The Cyclic Helix and Cyclic Coil Forms of Polyoma Viral DNA

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PNAS 1963;50;730-738
doi:10.1073/pnas.50.4.730

for its association with the chloroplast *in vivo*. Light microscope examination of the isolated chloroplast preparations indicates very low contamination with other cell particulates.

* Supported by grant E-1605 of the U.S. Public Health Service and institutional grant IN-4 of the American Cancer Society.

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THE CYCLIC HELIX AND CYCLIC COIL FORMS OF POLYOMA VIRAL DNA

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Communicated by R. Dulbecco, August 19, 1963

The DNA extracted from polyoma virus exhibits certain properties which have not been reported for other viral base-paired DNA's.[1] The DNA renatures monomolecularly. The loss of helical configuration does not impair biological activity. Heating at 100° for 10–20 min followed by rapid cooling does not reduce the infective titer. Dulbecco has given evidence suggesting that a fraction of the polyoma DNA molecules are cyclic.[2] This form would in part account for the above properties.

The results reported in this communication give strong evidence for cyclic polyoma DNA molecules; they also define certain properties of the cyclic and open forms. Some of the evidence reported here is similar to that obtained in an independent investigation by Dulbecco and Vogt.[3]

Materials and Methods.—Isolation and purification of the virus and extraction of the DNA: The virus was isolated and purified by the procedure described by Winocour.[4] The main visible band from the CsCl density gradient was collected, and the DNA extracted by a modified phenol procedure.[1] The homogenization step was omitted. The CsCl, Harshaw Chemical Company, Optical Grade, formed solutions which remained clear upon addition of alkaline buffers. Reagent grade inorganic chemicals and Merck's reagent grade formaldehyde were used.

Analytical ultracentrifugation: Sedimentation velocity analyses were performed in the Spinco analytical ultracentrifuge by band-centrifugation.[5] Boundary-velocity experiments were precluded by the limited supply of the virus. Buoyant density experiments were performed at 44,770 rpm at 25°C and analyzed by methods previously described.[6]

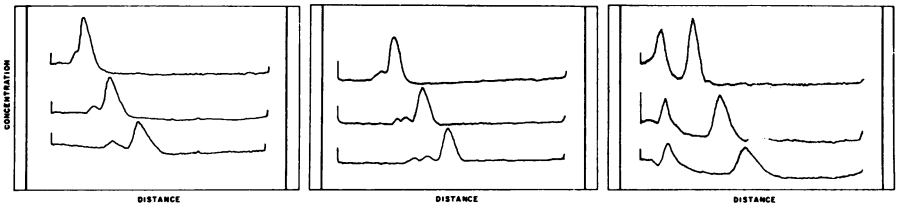
Preparation of the alkaline solutions: The alkaline CsCl solutions were prepared with the buffers described by Vinograd *et al.*[7] The alkaline NaCl solutions were prepared by mixing 1 volume of 4 *M* NaCl with 3 volumes of 0.11 *M* KOH.[7a] The pH measured with a small general-purpose Beckman glass electrode was 12.2 ± 0.1. DNA samples were made alkaline in the sample hole by addition of 1 volume of 1.1 *M* KOH[7a] to 9 volumes of DNA solution, and mixed by drawing the solutions back into the Kel-F tubing several times. The final pH was 12.5 ± 0.2. Pre-incubation for longer periods was performed in 0.032-in. I.D. Kel-F tubing sealed at both ends with 4-mm plugs of silicone grease.

Denaturation and renaturation: These experiments were performed as previously described.[1] Formaldehyde denaturations[8] were performed with 25 μl samples in 11% CH_2O, pH 8.3, ionic strength 0.01, in sealed glass melting-point tubes at 70–75°C 2–3 min followed by chilling at 0°C.

Sonication: The DNA solutions in thin 1-ml nitrocellulose test tubes were sonicated in a 9,000 cps Raytheon Sonicator Model no. S102A at the indicated power level.

Calculations: The molecular weight of anhydrous NaDNA was calculated from the standard deviation of the band.[6] The additional cesium ions and preferential hydration[7] introduced at high pH were included in the calculations. Buoyant densities were measured with *M. lysodeikticus* DNA, $\rho_0^0 = 1.732$ gm cm^{-3}, as a reference, a value based on $\rho_0^0 = 1.710$ gm cm^{-3} for DNA from *E. coli*.[9] This value disagrees with a recent redetermination by +0.006 gm cm^{-3}.[7]

Results.—Sedimentation analysis of DNA extracted from the virus: Band-centrifugation analyses of the DNA from six different virus batches all showed three components which we refer to as I, II, and III, in order of decreasing sedimentation coefficients. The values, $s^\circ_{20,w}$, in 1 *M* NaCl, 0.01 *M* Na_2HPO_4, pH 8.0 were 20.3 S ± 0.4 S.D., 15.8 S ± 0.4 S.D., and 14.4 S[10] (Fig. 1*A*). The component composition was determined in 3.5 *M* CsCl (Fig. 1*B*). In this solvent the band profiles are more symmetrical than in 1 *M* NaCl or 1 *M* CsCl. In all preparations



(A) (B) (C)

FIG. 1.—Band sedimentation velocity analyses of polyoma DNA in (*A*) neutral NaCl, (*B*) neutral CsCl, (*C*) alkaline NaCl. (*A*) Lamella: 15 μl, 24 μg/ml DNA; bulk solution: 1 *M* NaCl, 0.01 *M* Na_2HPO_4, pH 8.0. Densitometer records are from photographs taken at 16-min intervals. 35,600 rpm, 20°. (*B*) Lamella: 20 μl, 60 μg/ml DNA; bulk solution: CsCl, $\rho = 1.36$, 0.01 *M* Tris, pH 8.1. 16-min intervals, 35,600 rpm, 20°. (*C*) Lamella: 25 μl, 24 μg/ml DNA, pH = 12.75; bulk solution: 1.0 *M* NaCl, 0.10 *M* KOH, pH 12.2, 8-min intervals. 35,600 rpm.

I comprised 80–90 per cent of the DNA. Component II varied between 1 and 20 per cent and III between 1 and 10 per cent. DNA released from the virus in the sample hole, pH 11.2 or pH 12.3, gave patterns indistinguishable from those obtained with the phenol extract. The sedimentation pattern at neutral pH did not change upon storage at 4° for periods up to 20 weeks, or upon freezing and thawing. The DNA formed a single band at equilibrium in CsCl with a buoyant density of 1.709 gm cm^{-3}. All preparations formed bands which were skewed on the light side. It is shown later that this skewness is due to III.

The renaturation behavior of polyoma DNA suggested that the strands did not physically separate when heated under conditions in which strand separation normally occurs. To examine this problem we employed the procedure described by Freifelder and Davison.[8] Solutions of polyoma DNA were denatured in CH_2O and examined in sedimentation velocity experiments in 11 per cent CH_2O, 1 *M* NaCl. The DNA now sedimented in two components (80 per cent fast) with a ratio of sedimentation coefficients of 2.2. This result was not compatible with the hypothesis that the fast material represented singly cross-linked double strands, and the slow material single strands[8] because a ratio of $\sqrt{2}$ is to be expected. Strand separation was also examined at high pH, 12.2 ± 0.1, in 1 *M* NaCl. In this solvent, complete strand separation is to be expected.[7] All fresh polyoma DNA preparations, including those stored at −70°C, sedimented in two well-resolved components[11] (about three-fourths fast) with sedimentation coefficients, $s^{\circ}_{20,w}$ of 53.1 S ± 0.3 S.D. and 14.6 S ± 0.5 S.D. (Fig. 1*C*). We will refer to the alkali components as the 53 S and 15 S DNA's and will show below that these are double-stranded cyclic coils and single-stranded random coils, respectively. That both of these components can derive from I was shown in experiments in which the DNA was so dilute that II and III could not be detected at pH 8; in alkali, however, both the 53 S and 15 S components were observed.

The sedimentation velocity of the 15 S DNA was compared with the sedimentation velocity of φX-174 DNA (16.0 S) in the same solvent, and its molecular weight calculated with the relation for random coils, $S_1/S_2 = (M_1/M_2)^{0.5}$. With $M = 1.7 \times 10^6$ for φX-174 DNA[12] we obtain $M = 1.4 \times 10^6$ for the 15 S component. The same value is obtained if we use single-stranded T-7 DNA as a reference in this calculation, $s^{\circ}_{20,w} = 39$ S, $M = 9.5 \times 10^6$.[13]

Fresh polyoma DNA forms two well-separated bands in buoyant density experiments in alkaline CsCl pH 12.3. In *band-buoyancy* experiments (band-centrifugation performed with buoyant bulk solutions[5]) (Fig. 2), the 53 S DNA forms a band at a higher density than the 15 S DNA. The buoyant densities are 1.784 and 1.766 gm cm^{-3}, referred to 1.710 for *E. coli* DNA. The molecular weight calculated from several band profiles for the 53 S DNA was $2.4 \pm 0.3 \times 10^6$. The band formed by the 15 S DNA was skewed on the light side because of slowly sedimenting DNA and a small amount of III.

With a ratio of molecular weights $M_{53\ S}/M_{15\ S} \approx 2$, as indicated in the arguments in the above two paragraphs, we expect for random coils a ratio of sedimentation coefficients of 1.4. The high velocity, 53 S, in alkali of the double-stranded DNA has thus to be attributed to a drop in the frictional coefficient by a factor of 2.5 relative to a random coil containing two strands (Fig. 3). We therefore propose that the double-stranded cyclic helix denatures to form a double-stranded cyclic

coil in which the turns originally present in the helix are conserved. The topological constraint imposes a compact structure on this molecule (Fig. 3) and gives rise to an unusually high sedimentation coefficient and buoyant density.

Several physical and chemical agents can convert the cyclic helix, the linear helix and the double-stranded cyclic coil to single strands, as well as the cyclic helix to the double-stranded cyclic coil and to the linear helix.

(1) *Exposure to high pH:* After the initial rapid conversion of about one fourth of component I into single strands, a further conversion into single-stranded 15 S DNA occurs at a slow rate. For example, after 72 hr incubation at pH 12.1 in 0.01 *M* NaCl, the amount of 53 S DNA decreased to about one third with a concomitant increase in 15 S DNA. Unlike the usual 15 S DNA, this material contained two velocity components, a small fraction, presumably cyclic single strands, sedimenting about 10 per cent faster than the main component.[11] The patterns were similar to those observed in experiments with ϕX-174 DNA, which contained both linear and circular molecules.[14]

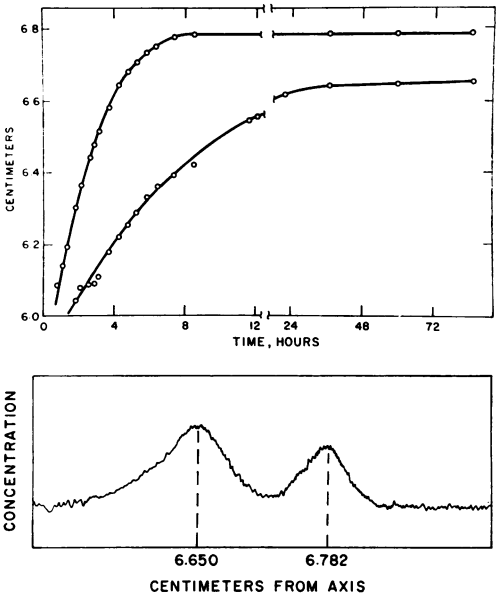


FIG. 2.—Band-buoyancy analysis of polyoma DNA in alkaline CsCl. Lamella: 20 μl, 24 μg/ml DNA, pH 12.75. Bulk solution: CsCl, ρ = 1.76, 0.04 *M* K_3PO_4, pH = 12.35. 44,770 rpm. *Upper:* position of 15 S and 53 S components at various times. *Lower:* densitometer records of bands at 72 hr, 25°.

(2) *Exposure to high CsCl concentrations:* The sedimentation velocity patterns of polyoma DNA in dilute CsCl solutions are skewed because of the effects of concentration-dependent sedimentation. At a density of 1.35 gm cm^{-3} the bands become symmetrical, presumably because of a reduction in the equivalent hydrodynamic volume.

At still higher concentrations of CsCl, ρ = 1.5 and ρ = 1.6, the relative amounts of the components changed in two of the three preparations studied. Component I became smaller and II larger. That this conversion is not rapidly reversible was shown in experiments in which polyoma DNA was incubated at 23°C in CsCl, ρ = 1.72, 0.006 *M* Na citrate pH 7.0 for 1, 24, and 44 hr, and subsequently analyzed, after dilution, in CsCl, ρ = 1.35 gm cm^{-3}. Analyses in neutral CsCl showed that irreversible conversion of I in all three DNA preparations had occurred to the extent of 50, 75, and 90 per cent, respectively. In one of the preparations of DNA it was confirmed that the conversion noted after 44 hr by analysis in neutral CsCl represented a ring cleavage. This was shown by a change in the relative amounts of fast and slow forms in alkaline CsCl, ρ = 1.36, 0.04 *M* K_3PO_4, pH = 12.1. The ratio of amounts changed from 0.6 to 0.1. The corrected sedimentation coefficients, $s^0_{20,w}\,\eta_{rel}$, for the double-stranded cyclic coils and the single-stranded coils

were 37 S and 11.6 S. These results indicate that preparative equilibrium sedimentation in CsCl at room temperature can lead to a substantial loss of the cyclic helical form.

(3) *Exposure to high CsCl concentration at high pH:* A series of buoyant density experiments were performed to examine the effects of pH. At pH 11,

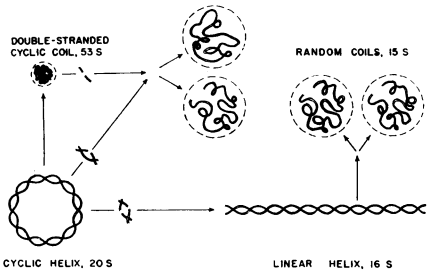


FIG. 3.—Diagrammatic representation of the two forms of polyoma DNA. The double-stranded cyclic helix denatures to form a double-stranded cyclic coil. The linear helix denatures to form two single-stranded random coils. The dashed circles indicate the relative equivalent hydrodynamic diameters. Possible links between chains or special bonds connecting chain ends are not considered. The sedimentation coefficients of the helices and coils were measured in neutral and alkaline NaCl solutions, respectively.

as at pH 8.1, the buoyant density was 1.709 gm cm^{-3}, and the band was skewed at the light side. At pH 11.8 a substantial fraction of the DNA titrated to form a band at 1.766 gm cm^{-3} that was skewed at the light side while the remainder, the intact cyclic helical DNA, formed a symmetrical band at 1.714 gm cm^{-3}.[11] At pH 12.2 the latter material denatured and titrated to form a band of double-stranded cyclic coils at 1.784 gm cm^{-3}. The results show that the cyclic helix is more stable than the linear helix to alkali denaturation. Both at pH 11.8 and 12.2 the relative amounts of the double-stranded cyclic DNA were smaller than the relative amount of 53 S DNA initially present in the preparations. A portion of the double-stranded cyclic DNA was therefore converted to single strands.

(4) *Sonication:* Sonication at 75 per cent full power for 1.5 and 5 min gave the same results. The relative amount and the sedimentation coefficient of I were essentially unchanged in neutral 1 *M* NaCl (Fig. 4). Components II and III formed a relatively broad band with decreased sedimentation coefficient, 12.2 S. At full power, sonication for 2 and 4 min resulted in the conversion of 50 and 80 per cent, respectively, of I into a slower material, 8.3 S. In previous studies with native DNA's, the resistance to shearing increases with decreasing sedimentation coefficient.[15] We have here the reverse effect, presumably because the extended length of the cyclic form is less than that of the linear form.

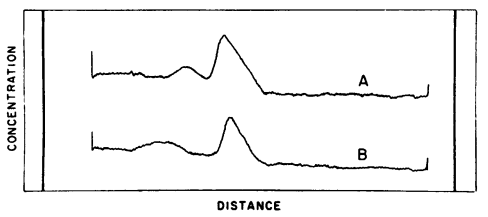


FIG. 4.—Effect of sonication of polyoma DNA. (*A*) untreated; (*B*) sonicated 1.5 min. Lamella: 15 μl, 25 μg/ml DNA. Bulk solution: 1.0 *M* NaCl, 0.01 *M* $NaHPO_4$, pH 8.0. Photographed 64 min after reaching full speed. 35,600 rpm, 20°.

(5) *Effects of heating at 100°C:* Component III is selectively melted out and becomes polydisperse in neutral sedimentation velocity experiments performed after heating solutions of polyoma DNA in 0.4 *M* NaCl 1–2 min at 100° followed by rapid cooling. The skewness disappears from the main band in buoyant density experiments at neutral pH. Component III forms a band of denatured DNA with a buoyant

density 1.721 gm cm^{-3}, 0.003 gm cm^{-3} less than the buoyant density of denatured polyoma DNA. In these experiments the relative amounts and the sedimentation velocities of I and II were unchanged. Longer heating leads to gradual conversion of I into a 16 S DNA.

When fresh polyoma DNA at a concentration of 1–2 μg/ml was heated at 100° 1–2 min in buffers of low ionic strength followed by rapid cooling, I renatured nearly completely, while an amount corresponding to II and III remained denatured as judged by buoyant density. This result indicates that intact cyclic coils renature readily. Upon further heating, increasing amounts of denatured DNA were formed. Heated materials analyzed in alkali contained decreasing amounts of 53 S DNA and increasing amounts of 15 S DNA. Depurination which occurs on heating and leads to chain scission in alkali[16–18] is in part responsible for the conversion of double-stranded cyclic coils to random coils.

Electron microscopy: The striking and important result is that cyclic molecules are observed (see *Appendix*).

Discussion.—The major fraction of polyoma DNA denatures in alkali and remains double-stranded. A smaller fraction denatures in alkali to form single strands with about half the molecular weight of the double-stranded form. Comparison of the sedimentation coefficients in alkali of these two denatured DNA's with each other and with the sedimentation coefficients in alkali of other DNA single strands of known molecular weight leads to the conclusion that the denatured double-stranded polyoma DNA has an abnormally low frictional coefficient. A simple model for the native DNA which can account for the properties mentioned is a cyclic helix with separately continuous strands (Fig. 3).

The requirement that the approximately 500 turns originally present in the helical structure be conserved as long as the strands remain continuous leads necessarily to a configuration for the denatured molecule which is more compact than the random-coil configuration. Any spatial rearrangement of the phosphodiester chains upon denaturation gives rise to supercoiling. This new type of DNA coil, which we refer to as the *double-stranded cyclic coil*, has an abnormally high sedimentation coefficient and a higher buoyant density in alkaline cesium chloride than the random coil.

The introduction of one discontinuity in one of the strands in the native or denatured double-stranded molecule results in the formation in alkali of single strands, one linear and one cyclic. The finding that the molecular weight is always first reduced by a factor of 2 upon degradation shows that the strands in polyoma DNA are separately continuous. None of the foregoing excludes the additional presence of a cross-link labile in alkali. Further evidence for a cyclic structure for component I is the relatively high resistance to shearing by sonication, and the presence in the electron micrographs of about 90 per cent cyclic molecules.

We now examine the evidence that II is an open or linear form of the cyclic helix I. The buoyant density of II is essentially the same as I. Upon thermal denaturation of II, a buoyant density shift characteristic for double-stranded DNA is observed. The alkaline buoyant density shift is also normal for a linear helix. We conclude therefore that II is a linear helix. I is converted into 16 S helical DNA by the action of concentrated CsCl or heating at 100° in 0.4 *M* NaCl.

The sedimentation coefficient of II is slightly lower than I, which is reasonable for the proposed relation between I and II. That II has the same molecular weight as I is indicated by the formation from II in alkali of 15 S single strands of one half the molecular weight of I. These arguments all suggest that II is an open or linear form of the cyclic helix I. Consistent with the above conclusion is the greater sensitivity of II to sonication, and the appearance in the electron micrographs of linear and cyclic molecules with the same mean length.

The origin of III, which was present in small amounts in all preparations, is not known. Its homogeneity with respect to sedimentation velocity and buoyant behavior rules out an adventitious contamination by host cell DNA. Component III was not found by Dulbecco and Vogt[3] in radioactively labeled polyoma DNA preparations, or by Crawford (cf. ref. 10) with boundary-sedimentation velocity experiments. Either the methods used by these investigators were not sufficiently sensitive or III represents a well-defined host cell DNA molecule which was synthesized prior to viral infection and thus remained unlabeled.

The thermal stability[1] of the infective titer of polyoma DNA upon heating at 100° can now be interpreted. The initial threefold increase in infective titer is the

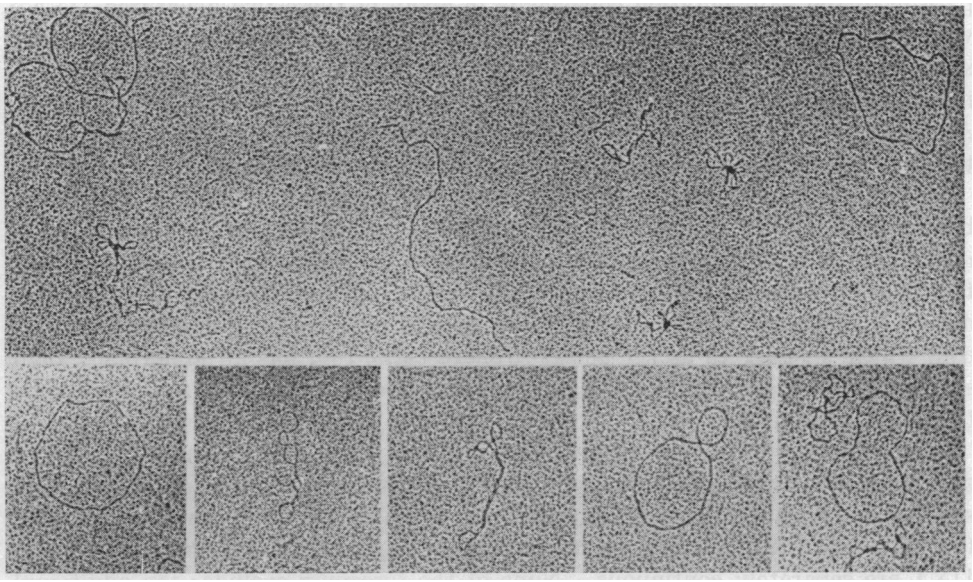


FIG. 5.—Electron micrographs of polyoma DNA, ×44,000. *Upper:* a field showing linear and cyclic forms. *Lower:* selected cyclic forms.

result of a more efficient attachment of the DNA to the mouse cells in the assay for infectivity.[1] The essentially unchanged titer during the next 10–20 min is attributed to a steady state between a thermal activation and an inactivation process. In the activation process the cyclic double-stranded molecule is opened to produce a molecule of higher infectivity. If a cleavage occurs at a phosphodiester bond, a pair of linear and cyclic single strands is formed. These separate unless held together by a cross link which we have not ruled out. If a cleavage at the site of a

linker connecting four chain ends occurs, either single- or double-stranded linear molecules are formed. When all cyclic double-stranded molecules have been opened, the reservoir of potential infectivity is exhausted. Further heating leads to an exponential decrease in the infective titer.

The opening of the ring at high pH or as a result of heating may proceed by well-known reactions such as hydrolysis or depurination followed by hydrolysis; and these may occur at any site along the chains. The slow cleavage of the ring in concentrated neutral CsCl solutions is a surprising result which suggests that polyoma DNA contains a labile site in the chain not present in other biologically active DNA's which have been routinely studied in and recovered from equilibrium density gradient experiments in concentrated CsCl solutions.

APPENDIX BY WALTHER STOECKENIUS

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Three different configurations of polyoma viral DNA are visible in the electron micrographs (Fig. 5): coiled molecules without visible free ends, fully extended cyclic molecules, and linear molecules. If a coiled form contained not more than two twists, its length could be accurately measured. In a given typical area, 83 coiled, 8 extended, and 9 linear molecules were observed. The mean lengths of 66 cyclic and 68 linear molecules were 1.58 $\mu \pm 0.16$ S.D. and 1.53 $\mu \pm 0.16$ S.D., respectively. These lengths correspond to a molecular weight of 3.0×10^6, if we assume that the mass per unit length of DNA molecules in the protein film is the same as in the B-form in fibers of NaDNA.[19-21]

Electron microscopy thus confirms that polyoma DNA molecules exist in linear and cyclic forms of equal molecular weight. The proportion of I and II by electron microscopy corresponds to that found by sedimentation analysis, as described below.

A striking feature of the electron micrographs is the high proportion of tightly coiled molecules. In otherwise identical preparations of T-7 DNA, linear molecules and a very few tightly coiled molecules were found. The latter form is probably due mainly to intramolecular cross-linking by protein.[21] The absence of free ends in the tightly coiled polyoma DNA molecules indicates that they are cyclic; the high proportion of such forms suggests that cyclic molecules are more readily cross-linked by protein. Component III could not be identified in the electron micrographs.

A sample containing 2 μg/ml polyoma DNA in 0.006 *M* KCl, 0.01 *M* Tris, pH 7.5 was shipped in dry ice, thawed, and frozen three times during these studies, and the remainder returned in dry ice. Re-examination (by R. W. and J. V.) at neutral pH in 1.0 *M* NaCl and 3.5 *M* CsCl showed an unchanged composition, 85% I and about equal amounts of II and III. In alkaline NaCl only slow polydisperse strands were found.

Grids were prepared by the method of Kleinschmidt and Zahn,[22] except that diisopropylphosphoryl trypsin (Worthington Biochemical Corp., no. TRL-DIP104) was used instead of cytochrome C and the solution was delivered to the surface via a glass rod.[23] One ml of solution (0.24 μg DNA, 100 μg DIP-trypsin in 1.0 *M* NH_4 acetate, pH 8.0) was delivered onto 0.015 *M* NH_4 acetate, pH 8.0. The resulting surface film was compressed to 1.0 dyne/cm at about 450 cm^2. Randomly selected portions of the surface film were picked up on platinum grids and shadowed with uranium at an angle of 7° while the specimen was rotating.

Summary.—The results obtained in this study show that polyoma viral DNA contains linear and cyclic helical molecules. The double-stranded cyclic helix de-

natures in alkaline solutions to form a double-stranded cyclic coil, a new type of coiled molecule in which all of the turns originally present in the helix are conserved.

It is a pleasure to thank R. W. Watson for expert technical assistance, R. D. Bruner for helpful discussions during the preparation of the manuscript, R. Dulbecco for providing the polyoma virus, R. L. Sinsheimer and P. F. Davison for gifts of materials, and R. Dulbecco and M. Vogt for a copy of their unpublished manuscript. This work was supported in part by grants HE 03394 and GM 08812 from the U.S. Public Health Service.

* Contribution 3018 of Gates and Crellin Laboratories of Chemistry.

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REPLICATION OF NUCLEIC ACIDS

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Communicated by Linus Pauling, July 15, 1963

Several model schemes have been proposed to explain the replication of nucleic acids, in particular the sequence selection of filial nucleotides when a double-stranded nucleic acid undergoes semiconservative replication. (These schemes usually are concerned with a Watson-Crick type DNA, although similar schemes might involve a filial RNA strand.) Two difficulties involved in previously proposed schemes are the unexplained accuracy of replication of nucleotide sequences