Observation on the Size and Morphology of Cauliflower Mosaic Virus Deoxyribonucleic Acid

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ABSTRACT

Two different procedures have been used to prepare deoxyribonucleic acid (DNA) from cauliflower mosaic virus for visualization in the electron microscope. With both procedures, DNA filaments exhibited a random orientation when immobilized on positively charged films fixed to electron microscope grids. Two types of molecules, circular and linear, with the same contour length ($2.31 \pm 0.24\,\mu$) were evident in all preparations examined. Phytopathology 61:188-193.

Additional key words: crucifer viruses, nucleic acids.

Cauliflower mosaic is a small, DNA-containing virus with isometric particles about 50 nm in diam (11, 13). The DNA extracted from purified virus exhibits properties characteristic of a double-helical form of nucleic acid. Viral nucleic acid, for example, is nonreactive with formaldehyde at ordinary temp, shows a sharp, cooperative-type melting profile, and has a nucleotide composition with the ratio of adenine:thymine and guanine:cytosine near unity which indicates complementary chains in its structure (12).

The double-stranded nature of the nucleic acid suggested that it might be amenable to observation and measurement with the electron microscope using the techniques developed initially by Kleinschmidt & Zahn (6). With this technique, a solution of the nucleic acid and a basic protein are deposited on the surface of an aq solution. The basic protein spreads on the surface of the liquid to form a monomolecular film to which negatively charged nucleic acid molecules become adsorbed and fixed. The film is deposited on a carbon-coated electron microscope grid and shadowed with a heavy metal at an acute angle from a number of directions to accentuate the immobilized nucleic acid. Information regarding size and morphology of nucleic acid molecules can be readily obtained with the technique. The nucleic acids of numerous animal and bacterial viruses and the double-stranded ribonucleic acids induced by turnip yellow mosaic virus and tobacco mosaic virus (16) and of wound tumor virus (4) have been visualized recently with this technique. The present report gives observations on the morphology and contour length of the double-stranded DNA of cauliflower mosaic virus using the conventional Kleinschmidt technique and various modifications introduced by others.

MATERIALS AND METHODS.—Virus was propagated in *Brassica campestris* (Tendergreen mustard) and purified as described previously (12). Viral DNA was isolated after digestion of whole virus with pronase in the presence of sodium dodecyl sulfate (SDS). For this digestion, the virus solution (1-3 mg/ml) was made to contain $1 \times$ SSC (0.15 M NaCl, 0.15 M sodium citrate, pH 7.0), 1 mg/ml pronase, and 0.25% SDS. The SDS had been recrystallized from ethanol. The pronase (Cal biochem) was filtered through a sterile Millipore filter (HA-0.45) to remove bacterial cells, and incubated for 2 hr at 37 C before use. The digestion to liberate the DNA was carried out for 2 hr at 37 C, followed by addition of SDS or another detergent, Sarkosyl (NL-32, Geigy Chem. Co.), to a 1% concn. The nucleic acid was purified further by procedures effective in removing protein. These consisted of emulsification 2-3 times with an equal volume of phenol followed by precipitation of the DNA with ethanol, resuspension in SSC, and extensive dialysis against the same medium, or alternatively, the virus digest was mixed with a sufficient amt of 65% (w/w) cesium chloride (CsCl) to give an initial solution density of 1.70 g/ml followed by centrifugation for 24-36 hr at 35,000 rpm in a Spinco SW 39 rotor. After centrifugation, the tube contents (5 ml) were fractionated into 0.2-ml portions and the DNA content of each was determined by removal of aliquots for ultraviolet spectrophotometry. The DNA banded near the center of these tubes. Fractions containing the DNA were pooled and dialyzed extensively against SSC and stored frozen.

Viral DNA was prepared for electron microscopy by the modification of the Kleinschmidt technique described by Mayor & Jordan (10) in which the nucleic acid diffuses to a cytochrome C film to which it adsorbs, and by the recently described method of Koller et al. (7). With the latter method, thin films are prepared by evaporation of carbon onto freshly cleaved plates of mica, then removed to 200-mesh electron microscope grids. The carbon-coated grids are then floated overnight on a solution of Zephiran (Winthrop Laboratories, New York, N.Y.) diluted 10^{-3} with water. The Zephiran consists of a dilute solution of a quaternary amine which adsorbs to the carbon film to produce a positively charged surface. The function of this film is essentially the same as that of cytochrome C in the Kleinschmidt method, which is to remove negatively charged DNA from solution by adsorption. The coated grids were dipped for 3-6 sec into a solution of the viral DNA at a concn of 2 µg/ml in 0.01 M ammonium acetate, pH 7.0, containing 0.001 M EDTA and 5 M urea. The grids were then floated for 1 hr on double-distilled water. The DNA was then stained by floating the grids for 1-2 min on a solution of uranyl acetate prepared by diluting a 1% aq solution of the salt with

3 volumes of absolute ethanol. After staining, the grids were rinsed for 5-10 sec in absolute ethanol and air dried.

Material prepared by using a cytochrome C film to immobilize the DNA was shadowed with platinum at a low angle (7 degrees) from two directions 90 degrees apart, or rotary shadowed.

The grids were observed in an RCA EMU 3 H electron microscope at an instrument magnification of approx 10,500. During each session in which the DNA was photographed, a carbon replica of an optical grating with 28,800 lines/inch was also photographed 2-3 times as a reference standard to determine the extent of magnification. The photographs were projected at a magnification of about 25-fold, and the image of individual DNA molecules was traced on paper. The contour length of these tracings were measured using a map-measuring device (A. Lietz Co., No. 3656-00).

RESULTS.—In early stages of this investigation, it was observed that many preparations of the DNA consisted largely of aggregates with a high degree of entanglement or collapse of the molecules into compact, rodlike masses. With some preparations, relatively few molecules were displayed as free, untangled individuals. It was found that precipitation of the DNA with ethanol during the course of purification contributed to the compact conformation. Figure 1-5 and 1-D (upper left hand corner) show the sort of compact masses exhibited by preparations precipitated by ethanol. These appear as short, fibrous masses lacking a uniform diam or length. Most were of such a size as to suggest that several molecules participate to form a single fibre. Some preparations consisted almost wholly of these collapsed molecules. Lang (8) has noted a similar collapsed conformation for T3 phage DNA precipitated with ethanol.

Owing to this problem, ethanol precipitation was avoided as a method for preparation of the DNA and as an alternative, preparations were subjected to buoyant density separations in CsCl without subsequent precipitation. With preparations of this sort, virtually all molecules appeared as randomly oriented filaments with little aggregation or indistinguishable folding.

Shape of DNA.—Two types of filaments, linear and circular, of approx equal length, were observed in all preparations of DNA examined (Fig. 1, 2). Both types of filaments appeared in completely random orientations. The filamentous types, though frequently folded or perhaps twisted to form small loops, were generally much less compact than circular molecules. The latter usually appeared as rather compact masses with several loops and intersections, and in general were more tangled than the linear molecules.

Circularity was observed too frequently to be ascribed to fortuitous occurrences in which the two ends of a linear molecule would be located near to each other by chance. For native DNA with the conformation of a random coil, Lang et al. (9) pointed out a method for calculating the expected number of circular molecules in a population of linear molecules depending on the length of the DNA and the resolution in the microscope. For a diffusion technique similar to that used to obtain the electron micrographs shown in Fig. 1, and an estimated resolution of 100 Å on a low power micrograph, they calculated that about 0.01% of the molecules would appear circular by chance for DNA with a length of about 2 μ. In contrast, DNA preparations used to obtain the micrograph and other data for Fig. 1 contained about 47% "circular" molecules. Of 396 molecules observed on various electron micrographs with this preparation, 186 individuals appeared circular, 170 appeared linear, and 40 were uncertain in shape. Moreover, several selected molecules, some of which are depicted in Fig. 1-B, C, D, E, had few indistinguishable kinks and appeared unequivocally as closed loops without ends. Other preparations which were subjected to more prolonged centrifugation in CsCl and dialysis against SSC consisted predominately of circular molecules.

Little positive evidence for supercoiling, which is a common feature of the cyclic genome of other small DNA-containing viruses, was observed in the various preparations of cauliflower mosaic virus DNA examined. Circular molecules commonly exhibited what appeared to be twisting of the strands about one another in small kinks and loops, but the molecules as a whole seldom appeared to be markedly twisted, as for example in electron micrographs with native polyoma virus DNA. In contrast, circular cauliflower mosaic virus DNA appeared to possess a more openly disposed nature resembling cyclic polyoma virus DNA treated with deoxyribonuclease sufficient to cause several single-stranded nicks (15).

Supercoiling caused by tertiary twists in the double-stranded DNA helix would depend upon complete covalent continuity of both polynucleotide strands in a circular molecule. This structure does not seem very probable for cauliflower mosaic virus DNA in view of the presence of linear individuals, unless the DNA had been exposed to nucleases during preparation for electron microscopy.

It seems more likely that the viral DNA is initially linear and possesses some mechanism for becoming circular in vitro after liberation from the virus particle.

Size of DNA.—The contour lengths obtained with four different preparations of the DNA are given in Table 1. The differences in contour length in different sessions can be attributed either to use of different preparations of DNA, to the different methods of preparation or to both these factors, since four different preparations of DNA were used for sessions 1, for 2 and 3, for 4 and 5, and for 6. The standard deviations varied in different sessions from about 6% of the contour length (session 6) to about 14% (session 2).

With a few exceptions, the measurement of intersecting molecules could not be avoided, particularly with circular molecules. An attempt was made, however, to avoid molecules with doubtful kinks and small loops. Many molecules were not suitable for measurement because of tangling or association with debris which led to an indistinguishable amount of twisting, or which
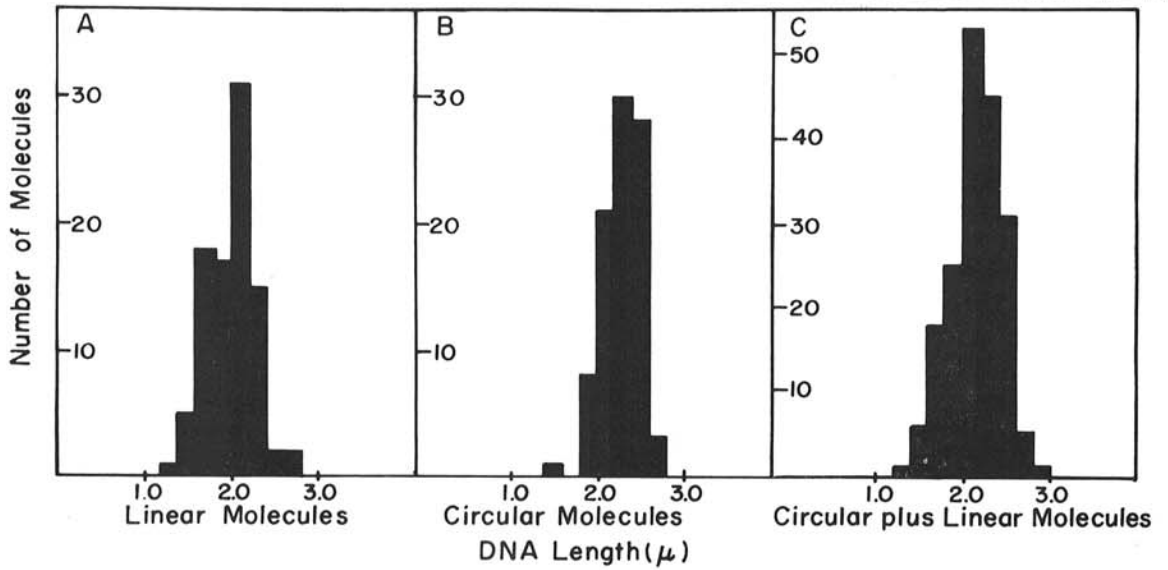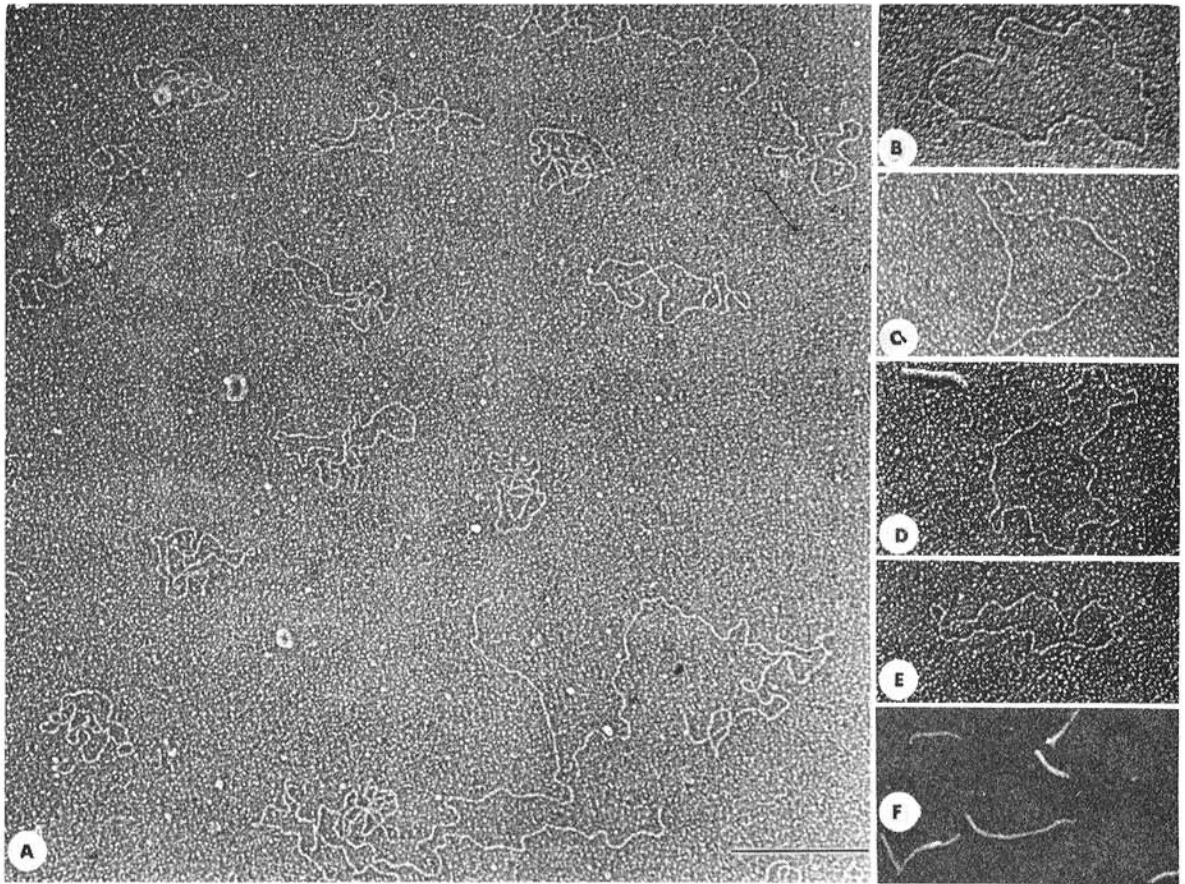
Fig. 1. (Above) DNA from cauliflower mosaic virus prepared for observation by the Kleinschmidt procedure (6). The bar in the lower right hand corner of A represents $0.5\,\mu$. B, C, D, and E show well-defined circular molecules. F shows collapsed molecules following precipitation with ethanol. These have been stained with uranyl acetate. (Below) Contour length distributions of 185 molecules after preparation by the Kleinschmidt procedure.
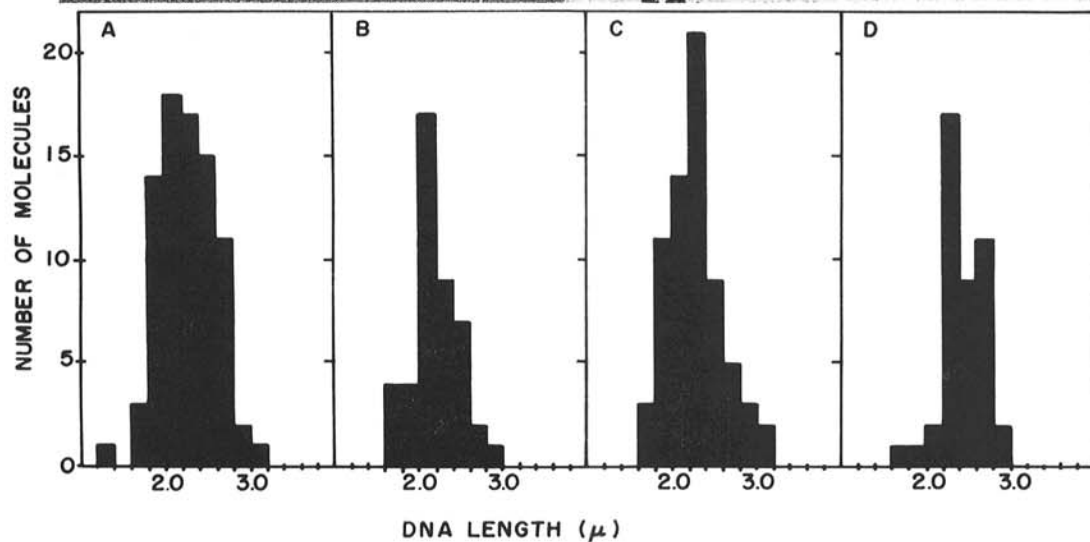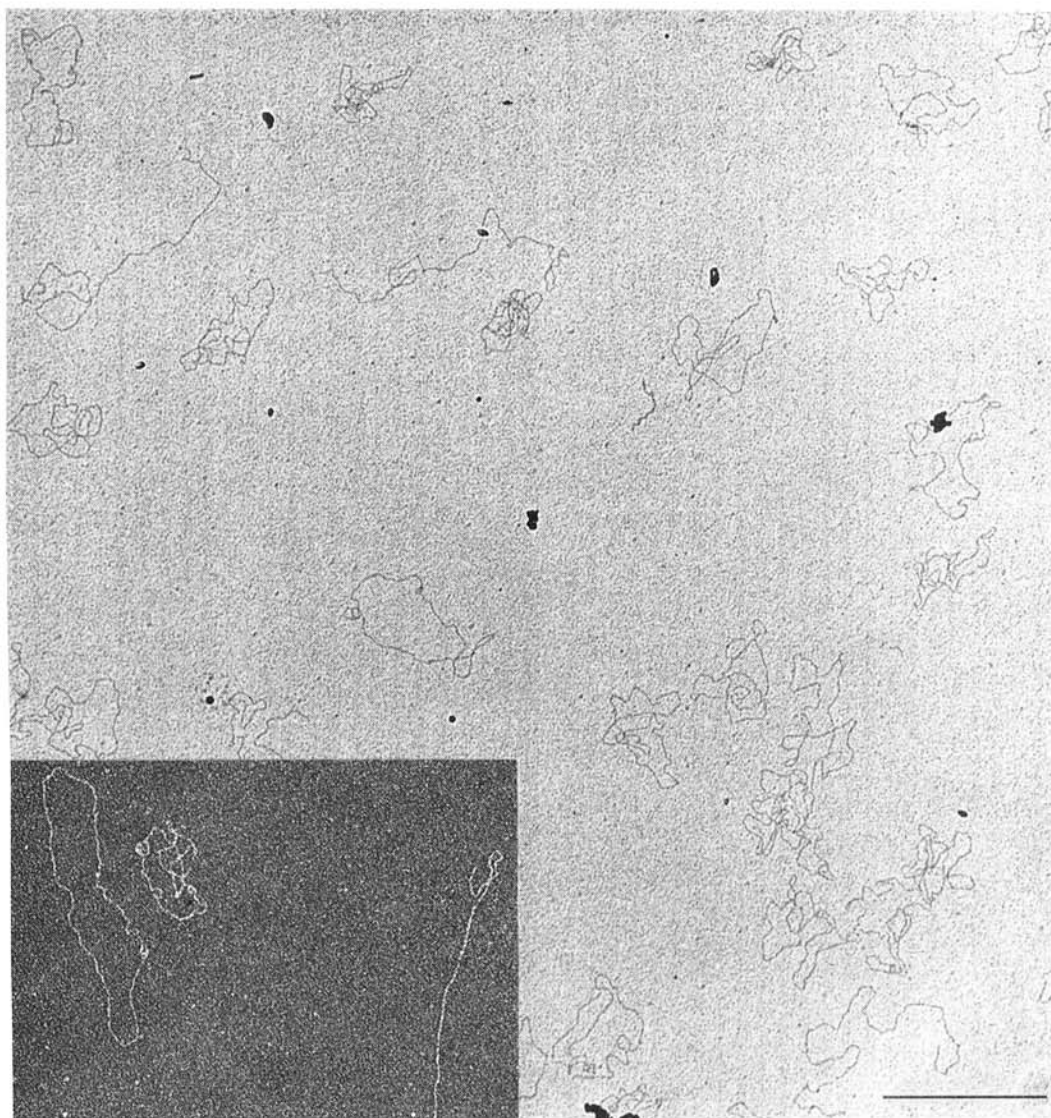
Fig. 2. (Above) DNA from cauliflower mosaic virus prepared for electron microscopy by the procedure of Koller et al. (7). The insert at the lower left shows DNA shadowed with platinum from two directions 90 degrees apart after preparation by the Koller procedure. The bar in the lower right hand corner represents 0.5 μ. (Below) Contour length distributions of the DNA in four different sessions with material prepared by the procedure of Koller et al. (7).

may have induced artificial distortion or obscured small loops in the molecule.

Frequency peaks for full-length DNA molecules are shown in the histograms in the lower portion of Fig. 1, which corresponds to session 1 using the Kleinschmidt procedure, and the lower portion of Fig. 2 in which A, B, C, and D correspond to sessions 2, 3, 4, and 5, respectively, using the Koller technique. Circular molecules possessed a more uniform length than linear molecules. The lower portion of Fig. 1 shows separate histograms for the size distribution of circular and linear molecules measured by the Kleinschmidt technique.

Fragments less than 1 μ in length were not included in the measurement data given in Table 1 or in the histograms in Fig. 1 and 2. Fragments were not common, however, and were estimated to represent about 1 to 2% of the total material. This is probably an overestimate of the proportion of fragmented material, as fragments would be expected to diffuse more rapidly to the basic protein or quaternary amine film than higher mol wt, full-length DNA.

Fragments are assumed to arise from breakage of the DNA filaments, perhaps through shearing forces, since no particular precautions were exercised during pipetting or other manipulations. It seems unlikely, however, that duplex DNA of such short length is susceptible to breakage by gentle shearing forces.

Measurement of the contour length of relatively uniform DNA permits calculation of the mol wt using an assumption for the weight per unit length. A mol wt of 192 daltons/Å appears to be valid for the sodium salt of double-stranded DNA at ionic strengths of 0.15-0.50 (9) whose configuration in electron micrographs is believed to correspond to the hydrated B form in X-ray diffraction analysis (14). With this assumption, the mol wt of cauliflower mosaic virus DNA can be estimated to be 4.4×10^6 daltons (Table 1).

DISCUSSION.—Of the two techniques used for visualization of the DNA, the technique of Koller et al. (7) is simple to use, as spreading on aq solutions and shadow casting is not necessary. In addition, the stained DNA appears as filaments of much smaller diam which facilitates visual separation of microloops and kinks.

The presence of linear and circular molecules in all preparations examined suggests that circularity may be a reversible phenomenon similar perhaps to that of phage λ DNA. The latter is linear when liberated from the virus, but possesses short, single-stranded segments at each end of the double-stranded molecule. The single-stranded segments at opposite ends of the molecule appear to bear complementary base sequences which undergo spontaneous hydrogen bonding to close the molecule into a circular configuration (3). More information is needed to establish this feature for cauliflower mosaic virus DNA.

The absence of supercoiling in circular molecules of cauliflower mosaic virus DNA is not clearly established by the results presented herein, as the compact tangled masses frequently observed with circular DNA in electron micrographs could possess supertwists. The results of equilibrium density separation of the DNA in CsCl gradients in the presence of the intercalating dye ethidium bromide, however, indicate the absence of covalently closed circles (12).

The DNA of cauliflower mosaic virus possesses approx the same length and mol wt as the DNA of the papilloma viruses of vertebrates. The reported contour lengths for these DNA's are as follows: Shope papilloma, 2.3-2.8 μ (5, 10); human papilloma, 2.6 μ (10); and bovine papilloma, 2.45 μ (9) as compared with 2.3 μ for cauliflower mosaic virus DNA. The DNA's of those viruses are supercoiled (1, 2), in contrast to cauliflower mosaic virus DNA which, based on presently available evidence, does not occur as covalently closed circles.

TABLE 1. Contour length and estimated molecular weight of cauliflower mosaic virus deoxyribonucleic acid (DNA) with two different methods for electron microscopy

Session no.[a]	No. molecules measured	Contour length with standard deviation (μ)	Estimated mol wt (daltons)
1	185	2.15 ± 0.28	4.1×10^6
2	84	2.31 ± 0.34	4.4×10^6
3	38	2.22 ± 0.20	4.3×10^6
4	60	2.30 ± 0.25	4.4×10^6
5	41	2.42 ± 0.21	4.7×10^6
6	20	2.50 ± 0.17	4.8×10^6
	Mean =	2.31 ± 0.24	4.4×10^6

[a] The method of Kleinschmidt & Zahn (6) was used for session 1, and the method of Koller et al. (7) for the others. In session 6, the films with adsorbed DNA were stained with uranyl acetate, then shadowed from two angles at 90 degrees to one another with platinum (see insert in the top portion of Fig. 2).

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