Physical and Topological Properties of Circular DNA

JEROME VINOGRAD and JACOB LEBOWITZ

From the Divisions of Biology and Chemistry, California Institute of Technology, Pasadena. Dr. Lebowitz' present address is the Department of Bacteriology and Botany, Syracuse University

ABSTRACT Several types of circular DNA molecules are now known. These are classified as single-stranded rings, covalently closed duplex rings, and weakly bonded duplex rings containing an interruption in one or both strands. Single rings are exemplified by the viral DNA from ϕX174 bacteriophage. Duplex rings appear to exist in a twisted configuration in neutral salt solutions at room temperature. Examples of such molecules are the DNA's from the papova group of tumor viruses and certain intracellular forms of ϕX and λ-DNA. These DNA's have several common properties which derive from the topological requirement that the winding number in such molecules is invariant. They sediment abnormally rapidly in alkaline (denaturing) solvents because of the topological barrier to unwinding. For the same basic reason these DNA's are thermodynamically more stable than the strand separable DNA's in thermal and alkaline melting experiments. The introduction of one single strand scission has a profound effect on the properties of closed circular duplex DNA's. In neutral solutions a scission appears to generate a swivel in the complementary strand at a site in the helix opposite to the scission. The twists are then released and a slower sedimenting, weakly closed circular duplex is formed. Such circular duplexes exhibit normal melting behavior, and in alkali dissociate to form circular and linear single strands which sediment at different velocities. Weakly closed circular duplexes containing an interruption in each strand are formed by intramolecular cyclization of viral λ-DNA. A third kind of weakly closed circular duplex is formed by reannealing single strands derived from circularly permuted T2 DNA. These reconstituted duplexes again contain an interruption in each strand though not necessarily regularly spaced with respect to each other.

In recent years it has become known that genes in chromosomes may be circularly arranged with respect to each other. Jacob and Wollman (1) in 1958 showed that distant markers in a donor strain of *E. coli* appeared in closely linked genes in a recipient strain after bacterial mating. Streisinger, Edgar, and Denhardt (2) in 1964 proved in genetic mapping experiments that the entire map of T4 bacteriophage is circular. It is not surprising therefore that DNA, the genetic macromolecule, should also occur in a circular form.

Since the demonstration in 1962 by Fiers and Sinsheimer (3) that the single-

stranded DNA from the small coliphage ϕX174 is in the form of a ring, there has been a rapid proliferation of types and examples of circular DNA. Cairns (4) observed in his well known autoradiograms that the DNA in an *E. coli* cell is arranged in one long circular unit. Hershey, Burgi, and Ingraham (5) found that the linear DNA from coliphage lambda had sticky ends which could be induced to form circular DNA. In the same year Dulbecco and Vogt (6) and Weil and Vinograd (7) showed that the DNA from the tumor virus, polyoma, was in the form of covalently closed circular duplex molecules. Also in 1963 Burton and Sinsheimer (8) and Kleinschmidt, Burton, and Sinsheimer (9) found that the replicating form of ϕX DNA was a covalently closed circular duplex. In the last 2 years Crawford and Black have shown that other viruses of the papova group, SV-40, rabbit papilloma and human papilloma, contained covalently closed circular DNA (10–12). Thomas and MacHattie (13) have found circular duplexes after reannealing the circularly permuted single strand of T2 DNA. Recently Young and Sinsheimer (14) and Bode and Kaiser (15) have found that the parental DNA molecules injected by lambda virus into susceptible and immune bacteria are first cyclized to form covalently closed duplexes.

In this paper we wish to review these rapid developments and to examine the physical and chemical properties of circular DNA with special reference to those properties associated with circularity.

The first DNA observed to be in a ring form was obtained from the virus ϕX174. This DNA had been shown to be single-stranded and noncomplementary by Sinsheimer in 1959 (16). The conclusion regarding the circularity was reached in the following experiments (3).

1. The DNA contained, at pH 12, two sedimentation velocity components differing by about 10% in velocity.
2. The fast form was converted into the slow form by treatment with pancreatic DNAase without forming products of intermediate sedimentation velocity.
3. The decrease in amount of the fast component and the increase in amount of the slow component followed the prediction of the Poisson relation for the effects of random scission of homogeneous rings to a homogeneous linear product and then to a wide mass distribution of linears formed by additional scissions (Fig. 1).
4. The fast component was resistant to exonucleases (17) while the slow component, formed by the action of pancreatic DNAase, was digested further by the *E. coli* exonuclease I.

There was little doubt in view of the above results that ϕX DNA was in the form of a ring, a conclusion corroborated in 1964 by the finding of circular forms of this single-stranded DNA in electron micrographs by Freifelder, Kleinschmidt, and Sinsheimer (18). It is not known whether knots are absent

in these rings. The diagrammatic representations of the circular forms presented here are the simplest ones necessary to account for the experimental results cited.

Hershey, Burgi, and Ingraham (5) in a sedimentation study of viral lambda

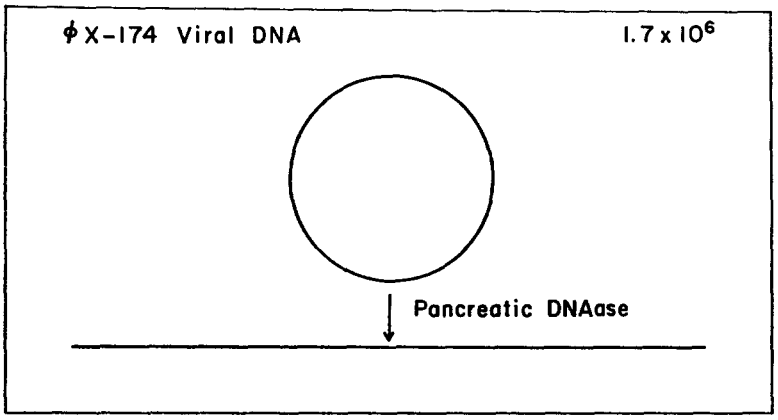


FIGURE 1. Diagrammatic representation of two forms of ϕX174 viral DNA.

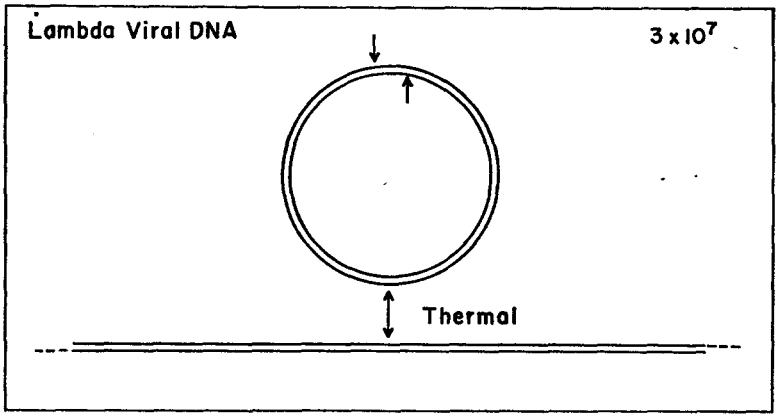


FIGURE 2. Diagrammatic representation of the formation of the weakly closed circular lambda DNA. The arrows indicate the region in which the complementary bases present at the ends of the linear molecule have paired in the circular molecule (19).

DNA observed a complex behavior which they successfully resolved in the following experiments. A single sedimenting species, linear λ-DNA, was obtained after heating dilute viral DNA solutions to 65°C and quick cooling in dilute saline. Slow cooling of DNA solutions at <10 μg/ml, led to the formation of a new species, 1.13 × faster than linear lambda DNA. The new form could be readily reconverted to linear lambda by a second heating and quick cooling cycle. When more concentrated solutions were slowly cooled, an addi-

tional species 1.41 × faster than linear lambda was formed. They reasoned that λ-DNA contained two cohesive regions located near the ends of the molecule. These regions react intramolecularly in dilute solution to form cyclic molecules, and polymerize in more concentrated solution to form tandem linear and cyclic polymers of lambda molecules. The low reaction temperatures suggested to them, as expressed in a later paper (19), that complemen-

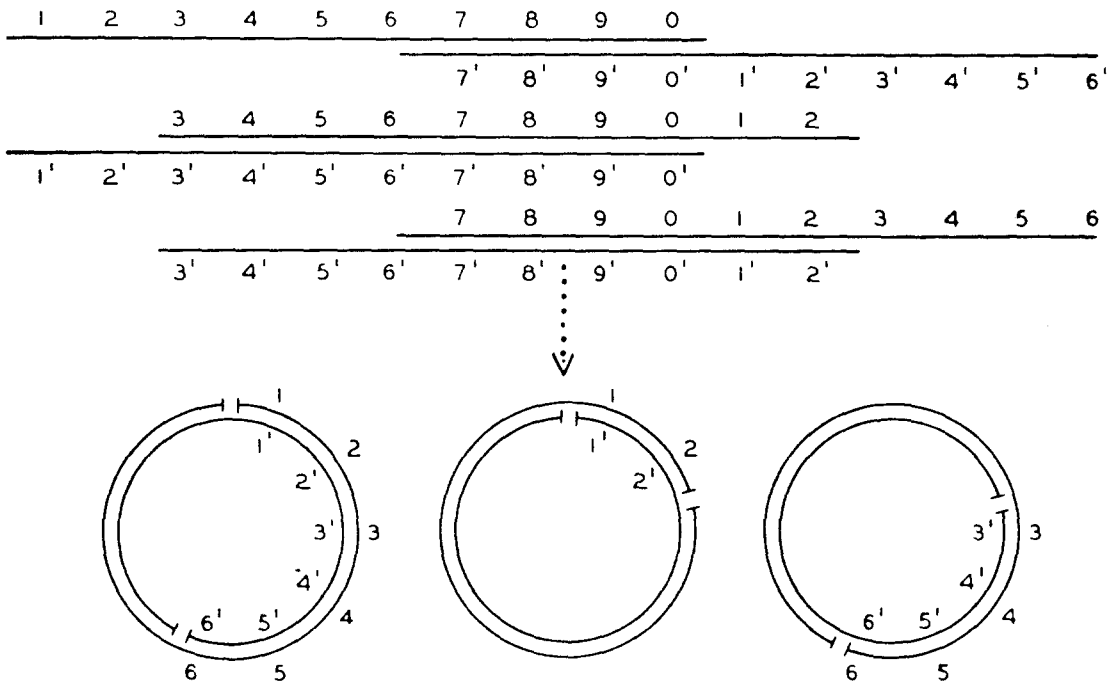


FIGURE 3. The diagram shows how a collection of linear duplex DNA molecules with sequences that are circular permutations of each other can produce circular molecules by chain separation followed by random association and reformation of the duplex structure. In principle nearly every final molecule could be circular. *Figure reprinted by permission from Proc. Nat. Acad. Sc., 1964, 52, 1297.*

tary hydrogen-bonding bases present as single-stranded regions at the ends of the DNA were responsible for the closure. Ris and Chandler (20) showed by electron microscopy that specimens of lambda DNA prepared by the method of Kleinschmidt and Zahn (20 *a*) indeed contained circular molecules of the length expected for this DNA. Thus viral lambda DNA forms weakly closed circular duplex molecules (Fig. 2).

Thomas and MacHattie (13) performed an ingenious experiment to test the hypothesis that T2 is a population of circularly permuted linear molecules. This can be visualized by imagining a population of linear molecules derived by random cleavage of identical circular duplex molecules.

For these experiments they brought T2 DNA into the single-stranded state and then after annealing observed the product in the electron microscope. Indeed it was highly populated with duplex circles which they have called "artificial" (Fig. 3).

In its simplest form the DNA from the tumor virus polyoma may be represented as an *extended* circular duplex with no special closure. The DNA consists of two complementary circular single strands intertwined in a Watson-

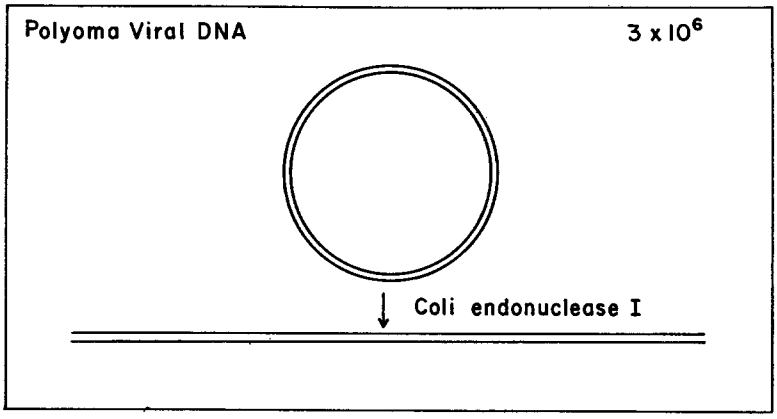


FIGURE 4. *E. coli* endonuclease I is known to produce a double chain scission of a duplex DNA (13 *a*). The first product of the action of the *E. coli* endonuclease I on circular polyoma DNA would be a homogeneous linear molecule. This linear molecule would be degraded by additional hits. This action of the enzyme on polyoma DNA was observed in sedimentation studies (25) and is further supported by unpublished electron micrograph data of the authors.

Crick structure. This DNA has been converted to a linear form by the action of endonuclease I from *E. coli* (13 *a*) (Fig. 4).

The critical experiments which led to discovery of the closed circular structure are now reviewed. Polyoma DNA was shown by Weil (21) to reanneal readily after heating to 100°C in 0.001 M NaCl. This result suggested that the single strands might be bonded to each other by covalent bonds. Dulbecco and Vogt (6) and Weil and Vinograd (7) arrived at the conclusion that the bonds between the strands were "topological" bonds as defined by Wasserman (22) who in 1960 prepared a catenane consisting of two interlocked 36 carbon chain rings. Unlike the fused rings in organic chemistry, Wasserman's topologically bonded ring pairs contain no shared atoms. Yet the rings could not be separated without breaking a covalent bond.

The analytical band sedimentation velocity pattern of polyoma DNA prepared by phenol treatment of the virus contains three sedimenting forms which we shall refer to as I, II, and III in order of descending sedimentation co-

efficients 20S, 16S, and 14.5S (Fig. 5) (7). Preparations contain variable amounts of II which can be kept as low as 1% by control of preparative variables. III is always observed in our preparations at the 5 to 10% level. Dul-

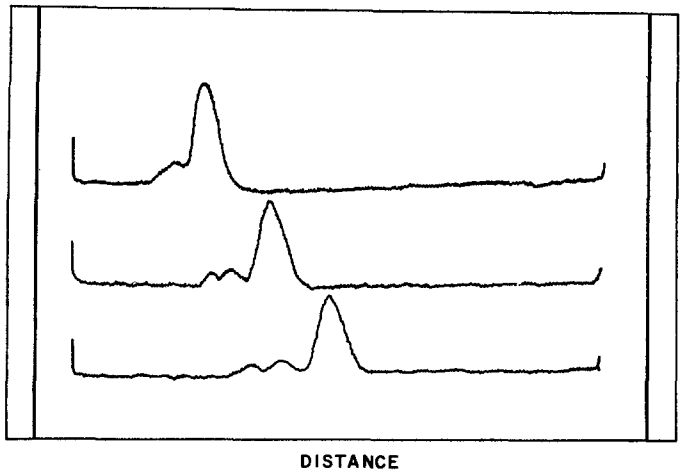


FIGURE 5. Band sedimentation velocity analyses of polyoma DNA in neutral 3 M CsCl. *Figs. 5, 7, and 8 are reprinted by permission from Proc. Nat. Acad. Sc., 1963, 50, 730.*

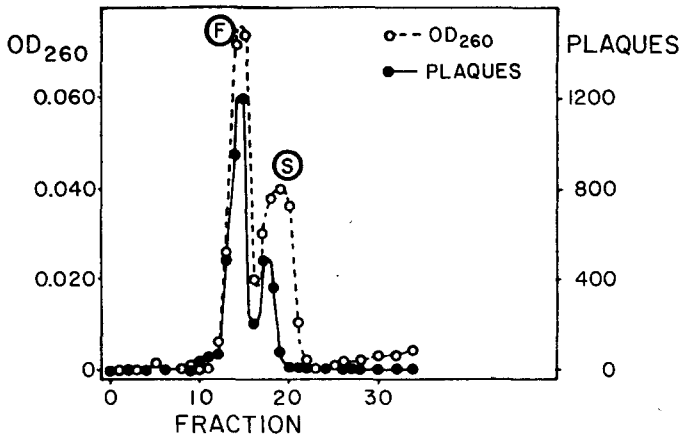


FIGURE 6. Band sedimentation of polyoma DNA at pH 7.5. Polyoma DNA extracted once with phenol and sedimented at 35 K RPM in CsCl of density 1.50 for 4 hr. *Figure reprinted by permission from Proc. Nat. Acad. Sc., 1963, 50, 236.*

becco and Vogt (6) showed that I and II were both infective in the plaque assay for the DNA (Fig. 6). It can be seen that the infectivity falls below radioactivity in the slow band in this preparative velocity separation indicating that the unresolved component III is noninfective.

The principal findings which gave rise to the proposal of the covalently

closed circular duplex were obtained from sedimentation studies of this DNA in alkaline CsCl solutions.

It had been shown earlier (23) that all guanine and thymine ring protons are titrated in buoyant CsCl at pH 12.4, and that in these solutions the single strands of linear DNA separate and form a new buoyant band, 0.06 density unit denser than the untitrated duplex. This increase in buoyant density is the result of the loss of secondary structure and the addition of one-half of a mole

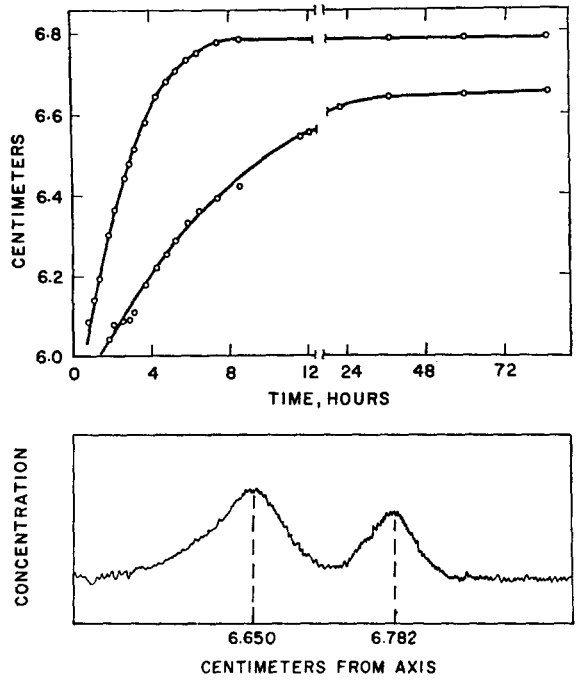


FIGURE 7. Band buoyancy analysis of polyoma DNA in alkaline CsCl. Upper, position of 16S and 53S components at various times. Lower, densitometer records of bands at 72 hr, 25°C. CsCl ρ = 1.76, 0.04 M K_3PO_4, pH 12.3.

of Cs ion per mole of nucleotide. The alkali shift was known to be independent of base composition of the DNA.

In more dilute alkaline CsCl solutions, 1 to 3 M, the sedimentation velocity behavior of fully titrated, separated, and nonaggregating single strands may be studied. This latter procedure derives in part from the earlier work of Freifelder and Davison (24) who studied the sedimentation velocity properties of the single strands of DNA after thermal treatment of duplex DNA in formaldehyde solutions.

When a 40/60 mixture of polyoma I and II is sedimented as bands into an almost buoyant alkaline CsCl solution, we observe both the velocity behavior and the buoyant behavior of these two materials (Fig. 7). Species I moves

rapidly from the top of the cell to a high buoyant density with a shift of 0.08 g/ml. Species II moves at a slower rate to the expected density with a shift of 0.06 g/ml. The properties of the neutral and alkaline components are summarized in Table I, in which it may be seen that the 20S material moves in alkali with a sedimentation coefficient of 53S as determined in 1 M NaCl pH 12.5, and that the neutral 16S material moves in alkali with a sedimentation coefficient of 16 to 18S which, as we shall see later, may be resolved into two species. The molecular weight of I estimated from the alkaline band width was approximately twice as large as the molecular weight of the single strands derived from II, as estimated from the sedimentation coefficient in alkali.

TABLE I

PHYSICAL AND BIOLOGICAL PROPERTIES OF POLYOMA DNA COMPONENTS

pH		I	II	III
8	Amount, %	80–90	1–20	5–10
	$s^0_{20,w}$	20.3	15.8	14.4
	ρ^0_0	1.709	1.709	1.69_9, 1.709
	Infectivity	+	+	−
12.5	$s^0_{20,w}$	53	16, 18	
	ρ^0_0	1.784	1.766	
	Infectivity*	+	−, +	

* Dulbecco and Vogt (6).

It was reasoned that II was a normal duplex DNA with dissociable single strands and that I was a modified molecule that was abnormal in that single strands did not dissociate from each other in alkali. The large ratio of velocities of the alkaline forms, 3.3 to 1, ruled out a covalent cross-link between strands because simple doubling of the molecular weight of a random coil by such bonding should increase the sedimentation coefficient by only a factor of approximately 1.4, based on the usual relations between S and M for random coils. It was argued that the 53S species was the compact denatured double-stranded form to be expected if I were a covalently closed circular duplex. The elements in the single strands are restrained in space now not only by bonds along the chain but also by the requirement that the strands be arranged around each and that each strand be circular. The structure is therefore more compact. This conclusion was supported by electron micrographs obtained by Stoeckenius (7) on the materials prepared by Weil and Vinograd (Fig. 8). The micrographs show several circular molecules, both extended and twisted, as shown in the selected fields below, as well as linear molecules of the same length as shown in the larger typical field above. It was assumed at this

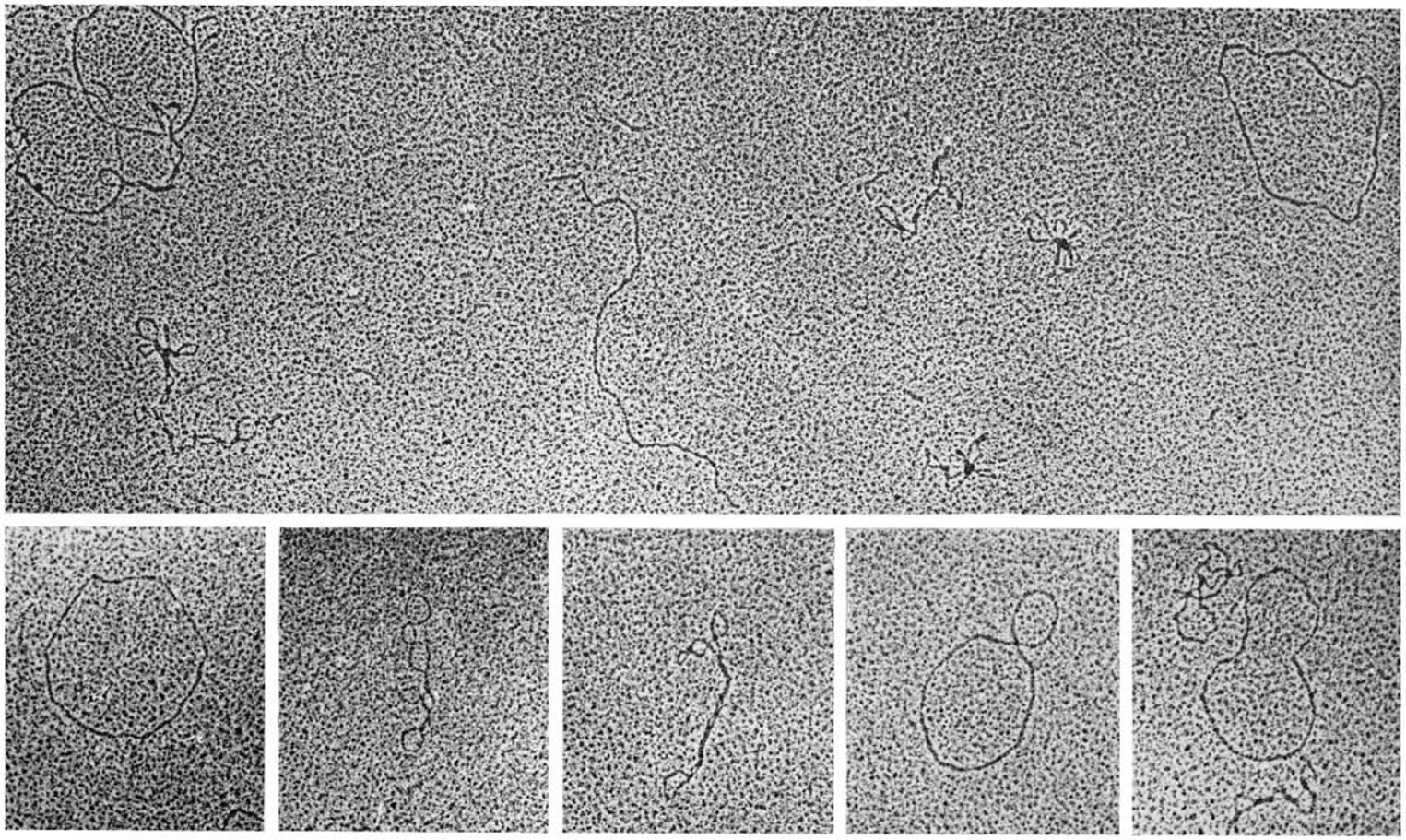
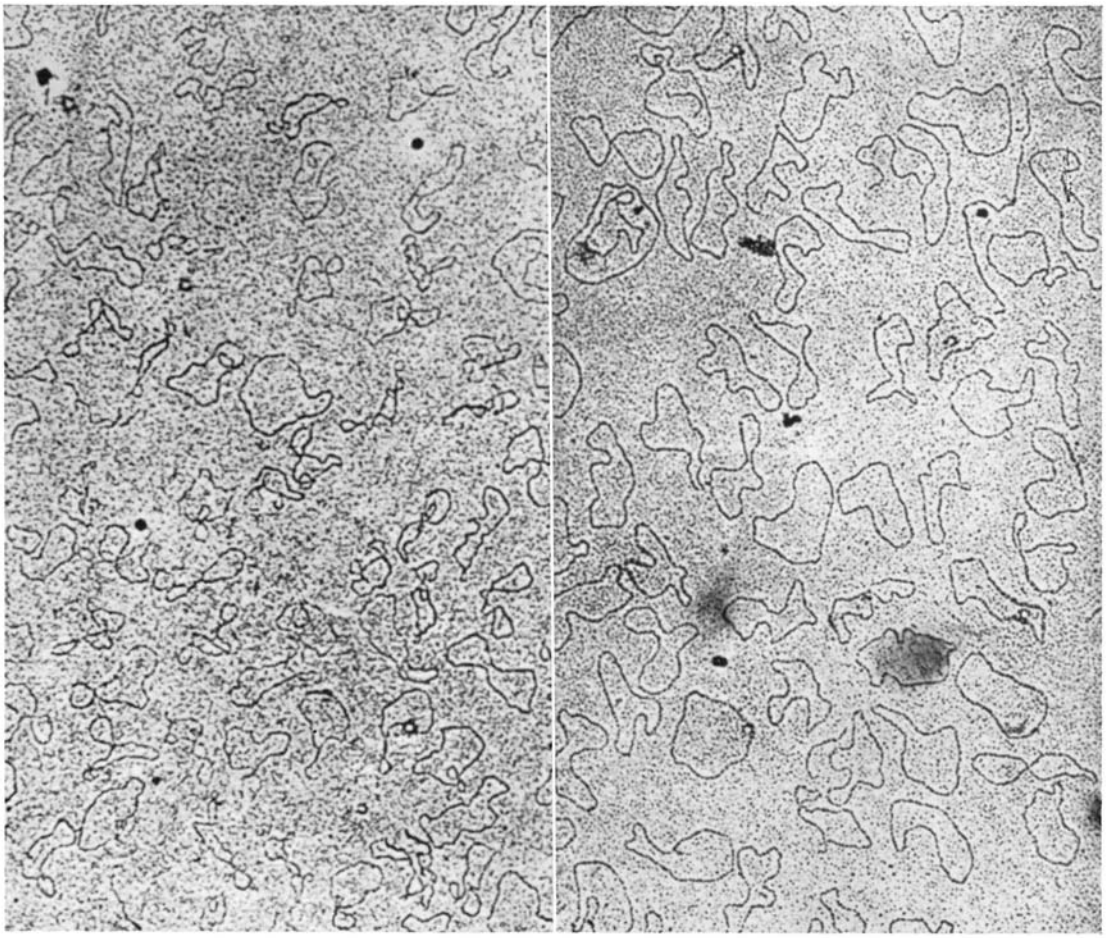


FIGURE 8. Electron micrographs of polyoma DNA. × 70,000. Upper, a field showing linear and cyclic forms. Lower, selected cyclic forms.

stage of the research that I was circular, that II was linear, and that III was a contaminating linear DNA of unknown origin.

Dulbecco and Vogt (6) found that treatment of polyoma I with low concentrations of pancreatic DNAase gave rise, with first-order kinetics, to a nonin-



(*a*) (*b*)

FIGURE 9. Electron micrographs of polyoma DNA. × 25,000. The materials in (*a*) and (*b*) were prepared by treatment of polyoma I with pancreatic DNAase. (*a*) was withdrawn from the reaction mixture after 5% conversion of I to II; (*b*) after 95% conversion. *Figs. 9, 11, 12, and 14 reprinted by permission from Proc. Nat. Acad. Sc., 1965, 53, 1104.*

fectious DNA with the sedimentation properties of polyoma II. They suggested that the circular duplex was under strain, and that a bond in the complementary strand opposite the single strand scission, introduced by the enzyme action, hydrolyzed under the influence of the strain to form a linear molecule.

In a further investigation (25) in our laboratory it was learned that

1. Component II is a circular duplex.
2. This slow circular duplex is formed by introducing *one* single strand scission in the faster circular duplex.

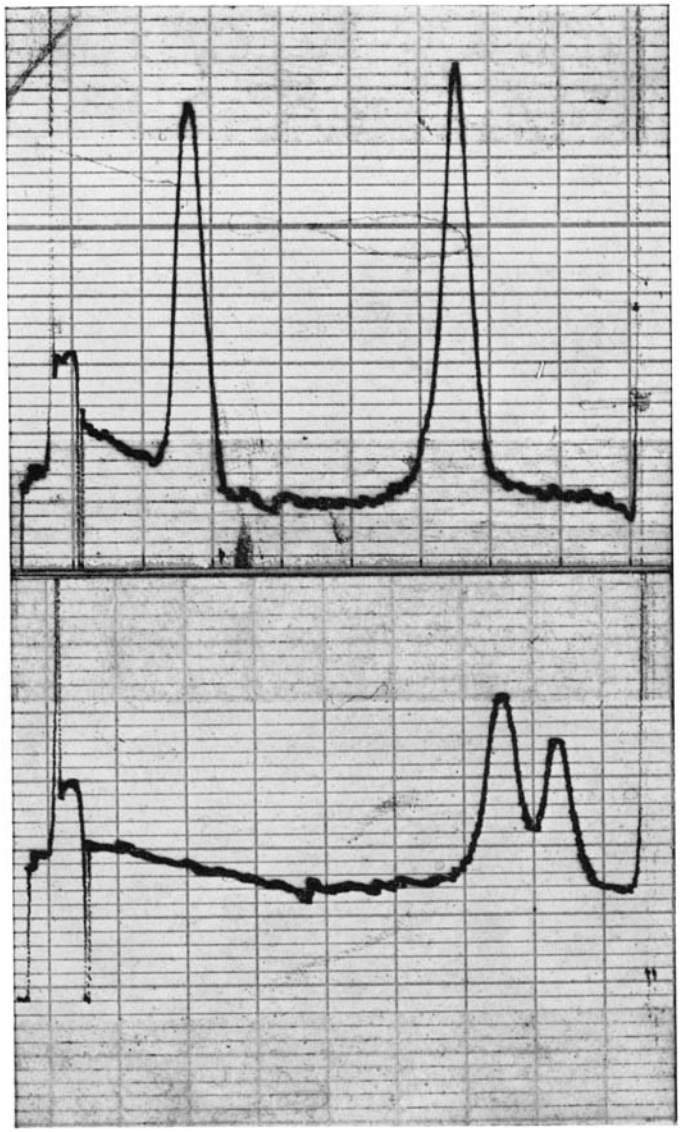


FIGURE 10. Sedimentation velocity patterns of polyoma DNA in alkaline CsCl. The field is directed toward the right. Upper, after 30 min of sedimentation at 44,000 RPM, the leading component is the 53S intact denatured polyoma I. The slow component is an unresolved mixture of 16S single linear and 18S single circular strands. Lower, after 90 min of sedimentation the slow component resolves into single linear 16S and 18S single circular strands.

3. Further single strand scissions do not affect the sedimentation coefficient of II.
4. The conversion products when not excessively nicked are infective.

The foregoing results raised a new problem. Why does the viral DNA, an intact duplex ring, sediment 25% faster than the same material containing one single strand scission? Before proposing a solution to this problem we will examine the experiments that lead to the above conclusions regarding the structure of II.

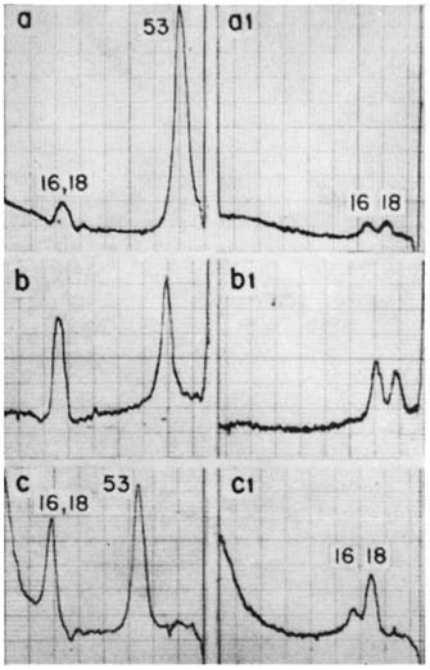


FIGURE 11. Sedimentation velocity patterns of polyoma DNA in alkaline CsCl. The left and right patterns are scans at about 30 min and 90 min after sedimentation begins. The field is directed toward the right, at 44,000 RPM. (*a*, *a*1), control, Component I isolated in a sucrose gradient treated identically as in (*b*) and (*c*) except for the absence of enzymes. (*b*, *b*1), pancreatic DNAase treatment, reaction stopped by shifting pH to 9.8 with 1 M glycine buffer. The leading band in (*a*), (*b*), and (*c*) is the 53S component. The resolved slower bands in (*a*1), (*b*1), and (*c*1) are the 16S and 18S components. (*c*, *c*1), effect of heat denaturation followed by *E. coli* exonuclease I treatment, product of (*b*) heated 5 min 100°C, cooled rapidly, and treated with the exonuclease for 90 min at 37°C. Reaction stopped with EDTA.

Both isolated natural component II, and II prepared by complete conversion of I with pancreatic DNAase, contained exclusively circular molecules in electron micrographs (Fig. 9). The figure shows the results obtained after treatment with the enzyme. The possibility that linear molecules were selectively excluded in the preparation of the specimens for electron microscopy was eliminated in a reconstruction experiment with a synthetic mixture of 90% linear molecules, isolated III, and circular II. The linear molecules were found in the expected relative amount.

Component II formed by light treatment with pancreatic DNAase should contain circular single strands which ought to be observable, based on the ϕX DNA results, as a sedimenting species in alkali about 10% faster than the fastest linear strands. An example of such a result is shown in Fig. 10 in which

the sedimentation pattern of polyoma DNA in alkali after a 60% conversion of purified component I to component II is shown. In the upper frame, after 30 min of sedimentation, the leading component is the 53S intact denatured molecule and the slow component is a mixture of 16S single linear and 18S

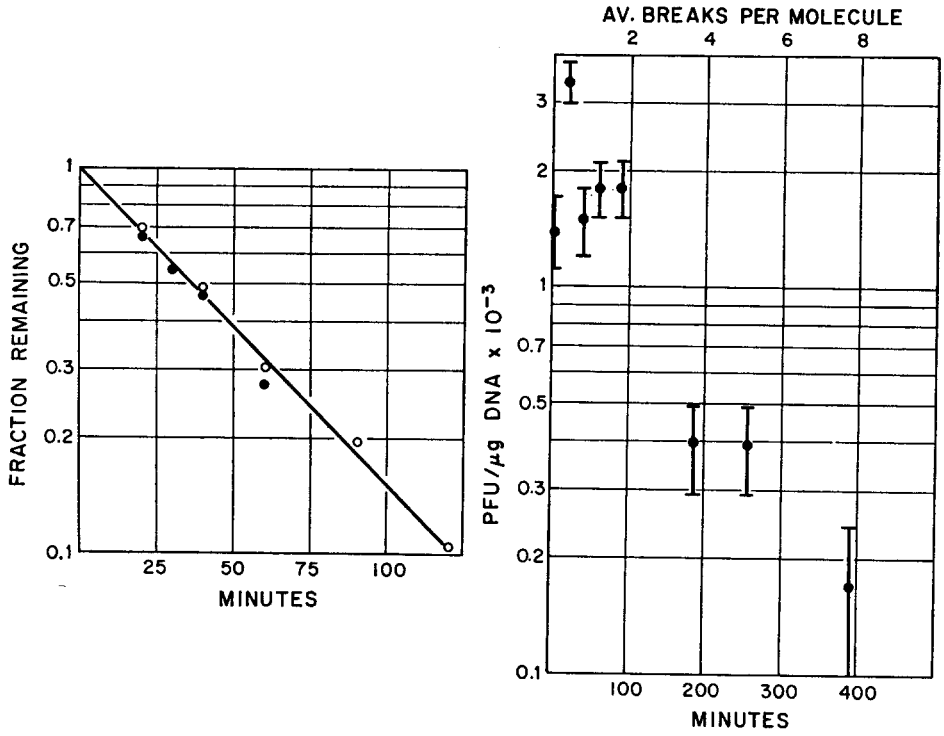


FIGURE 12. Chemical and biological effects of pancreatic DNAase treatment. (*a*) Analyses for single-stranded and double-stranded DNA. Extent of conversion was determined by band-sedimentation velocity experiments with photoelectric scanner. Open circles, (I)/(I + II) in neutral CsCl bulk solutions. Filled circles, 53S/(total) in alkaline CsCl, pH 12.3. Areas under bands were corrected for radial dilution. Aliquots were withdrawn at the indicated times and the reaction was stopped with EDTA. (*b*) Infectivity of samples withdrawn from incubation in (*a*). The time for a unit average number of hits was obtained from (*a*) at 63% conversion. The error bars give the standard deviation from 16 replicate plates.

single circular strands which resolve after 90 min as seen in the lower frame. Sedimentation proceeds from left to right. There are fewer single rings than single linear strands as one would expect if the sites of enzymatic hydrolysis were randomly distributed along the molecule.

The two slower alkaline components have been identified as single linear and single circular strands by a variation of the experiment originally performed by Fiers and Sinsheimer (17) (Fig. 11). An aliquot of the product of

the pancreatic DNAase digestion (40% conversion) was heat-denatured and treated with *E. coli* exonuclease I. The upper 30 and 90 min frames are patterns obtained at pH 12.5 for the untreated, but heated material. The middle frames are the patterns obtained with the enzymatically treated sample in which the elevation in the amount of strand-separated material can be seen in the slower band profiles. In the bottom frames observe the decrease in the relative amount of 16S linear material after treatment with exonuclease I. It may be concluded that 18S material contains a single-stranded covalently closed ring similar to that of ϕX DNA.

We now examine the postulate that one single strand scission in the duplex is adequate to convert circular polyoma I to circular II. If only one chain scission is necessary, the rate of conversion of I to II should be the same as the rate of conversion of the 53S component to the slower moving single-stranded molecules in alkali. If more than one break were necessary to convert I to II, a faster rate of conversion would be seen in alkali.

It is seen in Fig. 12 that the alkaline analyses, dark points, and the neutral analyses, light points, give within the experimental error the same extent of conversion. Therefore, the conversion of I to II occurs whenever the first single strand scission is introduced. The conversion, moreover, appears to be first order. While the infectivity declines at a slower rate than the conversion of I to II, the scatter in the data precludes firm conclusions regarding the kinetics of inactivation. It is clear, however, that the first single strand scission in this duplex DNA is not lethal.

More extensive treatment with pancreatic DNAase so as to completely convert I to II (>4 average breaks per molecule) caused no detectable change in the sedimentation coefficient of II.

The above experiments clearly established that component II is a circular duplex with one or more single strand scissions. We now turn attention to the structure of component I. The high sedimentation coefficient of I relative to II indicates that the viral component is either more compact or larger in mass than the circular conversion product. In the extreme case of constant friction a 20% reduction in mass is required to account for the change in S. An excision of viral DNA without a loss in infectivity is regarded as being unlikely. The identical buoyant densities of I and II ruled out the loss of protein or other nonDNA mass to the extent of 20%.

Two kinds of experiments suggest that a particular kind of compact structure—a *twisted circular form*—is responsible for the high sedimentation coefficient of polyoma DNA as a duplex at neutral pH.

1. A study of the sedimentation velocity in 3 M CsCl of a mixture of the three components of polyoma DNA as a function of pH revealed a complicated pH-melting curve for component I (Fig. 13). Component II behaved normally, and moved faster as denaturation increased until strand separation

occurred with an attendant sudden drop corresponding to the formation of separated linear and circular single strands at pH 11.8. Component I, like II, was at first insensitive to pH. At pH 11.5, however, the sedimentation coefficient first dropped, and then in the pH range 11.6 to 11.8 was the same as for polyoma II. Only one moving band is observed in this pH range. The sedimentation coefficient of I then increased to the very high value characteristic

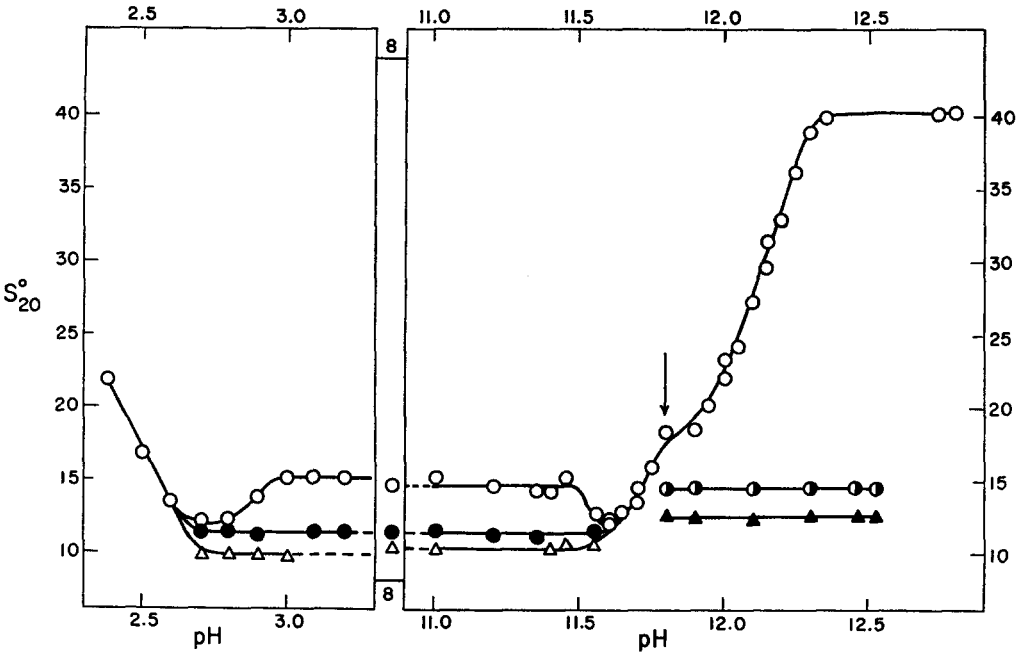


FIGURE 13. Sedimentation velocity–pH titration of the three components in polyoma DNA. I, open circles; II, filled circles; III, open triangles, 29,500 RPM. 18S single circular strands, half-filled circles, 16S single linear strands, filled triangles, 44,770 RPM. Sedimentation coefficients at 20°C in CsCl, ρ = 1.35 g cm^{-3}, are not corrected for solvent viscosity η_r = 0.925, or buoyancy effects. The values at pH 8.0 and 12.4 are the means of 12 and 7 determinations, respectively. The data in the acid region were obtained by S. Mickel, unpublished.

of the *double-stranded cyclic coil*. Essentially the same results were obtained in alkaline 1.0 M KCl solutions. The dip in the sedimentation velocity–pH curve, which was also observed just prior to acid denaturation, was initially unexplainable. If, however, polyoma DNA I contains right-handed twists, such a dip in the pH-melting profile would be required (Fig. 14). In the early stages of denaturation some of the duplex turns, which are known to be right-handed, unwind. To preserve the number of turns in the topologically invariant structure, the unwinding of the duplex is accompanied by an unwinding of the twists of the twisted circular structure. If the tertiary turns

were originally right-handed, progressive duplex unwinding would pass through a stage, I′, in which tertiary turns would be totally absent. The extended configuration I′ is similar to that in polyoma II, and both I′ and II would have similar sedimentation velocities. The above mechanism requires that this sedimentation velocity dip occur under all denaturing conditions. Crawford and Black (10) reported the same behavior upon heating polyoma

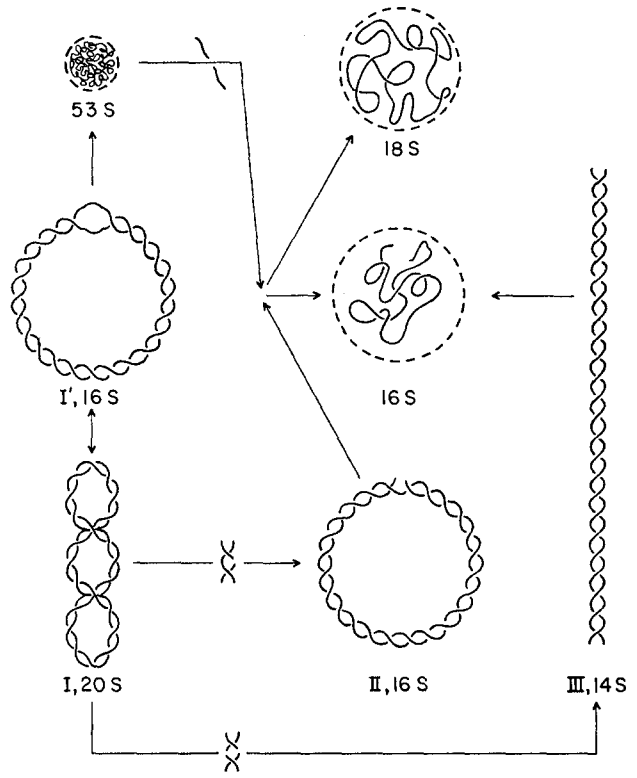


FIGURE 14. Diagrammatic representation of the several forms of polyoma DNA. The dashed circles around the denatured forms indicate the relative hydrodynamic diameters. The sedimentation coefficients were measured in neutral and alkaline NaCl solutions. The twist in I should be right-handed.

and SV40 DNA in formaldehyde solutions to various temperatures (Fig. 15). Under the variety of denaturing conditions employed we always observe the same characteristic sedimentation velocity-denaturation curve. In closed double circular DNA, the number of degrees of angular rotation of one strand around the other is invariant.

2. The twisted circular structure provides a satisfactory explanation for the configurational change that occurs when one single strand scission is introduced into the molecule. Such a scission generates a swivel (4), i.e. a site for

free rotation in the complementary strand opposite the break and relieves the topological restraint responsible for the twisted configuration.

In an earlier publication the appearance of twisted molecules in electron micrographs of specimens prepared by the Kleinschmidt-Zahn (20 *a*) method was regarded as indicating the existence of the twisted form in solutions containing polyoma I. It was noted that no twisted forms were observable in micrographs prepared with polyoma II. Recently Kleinschmidt *et al.* (25 *a*)

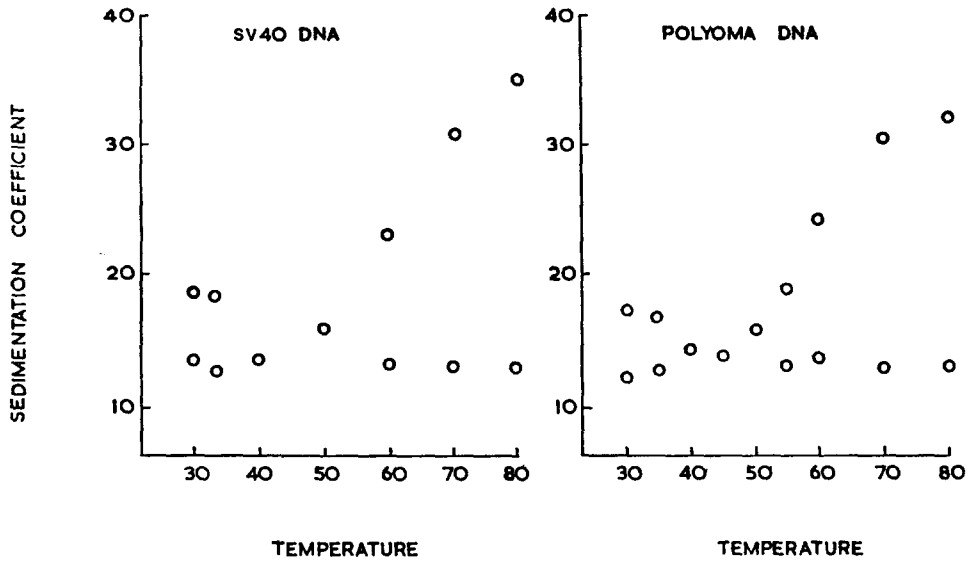


FIGURE 15. The effect of heating SV40 and polyoma DNA in formaldehyde. The sedimentation coefficients were determined at 20°C in M NaCl containing 0.03 M phosphate, pH 7.8, and 2% formaldehyde. They are presented without correction for buoyancy or viscosity of the solvent. *Figure reprinted by permission from Virology, 1964, 24, 388.*

have suggested, in order to explain the nonconstant lengths of Shope papilloma DNA in various preparations of DNA, that a small amount of local denaturation occurs during preparation of the specimens for electron microscopy. Such an effect, which we cannot exclude, could cause the formation of twisted molecules in electron micrographs prepared with polyoma I. The twists in such molecules would be left-handed and not right-handed as we have proposed. Unfortunately the direction of twists cannot be observed in electron micrographs prepared by the present procedures. In polyoma II these left-handed twists would not form because of the presence of the swivel.

Enhanced Stability of Closed Circular Duplex DNA

It was noted in the earlier papers (6, 7) on polyoma DNA that I was more resistant to alkaline denaturation than II. This observation has been extended

(26) in a study of the thermal and alkaline-induced melting of polyoma DNA. Since the conformational entropy of unwinding and the entropy of strand separation are driving forces for the helix-coil transition in the denaturation of DNA, it is to be expected that polyoma I, in which these entropic effects are forbidden by topology, will be thermodynamically more stable than II.

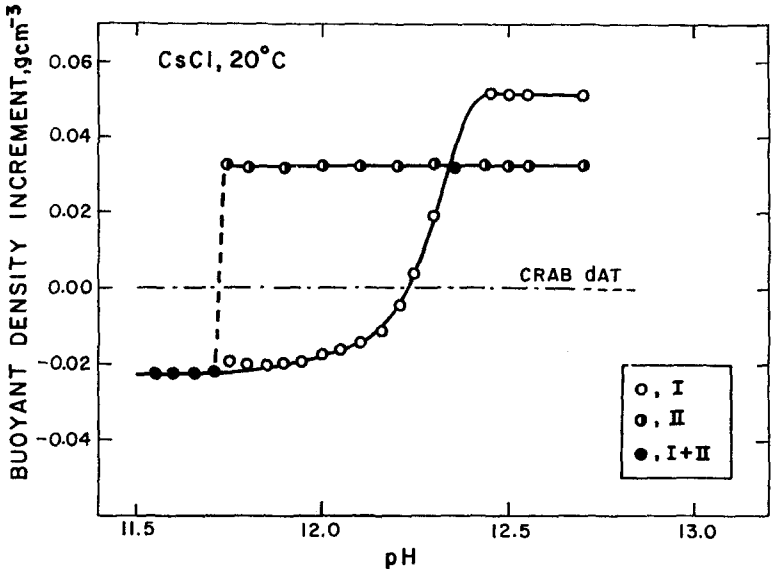


FIGURE 16. Buoyant density titration of polyoma DNA containing 70% component I and 30% component II. All measurements were made relative to crab dAT DNA as a marker (26).

Buoyant Density Titration

The results of the buoyant density titration are seen in Fig. 16. It may be seen that the midpoint of the transition of the doubly closed form occurs at 0.56 pH unit higher than for the strand-separable molecule. The higher buoyant density of titrated intact II relative to the single strands noted in the earlier work is confirmed here.

The transition for the closed form is less cooperative. The breadth of the transition (10 to 90%) is 0.32 pH unit compared to less than 0.05 pH unit for the nicked form.

Thermal Denaturation Studies

In studies of absorbance at 260 mμ vs. temperature it is necessary to know that single strand scissions are not introduced during the course of its measurements. For this reason neutral and alkaline sedimentation velocity analyses were performed at the completion of each experiment.

As in the alkaline titration the closed double-stranded form was substantially more stable than the nicked form (Fig. 17). In standard saline citrate, the melting point of the nicked form was 89°C in agreement with the guanine-cytosine content estimated from the buoyant density. The intact form, however, first begins to melt at this temperature but the transition is less than 10% complete at 100°C. At lower ionic strengths, SSC/10, we observed that about 40% of the molecules underwent a reaction that results in chain scission after heating to 100°C at 0.66° per minute.

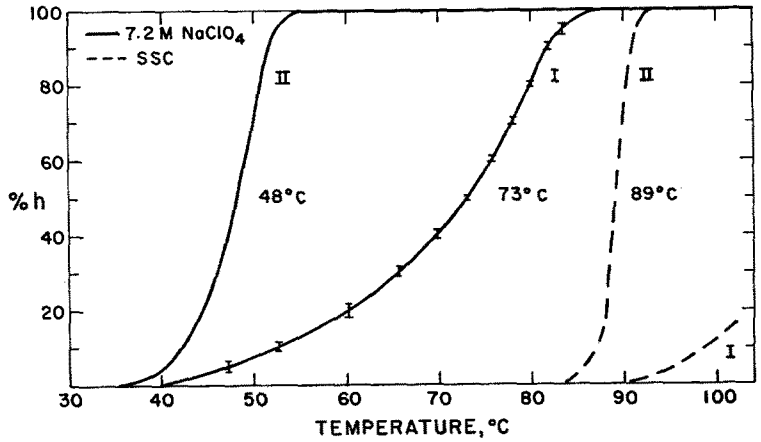


FIGURE 17. Thermal denaturation of polyoma DNA in 7.2 M $NaClO_4$ and SSC (0.15 M NaCl, 0.015 M Na citrate). Unbroken line, 7.2 M $NaClO_4$; broken line, SSC. The T_m values obtained for several control DNA's of varying G-C content showed excellent agreement with previously published measurements (26).

Hamaguchi and Geiduschek (27) in an investigation of the melting of DNA in various salt solutions showed that T_m, the melting temperature of DNA, in 7.2 M $NaClO_4$ is reduced about 40°C relative to the value in 0.15 M NaCl. Moreover, the T_m was again linearly related to the base composition with the slope being 0.56°C/per cent GC compared with 0.41°C/per cent GC in SSC. The curves on the left-hand side of Fig. 16 give the means of 5 separate experiments for polyoma I and II. The melting temperatures in $NaClO_4$ are 58° and 73°C which would correspond in SSC to 88° and 107°C. The breadth of the transition is again large for the intact molecule and the corresponding values are 6° and 25°C.

The effect of the covalent closure thus corresponds to an increase of 19°C in the melting temperature in standard saline citrate and an increase of 0.56 pH unit in the pH of melting at 20°C in alkaline buoyant CsCl. If it is assumed that the increase in melting temperature is due solely to entropic effects, i.e. the enthalpy of the transition is the same for the intact and singly nicked forms, we find that entropy of unwinding and of strand separation accounts

for about 5% of the total entropy of the transition, and 10 to 20% of the change in configurational entropy (28) that takes place during melting of DNA.

The Common Properties of Covalently Closed Circular Duplex DNA

The large effects of the covalent closure in circular duplex DNA described above for polyoma DNA should be observable with all intact covalently closed duplexes regardless of the size of the molecule. These special properties,

TABLE II

PROPERTIES OF COVALENTLY CLOSED CIRCULAR DUPLEX DNA

	Polyoma	SV40	Papilloma		RF-ϕX	λ injected
			Rabbit	Human		
Elevated sedimentation coefficient* in strand-separating solvents	+ (6, 7)	+ (10, 29)	+ (11)	+ (12)	+ (8, 30)	+ (14, 15)
Elevated buoyant density‡ in alkaline CsCl	+ (7)	+ (29)	...	...	+ (8, 30)	...
Elevated T_m§	+ (26)	+ (29)	...	...	...	...
and pH_m‖	+ (6, 7, 26)	+ (29)	...	...	...	...
Circular molecules in electron micrographs¶	+ (7)	+ (29)	+ (25 *a*)	...	+ (9, 30)	...

* Sedimentation coefficients relative to separated single strands. The ratio is approximately 3.5.

‡ Buoyant density relative to buoyant density of single strands. The difference is approximately 0.02 g cm^{-3}.

§ T_m relative to T_m of material containing single strand scissions. The effect for polyoma and SV40 DNA is about 25°C in 7.2 M $NaClO_4$.

‖ Midpoint (pH_m) of the pH melting curve in alkaline CsCl relative to the pH_m for material containing single strand scissions. The effect for polyoma and SV40 DNA is about 0.5 pH unit.

¶ This is a necessary but not sufficient indication for covalently closed duplex rings.

summarized in Table II, are the high sedimentation coefficient in strand-separating solvents, the elevated buoyant density in alkaline CsCl, and the elevated midpoints of the thermal and pH melting curves. All the foregoing properties may be understood as a consequence of the inability of the strands to unwind in a topological sense and the inability to separate physically from each other. The results given in Table II show that the above common properties are indeed shared by all such covalently closed duplexes so far described.

The elevated sedimentation coefficients in alkali or after heat treatment in formaldehyde are readily observed and have served as a principal method for the detection of this DNA structure in viruses or in infected cells.

The Common Properties of Twisted Circular Duplex DNA and the Origin of the Tertiary Turns

The surprising presence of a form of tertiary structure in intact polyoma DNA immediately raised the problem of how this structure arose in the host cell.

The authors and their collaborators have suggested that if the final closure were made in a completely complementary DNA while the structure, for some unknown reason, were partially unwound a right-handed twisted structure would form spontaneously as soon as the DNA was brought into an environment in which the Watson-Crick structure is the stable form. This is illustrated as the conversion I′ to I in Fig. 14. The spontaneous formation of the tertiary turns is thus the consequence of the topologically required invariance of the winding number in closed circular duplex structures. Polyoma DNA contains about 5000 base pairs. As a linear molecule it contains 500 right-handed turns, based on the Watson-Crick structure of one turn (360°) per 10 base pairs. If at the time of the final closure in the host cell the mole-

TABLE III

COMMON PROPERTIES OF TWISTED CIRCULAR DUPLEX DNA

			Papilloma			
	Polyoma	SV40	Rabbit	Human	RF-ϕX	λ injected
Usually found with slower sedimenting circular DNA at neutral pH	+ (6, 7)	+ (10, 29)	+ (11)	+ (12)	+ (8, 30, 31)	+ (15)
Dip in the sedimentation velocity-melting curve	+ (10, 25)	+ (10)	...	+ (12)	...	...
Converted to a slower sedimenting circular DNA by one or more single strand scissions	+ (25)	...	...	+ (12)	+ (31)	...

cule contained, for example, only 480 right-handed turns it would have a winding number of 480. Formation of 500 duplex right-handed turns then requires the formation of 20 superhelical turns in a left-handed sense. Molecules which contain left superhelical turns (such a molecule may be visualized as a circular duplex wound around a torus) convert spontaneously into right-handed twisted structures. We were unaware of the change in handedness in the first publication (25) on the twisted form of polyoma DNA.

In the first paper on this subject (25) it was suggested that a "blister" as shown in I′ (Fig. 14) might be present to accommodate the closing enzyme(s). If the final closure were made with these present and the enzyme(s) were then removed, I′ would spontaneously wind into a complete Watson-Crick structure I. The foregoing proposal leads to the expectation that all closed circular DNA's should have a similar number of twists and that the change in the frictional coefficient caused by tertiary turning should be smaller for larger DNA's.

The finding of Bode and Kaiser (15) that the ratio of sedimentation coefficients of I/II for closed circular lambda DNA was essentially the same as

the value for polyoma DNA even though the molecular weight of lambda DNA is approximately 10 times greater than that of polyoma DNA, makes the foregoing suggestion appear to be unlikely.

An obvious alternative suggestion is that the pitch of the duplex helix of the DNA in the cell at the time of final closure is larger by a few per cent than the pitch of the helix in the Watson-Crick structure in the purified DNA. The number of twists that would arise for such a reason would be proportional to the length of the DNA.

All the known closed circular duplex DNA's appear to contain twists. The properties associated with the tertiary structure are summarized in Table III. It is seen that no exceptions have as yet been reported.

The Swivel in Circular Duplex DNA

Cairns (4) recognized that semiconservative replication of circular DNA required the presence of a "swivel" which would allow the unreplicated duplex ahead of the growing point to rotate around the duplex axis so as to permit unwinding of the parental strands. A swivel was similarly invoked by the authors and their colleagues (25) to explain the effect of a single strand scission which allows the tertiary turns in the twisted circular duplex to unwind and relax the circular structure. The swivel is to be found in the rotatable covalent bonds in the complementary strand in a region across the duplex from the site of the single strand scission.

This paper is Contribution 3347 from the Gates and Crellin Laboratories of Chemistry.
It is a pleasure to thank P. Laipis, R. Watson, and S. Mickel for permission to quote their unpublished results, R. Kent for assistance with the manuscript and J. Petruska, W. R. Bauer, and R. Radloff for helpful discussions.
This work was supported in part by grants HE 03394 and CA 08014 from the United States Public Health Service.

REFERENCES

1. JACOB, F., and WOLLMAN, E. L., *E. coli, Symp. Soc. Exp. Biol.*, 1958, **12**, 75.
2. STREISINGER, G., EDGAR, R. S., and DENHARDT, G. H., *Proc. Nat. Acad. Sc.*, 1964, **51**, 775.
3. FIERS, W., and SINSHEIMER, R. L., *J. Mol. Biol.*, 1962, **5**, 424.
4. CAIRNS, J., *J. Mol. Biol.*, 1963, **6**, 208.
5. HERSHEY, A. D., BURGI, E., and INGRAHAM, L., *Proc. Nat. Acad. Sc.*, 1963, **49**, 478.
6. DULBECCO, R., and VOGT, M., *Proc. Nat. Acad. Sc.*, 1963, **50**, 236.
7. WEIL, R., and VINOGRAD, J., *Proc. Nat. Acad. Sc.*, 1963, **50**, 730.
8. BURTON, A., and SINSHEIMER, R. L., *Science*, 1963, **142**, 962.
9. KLEINSCHMIDT, A. K., BURTON, A., and SINSHEIMER, R. L., *Science*, 1963, **142**, 961.
10. CRAWFORD, L. V., and BLACK, P. H., *Virology*, 1964, **24**, 388.
11. CRAWFORD, L. V., *J. Mol. Biol.*, 1964, **8**, 489.
12. CRAWFORD, L. V., *J. Mol. Biol.*, 1965, **13**, 362.
13. THOMAS, C. A., JR., and MACHATTIE, L. A., *Proc. Nat. Acad. Sc.*, 1964, **52**, 1297.

13 *a.* LEHMAN, I. R., ROUSSOS, G. G., and PRATT, E. A., *J. Biol. Chem.*, 1962, **237**, 819.
14. YOUNG, E. T., III, and SINSHEIMER, R. L., *J. Mol. Biol.*, 1964, **10**, 562.
15. BODE, V. C., and KAISER, A. D., *J. Mol. Biol.*, 1965, **14**, 399.
16. SINSHEIMER, R. L., *J. Mol. Biol.*, 1959, **1**, 43.
17. FIERS, W., and SINSHEIMER, R. L., *J. Mol. Biol.*, 1962, **5**, 408.
18. FREIFELDER, D., KLEINSCHMIDT, A. K., and SINSHEIMER, R. L., *Science*, 1964, **146**, 254.
19. HERSHEY, A. D., and BURGI, E., *Proc. Nat. Acad. Sc.*, 1965, **53**, 325.
20. RIS, H., and CHANDLER, B., *Cold Spring Harbor Symp. Quant. Biol.*, 1963, **2**, 306.
20 *a.* KLEINSCHMIDT, A. K., and ZAHN, R. K., *Z. Naturforsch.*, 1959, **14b**, 770.
21. WEIL, R., *Proc. Nat. Acad. Sc.*, 1963, **50**, 730.
22. WASSERMAN, E., *J. Am. Chem. Soc.*, 1960, **82**, 4433.
23. VINOGRAD, J., MORRIS, J., DAVIDSON, N., and DOVE, W. F., JR., *Proc. Nat. Acad. Sci.*, 1963, **49**, 12.
24. FREIFELDER, D., and DAVISON, P. F., *Biophysic. J.*, 1963, **3**, 49.
25. VINOGRAD, J., LEBOWITZ, J., RADLOFF, R., WATSON, R., and LAIPIS, P., *Proc. Nat. Acad. Sc.*, 1965, **53**, 1104.
25 *a.* KLEINSCHMIDT, A. K., KASS, S. J., WILLIAMS, R. C., and KNIGHT, C. A., *J. Mol. Biol.*, 1965, **13**, 749.
26. LEBOWITZ, J., WATSON, R., and VINOGRAD, J., data to be published.
27. HAMAGUCHI, K., and GEIDUSCHEK, E. P., *J. Am. Chem. Soc.*, 1962, **84**, 1329.
28. DEVOE, H., and TINOCO, I., JR., *J. Mol. Biol.*, 1962, **4**, 500.
29. LAIPIS, P., unpublished results.
30. BURTON, A., and SINSHEIMER, R. L., *J. Mol. Biol.*, 1965, **14**, 327.
31. JANZ, H. S., and POUWELS, P. H., *Biochem. and Biophysic. Research Commun.*, 1965, **18**, 589.

Discussion

Dr. Charles Thomas: I wonder whether you would explain again how you calculated the number of superhelical turns and how you came by this number.

Dr. Vinograd: I hope I did not give the impression that the number of tertiary turns shown in Fig. 14 was a measured number. It was adopted arbitrarily for the purpose of drawing the figure. We do not know, at the present time, the number of tertiary turns in the molecule. It is clearly not a large number because the tertiary turns disappear when a small amount of denaturation occurs. We have various types of experiments in progress which we hope will contribute information on this matter.