but which on occasion enters the vegetative state, producing more or less complete phage particles and the R body as well. Although the host kappa is killed in the process, it acquires toxic activity capable of killing sensitive paramecia.

Until now the evidence that the phage-like particles are truly bacteriophages has rested primarily on a consideration of their fine structure in the electron microscope. In this paper we report the isolation of the phage-like particles from kappa of stock 562. Analyses of isolated phage-like particles as well as cytochemical tests indicate that they are composed of protein and DNA, further strengthening the view that they are bacteriophages.

Material and Methods

Stock 562 is a killer of syngen 2 of *Paramecium aurelia* from Milan, Italy isolated by G. H. Beale. It originally contained both kappa and the macronuclear symbiont, alpha (Preer, 1969). An alpha-free strain obtained from the original stock was used in these experi-

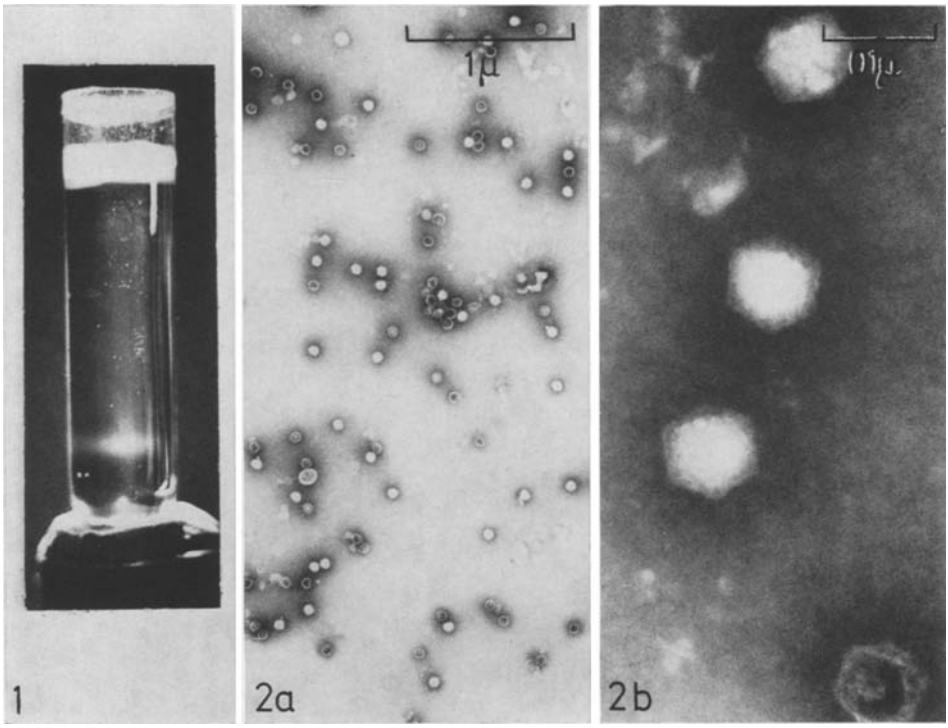


Fig. 1. Banding of the bacteriophage-like particles from disrupted kappa in a CsCl gradient. Kappa from 6 ml of packed cells was ultrasonated and centrifuged at 12000 × *g* for 5 minutes. The supernatant was brought to a volume of 4.32 by adding 0.01 M Na-K phosphate buffer pH 7, and 2.65 g CsCl were added to yield a density of 1.40. After centrifugation of this preparation at 38000 RPM at 4° C for 20 hours in the SW39 rotor of a Spinco Model L centrifuge, a well-defined turbidity was observed by reflected light in the lower third of the tube. The turbid band consisted of bacteriophage-like particles (see Fig. 2a, b)

Fig. 2a and b. Bacteriophage-like particles found in the turbid band shown in Fig. 1. Fractions from the CsCl density gradient centrifugation were collected. Each fraction was diluted with buffer and centrifuged 45 minutes at 38000 RPM. Each precipitate was resuspended and the wash repeated. The fractions were observed after negative stain with 2.5% phosphotungstic acid at pH 7. In this photomicrograph of material from fractions containing the turbid band in the lower portion of the tube "empty" (dark) and "full" (light) particles are seen. a × 20200. b × 151000

ments. Paramecia were cultured at 30° C in Cerophyll medium (Sonneborn, 1970) at the rate of two doublings per week. Cells were harvested, homogenized, and kappa was isolated with the use of Ecteola, as described elsewhere (Sonneborn, 1970).

After the kappa from 2–6 ml of packed paramecia was isolated it was centrifuged at 12,000 × *g* for five minutes and resuspended in 1.5 ml 0.01 M sodium-potassium phosphate at pH 7 and placed into a stainless steel test tube cap 18 mm in diameter. A Biosonic II (Bronwill Scientific, Rochester, New York) ultrasonic probe was used to disrupt the kappa. The test tube cap was set into ice water and sonication was carried out in 60 second bursts with two minute intervals between bursts to allow cooling. Sonication was continued until microscopic examination revealed that at least 90% of the kappas were disrupted (generally about two minutes of sonication).

Protein determinations were by the method of Lowry *et al.* (1951). Keck's (1956) technique was used to measure DNA. RNA was determined by spectrophotometry of alkaline extracts by the Schmidt-Thannhauser methods as recommended by Munro and Fleck (1966).

Counts of negatively stained phage-like particles were made using standard latex spheres (0.5 microns in diameter, standardized by counting with a Petroff-Hausser bacterial counter) and spraying an appropriate mixture of particles, latex, 2% phosphotungstic acid and 1% bovine serum albumen on electron microscope grids according to the method of Watson (1962).

The presence of DNA in the phage-like particles in sectioned material viewed with the electron microscope was detected by the method of Bernhard (1969), and is described in the captions for Figs. 6 and 7.

Results

When disrupted kappa was centrifuged in a CsCl density gradient a band of turbid material was observed in the lower third of the tube (see Fig. 1). Fractions were collected, washed by centrifugation, and observed with the electron microscope. Fractions containing the turbid material in the lower portion of the tube were found to contain relatively pure bacteriophage-like particles (see Fig. 2a, b). Those fractions near the meniscus contained "empty" bacteriophage-like particles and fragments of the cell wall of kappa.

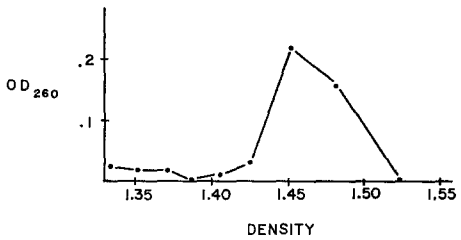


Fig. 3. Fractions from a CsCl density gradient centrifugation carried out at 4°C. The O.D.$_{260}$ of the fractions is plotted against the solution density (corrected to 4°C) as computed from refractive indices determined at 25°C

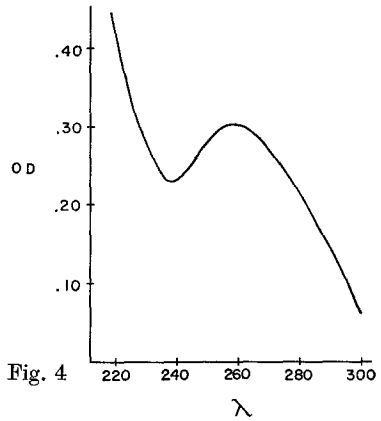


Fig. 4

Fig. 4. Scan of the optical density values of a suspension of bacteriophage-like particles from kappa obtained by centrifugation in a CsCl gradient

The O.D.$_{260}$ values of fractions from a CsCl density gradient centrifugation made at 4°C and plotted against the solution density of the fractions at 4°C indicates that the bacteriophage-like particles have a buoyant density of about 1.47 (see Fig. 3). The particles banded at approximately the same density in a similar experiment in which the centrifugation was carried out at 20°C. A scan of the optical density of a suspension of the particles is shown in Fig. 4.

Counts of particles and analyses of protein and DNA reveal in replicate determinations per unit of one O.D.$_{260}$ a mean $\pm$ standard error of $26 \pm 4.0 \times 10^{10}$ particles, 51 ± 0.9 μg of protein, and 42 ± 4.7 μg of DNA. No RNA was detected. It is calculated that the O.D.$_{260}$ per particle is 0.4×10^{-11}, the mean amount of DNA per particle is 1.6×10^{-16}g and the mean amount of protein per particle is 2.0×10^{-16}g.

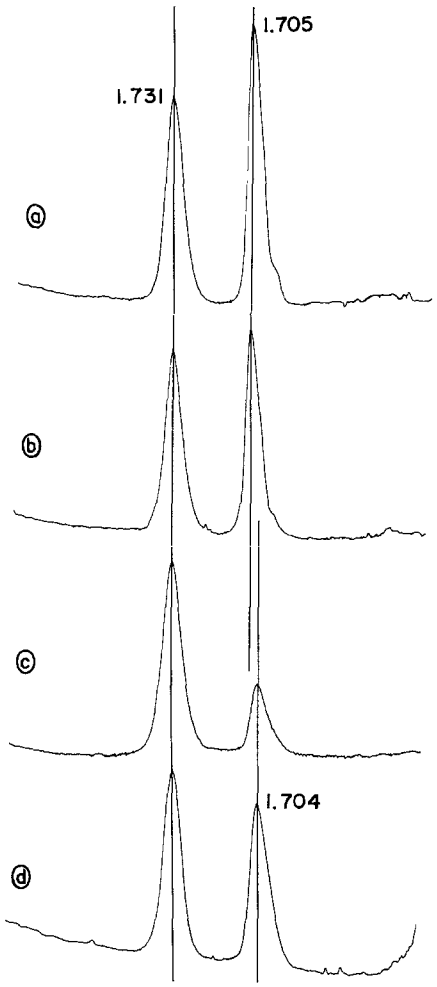
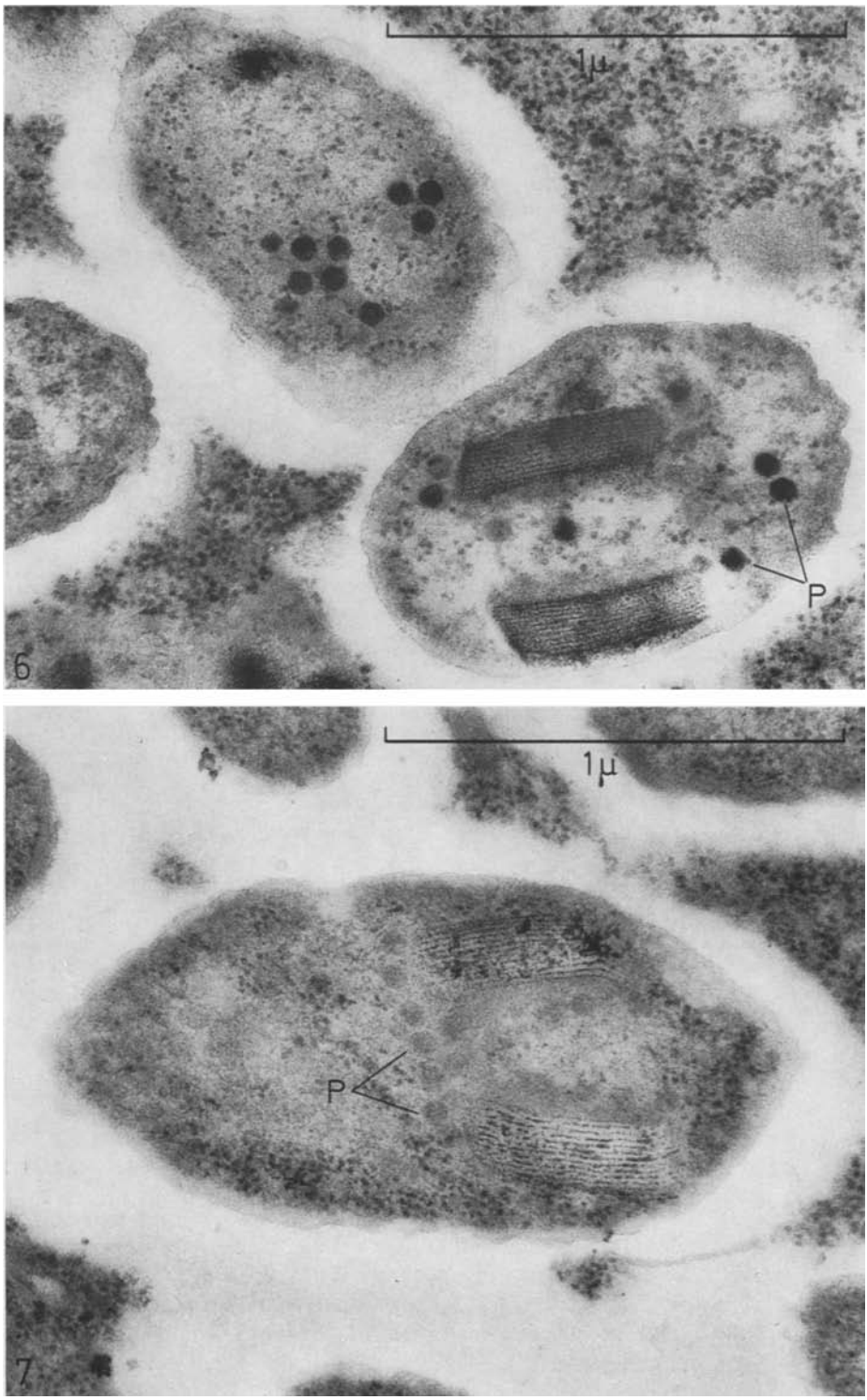


Fig. 5a–d. Microdensitometer tracings of (a) and (b) two different preparations of whole 562 kappa DNA ($\varrho = 1.705$) and (c) and (d) two different preparations of phage-like particle DNA ($\varrho = 1.704$) in CsCl density gradients formed by centrifuging at 44770 RPM for 20 hours. The band at the left is standard reference DNA of *Micrococcus lysodeikticus* (ϱ taken as 1.731). DNA was freed by lysing kappa and phage-like particles by heating for minutes at 60°C in 2% of sodium lauryl sulfate in saline EDTA. DNA was isolated from the kappa lysates using phenol and ribonuclease (Suyama and Preer, 1965). The whole lysates of the phage-like particles were centrifuged in CsCl

Although a thorough characterization of the DNA from the phage-like particles has not been possible, its buoyant density in CsCl was calculated to be 1.704, while the DNA from whole 562 kappa was 1.705 (Fig. 5). Although the difference between the two is very small (0.0015), the reproducibility of the runs suggests that the difference may be significant. The small shoulder on the whole kappa band may be produced by the presence of the phage DNA. It is noted that both DNA bands are somewhat asymmetrical, indicating heterogeneity.



Figs. 6 and 7

Verification of the presence of DNA in the phage-like particles was obtained cytochemically using the method of Bernhard (1969). In the conventional staining method used in electron microscopy, uranyl acetate followed by lead citrate stains both DNA and RNA areas with the same electron density (Fig. 6). Treatment with McIlvaine buffer (1921) at pH 5 or with 0.02 M EDTA solution at pH 6.5 after staining with uranyl acetate and before staining with lead citrate renders the DNA-containing phage-like elements unstained whereas RNA-containing ribosomes are only slightly destained (Fig. 7). This result of the procedure was confirmed by comparing the appearance of the macronuclear DNA and RNA-containing areas.

Discussion

It is assumed that the numerous "unfilled" phage-like particles banding with "filled" particles in the band at density 1.47 were actually filled with DNA when they banded in the gradient, and were emptied either during subsequent washing or during negative staining. This possibility, of course, casts some doubt on the reliability of the quantitative measurements of the DNA content of the particles. Difficulties in determining by negative staining whether viruses are full or empty have long been recognized (Crawford *et al.*, 1962).

The buoyant density of the virus-like elements, their absorption spectrum, DNA and protein content, the buoyant density of the nucleic acid extracted from them, and finally their cytochemical properties show clearly that their chemical composition is typical of numerous DNA-containing phages. Thus the bacteriophage T2 (Hershey, 1955) has an $O.D._{260}$ of 1×10^{-11} per particle (compared to 0.4×10^{-11} for the phage-like particle of 562 kappa), 2.0×10^{-16}g DNA (compared to 1.6×10^{-16}g), 2.7×10^{-16}g protein (compared to 2.0×10^{-16}g). The density of T4 is about 1.45 (compared to 1.47) (Kozinski and Kozinski, 1963).

Since it is now clear that kappa is a bacterium, it seems reasonable that the virus-like elements should be considered bacteriophages. Whether they are infective or not, is not known. However in view of the very numerous incomplete structures, particularly in related kappas, and in view of their apparent symbiotic nature, it would not be surprising if they eventually prove to be defective phages.

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Fig. 6. Kappa particles fixed with 1.5% glutaraldehyde in phosphate buffer, embedded in Araldite (Durcupan), sectioned, and stained with 2.5% uranyl acetate for 10 minutes followed by Reynolds' lead citrate solution for 10 seconds. Phage-like particles (*p*) are heavily stained. ×64.500

Fig. 7. Kappa particles of the same stock fixed at the same time and embedded in the same block as that in Fig. 6, but sections were treated with McIlvaine buffer at pH 5.0 for 30 minutes after staining with uranyl acetate (2.5%) and before staining with Reynolds' lead citrate solution. The phage-like particles appear pale grey due to greatly reduced stainability with lead citrate after chelating with McIlvaine buffer. ×64500

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