# *ADENOVIRUS DNA, I. MOLECULAR WEIGHT AND CONFORMATION**

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The 31 human adenovirus (Ad) DNA's have been isolated in highly purified form in 60–80 per cent yields and several of their properties described.[1, 2] Based on thermal denaturation temperature and buoyant density measurements, the adenovirus DNA's are double-stranded and range in G+C content from 48 to 61 per cent. A correlation has been established between base composition of viral DNA and viral carcinogenicity.[2, 3] We reported previously $s^0_{20,w}$ values of 30 to 32 for Ad 2 and 12 DNA's which correspond to molecular weights of 20 to 23 million assuming that adenovirus DNA's are linear duplex molecules.[1] However, circular DNA's have been described for the oncogenic papovaviruses, including polyoma virus,[4, 5] simian virus 40,[6] and Shope papilloma virus,[7] suggesting the possibility that adenovirus DNA's likewise may be circular. Smith[8] observed circular DNA forms of 2.5 microns in length, corresponding to $5 \times 10^6$ daltons, by electron microscopy of detergent-treated Ad 2, 7, and 12 preparations. In this paper[9] we present the results of analytical zone centrifugation, exonuclease III digestion followed by annealing, and electron microscopy of the DNA's isolated in high yields from purified Ad 2, 4, 7, 12, 18, and 21. These DNA's are shown to consist of linear duplex molecules of molecular weight 20 to 25 million.

*Experimental Procedures.—Isolation of adenovirus DNA's:* Ad 2, 4, 7, 12, 18, and 21 were grown in KB suspension cultures, purified, and viral DNA was extracted in 60–80% yields as previously described.[1, 2]

*Analytical zone centrifugation:* Sedimentation measurements were made with a Spinco model E ultracentrifuge using ultraviolet optics and an alternator to allow automatic photography of two cells at one time. The method of Vinograd, Bruner, Kent, and Weigle[10] was employed. Twenty $\mu$l of viral DNA in 0.1 × SSC (SSC = 0.15 *M* NaCl–0.015 *M* sodium citrate) were "layered" during centrifugation on a solution of either 1 *M* NaCl–0.01 *M* Tris buffer, pH 8.1, for sedimentation of native DNA, or 0.9 *M* NaCl–0.1 *N* NaOH for sedimentation of denatured, single-stranded DNA. Purified intact virus was layered on 0.8 *M* NaCl–0.2 *N* NaOH to release viral DNA during centrifugation. Corrections for viscosity and density, and formulas relating $s^0_{20,w}$ to molecular weight are described by Studier.[11] Ultracentrifuge cells containing 12 mm Kel F centerpieces modified for zone centrifugation were employed. Sedimentation was performed at 35,600 rpm in a rotor maintained at 20°. Pictures were taken on Kodak commercial film and films were traced with a Joyce-Loebl microdensitometer. $s^0_{20,w}$ values were calculated using the point of maximum concentration of the sedimenting bands.

*Enzymatic digestion and annealing:* $P^{32}$-labeled viral DNA molecules were degraded by *E. coli* exonuclease III,[12] and the extent of degradation to acid solubility was measured as previously described.[13] Annealing of degraded samples and undegraded controls was done at a DNA concentration of 2 $\mu$g/ml in 2 × SSC at 65°C for 60 min.

*Electron microscopy of adenovirus DNA's:* The Kleinschmidt protein monolayer technique, as recently described,[14] was used at St. Louis University. The modification of the Kleinschmidt technique used at the Johns Hopkins University has been described.[15] The incidence of circular molecules was estimated by examining the first 100 whole molecules seen in the electron microscopy. All molecules which were not readily identifiable as linear were photographed, examined for continuity, and measured, along with a sampling of linear molecules.

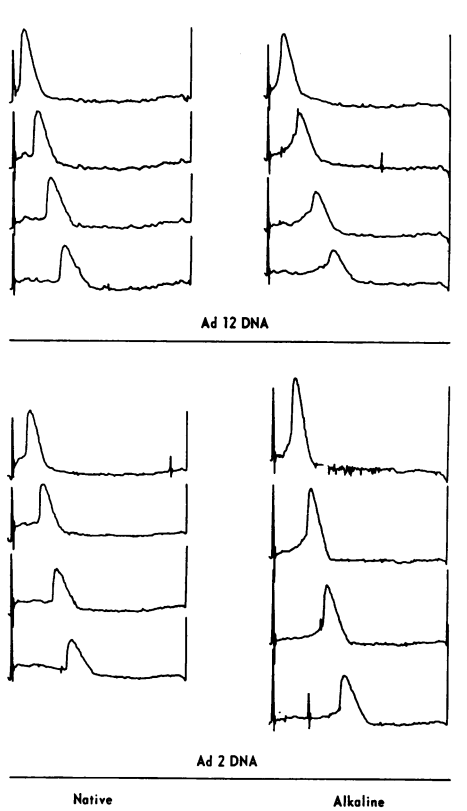


FIG. 1.—Analytical zone sedimentation of native and alkali-denatured Ad 2 and 12 DNA's. Microdensitometer tracings. Intervals between patterns is 8 min, sedimentation is from left to right, rotor speed 35,600 rpm. Initial lamella concentration: native Ad 2 DNA, 30 $\mu$g/ml; alkali-denatured, 40 $\mu$g/ml; Ad 12 DNA, native and alkali-denatured, 30 $\mu$g/ml.

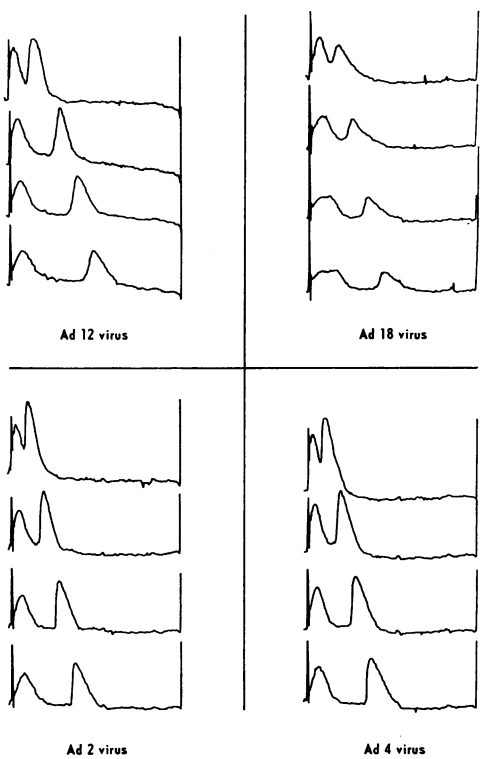


FIG. 2.—Analytical zone sedimentation of Ad 2, 4, 12, and 18 DNA's liberated from intact virus. Microdensitometer tracings. Virus preparations in 0.01 *M* Tris buffer, pH 8.1, at a concentration of 40 $\mu$g of viral DNA/ml for Ad 2, 4, and 12 and 20 $\mu$g/ml for Ad 18 were layered above 0.74 ml of 0.8 *M* NaCl–0.2 *N* NaOH and sedimented at 35,600 rpm. Interval between patterns is 8 min; sedimentation is from left to right. The rapidly moving band is viral DNA; the slowly moving band is viral protein.

*Results.—Sedimentation of native and alkali-denatured adenovirus DNA's:* Analytical zone centrifugation measurements were performed on adenovirus DNA's in neutral and alkaline NaCl solutions. The sedimentation coefficients were used to calculate the molecular weights of native, double-stranded DNA and alkali-denatured, single-stranded DNA and to determine whether circular DNA forms, such as those present in the papovaviruses, occur in adenoviruses. Microdensitometer tracings of the sedimenting bands of native and alkali-denatured Ad 2 and Ad 12 DNA's reveal in each case a single, apparently homogeneous component (Fig. 1). Similar sedimentation patterns were obtained with native and alkali-denatured Ad 4 and Ad 18 DNA's. Alkaline denaturation of adenovirus DNA's did not yield fast-sedimenting forms such as occur when the un-nicked circular DNA molecules of polyoma[4] and papilloma[7] virus DNA's are denatured. Finally, *s* values of native and alkali-denatured adenovirus DNA's are consistent with a conventional

TABLE 1

$s^0_{20,w}$ AND MOLECULAR WEIGHTS OF ADENOVIRUS DNA'S

| DNA | $s^0_{20,w}$ Native[a] | $s^0_{20,w}$ Alkaline[b] | $s^0_{20,w}$ Whole virus[b] in alkali | Molecular weight $\times 10^{-6}$ Single strand[c] | Molecular weight $\times 10^{-6}$ Double strand[d] |
|---|---|---|---|---|---|
| Ad 2 | 31.1 (4)[e] | 34.1 (3) | 33.5 (1) | 10.5 | 23.0 |
| Ad 4 | 31.0 (2) | 34.7 (1) | 34.1 (1) | 10.8 | 22.8 |
| Ad 12 | 30.6 (4) | 33.5 (5) | 32.9 (4) | 10.0 | 22.0 |
| Ad 18 | 29.4 (2) | 33.1 (2) | 33.3 (1) | 10.0 | 19.6 |

[a] Values obtained by extrapolating to zero concentration.
[b] Initial DNA concentration in lamellae was 15–50 μg/ml; no correction for concentration is necessary.
[c] Calculated from relationship $s^0_{20,w} = 0.0528\ M^{0.400}$ (ref. 11). $s^0_{20,w}$ values obtained by alkaline sedimentation of viral DNA and whole virus were averaged for this calculation.
[d] Calculated from relationship $s^0_{20,w} = 0.0882\ M^{0.346}$ (ref. 11) using $s^0_{20,w}$ for native DNA.
[e] Number of determinations.

linear structure since molecular weights calculated for native DNA's are twice those of denatured DNA's (Table 1).

*Sedimentation of alkali-treated adenovirus:* It may be argued that circular forms of adenovirus DNA are converted to linear molecules during the isolation process which involves treatment of the virus with papain, sodium dodecyl sulfate, and phenol. To test this possibility, fresh virus preparations of Ad 2, 4, 12, and 18 were layered on alkaline NaCl in an analytical cell to liberate viral DNA during centrifugation. The release of viral DNA from the virion and its subsequent sedimentation were followed by ultraviolet photography. The tracings in Figure 2 reveal a single sharp sedimenting band of alkali-denatured, single-stranded DNA for each virus; the slow-sedimenting band near the meniscus is viral protein. $s^0_{20,w}$ of DNA released from virus by alkali is identical in each case to that of isolated DNA denatured by alkali (Table 1).

*s and DNA concentration:* The $s_{20,w}$ values of Ad 2 and Ad 12 DNA's in neutral 1 *M* NaCl solution show a concentration dependence not previously found for other DNA's:[11] with a 20-μl lamella, to convert $s_{20,w}$ to $s^0_{20,w}$ a 2.5 per cent correction is required at 10 μg DNA/ml, 5 per cent at 20 μg/ml, 7.5 per cent at 30 μg/ml, and 10 per cent at 40 μg/ml (Fig. 3). The concentration dependence is one half that found for boundary centrifugation.[1] In alkali, no concentration dependence was found at levels of DNA up to 50 μg/ml (Fig. 3), although skewing of the sedimenting band (Fig. 2) characteristic of concentration dependence[10] is evident.

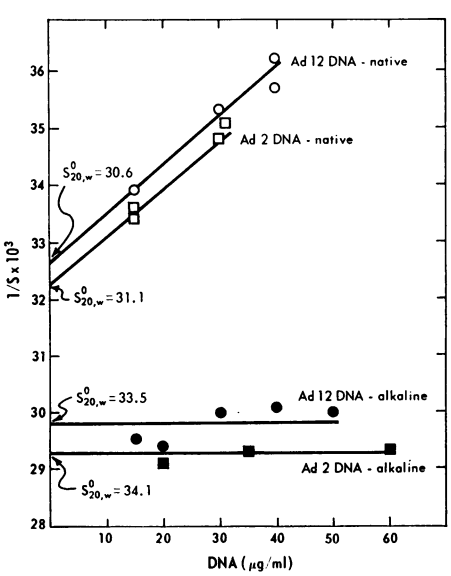


FIG. 3.—Relationship between $1/s_{20,w}$ and concentration of viral DNA.

*Molecular weight of adenovirus DNA's from s values:* $s^0_{20,w}$ values of 29 to 31 were obtained for native Ad 2, 4, 12, and 18 DNA's by extrapolation to zero concentration (Table 1); these correspond to molecular weights of 20 to 23 $\times$ $10^6$ using the relationship for duplex DNA.[11] $s^0_{20,w}$ values of 33 to 35 were found for alkali-denatured Ad 2, 4, 12, and 18 DNA and

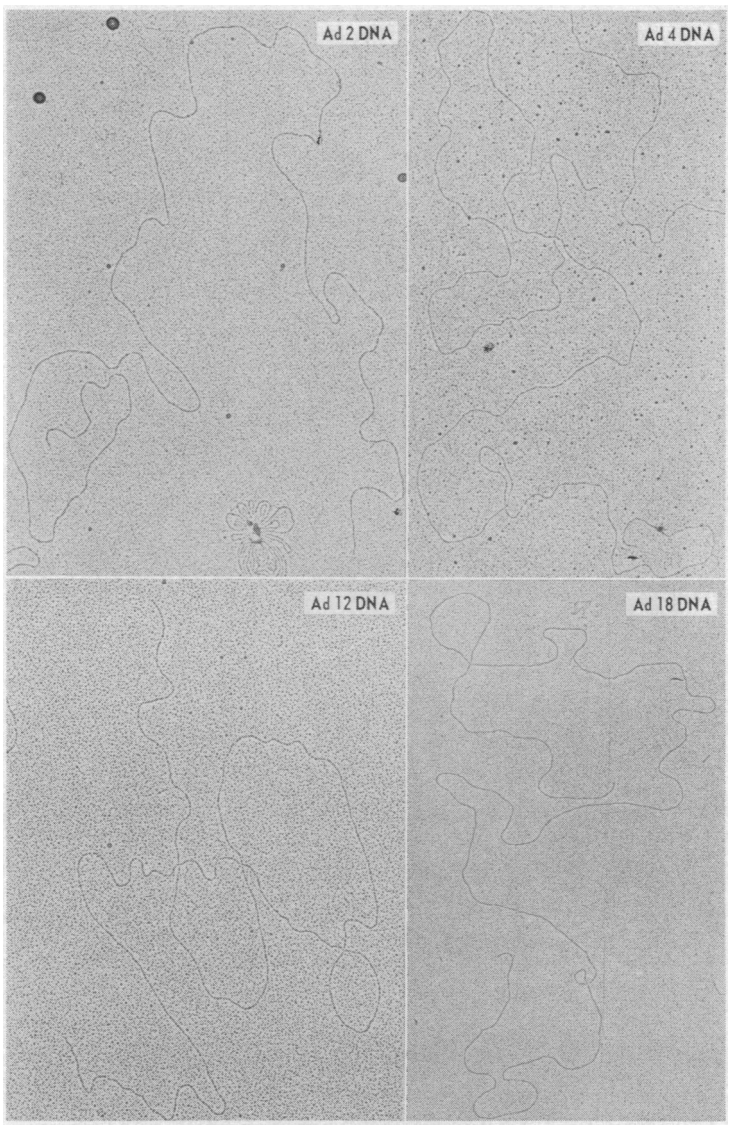


FIG. 4.—Electron micrograph of linear molecules of Ad 2, 4, 12, and 18 DNA's; lengths given in Table 2. Magnification to photographic plate, 7000×.

for DNA liberated from whole virus by treatment with alkali. These correspond to molecular weights for single-stranded DNA of 10 to 11 × $10^6$, values one half that of native DNA. Thus, most of the DNA molecules consist of two uninterrupted polynucleotide chains.

*Electron microscopic length measurements:* The DNA's of Ad 2, 4, 7, 12, 18, and 21 were visualized by the Kleinschmidt technique.[14] Linear molecules in the range 11 to 13 microns were found; representative molecules of Ad 2, 4, 12, and 18 DNA are shown in Figure 4.

Length distributions of Ad 2, 4, 7, 12, and 18 DNA's are plotted in Figure 5.

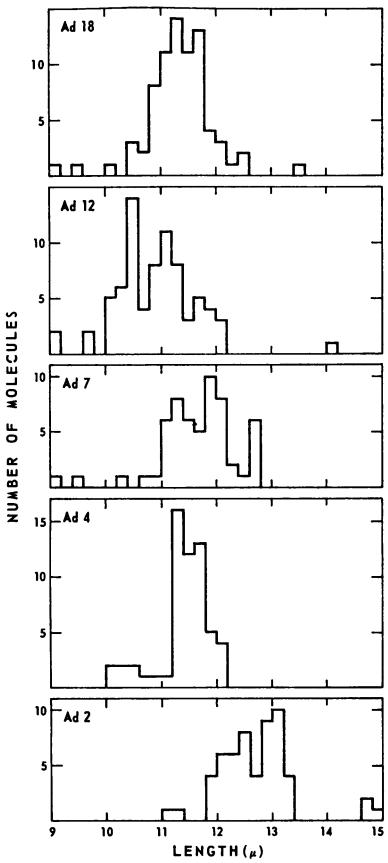


FIG. 5.—Distribution of molecular lengths of adenovirus DNA's. The lengths of all DNA structures were measured and only those molecules with lengths of 9–15 μ are plotted. Ad 2, 56 molecules plotted, two fragments of 8.8 and 7.1 μ not plotted; Ad 4, 59 molecules are plotted, 14 fragments from 1.6 to 9 μ not plotted; Ad 7, 57 molecules plotted, 29 fragments from 1.2 and 7.7 μ not plotted; Ad 12, 75 molecules plotted, 32 fragments from 1.6 and 8.8 μ and two 16.6 and 17.3 μ not plotted; Ad 18, 76 molecules plotted, 11 fragments from 4.1 and 8.2 μ not plotted.

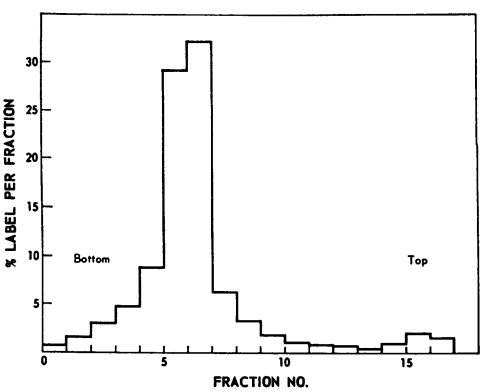


FIG. 6.—Zone centrifugation of $P^{32}$-labeled DNA released from Ad 2. Two hundred μl of $P^{32}$-labeled Ad 2 (15,070 cpm) were layered above 16 ml of a 5–20% linear sucrose gradient in 0.8 *M* NaCl–0.2 *N* NaOH and centrifuged in a Spinco SW 25.3 rotor at 25,000 rpm for 15 hr at 5°. One-ml fractions were collected, 100 μg carrier DNA plus 5 ml of cold 0.6 *M* trichloroacetic acid were added, the DNA was collected on a Schleicher and Schuell membrane filter and counted in a Packard scintillation counter; 98% of the input counts were recovered from the gradient.

Sixty to 110 molecules of each adenovirus DNA were measured, but only those from 9 to 15 microns in length are plotted; variable proportions of shorter molecules were found, from 3 per cent for Ad 2 DNA to 33 per cent for Ad 7 DNA, as listed in the legend to Figure 5. It seems likely that these small molecules resulted from breakage during grid preparation and were not present in the original DNA preparation. This interpretation is supported by zone centrifugation experiments with radioactive viral DNA which consistently yielded less than 5 per cent of the DNA outside of the main distribution (e.g. Fig. 6).

Table 2 lists the molecular lengths of the DNA's of Ad 2, 4, 7, 12, 18, and 21 and the corresponding molecular weights based on a linear density of 1.92 daltons/Å, a value that is appropriate for these spreading conditions and grid preparations. Molecular weights of Ad 2, 4, 12, and 18 DNA's of 21 to 25 $\times$ $10^6$ daltons are in agreement with the values of 20 to 23 $\times$ $10^6$ derived from sedimentation analysis.

*Zone sedimentation of DNA released from adenovirions in the centrifuge tube:* Analytical zone centrifugation and electron microscopy show that adenovirus

TABLE 2

LENGTH AND MOLECULAR WEIGHT OF ADENOVIRUS DNA MOLECULES

| DNA from | Length (μ) | | Molecular weight* × $10^{-6}$ |
|---|---|---|---|
| | Johns Hopkins† | St. Louis‡ | |
| Ad 2 | 12.6 ± 0.8 (100)§ | 12.6 ± 0.3 (76) | 24.4 |
| Ad 4 | 11.6 ± 0.4 (60) | 12.9 ± 0.1 (13) | 22.3–24.8 |
| Ad 7 | 11.8 ± 0.8 (100) | — | 22.7 |
| Ad 12 | 11.0 ± 1.0 (100) | 12.8 ± 0.3 (59) | 21.1–24.6 |
| Ad 18 | 11.4 ± 0.5 (74) | 11.9 ± 0.8 (71) | 21.9–22.8 |
| Ad 21 | 13.0 ± 2.0 (100) | — | 25.0 |

* Calculated using 1.92 × $10^6$ daltons per micron.
† Determined at Johns Hopkins University.
‡ Determined at St. Louis University on different DNA preparations.
§ Number of molecules measured.

DNA's isolated from virus particles are linear, duplex structures of molecular weight 20 to 25 × $10^6$. To test for the possible existence of minor DNA components not recovered by our standard purification procedure, $P^{32}$-labeled Ad 2 virions were layered onto an alkaline sucrose gradient, and centrifuged into it. The alkali in the gradient disrupted the virions, releasing and denaturing the DNA, which then sedimented as isolated single chains (Fig. 6). Under conditions where 96–98 per cent of the radioactivity was recovered from the gradient in two experiments, less than 5 per cent of the radioactive DNA sedimented outside of the main peak of viral DNA.

*Tests for terminal repetition of base sequence:* Electron microscopy of native Ad 2, 4, 7, 12, 18, and 21 DNA molecules after annealing in 2 × SSC at 65°C for 60 minutes revealed only linear molecules. Thus there is no evidence of an "exposed" terminal repetition of the sort found in bacteriophage λ and its relatives.[16] To test for a duplex terminal repetition of the sort found in bacteriophages $T_2$,[13] $T_3$,[17] and $T_7$[17] DNA's, the molecules were degraded by exonuclease III to various extents before the reannealing treatment. The results (Table 3) show a marginally low (1–2%) incidence of circles after this treatment in Ad 2, 4, 7, and 18, and no circles in 100- to 200-molecule samplings of Ad 12 and 21. Examples of Ad 2, 4, and 18 circles are shown in Figure 7*a*, *b*, and *c*.

*Discussion.*—Three independent measurements, (1) the sedimentation coef-

TABLE 3

DIGESTION OF ADENOVIRUS DNA BY EXONUCLEASE III

| DNA from | Degradation (%) | No. of Circles | | Total no. of molecules inspected |
|---|---|---|---|---|
| | | Distinct | Questionable | |
| Ad 2 | 0 | 0 | 0 | 100 |
| | 1.72 | 1 | 1 | 100 |
| | 8.5 | 0 | 1 | 100 |
| | 13.9 | 0 | 0 | 22 |
| Ad 4 | 0 | 0 | 0 | 60 |
| | 4.64 | 2 | 1 | 100 |
| | 6.4 | 1 | 1 | 75 |
| Ad 7 | 0 | 0 | 0 | 100 |
| | 2.66 | 3 | 0 | 100 |
| | 17.1 | 0 | 0 | 50 |
| Ad 12 | 0 | 0 | 0 | 100 |
| | 1.06 | 0 | 0 | 80 |
| | 4.85 | 0 | 0 | 100 |
| Ad 18 | 0 | 0 | 0 | 74 |
| | 3.8 | 1 | 1 | 100 |
| Ad 21 | 0 | 0 | 0 | 100 |
| | 2.07 | 0 | 0 | 100 |

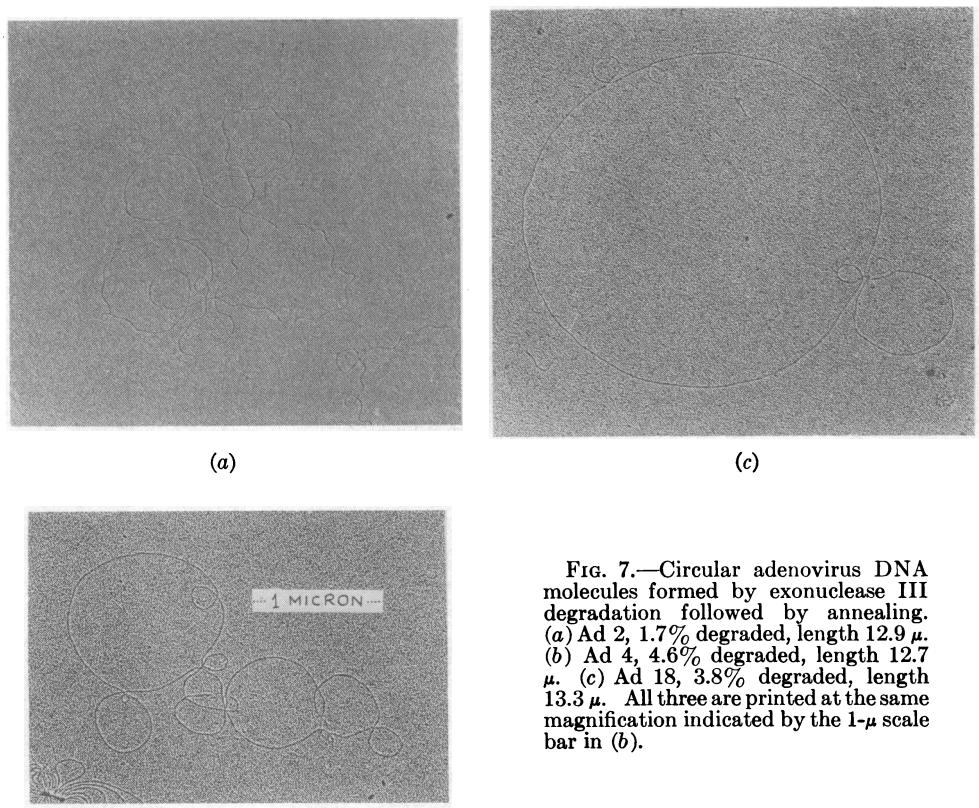


FIG. 7.—Circular adenovirus DNA molecules formed by exonuclease III degradation followed by annealing. (*a*) Ad 2, 1.7% degraded, length 12.9 $\mu$. (*b*) Ad 4, 4.6% degraded, length 12.7 $\mu$. (*c*) Ad 18, 3.8% degraded, length 13.3 $\mu$. All three are printed at the same magnification indicated by the 1-$\mu$ scale bar in (*b*).

ficient of native DNA, (2) the sedimentation coefficient of denatured DNA, and (3) the molecular length of native DNA, provide conclusive evidence that the DNA's isolated from the human adenoviruses are double-stranded, linear molecules 20 to 25 $\times$ $10^6$. These results are incompatible with the report[8] that the DNA's of Ad 2, 7, and 12 are circular molecules of 5 $\times$ $10^6$ daltons.

The size of adenovirus DNA and the 13 per cent DNA content of the virion[2] provide a reliable estimate of the molecular weight of the virion of 175 million (23 million divided by 0.13).

Only a small proportion of some adenovirus DNA molecules appear to form circles upon exonuclease III degradation followed by annealing: Three possible interpretations are that (*a*) terminal repetition may occur in only a small proportion of adenovirus DNA molecules, (*b*) a short terminal repetition initially present in all the viral DNA molecules may be particularly susceptible to elimination by mechanical or chemical effects during DNA extraction or subsequent treatments, or (*c*) exonuclease III degradation may sometimes confer on the ends of some DNA molecules a slight predisposition to stick together in some nonspecific way. Thus it is impossible to say whether DNA molecules that are isolated from adenovirions are terminally repetitious or not.

Although the DNA's isolated from the adenoviruses are linear molecules, the conformation of DNA within the virion is unknown. Purified adenovirus DNA is

not infectious: the only infectious animal virus DNA's isolated are the circular DNA molecules of the papovaviruses.[18]

*Summary.*—We have studied the intact DNA molecules isolated from six different human adenovirions, including the "highly" carcinogenic adenoviruses (Ad) 12 and 18, the "weakly" carcinogenic Ad 7 and 21, and the noncarcinogenic Ad 2 and 4. In all cases the molecules are linear, 11 to 13 microns in length, as seen by protein-film electron microscopy. The molecular weights calculated from sedimentation coefficients agree with the observed lengths. The sedimentation rates under alkaline conditions (where the composite single chains of many other viral DNA molecules completely separate) predict molecular weights that are one half those of the duplex adenovirus DNA molecules. Thus the majority of duplexes consist of uninterrupted polynucleotide chains. Finally, sedimentation under alkaline con ditions reveals no detectable "supercoil"—that compact structure formed by the denaturation of an uninterrupted circular helix.

Thus we conclude that the DNA's isolated from adenovirions are linear duplex DNA molecules of 20–25 million daltons.

Partial degradation of these molecules by exonuclease III followed by annealing rendered only 1 or 2 per cent of them circular in the cases of Ad 2, 4, 7, and 18, and none circular in Ad 12 and 21. The significance of this low incidence of artificial circles is uncertain.

*Note added in proof:* After this manuscript was completed, a preliminary note appeared by A. J. Van der Eb and L. W. van Kesteran (*Biochim. Biophys. Acta*, **129**, 441 (1966)) describing sedimentation data on Ad 5 DNA which quantitatively confirm some of the experiments presented here with Ad 2, 4, 12, and 18 DNA's.

* A brief description of some of these results has been published[18] and has been presented at the International Tumor Virus Symposium held in Nagoya, Japan, in 1966. Supported by USPHS grant AI-01725 and contract PH43-64-928 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland. The work at Johns Hopkins has been supported by the AEC (AT-30-1) 2119, NSF (G-10726), and the NIH (E-3233).

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‡ Career Development Awardee (K3-GM-25,342).

[1] Green, M., and M. Piña, these PROCEEDINGS, **51**, 1251 (1964).

[2] Piña, M., and M. Green, these PROCEEDINGS, **54**, 547 (1965).

[3] Green, M., M. Piña, G. Tockstein, and H. Thornton, manuscript in preparation.

[4] Weil, R., and J. Vinograd, these PROCEEDINGS, **50**, 730 (1963).

[5] Dulbecco, R., and M. Vogt, these PROCEEDINGS, **50**, 236 (1963).

[6] Crawford, L. V., and P. H. Black, *Virology*, **24**, 388 (1964).

[7] Crawford, L. V., *J. Mol. Biol.*, **8**, 489 (1964).

[8] Smith, K. O., *Science*, **148**, 100 (1965).

[9] Some of these studies were begun in collaboration with Dr. J. Vinograd in August 1964. One of us (M.G.) wishes to thank Dr. Vinograd for his hospitality during this brief stay.

[10] Vinograd, J., R. Bruner, R. Kent, and J. Weigle, these PROCEEDINGS, **49**, 902 (1963).

[11] Studier, F. W., *J. Mol. Biol.*, **11**, 373 (1965).

[12] Richardson, C. C., and A. Kornberg, *J. Biol. Chem.*, **239**, 242 (1964).

[13] MacHattie, L. A., D. A. Ritchie, C. A. Thomas, Jr., and C. C. Richardson, *J. Mol. Biol.*, **23**, 355 (1967).

[14] Kleinschmidt, A. K., S. S. Kass, R. C. Williams, and C. A. Knight, *J. Mol. Biol.*, **13**, 749 (1966).

[15] MacHattie, L. A., and C. A. Thomas, Jr., *Science*, **144**, 1142 (1964).

[16] Strack, H. B., and A. D. Kaiser, *J. Mol. Biol.*, **12**, 36 (1965).

[17] Ritchie, D., C. A. Thomas, Jr., L. MacHattie, and P. Wensink, *J. Mol. Biol.*, **23**, 365 (1967).

[18] Green, M., in *Viral and Rickettsial Infections of Man*, ed. F. L. Horsfall, Jr., and I. Tamm (Philadelphia: J. B. Lippincott, 1965), chap. 6, p. 145.