JOURNAL OF BACTERIOLOGY, May, 1966
Vol. 91, No. 5

Structure of *Bacillus subtilis* Bacteriophage ϕ29 and the Length of ϕ29 Deoxyribonucleic Acid

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Received for publication 12 February 1966

ABSTRACT

ANDERSON, D. L. (University of Minnesota, Minneapolis), D. D. HICKMAN, AND B. E. REILLY. Structure of *Bacillus subtilis* bacteriophage ϕ29 and the length of ϕ29 deoxyribonucleic acid. J. Bacteriol. **91**:2081–2089. 1966—*Bacillus subtilis* bacteriophage ϕ29 were negatively stained with phosphotungstic acid. The head of ϕ29 has a hexagonal outline with a flattened base, and is about 315 A wide and 415 A in length. The virus has an intricate tail about 325 A in length. Twelve spindle-shaped appendages are attached to the lower of two collars which comprise the proximal portion of the tail. The distal 130 A of the tail axis has a diameter of about 60 A and is larger in diameter than the axis of the upper portion of the tail. Comparison of electron microscopic counts of ϕ29 with plaque-forming units indicated that about 50% of the microscopic entities were infective. Phenol-extracted ϕ29 deoxyribonucleic acid (DNA) molecules were prepared for electron microscopy by the cytochrome *c* film technique of Kleinschmidt et al. Measurement of contour lengths of DNA molecules from three preparations gave skewed distributions of lengths with observed modal class values ranging from 5.7 to 5.9 μ. Assuming that ϕ29 DNA is a double helix in the B form, the corresponding molecular weights would be 10.9×10^6 to 11.3×10^6 daltons. The largest DNA molecules would have a volume of 1.9×10^7 A^3 which is about 25% greater than the estimated 1.4×10^7 A^3 internal volume of the phage head.

Reilly and Spizizen recently described infection of competent *Bacillus subtilis* by deoxyribonucleic acid (DNA) from bacteriophages ϕ1, ϕ25, and ϕ29 (15). Phage ϕ29 was of particular interest because of the small size of the virus and the relatively high efficiency of infection with ϕ29 DNA. This paper describes the morphology of ϕ29 and the contour lengths of phenol-extracted DNA molecules from the virus.

MATERIALS AND METHODS

Phage and host. Phage ϕ29 were grown on *B. subtilis* strain H according to Reilly and Spizizen (15).

Purification of phage ϕ29. Lysates were clarified by centrifugation at 8,000 $\times$ *g* for 20 min and the supernatant fluid was filtered through an 03 Selas filter. The phage were sedimented from the filtrate by centrifugation for 3 hr at 48,000 $\times$ *g* (maximum) with the use of the no. 19 rotor of the Spinco L2 ultracentrifuge, and the pellets were resuspended in a phage diluent containing 0.05 M potassium phosphate buffer (*p*H 7.0), 0.1 M NaCl, and 0.005 M $MgSO_4$. The phage suspensions were pooled and concentrated to 4 ml in dialysis tubing surrounded by polyethylene glycol. Cesium chloride (Matheson Coleman & Bell, Norwood, Ohio) was added to the concentrated phage suspension to a final concentration of 41.5% (w/w), and the mixture was centrifuged for 18 hr at 70,000 $\times$ *g* with the use of the SW39L rotor of a Spinco L preparative ultracentrifuge. The phage band, located about 14 mm from the meniscus, was separated from gel-like floating material and the pellet. Cesium chloride was removed by a 16-hr dialysis against 2 liters of phage diluent.

Extraction of phage DNA. Viral DNA was extracted with phenol by use of the procedure of Reilly and Spizizen (15), or by a combination of the procedures used by Frankel (7) for isolation of high molecular weight DNA from T2-infected bacteria and by Berns and Thomas (3) for extraction of *Haemophilus influenzae* DNA. Phage were treated with 8 M urea for 20 min and dialyzed overnight against 0.15 M NaCl-0.015 M sodium citrate (SSC) at 4 C. Pronase (Calbiochem, B grade) was added to a final concentration of 1 mg/ml, and the mixture was incubated for 7 hr at 37 C. The preparation was then extracted twice with an equal volume of SSC-saturated phenol (Mallinckrodt Chemicals, St. Louis, Mo.; chromatography grade) by rolling at 50 rev/min in tubes (17 by 125 mm) positioned about 10° from the horizontal.

Finally, the aqueous layer was extracted once gently with an equal volume of nonanhydrous ether and dialyzed overnight against SSC at 4 C. DNA preparations were stored at 4 C after addition of 1 drop of chloroform.

Electron microscopy. All micrographs were taken with a Siemens Elmiskop IA electron microscope.

Phage were negatively stained by use of 2% (w/v) phosphotungstate (PTA) solutions adjusted to *p*H 7.0 with 1 N KOH (5). Specimens were supported on fenestrated carbon films (8). The virus was also negatively stained with 2% (w/v) uranyl acetate for comparison.

Electron microscopic counts of ϕ29 were made according to the procedure used by Anderson, Pollock, and Brower (1) to count mycoplasma. Phage and 0.088-μ polystyrene latex (Dow Chemical Co., Midland, Mich.) were mixed in equal volumes and sprayed on carbon-coated Formvar films by use of an EFFA spray mounter (E. F. Fullam, Inc., Schenectady, N.Y.). Micrographs of droplet patterns containing phage and reference particles were made at instrumental magnifications of 8,000 to 9,000 ×.

DNA molecules were prepared for electron microscopy by spreading 0.2 ml of a 1 M ammonium acetate solution containing 0.01 or 0.02% (w/v) cytochrome *c* (Calbiochem; A grade) and approximately 2 μg/ml of DNA onto an 80 cm² surface of 0.1 M ammonium acetate. The film was compressed and prepared for electron microscopy according to the procedure of Kleinschmidt et al. (12).

Measurement of DNA molecules. Lengths of phenol-extracted molecules of ϕ29 DNA were measured by use of techniques similar to those used by Kleinschmidt et al. (12), Ris and Chandler (16), MacHattie and Thomas (14), Kaiser and Inman (10), and Caro (6), in studying DNA molecules from coliphages. Electron micrographs were taken at magnifications of 20,000 to 30,000 ×, and the contour lengths of projections enlarged to about 110,000 × were measured with a map measure (Eugene Dietzgen, Chicago, Ill.; model 1718). Two or three measurements were made on each molecule. The electron microscope was calibrated from micrographs of a carbon grating replica (E. F. Fullam, Inc., Schenectady, N.Y., 54,800 lines per inch).

Errors of measurement of DNA molecules prepared for electron microscopy by the cytochrome *c* film technique have been described in detail by Kleinschmidt et al. (12). In the present study, estimated errors in peripheral distortion, enlargement, and tracing yielded a composite error in length measurements of ϕ29 DNA molecules of less than 3%.

RESULTS

Size and morphology of ϕ29. The structure of ϕ29 is shown schematically in Fig. 1, and negatively stained in Fig. 2–10. In Fig. 3, phage ϕ29 is shown with coliphage T2hr⁺ for size comparison. The head of ϕ29 has a hexagonal outline with a flattened base and is about 315 A wide and 415 A long. The top of the head often appears flattened in outline (Fig. 2), and may represent

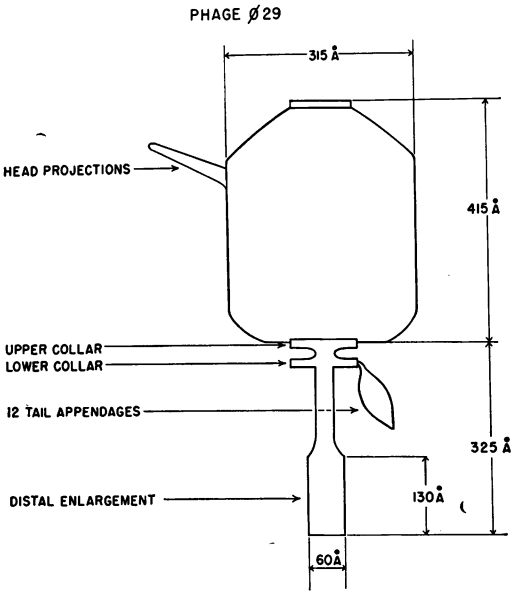

FIG. 1. *Schematic diagram of phage ϕ29.*

a differentiation of the outer surface of the head membrane. Also, thin projections, about 20 by 140 A, radiate from the head (Fig. 2, 4). The thickness of the head membrane of "ghosts" is about 25 A. The tail of ϕ29 is about 325 A in length. The upper portion of the tail consists of two collars of approximately equal diameter. The top collar is located at the point of attachment to the head, has a diameter of about 110 A, and is usually visible only on detached tails (Fig. 7). It is not yet clear whether this upper collar fits against the exterior of the head membrane or is recessed to become a part of the membrane. The second collar is located along the tail axis just below the top collar. Attached to this lower collar are 12 spindle-shaped appendages, symmetrically arranged around the periphery of the collar and extending toward the base of the tail (Fig. 2, 4, 5). In the usual air-dried, negatively stained preparations, the appendages extend downward approximately parallel to, or at a slight angle from, the tail axis. When the negatively stained preparations are freeze-dried (Fig. 6), the appendages extend inward toward the tail, and are so closely grouped that the individual appendages cannot be distinguished; this is probably a closer approximation of their normal orientation. Only three to five of the appendages are usually distinct in lateral views of most phage particles, although six are occasionally distinguishable (Fig. 5). However, a distal view of the 12 appendages is shown in Fig. 8, which also shows an opening of about 20 A in the center of the tail axis, and in Fig. 9 the 12 appendages are arranged radially

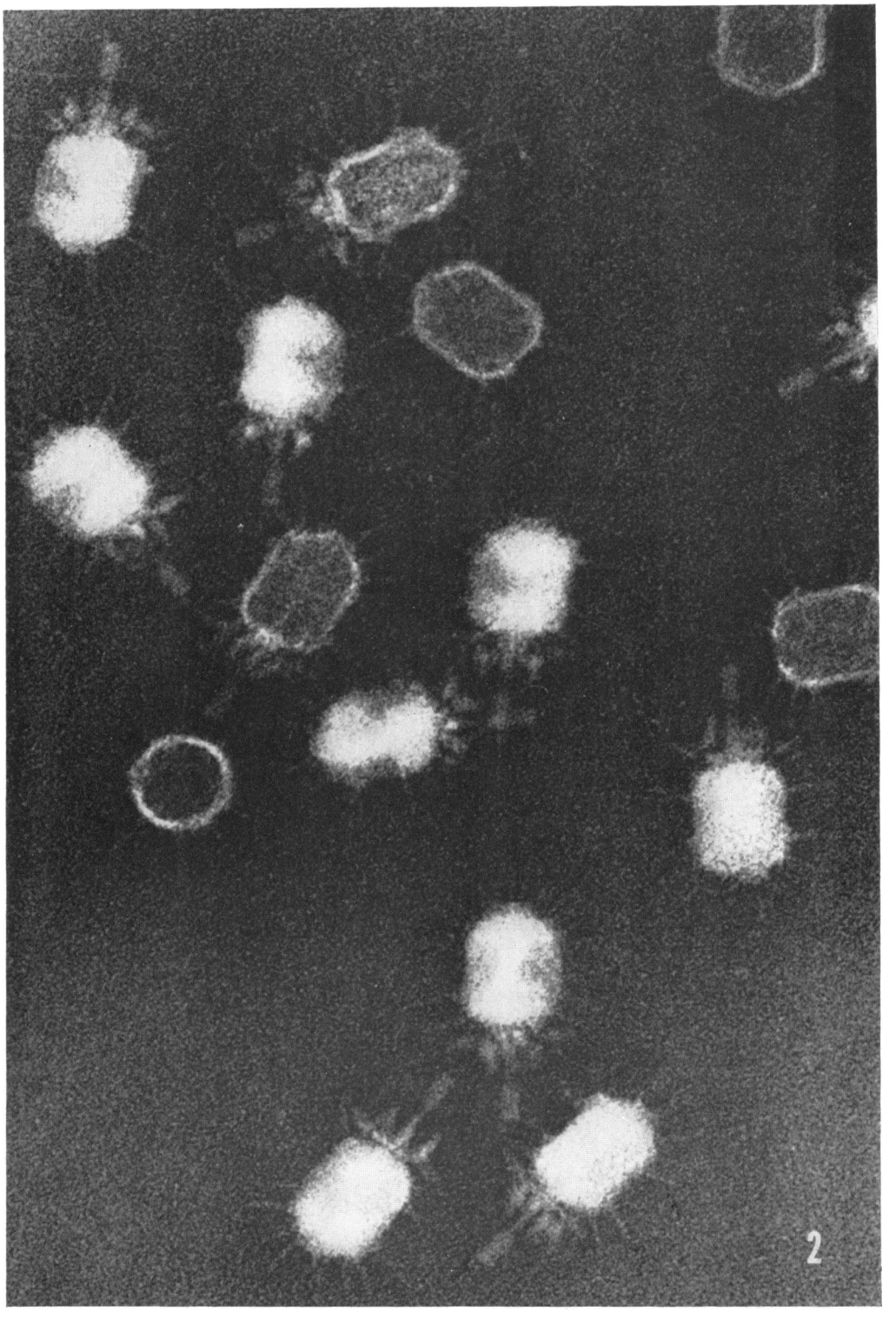

FIG. 2. *Phage ϕ29 negatively stained with phosphotungstate. × 460,000.*

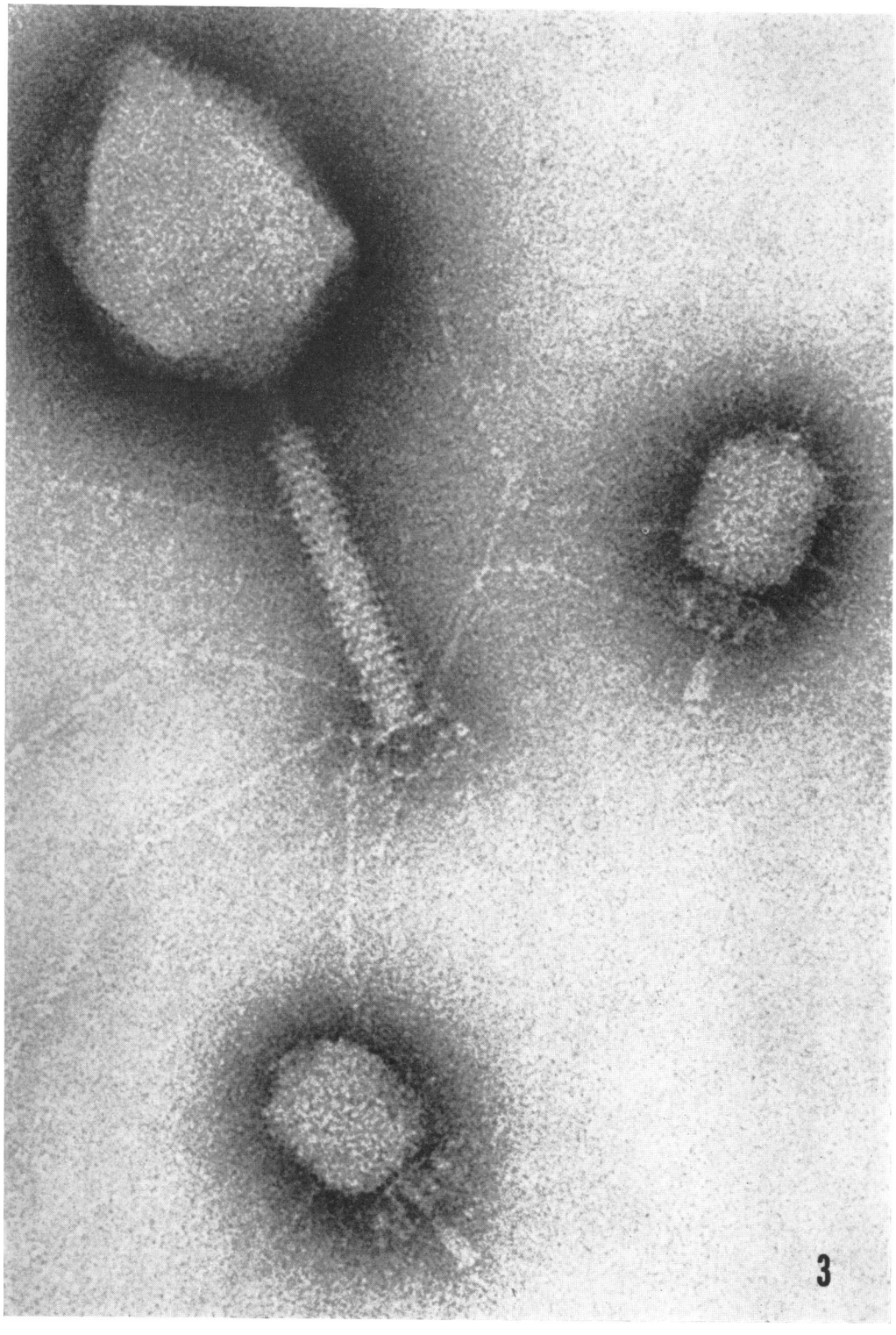

FIG. 3. *Phage φ29 with phage T2.* × *575,000.*

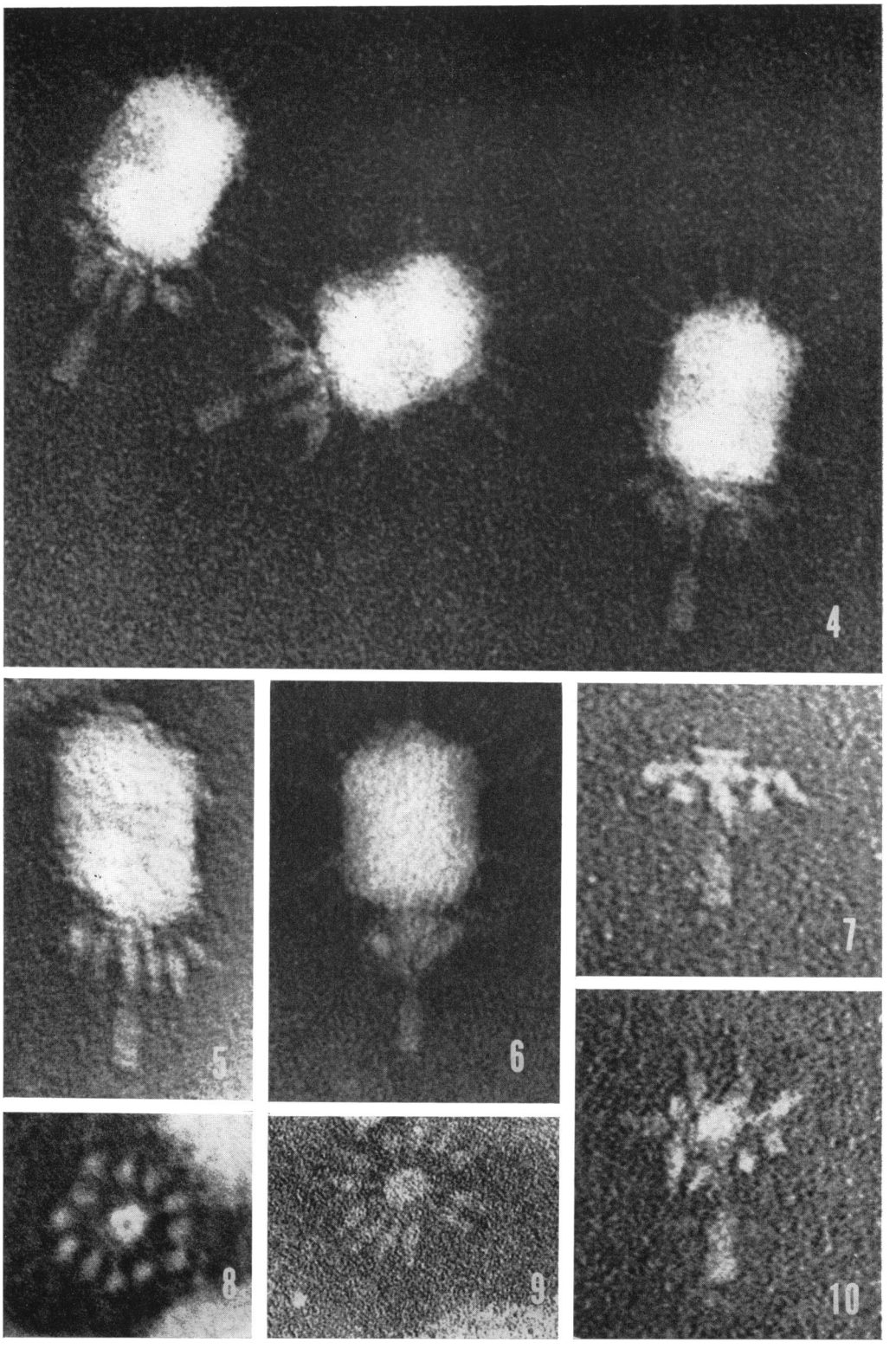

FIG. 4. *Phage φ29. × 700,000.*
FIG. 5. *Phage φ29 showing six appendages. × 700,000.*
FIG. 6. *Phage φ29 from a freeze-dried negatively stained preparation. × 700,000.*
FIG. 7. *Detached tail of φ29 showing upper collar at the top of the tail and lower collar with attached appendages. × 700,000.*
FIG. 8. *Distal view of detached tail of φ29 showing the twelve appendages symmetrically arranged around the tail. × 700,000.*
FIG. 9. *Detached tail of φ29 showing the twelve appendages radiating from the lower collar. × 500,000.*
FIG. 10. *Detached tail of φ29 with nine appendages arranged at different angles. × 700,000.*

around the collar. The distal 130 A of the tail axis has a diameter of about 60 A, and is larger in diameter than the axis of the upper portion of the tail. However, the presence of the appendages prevents accurate measurement of the diameters of both the lower collar and the upper tail axis. Cross striations are observed occasionally in the distal portion of the tail. There is no indication that the tail is contractile. Phage ϕ29 negatively stained with uranyl acetate were similar in structure and overall size to phosphotungstate-stained particles.

The head of ϕ29 has an approximate internal volume of 1.4×10^7 A^3 if a bipyrimidal shape with a flattened base is assumed, as compared with a volume of 3.3×10^8 A^3 for the head of T2 as calculated by Bendich and Rosenkranz (2). Shadowed preparations of ϕ29 confirmed the prismatic nature of the head structure. Thus the head volume of ϕ29 is approximately 4% that of T2.

Comparison of electron microscopic counts with plaque-forming units (PFU). Electron microscopic counts of ϕ29 purified according to Reilly and Spizizen (15) were compared with counts of PFU by use of 0.088-μ polystyrene latex as reference particles. Latex and ϕ29 could not be counted when mixed in approximately equal numbers because of excessive clumping; homogeneous distributions of particles in droplet patterns were obtained only when ϕ29 and 0.088-μ latex were mixed in a ratio of about 10:1. Enumeration of 2,789 phage and 222 latex in six droplet patterns yielded a microscopic count of 4.2×10^{12} phage per milliliter when the calculated latex concentration was 3.34×10^{11} per milliliter. The mean ratio of ϕ29 to latex was 13.61, giving a standard deviation of 2.13 and a coefficient of variation of about 16%. This same phage suspension used in the microscopic counts was titered three times for PFU, and yielded averages of 263, 227, and 230 plaques from six plates in each experiment. The mean number of PFU in the suspension was 2.4×10^{12} per milliliter, and the coefficient of variation of the mean PFU titer in the three experiments was about 5%. Considering these variations in the electron microscopic and infective counts, approximately 57% of the microscopic entities were infective.

Length of ϕ29 DNA molecules. Phage ϕ29 DNA molecules extracted with phenol by rolling at 50 rev/min were measured to determine lengths of the molecules and variability in these lengths. Of 101 molecules of one preparation, 48 were 5 μ or longer and 30 of the molecules were between 5.4 and 5.8 μ in length (Fig. 11a). The majority of the molecules appeared as threads of variable diameter with two free ends (Fig. 12), although 32 of the 101 molecules were multiple-looped structures or "flowers."

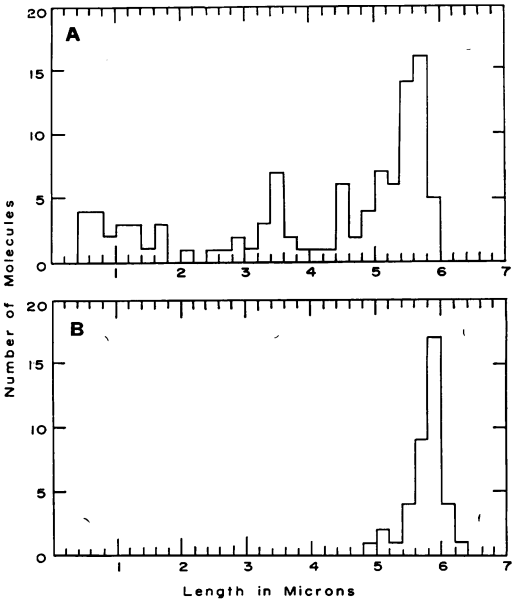

FIG. 11. *Distribution of lengths of DNA extracted by rolling with phenol; A and B represent separate preparations.*

Micrographs were made of 39 of the longest molecules from a second preparation with an estimated overall distribution of molecular lengths similar to that of the first preparation. Of the 39 molecules, 34 had contour lengths of 5.4 to 6.2 μ, and 26 of the 39 molecules were 5.6 to 6.0 μ in length (Fig. 11B). The observed modal class values for the two preparations were about 5.7 and 5.9 μ, respectively. In the first preparation, the mean length of molecules longer than 5 μ was 5.50 ± 0.23 μ, and the mean length of molecules longer than 4 μ was 5.30 ± 0.45 μ (Fig. 11A). Molecules in the skewed portion of the distribution were presumed to be fragments of longer whole molecules. Additionally, 12 molecules from a preparation extracted with phenol by the procedure of Reilly and Spizizen (15) had a modal class value of about 5.7 μ. Assuming that ϕ29 DNA is a double helix sodium salt in the B crystallographic form and that base pairs are 3.4 A apart (13), the molecular weight of the longest phenol-extracted ϕ29 DNA molecules in the three preparations, calculated from the observed modal class values, would be 10.9×10^6, 11.3×10^6, and 10.9×10^6 daltons, respectively.

DISCUSSION

Phage ϕ29 is smaller than other *B. subtilis* phages described previously, including the phages

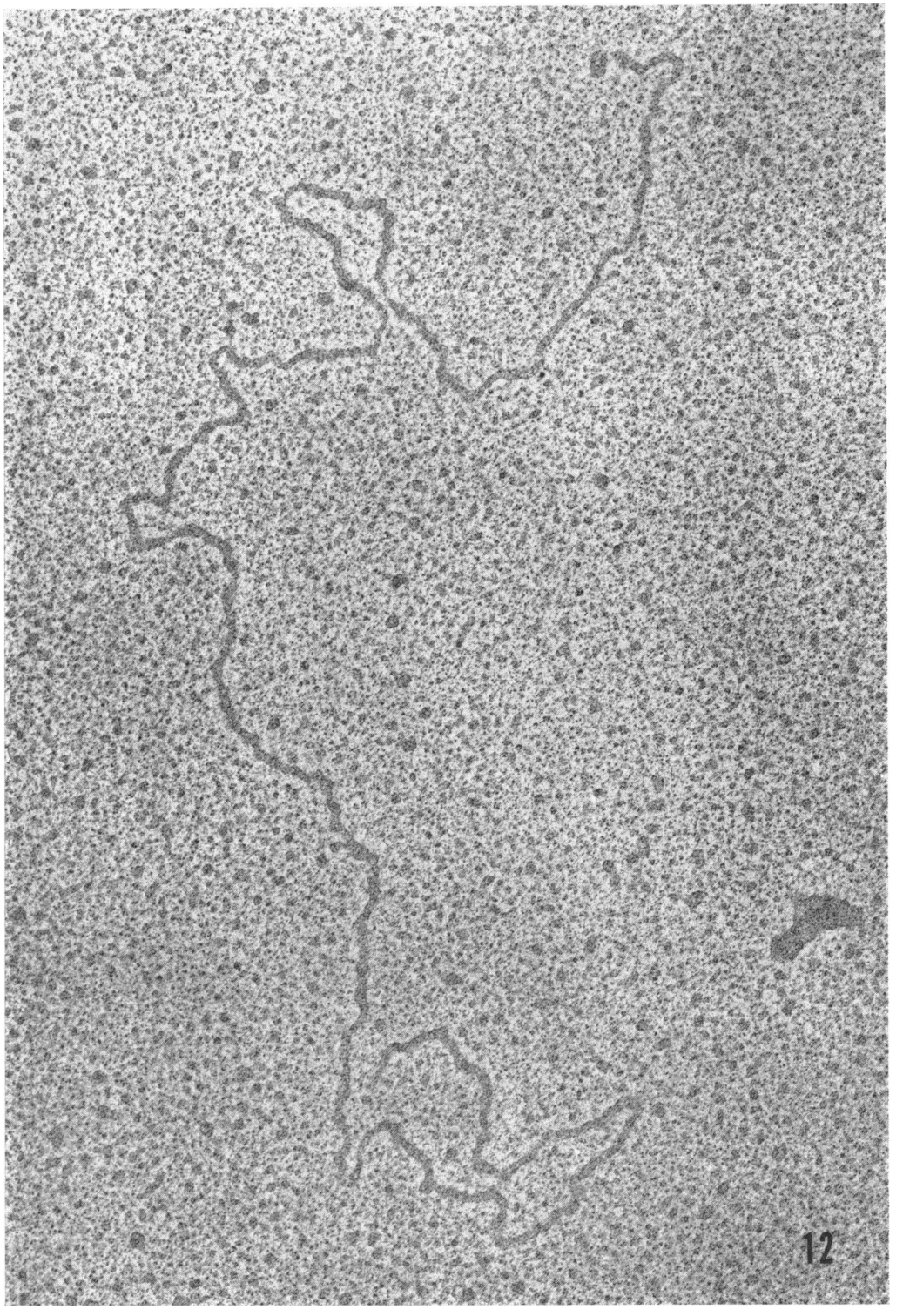

FIG. 12. *Phage ϕ29 DNA extracted with phenol by shaking.* × *95,000.*

SPα (Eiserling, Ph.D. Thesis, Univ. California, Los Angeles), PBSX (17), and μ (9) which have head diameters of about 450 A. Dimensions of *B. subtilis* phages, as determined by electron microscopy, have been summarized by Reilly (Ph.D. Thesis, Western Reserve Univ., Cleveland, Ohio). The head dimensions of ϕ29 are less than half the head dimensions of *B. subtilis* phages SP3, SP5, SP6, SP7, SP8, SP9, SP10, SP13, and PBS1 as described by Eiserling. Phage ϕ29 is smaller than, but somewhat structurally similar to, the *B. subtilis* phage GA-1 recently described by Bradley (4).

Phage ϕ29 were often observed attached to debris by the distal portion of the tail, indicating an apparent attachment function for this organelle. If a double helix of DNA passes through the tail during the infection process, the walls of the tail between the distal enlargement and the proximal end must be very thin, since this structure was estimated to have an outer diameter of only about 25 to 35 A.

Kleinschmidt et al. (12), in measuring DNA molecules representing the complete DNA content of phage T2, questioned whether the theoretical errors of $\pm$ 3.2% in a single measurement could account for the observed $\pm$ 8.6% mean variation of length, and concluded that individual length differences might exist between DNA molecules of T2 phage. Recently, Kleinschmidt et al. (11), studying DNA of Shope papilloma virus, concluded that differences in preparation might introduce substantial changes in observed contour lengths. The modal values of the longest ϕ29 DNA molecules from the preparations shown in Fig. 11 differed by 3.4%. Although the variability in molecular lengths within single preparations was great, the coefficients of variation of molecules over 5 μ in length in the two preparations obtained by gentle phenol extraction were 4.2 and 4.8%, respectively.

Distributions of 101 and 39 ϕ29 DNA molecules extracted gently with phenol had observed modal class lengths of 5.7 and 5.9 μ. The calculated molecular weight values of 10.9×10^6 and 11.3×10^6 for 5.7- and 5.9-μ molecules are based on an average molecular weight per base pair of 662 and the assumption that the B configuration of the DNA is justified. A recent observation that actinomycin had no observable effect on the measured length of intact polyoma DNA molecules prepared with the cytochrome *c* film technique was presented as evidence that transition from the B to A form did not occur upon drying (6).

The volumes of ϕ29 DNA molecules of the observed modal class lengths 5.7 and 5.9 μ can be compared with the space available in the head of the virus. Assuming the DNA molecules to be cylinders approximately 20 A in diameter, their corresponding volumes would be about 1.8×10^7 A^3 and 1.9×10^7 A^3. These volumes are about 25% greater than the internal volume of the head of ϕ29, which was estimated at 1.4×10^7 A^3.

The uniformity of the longest DNA molecules of ϕ29 supports the supposition that these molecules represent the complete DNA content of the virus and are not half molecules. Kaiser and Inman (10) reported a sharp, unimodal length distribution for whole molecules of λ DNA which centered at 14.5 to 15 μ, while half molecules of λ DNA obtained by hydrodynamic sheer had a broad length distribution terminating at 10 μ.

The molecular weights of ϕ29 DNA molecules, calculated as above, are less than half the molecular weight of ϕ29 DNA as determined by sedimentation velocity in preliminary studies (15). In any event, the specific infectivity of extracted viral DNA is low. In the transfection assay, usually less than one infection occurs per 10^4 phage equivalents of added ϕ29 DNA. Thus, the physical and chemical properties of the molecules of the bulk solution of DNA are not necessarily those of the biologically active DNA molecules.

ACKNOWLEDGMENTS

We are grateful to Frances Greene for growing the phage used in this study.

This investigation was supported by Grant 406-0303-4909-02 from the Graduate School of the University of Minnesota and by Public Health Service Research Grant AI-07003 from the National Institute of Allergy and Infectious Diseases.

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