Electron Microscopy of DNA from Adeno-associated Virus Type I

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Phenol-extracted DNA from adeno-associated virus (AAV) types 1 and 4 consists of duplex strands with molecular weights of $3.6 \times 10^6$ and $3.0 \times 10^6$, respectively (Rose, Hoggan & Shatkin, 1966; Parks et al. 1967). Parks and his co-workers have shown 1.5 μm. duplex strands in electron micrographs of AAV-4-DNA. However, on comparing the DNA from AAV-1 with the DNAs from a minute virus of mice and from bacteriophage φX174, Crawford et al. (1969) concluded that the molecular weight of the AAV-DNA should not exceed $1.7 \times 10^6$ and suggested that two populations of virus particles might contain complementary single strands.

The recent studies of Rose et al. (1969) and Mayor et al. (1969) have shown that preparations of AAV types 3 and 4 contain two populations of complementary single-stranded DNAs, which form duplex molecules after extraction from the virus particles; Mayer et al. (1969) showed an electron micrograph of AAV-4-DNA during renaturation, with partly duplex molecules.

The present study describes attempts to characterize further the DNA of AAV-1 by electron microscopy, using a technique that minimizes duplex formation by single strands of DNA.

Adenovirus-associated virus type I was grown in human embryonic kidney cells with adenovirus type 12 as helper. The source of AAV and the method of growth have been described (Hoggan, Blacklow & Rowe, 1966; Blacklow, Hoggan & Rowe, 1967). The AAV was purified by gel filtration on Sepharose 2B followed by two isopycnic centrifugations on CsCl gradients, as described by Neurath et al. (1969). AAV-containing fractions, found by electron microscopy to be free of adenovirus, were dialysed against 0.01 M-tris buffer, pH 7.2.

Purified AAV was shocked osmotically by the Kleinschmidt technique (Kleinschmidt et al. 1962, 1965). Virus suspended in 5.4 M- to 7.3 M-ammonium acetate with 0.02 % cytochrome C was spread on a hypophase composed of triple distilled water or 0.1 M-ammonium acetate with 0.05 % isopropanol. In some experiments, adenovirus type 12, purified as above, was mixed with the AAV to provide a direct comparison of the adenovirus double-stranded DNA with the AAV-DNA. In another experiment, bacteriophage φX174 (Miles Laboratories, Inc., Kankakee, Illinois) was mixed with the AAV in 8 M-ammonium acetate and heated for 30 min. at 65° before being spread on 1.0 % formaldehyde. The films were lifted on carbon-coated copper specimen grids, rinsed in ethanol and dried with filter paper. The specimens were rotary-shadowed with platinum-carbon at an angle of 9.5° under a vacuum of $10^{-5}$ Torr. They were observed in an RCA EMU 3H electron microscope, equipped with double condenser illumination, at a magnification of 15,560 at 50 kv. The microscope was calibrated with a carbon grating having 2160 lines/mm. (Ladd Research Industries, Burlington, Vermont). Measurements of DNA strands were made with a map measurer on enlarged prints.

Various adaptations of the Kleinschmidt technique, usually employing denaturing reagents, were found to be unsatisfactory (Crawford et al. 1969; Davis & Davidson, 1968; Freifelder & Kleinschmidt, 1965; Freifelder, Kleinschmidt & Sinsheimer, 1964; Inman,
Fig. 1. (a) to (i), AAV spread on water. DNA strands of various lengths in μm: (a) 1.1; (b) 1.6; (c) 1.3, crossing itself; (d) 1.3; (e) 1.8; (f) 0.9; (g) incompletely extruded strand (0.5); (h) a rare circular strand, 0.6; (i) monocentric rosette with at least four free ends; (j) adenovirus type 12 and AAV spread on water. The adenovirus DNA is thicker and better contrasted than the AAV-DNA (arrow).
1966; Knippers et al. 1969; Robinson & Hetrick, 1969). Even with our method, the degree of virus lysis varied from area to area on the protein film, and from square to square on the specimen grids. Lysed virus particles were most common on films obtained near the glass ramp.

The DNA spread on water was unbranched with few sharp bends; about 20% of the observed molecules were in rosette form (Fig. 1). Particles spread on ammonium acetate...
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extruded DNA strands that were unbranched, sharply bent, and usually contained hyper-coiled or pooled segments, characteristics common to single-stranded DNA (Fig. 2). Rare circular forms were seen on both hypophases. The DNA of adenovirus type 12 is a duplex molecule and appeared in better contrast, and 20 to 30 % thicker, than AAV-DNA when the two viruses were mixed before spreading (Fig. 1j). On one grid, the average shadow width for adenovirus DNA was 24 nm., for AAV-DNA, 18 nm.

The contour length distributions for AAV-DNA are shown in Fig. 3. Not included are rosettes and the eleven strands of ≥2 μm. or more in length, which either were rosettes, usually with more than two free ends, or appeared to be complexes of more than one molecule; pooling often prevented an estimate of the number of molecules present. In the estimate of molecular length, the histograms are divided between 1.1 and 1.2 μm. to minimize fragment measurements. The mean length for the longer populations of DNA on either hypophase is 1.5 ± 0.2 μm, as obtained by Parks et al. (1967) for extracted, duplex DNA from AAV-4. Unexpectedly, no significant differences were found between the two hypophases (Inman, 1967; Lang et al. 1967).

![Fig. 3. Distributions of contour length of AAV-DNA on two hypophases. The dark areas represent minimal lengths of molecules. Not included are strands less than 0.2 μm. long, rosettes, and the few strands ≥2 μm. or longer. (a) Water hypophase; (b) ammonium acetate hypophase.](image)

The high frequency (52 %) of 0.5 ± 0.2 μm. strands suggests two possibilities. The DNA may be extremely fragile, especially at a point roughly one-third of the distance from the end of the molecule. Alternatively, the smaller strands may represent a unique virus genome population. The wide and continuous distribution of filament lengths argues against the latter, although random fragmentation of longer strands could obscure a gap between long and short populations. Fragility of the DNA has been demonstrated in the production of an average of three breaks in duplex AAV-3-DNA by shearing (Rose et al. 1969) and by the finding of a short (0.1 to 0.3 μm.) population of AAV-4-DNA strands (Parks et al. 1967). Fragmentation of 0.5 μm. strands from 1.8 μm. molecules could account for the peak of 1.3 μm. filaments found on water hypophases.

The circular, single-stranded DNA of bacteriophage φX174 was compared directly with
AAV-DNA. Most of the phage DNA was tangled and aggregated, but the mean length of 13 measurable, circular molecules was 1.8 ± 0.1 μm. The length distribution of 42 linear DNA strands on the same grids was similar to those obtained for AAV-DNA alone, and the mean length for the longer population was 1.5 ± 0.1 μm. Only 9% of measurable strands were linear when φX174 was spread alone. The molecular weight of φX174 DNA is 1.7 × 10^6 (Sinsheimer, 1959), or 0.94 × 10^6/μm for the DNA examined in this study. Assuming the same weight: length ratio for AAV-DNA, we estimate that the latter has a molecular weight of 1.4 × 10^6 (Fig. 2i,j).

We have found no evidence that virus particles contain more than one DNA strand, as suggested by Rose et al. (1969). Thus the DNA of AAV-1 appears to be a linear, single-stranded molecule of length approximately 1.5 μm.

In a recent paper, Torikai et al. (1970) show electron micrographs of AAV-4-DNA obtained by a method similar to that described here. The possibility that populations of AAV-1 also contain DNA-deficient, 'light' particles (see Neurath et al. 1969) may account for our population of short DNA strands.

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