

A STUDY OF TWISTED CIRCULAR DNA'S FROM ONCOGENIC VIRUSES

by

Philip Laipis

THE RESEARCH REPORTED HERE COVERS THE PERIOD JUNE, 1964, TO
THE PRESENT DATE, MAY, 1966. THIS RESEARCH IS STILL IN PROGRESS.

Approved
Jerome W. Maynard
May 27, 1966

TABLE OF CONTENTS

I. Polyoma Studies	page 1
II. Simian Virus 40 Studies	
A. Preparative Methods	3
B. Experimental Work	6
III. Appendix — <u>Vinograd et al, Proc. Natl. Acad.</u> <u>Sci. U.S., 53:1104 (1965)</u>	10
IV. References	18

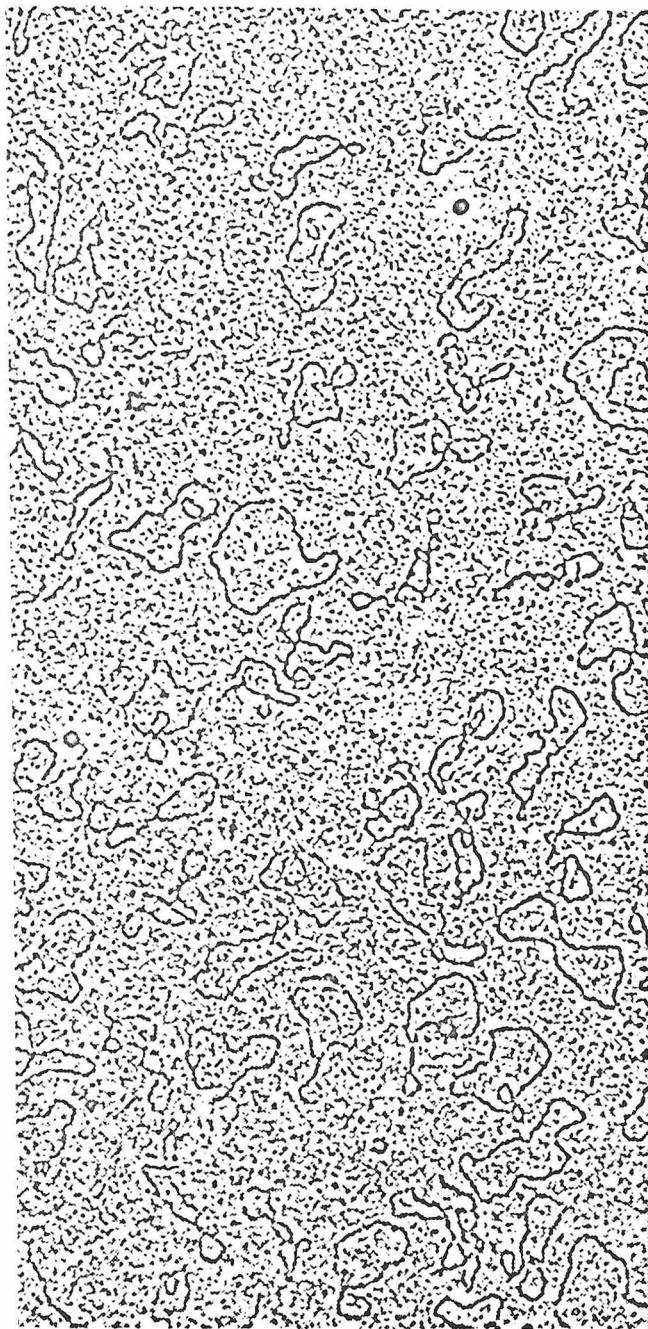
Research was originally begun on the problem of obtaining electron micrographs of alkaline pH DNA samples, by suitable modification of the Kleinschmidt procedure.¹ During the course of this work, several confusing aspects of the material under study, Polyoma viral DNA, became of greater interest, and the original project was dropped.

The original paper of Weil and Vinograd² presented evidence for the existence of a circular form of Polyoma viral DNA. By neutral sedimentation velocity analysis, Polyoma DNA contained three components: I, 20.3 S; II, 15.8 S; III, 14.4 S. Component I was believed to be a circular duplex molecule, II was the linear form of this molecule, and III was believed to be an impurity since it was generally present in small (5%) amounts. Electron micrographs of whole DNA preparations, prepared by the Kleinschmidt method, showed many circular forms. This result was interpreted as proof of the hypothesis.

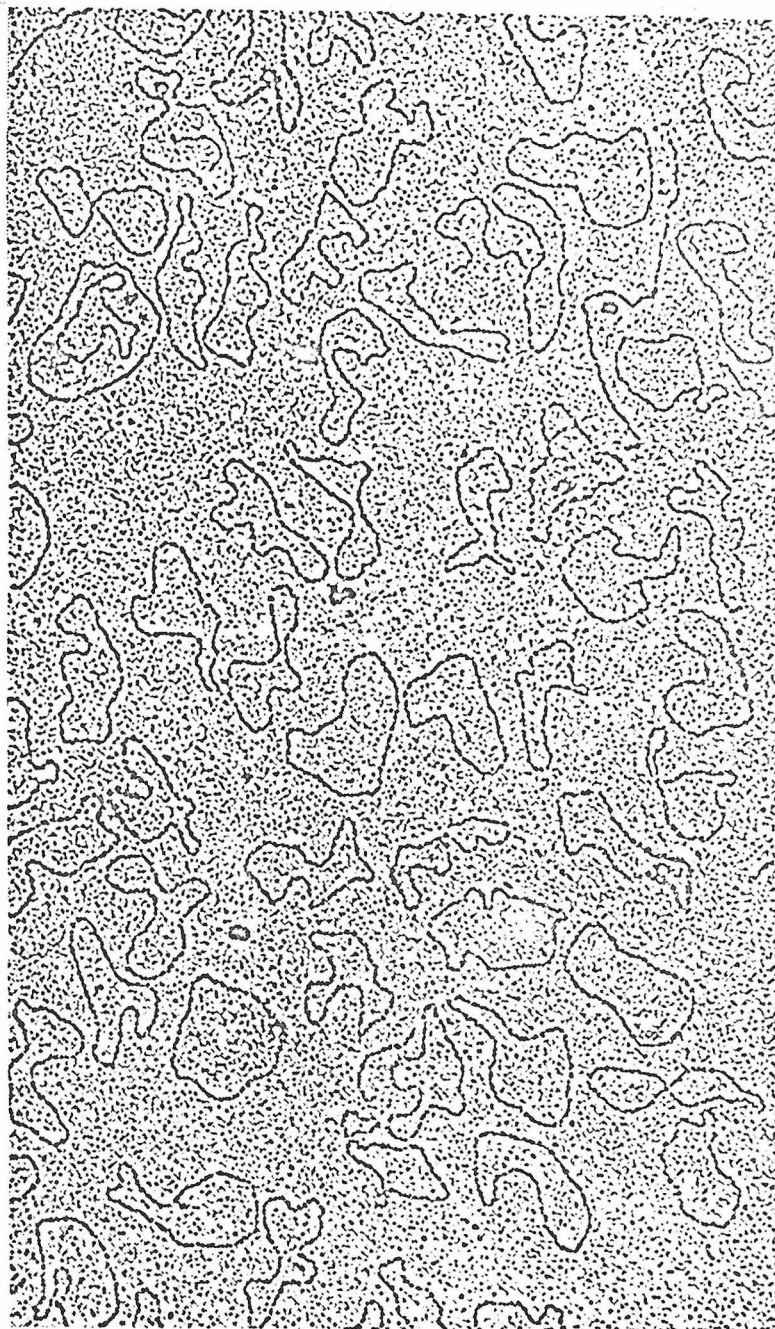
In the early summer of 1964, experiments by Lebowitz and Watson, of this laboratory, showed that a variety of mild reducing agents converted Py I into Py II, by sedimentation velocity analysis. In collaboration with Don Blair, I examined many samples of this converted material by electron microscopy. Micrographs of this converted material showed it to be all circular, with none of the expected linear forms. Repetition, with Pancreatic DNAase, which is known to introduce single-strand cuts into the double helix, gave similar results;

Py II was a circular molecule. Vinograd et al³ explained these experiments by proposing that Py I was a circular duplex molecule, with a small number of locked-in right-handed twists. Py II was a circular duplex molecule, with at least one single-strand scission, which acted as a swivel, allowing the twists to unwind. Py III was a linear duplex molecule. Electron microscopy of naturally occurring Py III, separated by a sucrose velocity experiment, and of Py III prepared by reaction of Py I with the E. coli endonuclease I⁴, confirmed this prediction of the linearity of Py III. This model provides a satisfactory explanation of the electron micrographs, figures 1 and 2, and of the sedimentation velocity and buoyant density studies of Lebowitz et al.⁵ Figure 3, from Vinograd et al (1965), shows the proposed model in detail. A complete description of the electron micrographs, and the other experiments leading to this model is contained in the appendix.

FIGURE I



(a)



(b)

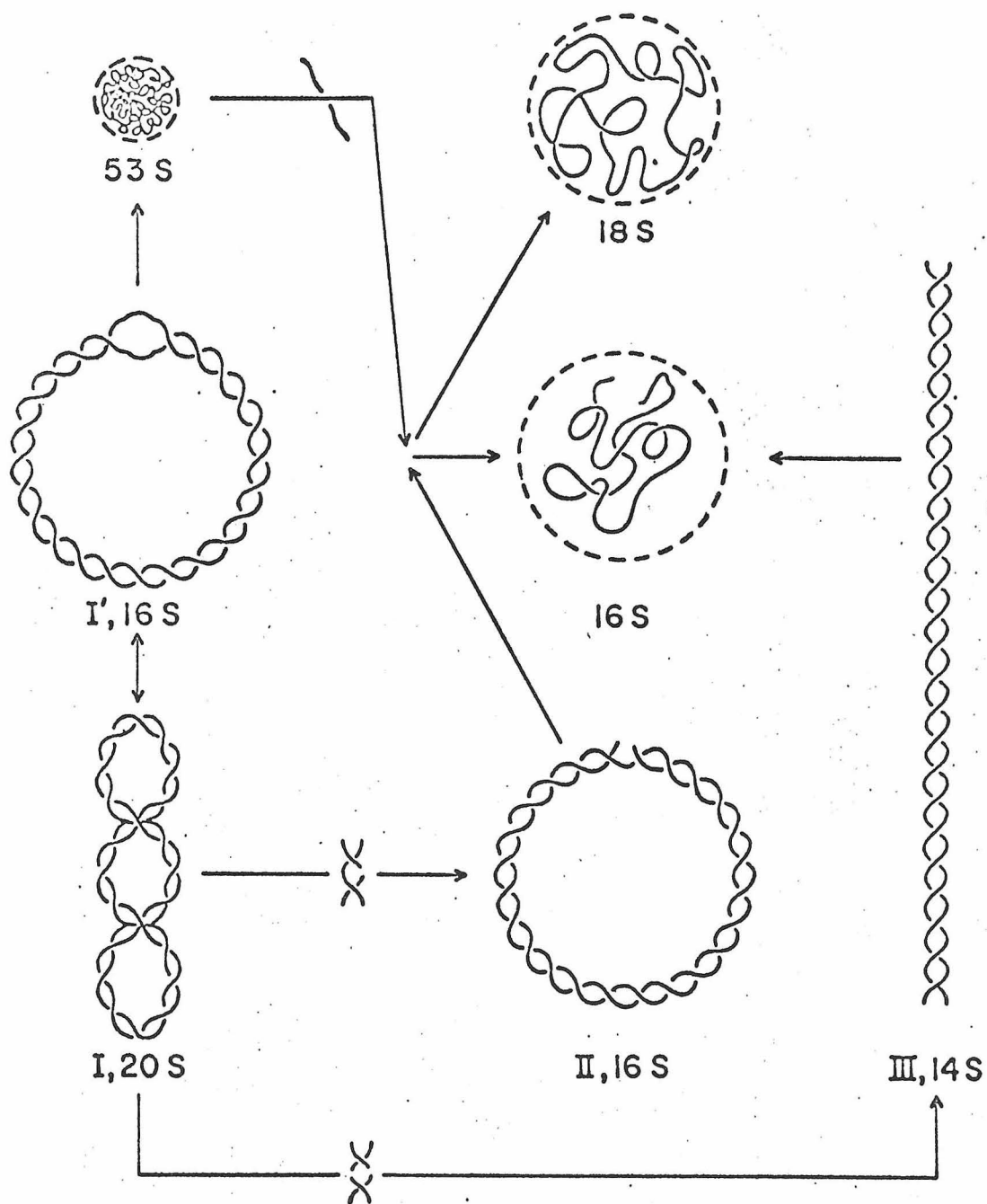
Electron micrographs of Polyoma DNA x 35,000. The materials in (a) and (b) were prepared by treatment of Py I with pancreatic DNAase. (a) was withdrawn from the reaction mixture after 5% conversion of I to II; (b) after 95% conversion.

FIGURE 2



Electron micrograph of naturally occurring Polyoma DNA, component III, x 38,000. This DNA was obtained from a sucrose velocity experiment.

FIGURE 3



Diagrammatic representation of the several forms of Py DNA. The duplex segments shown contain 12 turns, about one fortieth of the total number. 8% of the right-hand duplex turns in the model are unwound to form I'. The dashed circles around the denatured forms indicate the relative hydrodynamic diameters.

The existence of the twisted circular form of Polyoma DNA raised a question concerning the form of the DNA of Simian Virus 40, another small DNA virus. SV40 is similar in size and behavior to Polyoma and is considered a member of the Papova virus group,⁶ to which Polyoma belongs. A program of investigation of this viral DNA was begun, and is still continuing.

SV40 virus was first isolated from monkey kidney cells, of various species,⁷ although the cytopathic effect of the virus is visible only in the African green monkey (*Cercopithecus aethiops*). It has been shown to be oncogenic for the Syrian hamster⁸ and the multimammate rat.⁹

A sample of frozen SV40 lysate was obtained from Dr. Roger Weil, and the virus was purified by a series of differential centrifugations. Sedimentation velocity analysis in alkaline CsCl revealed a fast form similar to the 53S form of Py I. Electron micrographs of DNA extracted by a modified SDS procedure¹⁰ showed circular forms. Encouraged by these results, we began a program of virus production.

SV40 virus was obtained as a gift from Dr. Tom Benjamin. The virus was twice plaque-purified¹¹ to remove possible impurities. BSC-1 cells (African green monkey kidney) were obtained from Flow Laboratories, Inc. Cells were grown in Eagle's medium plus 10% calf serum; when confluent, the cultures were transferred, with a dilution factor of 5.

Virus production is as follows:

Twenty plates of confluent BSC-1 cells are transferred to 120 plates. Twenty are reserved as the cell line, with a reserve of 1 additional plate transferred on a different day as insurance against loss of the line through accident. When confluent, (about 72 hrs.) the 100 plates remaining are infected with approximately 10^7 PFU (plaque forming units). After 72 hrs., most of the cells have rounded up and are beginning to detach from the plate. The supernate is now removed and frozen; the cells are washed free of the plate, spun down, and frozen. Virus is present in both portions and is extracted from both, with different procedures. This method of virus production is adapted to a single person working with the tissue culture; it is easily scaled up.

Virus extraction from the supernate is most easily accomplished by addition of one-half volume of anhydrous methyl alcohol (-20°C) to the cold supernate, with stirring. The solution is now allowed to sit in the cold, with stirring, for a minimum of 4 hrs. The precipitate, containing the virus, is now spun down for 45' @ 6000 rpm.

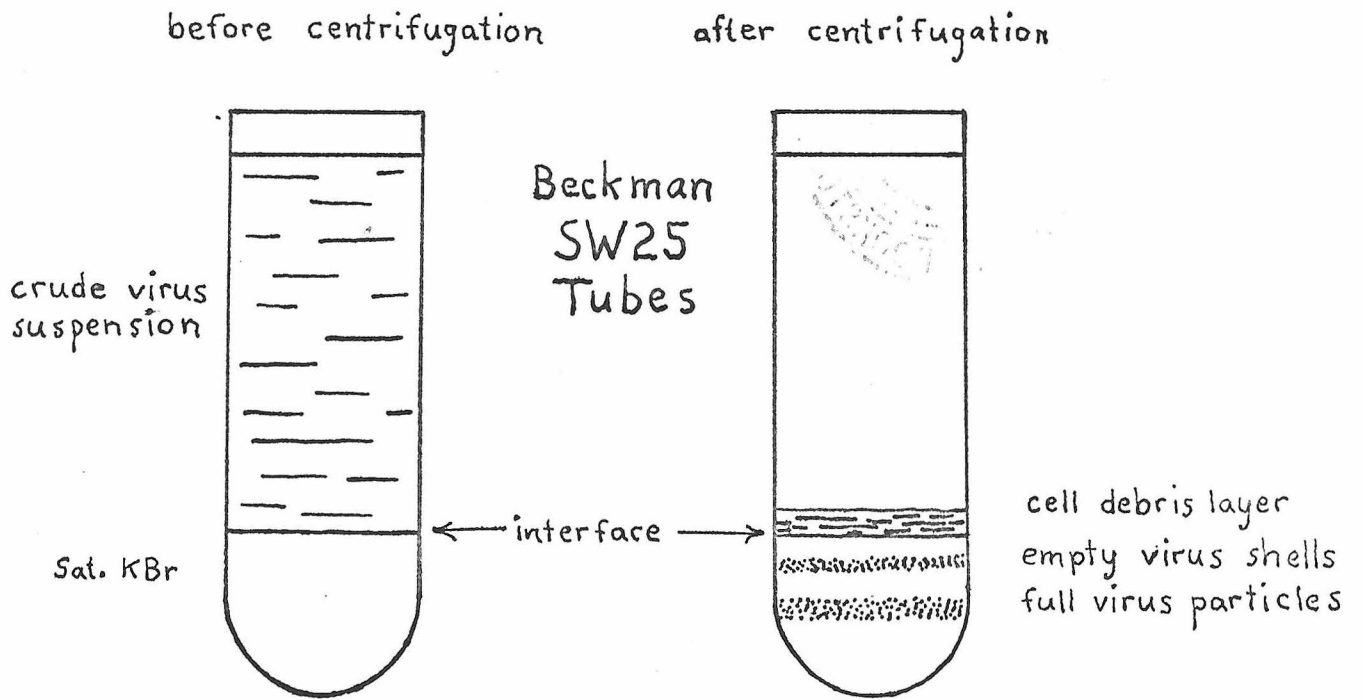
The whole cell pellet is sonicated (7' @ full power) or freeze-thawed three times to disrupt the cells.

Both preparations are now diluted to a convenient volume (50ml), incubated @ 37°C for one-half hour with 1% sodium deoxycholate and .01% trypsin. The trypsin is stopped by either addition of calf serum (to 20%) or soybean trypsin

inhibitor. Debris is removed by a low speed spin (10' @ 1000 rpm), and the supernate layered over 8 ml cold saturated KBr in .01 M Tris, pH 8, placed in the bottom of SW25 tubes. These tubes are spun cold for 4 hrs. @ 23,000 rpm, without braking. Appearance of the tubes is shown in figure 4.

The virus band is removed by collecting drops from the bottom of the tube, and after dialysis against .01 M Tris, SSC/10, pH 8, further purified by sedimentation to equilibrium in ρ 1.33 CsCl. The virus band is collected, dialysed, and the DNA extracted by the phenol method of Vinograd et al (1965). After dialysis to remove the phenol (.01 M Tris, SSC/10, pH 8), the DNA was stored at -20° C. DNA yields, per 100 plates, varies between .2 and 1 mg; this variance is unexplained.

FIGURE 4



A number of experiments have been carried out with this material.

The sedimentation velocity of SV40 DNA has been studied by the band centrifugation method.¹² In neutral NaCl, and in neutral CsCl (ρ 1.35), SV40 DNA sediments as three components. Component I ($20.5 \pm .3$ S) is the major (90%) portion of freshly extracted DNA. Component II ($16.5 \pm .3$ S) makes up the remainder of the DNA. In several preparations, where I has been extensively degraded, a minor third component has been observed. This component III has an $S_{20,w}^0$ of $14.4 \pm .5$.

Component I is slowly converted into component II on storage; the conversion is greatly accelerated by handling, indicating contamination is responsible for the conversion.

Analysis of the sedimentation velocity, in alkaline solution, of purified SV40 I revealed three components. A very fast component of 51.8 S, and two components of intermediate speed were found, SF (tracings too faint to allow S determination, but approximately 19 S), and S ($17.6 \pm .8$ S).

Buoyant densities were measured in neutral and alkaline CsCl solutions, using crab dAT as a marker (ρ 1.669 g/ml, pH 8; ρ 1.727 g/ml, pH 12.5; referred to E. coli ρ 1.704 g/ml, pH 8). Whole DNA preparations banded as a single peak in neutral CsCl at density 1.694 g/ml. Two peaks were visible in alkaline CsCl, and were shown by a band buoyancy experiment to correspond to SV40 I (ρ 1.772 g/ml) and SV40 II (ρ 1.754 g/ml).

All buoyant densities are $\pm .001$ g/ml.

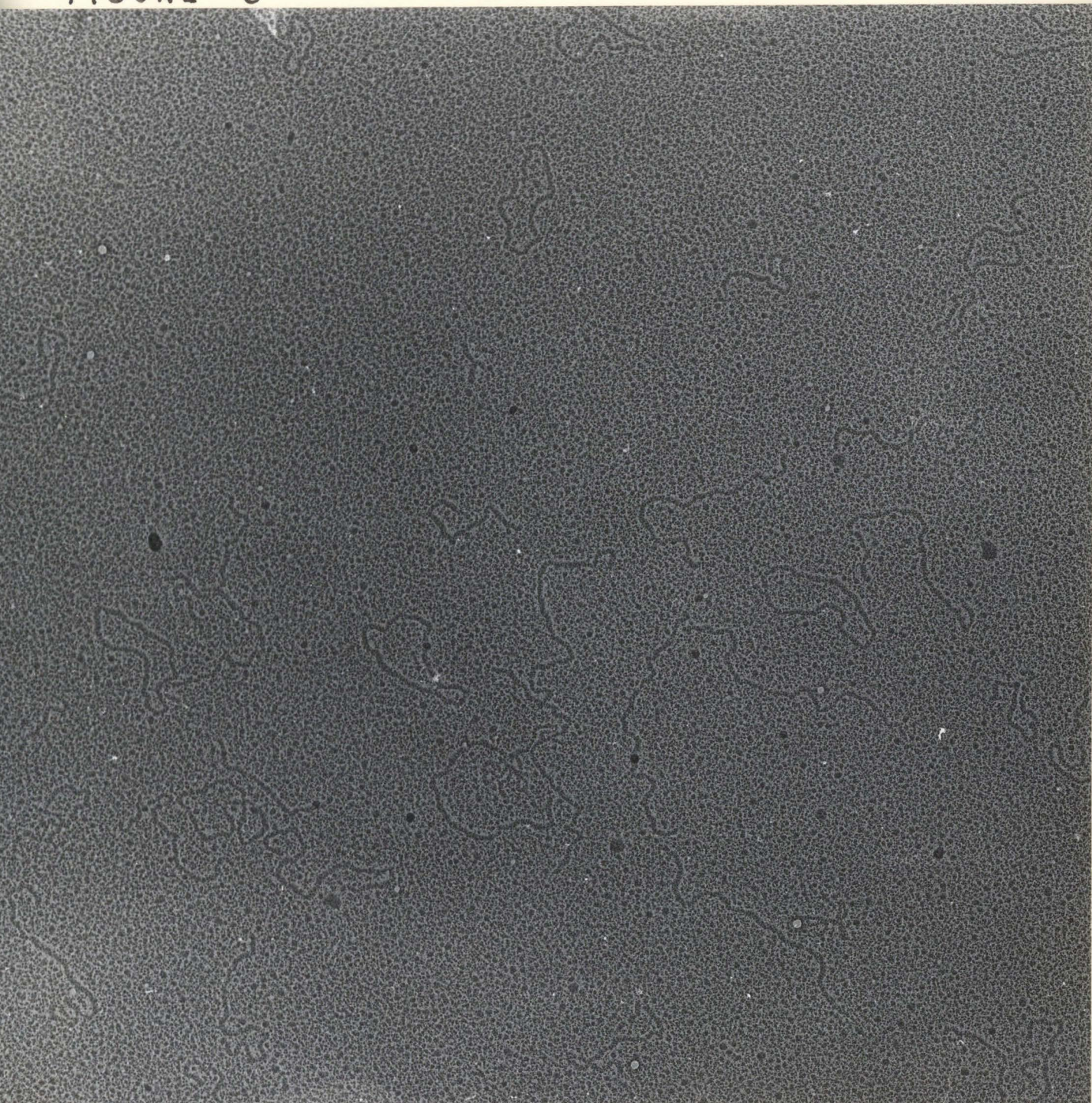
The DNA was photographed in the electron microscope by the Kleinschmidt procedure, figure 5. A small number of the circular molecules were measured, giving a length of $1.44 \pm .10 \mu$.

The molecular weights, estimated from the sedimentation velocity¹³ of III and S, the single-stranded linear molecule, were respectively 2.5×10^6 amu and 1.75×10^6 amu (single strand). Calculation of the molecular weight from the electron microscope length determination gave a value of 2.7×10^6 amu, assuming the helix is in the β form, and has an average residue weight of 326 amu. These values are in reasonable agreement, considering the accuracy of the data, and agree with the values of 2.7×10^6 (band width) and 3.2×10^6 (sedimentation velocity of II) obtained by Crawford and Black.¹⁴ The value of 41% GC, calculated from the buoyant density¹⁵, also agrees with Crawford and Black (1964).

The sedimentation velocity components, and density components correspond exactly to the components of Polyoma viral DNA. This correspondence, and the presence of circular forms by electron microscopy, makes it reasonable to assume SV40 I is a twisted circular form.

Since the tissue culture techniques required for SV40 production are simple, and easily suited for producing large amounts of virus, it was decided to study the behavior of SV40 I and II through the alkaline pH transition, by optical means. The hyperchromic effect is due entirely to breakage of hydrogen bonds between bases; therefore, an optical titration

FIGURE 5



Electron micrograph of SV40 DNA x 38,000. SDS extraction of a virus preparation containing 40% I, 50% II, and 10% III; the original sample of virus received from Dr. Roger Weil.

would reflect only the chemical changes occurring during the transition, and not the configurational changes. It should be possible to relate the sedimentation velocity titration curve to the optical titration curve, and thus separate the effects, in the sedimentation velocity, caused by titration of bases from those caused by unwinding of the double helix. A comparison of the optical titration with the buoyant density titration would make possible a direct calculation of buoyant density shift per percentage of hydrogen bonds broken.

Optical titrations were carried out in a Gilford spectrophotometer, equipped with a thermostatically-controlled cell block. Absorbance of the titrated solution was recorded on the integral recorder of the Gilford, using air as a blank. Spectrophotometer error is $\pm .001$ O.D. unit. A Beckman Research Model pH meter was used to measure pH in the titrant cell. Figure 6 shows the cell block and the internal arrangements of gas lines, titrant lines, and the Beckman miniature combination electrode.

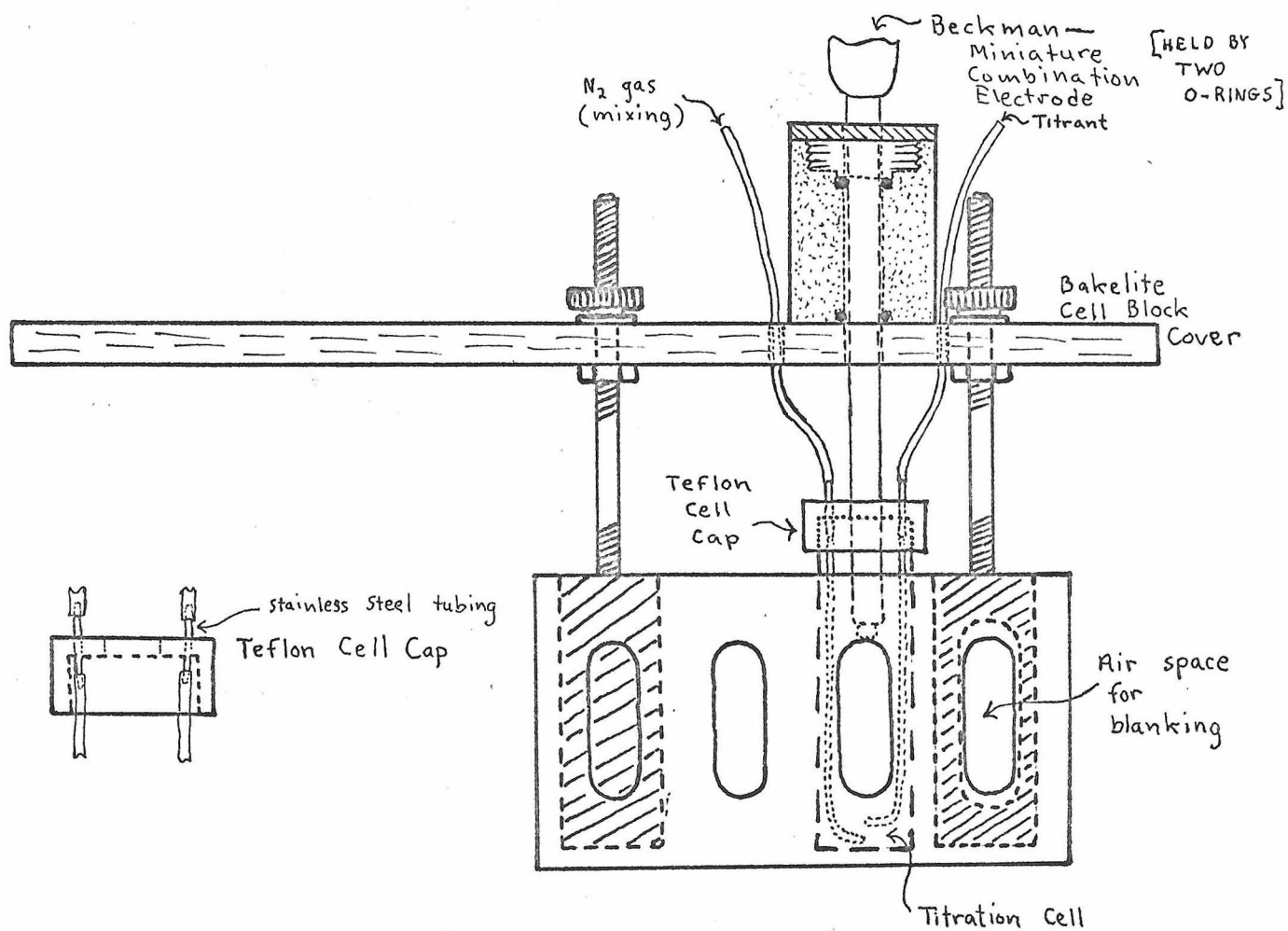
The major difficulties are the pH electrode, and premature denaturation caused by momentary high local concentration of added base.

The Beckman electrode, while conveniently small, has an appreciable lag time caused by the fiber junction of the reference portion of the electrode. This lag causes long delays in standardization, and errors in the readings during the titration. A ground-glass junction reference electrode

FIGURE 6

Titration cell assembly for the

Gilford Spectrophotometer



is being incorporated into the system and appears to be much more satisfactory.

Premature denaturation is not a factor in the titration of SV40 I, as this DNA is completely reversible. The titration of SV40 II shows a rising base-line caused by this premature melting which occurs in regions of high local pH. Preliminary experiments with a new design of inlet system, figure 7¹⁶, seems to have much less premature melting.

Figure 8 shows the two titration curves of SV40 I and II obtained thus far. The stabilization of I, caused by the inability of this component to unwind, is clearly evident. Repetition of this experiment, with the revised equipment, is now under way; the sedimentation velocity and buoyant density titrations are being planned.

FIGURE 7

Modified titrant - gas injection
system

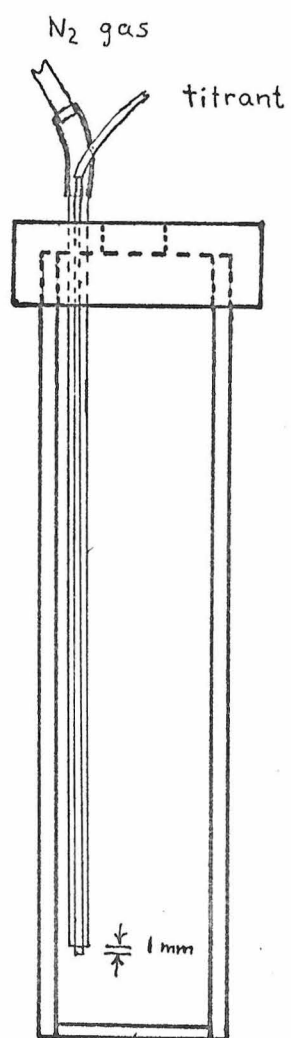
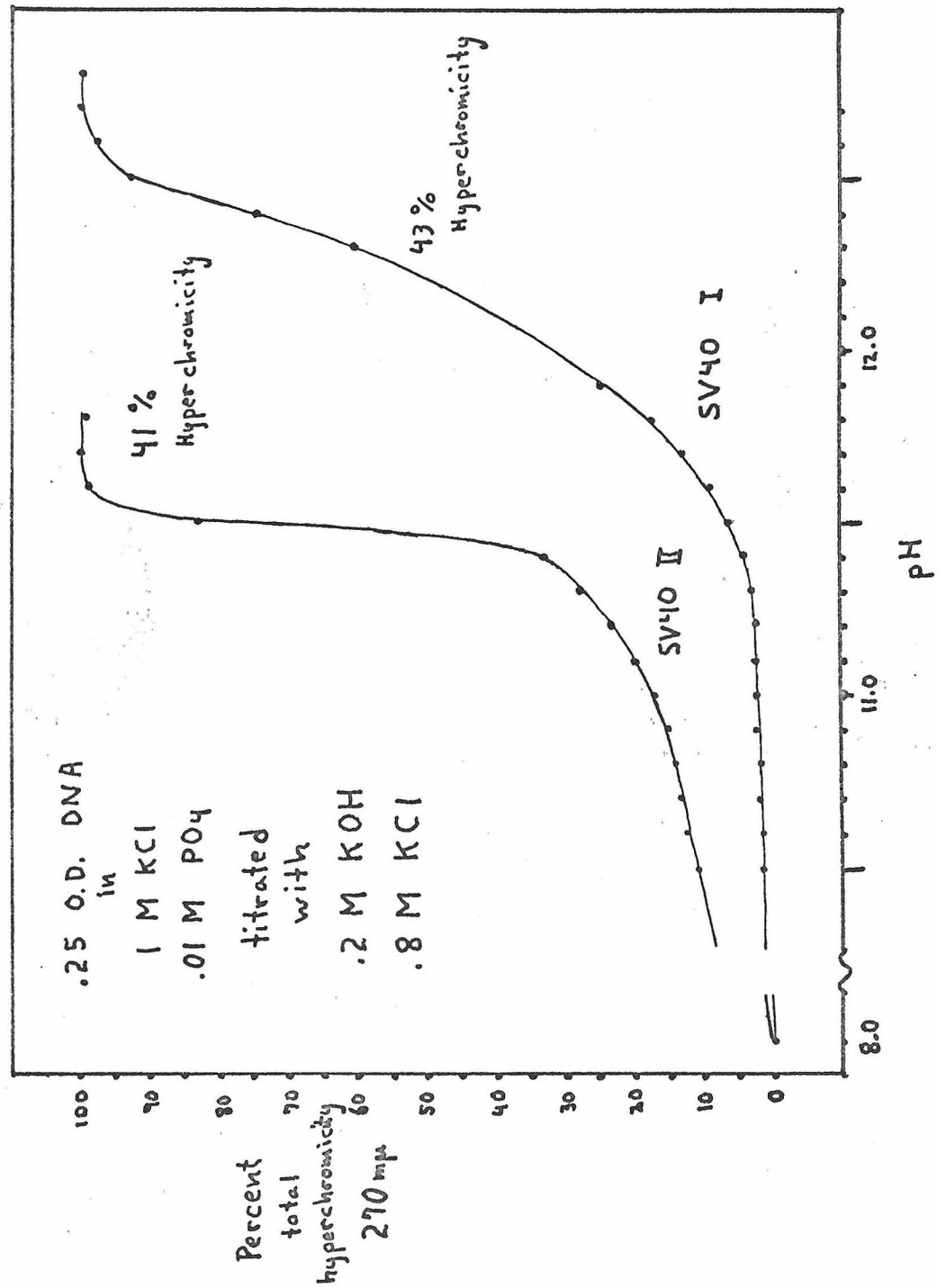


FIGURE 8



THE TWISTED CIRCULAR FORM OF POLYOMA VIRAL DNA*

By J. VINOGRAD, J. LEBOWITZ, R. RADLOFF, R. WATSON, AND P. LAIPIS

GATES AND CRELLIN LABORATORIES OF CHEMISTRY AND
THE NORMAN W. CHURCH LABORATORY OF CHEMICAL BIOLOGY,
CALIFORNIA INSTITUTE OF TECHNOLOGY

Communicated by Norman Davidson, March 30, 1965

The major part of the DNA from polyoma virus has been shown to consist of circular base-paired duplex molecules without chain ends.¹⁻³ The intertwined circular form accounts for the ease of renaturation⁴ of this DNA and the failure of the strands to separate in strand-separating solvents.¹⁻³

In previous studies¹⁻³ a minor component, II, observed in variable amounts in sedimentation analyses of preparations of polyoma DNA at neutral pH, was regarded to be a linear form of the viral DNA. Both the major component I (20S) and II (16S) were infective.¹⁻⁵ In our further investigations of the minor component the following results, which are reported below, have been obtained: (1) The minor component is a ring-shaped duplex molecule. (2) It is generated by introducing one single-chain scission in component I by the action of pancreatic DNAase or chemical-reducing agents. (3) The sedimentation coefficient of II is insensitive to several single-strand scissions. (4) The conversion products, when not excessively attacked, are infective.

The foregoing results raised a new problem. Why does the viral DNA, an intact duplex ring, sediment 20 per cent faster than a similar duplex ring containing one or more single-strand scissions? Experiments bearing on this problem, presented below, indicate the presence of a *twisted circular structure* in polyoma DNA I. A mechanism for the formation of this locked-in twisted structure is proposed.

Methods.—*Isolation and purification of the virus and extraction of the DNA:* Two methods^{6,7} for purification of the virus were used. The DNA was isolated by Weil's method⁴ except that the phenol was freshly distilled under argon.

Ultracentrifugation: Sedimentation analyses were performed in a Spinco model E ultracentrifuge by band centrifugation.⁸ Some of the results were recorded with the photoelectric scanning attachment.^{9,10} Sucrose density gradient experiments were performed at 4°, 30,000 rpm, and 9 hr. The 3% and 20% sucrose solutions contained SSC (0.15 M NaCl and 0.015 M Na citrate) and 0.05 M Tris chloride pH 8.0.

Enzymes: Pancreatic DNAase, 1 × crystallized, was obtained from Worthington Biochemicals Corp. *E. coli* endonuclease I, 1000 units/ml,¹¹ and *E. coli* phosphodiesterase, 2000 units/ml,¹² were gifts from Professor I. R. Lehman. BSA, 30% bovine albumin solution, sterile, was obtained from Armour Pharmaceutical Co. The endonuclease I, 0.12 units/μg DNA, converted 60% of I into linear molecules in 8 min at 20° in the incubation mixture described by Lehman.¹¹

Sedimentation velocity-pH titration: Fifteen μl, 40 μg/ml DNA in SSC/10, flowed from the sample well of the type III¹³ band-forming centerpiece onto an alkaline CsCl bulk-solution. This solution was prepared by titrating 10 ml (Harshaw Chemical Co.) optical grade CsCl, $\rho = 1.35$, with 1 M KOH in CsCl, $\rho = 1.35$, at 20° under argon, and was transferred to the cell assembly under argon. Usually four samples in a pH series were analyzed simultaneously. A Beckman research model pH meter, a general purpose probe glass electrode, and a calomel reference electrode modified with a ground glass junction¹⁴ were used.

Plaque assay: Infectivity of polyoma DNA was measured as described by Weil.⁴

Electron microscopy: Specimens were prepared by the method of Kleinschmidt and Zahn.¹⁵

Results.—*Preparation of polyoma DNA II:* Polyoma II can be prepared from I by treatment with several mild chemical-reducing agents (Table 1). These reagents

TABLE 1
ACTION OF REDUCING AGENTS ON POLYOMA DNA

Reagent	M^a	pH	Time (min)	Conversion ^b of I to II (%)
Hydroquinone ^c	0.0002	8.5	30	100
FeCl ₂	0.001	8.6	30	90
Na ₂ SO ₃ (1×10^{-3} M)				
Cu ₂ SO ₄ , 0.1 M	0.01	10.8	60	45
NH ₄ OH) ^d				
Thiols ^e	0.01-0.02	3.8-4.2	60	80-90

^a Final concentration of reducing agents which were diluted fivefold with SSC/10, 0.01 M Tris pH 8.5 containing 40 μ g/ml DNA. The thiols were first dissolved in 0.4 M acetic acid before addition to the DNA solution.

^b Sedimentation analysis at neutral pH.

^c The reaction product was assayed for infectivity: 0.5×10^3 pfu/ μ g DNA compared with 1.5×10^3 obtained for untreated DNA in the same assay.

^d Cu⁺⁺ acts as a catalyst.¹⁶ In the absence of Cu⁺⁺ or NH₄OH 5% conversion was observed. In the absence of SO₃²⁻ no conversion occurred.

^e Mercaptoethanol, cysteine, and glutathione. In experiments with acetic acid without thiols, 10% conversion occurred.

were suggested by the observation that rigorous exclusion of impurities from the phenol, used in the isolation of the DNA, diminished the amount of the minor component II in the final DNA preparation. A simple conversion of I to II without intermediates and without detectable degradation products was observed in sedimentation analyses at pH 8.0 (Table 1). Based on the earlier assignment^{1, 2} of a linear form to component II, the reactions with reducing agents and the infective nature of the products indicated a specific duplex cleavage. Dulbecco and Vogt¹ reported a similar conversion of I to II with low concentrations of pancreatic DNAase. These authors postulated that a bond opposite the single-strand scission introduced by the enzyme hydrolyzed under the influence of ring strain. The foregoing puzzling results are clarified by the experiments below.

Structure of Polyoma II.—The products from the action of pancreatic DNAase and of the reducing agents (Table 1) were examined in the electron microscope. They were found to be in the circular form. Figure 1b is typical of electron micrographs of materials obtained by treatment of I with pancreatic DNAase or with the several reducing agents. The possibility that linear molecules were selectively excluded in the preparation of the grids was eliminated by the results of a reconstruction experiment. A synthetic mixture of 10 per cent II and 90 per cent linear polyoma III (cf. below) showed the expected proportions of linear molecules.

The circular form for II is compatible with the proposal that the reducing agents and pancreatic DNAase introduce single-strand scissions into polyoma DNA I. It may be calculated that the circular form of the molecule should be retained until on the average about 50 single-strand scissions per molecule have been introduced.¹⁷ The material shown in Figure 1b contained, on the average, about three breaks per molecule as calculated from the Poisson distribution.

A still milder treatment with pancreatic DNAase should give rise to single-stranded rings and uniform single-stranded linear molecules in strand-separating solvents, such as alkaline NaCl or CsCl.^{2, 18} An example of such a result with 0.6 breaks per molecule is given in Figure 2b. These two components, $s_{w,20}^0 = 18.4S \pm 0.4$, $15.7S \pm 0.3$ for the alkaline Na DNA, have been identified as single-stranded rings (18S) and single-stranded linear molecules (16S), respectively, by the following variation of an experiment originally performed with ϕ X DNA by Fiers and Sinsheimer.¹⁹ An aliquot of the product of the pancreatic DNAase digestion

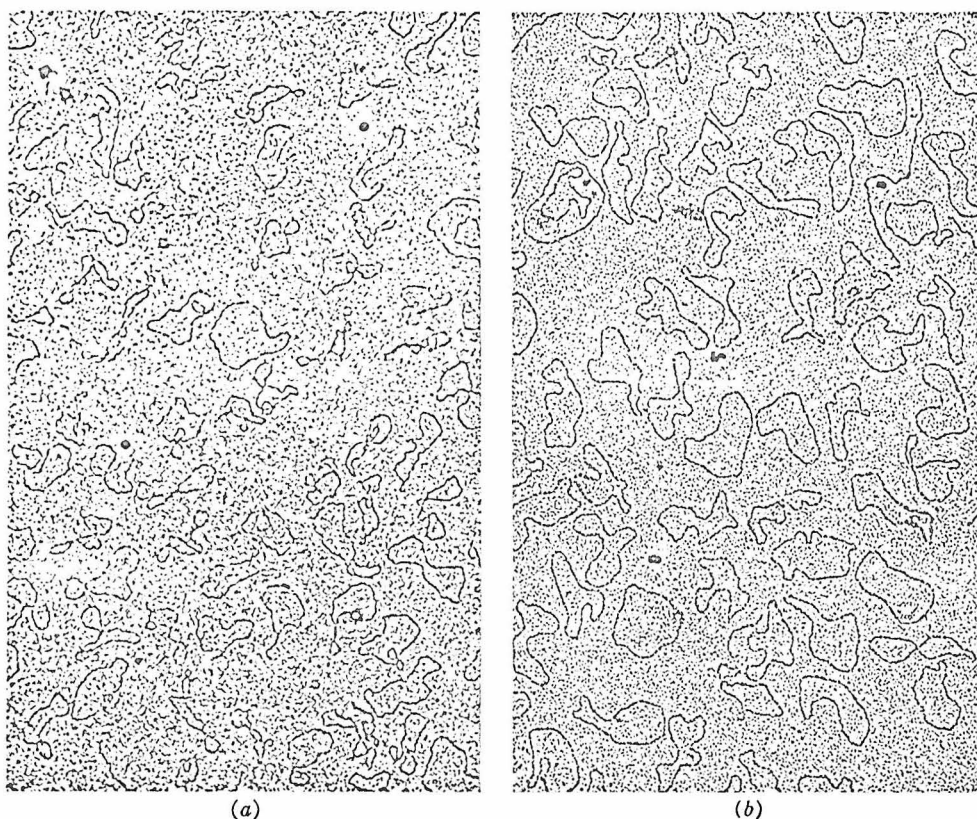


FIG. 1.—Electron micrographs of polyoma DNA $\times 21,000$. The materials in (a) and (b) were prepared by treatment of polyoma I with pancreatic DNAase, as described under Fig. 3. (a) was withdrawn from the reaction mixture after 5% conversion of I to II; (b) after 95% conversion.

(40% conversion) was heat-denatured and treated with *E. coli* phosphodiesterase. This enzyme attacks single-stranded DNA with a free 3'OH group. It is seen that the amount of the 16S component was substantially diminished, while the 18S component was resistant to the exonuclease (Fig. 2c). Thus polyoma DNA II can contain a wholly intact circular strand. At this level of digestion the second strand in the molecule will contain only one or two breaks if the attack is statistical.

We now examine the possibility that one single-strand scission in the duplex is adequate to convert polyoma ring I to ring 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 (intact, denatured, double-stranded, cyclic molecules) 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. Dulbecco and Vogt¹ have already reported that the two rates are alike. In view of the importance of the result, we have repeated this experiment with the analyses performed in the analytical ultracentrifuge.

In Figure 3a it is seen that the alkaline analyses and the neutral analyses give, within the experimental error, the same extent of conversion, a result which confirms the Dulbecco and Vogt finding. Therefore, the conversion of I to II occurs

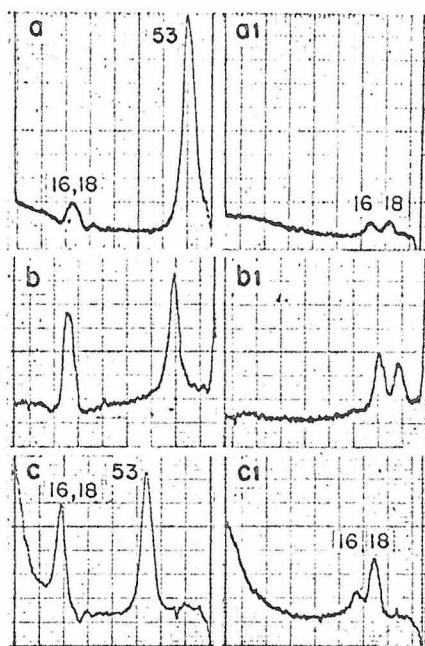


FIG. 2.—Sedimentation velocity patterns of polyoma DNA in alkaline CsCl. The left and right patterns are scans at about 30 min and 90 min after sedimentation begins. The field is directed toward the right. CsCl, $\rho = 1.35 \text{ gm cm}^{-3}$, pH 12.5, 44,000 rpm. (a, a1) Control: Component I isolated in a sucrose gradient experiment treated identically as in (b) and (c) except for the absence of enzymes and BSA. Separate experiments showed the BSA to be free of DNAase activity under the conditions used. (b, b1) Pancreatic DNAase treatment: 106 $\mu\text{g/ml}$ pure I in 0.048 M NaCl, 0.0075 M MgCl_2 , 0.01 M Tris pH 8.0, 40 $\mu\text{g/ml}$ BSA, and $2.7 \times 10^{-4} \mu\text{g/ml}$ enzyme, 20 min at 20° . Reaction stopped by $1/10$ volume 1 M glycine buffer, pH 9.8. The leading band in (a), (b), and (c) is the 53S component. The resolved slower bands in (a1), (b1), and (c1) are the 16S and 18S components. (c, c1) Effect of heat denaturation followed by *E. coli* phosphodiesterase treatment: Product of (b) heated 5 min 100° , cooled rapidly. 70 $\mu\text{g/ml}$ DNA in 0.03 M NaCl, 0.005 M MgCl_2 , 0.007 M Tris, 0.067 M glycine pH 9.8, 0.90 mg/ml BSA, 71 units/ml *E. coli* phosphodiesterase, 90 min at 37° . Reaction stopped by $1/10$ volume 0.1 M EDTA.

whenever the first single-strand scission is introduced. The conversion appears to be first order. While the infectivity (Fig. 3b) declines at a slower rate than the conversion of I to II, the scatter in the data precludes any 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 or the chemical-reducing agents so as to completely convert I to II (>4 average breaks per molecule) caused no detectable change in the sedimentation coefficient of II.

Preparation of Linear Polyoma DNA with *E. coli* Endonuclease I.—Polyoma I was partially converted into the linear form with *E. coli* endonuclease I, which is known to cleave duplex DNA.¹¹ The sedimentation velocity of the homogeneous linear molecules was 14.5S

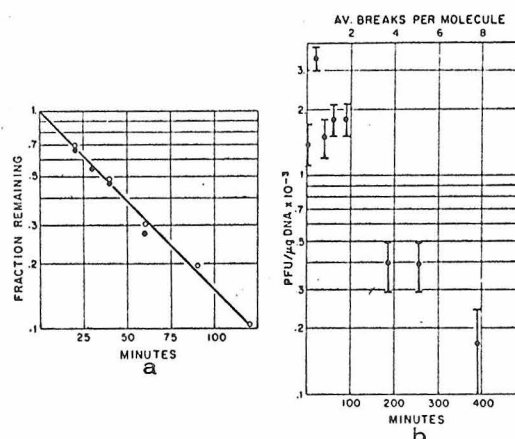


FIG. 3.—Chemical and biological effects of pancreatic DNAase treatment. (a) Analyses for single-stranded and double-stranded DNA. Extent of conversion was determined by band-sedimentation velocity experiments with photoelectric scanner. $\circ$, (I)/(I + II) in neutral CsCl bulk solutions. $\ominus$, (53S)/(total) in alkaline CsCl pH 12.3. Areas under bands were corrected for radial dilution. Incubation mixture and conditions were the same as those given in legend to Fig. 2b, except for enzyme concentration, $2.0 \times 10^{-4} \mu\text{g/ml}$. 20- μl samples were withdrawn at the indicated times and added to 4 μl 0.1 M EDTA pH 8.5. The samples were frozen prior to analyses. (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 deviations from 16 replicate plates.

at pH 8.0, the same as previously reported² for the minor component III, and 16S in alkali. Polyoma II was not produced in detectable amounts in the above conversion of I to linear molecules. Electron micrographs confirmed the assignment of a linear form to the enzymatic product and also to the minor component III isolated by sucrose gradient sedimentation.

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 no increase in friction, a 20 per cent reduction in mass is required to account for the change in *s*. An equal amount of mass would have to have been lost as a result of the action of pancreatic DNAase and the variety of reducing agents used. An excision of viral DNA would have been detected by Dulbecco and Vogt,¹ who were unable to find small fragments of labeled DNA after preparative band sedimentation of polyoma DNA treated with pancreatic DNAase. The identical

buoyant densities of I and II² make it unlikely that a nonlabeled, non-DNA mass is removed.

Three 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. (1) The electron micrographs of the grids prepared from polyoma I contained¹ twisted circles to a variable extent (Fig. 1a). Grids prepared from polyoma II contained extended circles and no twisted configurations (Fig. 1b). The DNA samples used to prepare the grids for Figures 1a and b were identical except for the time of incubation with the enzyme. The spreading forces acting on the macromolecules in the monolayer appear to remove loops and crossovers that are not locked in the structure of the DNA.

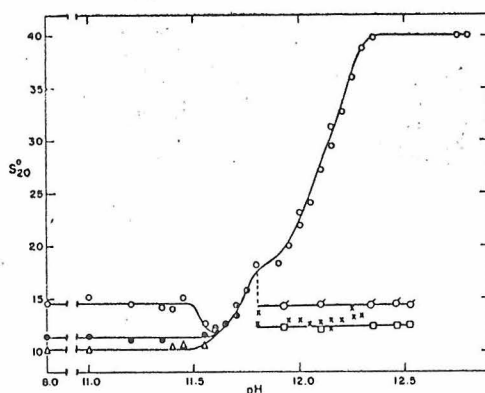


FIG. 4.—Sedimentation velocity-pH titration of the three components in polyoma DNA. I, $\circ$; II, $\square$; III, $\triangle$; $\bullet$ only band present, cf. text; mixture of unresolved single strands in alkali, $\times$; 29,500 rpm. Single linear, $\square$, and single circular, $\circ$, strands in alkali, 44,770 rpm. Sedimentation coefficients at 20° in CsCl, $\rho = 1.35 \text{ gm cm}^{-3}$, are not corrected for solvent viscosity, $\eta_r = 0.925$, or buoyancy effects. The values at pH 8.0 and 12.4 are the means of 12 and 7 determinations, respectively.

(2) 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 from 8 to 12.5 revealed a complicated pH-melting curve for component I (Fig. 4). Component II behaved normally^{18, 20} and moved faster as denaturation increased until strand separation occurred with an attendant sudden drop in sedimentation velocity 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 was observed in this pH range. The sedimentation coefficient of I then increased to the very high value characteristic of the *double-stranded cyclic coil* previously reported.² Essentially the same results were obtained in 1.0 M KCl solutions. The dip in the sedimentation velocity-

pH curve was initially unexplainable. If, however, polyoma DNA I contains left-handed tertiary turns, such a dip in the pH-melting profile would be required. In the early stages of denaturation some of the duplex turns, which are known to be right-handed, unwind. The unwinding of the duplex must be accompanied by a right-handed twisting of the remainder of the molecule. If the tertiary turns were originally left-handed (Fig. 5), progressive unwinding would cause the molecule to pass through configuration I' characterized by the absence of tertiary turns. The extended configuration I' is similar to that in polyoma II (Fig. 5) and both I' and II would have similar sedimentation velocities. Further unwinding of duplex I' is accompanied by continued right-hand twisting of the whole molecule until finally the double-stranded cyclic coil² configuration develops.

(3) 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 site for the rotation of the helix in the complementary strand opposite the break. The swivel relieves the topological restraint responsible for the twisted configuration.

Discussion.—A mechanism for the formation of the twisted circular structures suggested by the above analysis of the pH-melting curve. According to this mechanism the last closure of chain ends occurs before all of the winding of the two DNA strands into the Watson-Crick structure is completed. The closing leaves the duplex in the configuration I' (Fig. 5) restrained from converting to I by an as yet unknown factor participating in the DNA synthesis. Removal of the restraint then allows I' to wind spontaneously into a complete Watson-Crick structure and form the twisted circular structure, I, with no change in winding number.

An alternative proposal is that the molecule in form II, which contains a swivel, is twisted by some organizer, e.g., the virus protein. The last covalent backbone bond is then made while the DNA remains twisted under the constraint of the organizer. This alternative is unlikely in view of Dulbecco's²¹ finding that the polyoma DNA made before virus production begins has the sedimentation velocity of component I.

It is not possible at the present time to estimate reliably the number of tertiary turns. A turn is defined as a 360° rotation of the helix. The electron micrographs of I usually contain some molecules that are completely extended; these

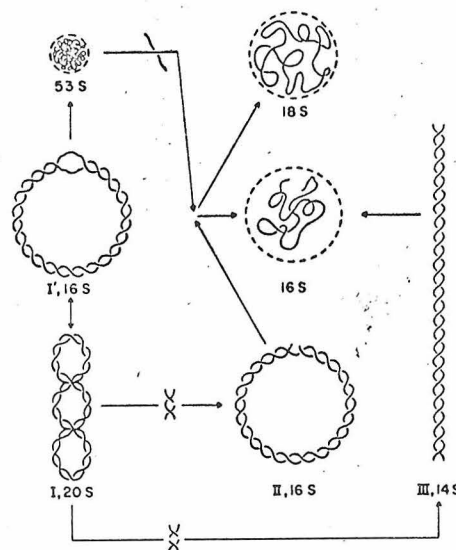


FIG. 5.—Diagrammatic representation of the several forms of polyoma DNA. The duplex segments shown contain 12 turns, about one fortieth of the total number. The twisted circular duplex shown contains one left-hand tertiary turn. 8% of the right-hand duplex turns in the model are unwound to form I'. The dashed circles around the denatured forms indicate the relative hydrodynamic diameters. The sedimentation coefficients were measured in neutral and alkaline NaCl solutions.

may have suffered a single break during grid preparation or may have been in state I' due to denaturation induced by the spreading forces. This latter action would result in the unwinding of the twisted circles to form extended circles. The maximum number of crossovers that can be distinguished is 8, which corresponds to 4 turns or the unwinding of 40 base pairs. This limit may be low because it is difficult to count crossovers in tightly coiled forms. That the number of tertiary turns in the molecule is not large is suggested by the fact that the transition of I to I' occurs before substantial melting of II takes place, as indicated by the *s* versus pH plot.

With the new assignment of structure to the three components of polyoma DNA, it is found that satisfactory agreement obtains between the observed sedimentation coefficient of the linear form, 14.5S, and the $15.3 \pm 0.5S$ predicted by Studier's relation¹⁸ for a molecular weight² of $3.0 \pm 0.3 \times 10^6$. The values, 18.4S and 15.7S for the alkaline single-stranded circular and linear forms similarly agree with the predicted values of 17.4S and 15.6S, respectively. The effect of ring closure of III to form component II is to increase *S* by 10 per cent. An effect of similar magnitude has been reported²² for the cyclization of λ DNA.

The twisted circular structure observed here for polyoma DNA may be a common characteristic of covalently closed, circular duplex DNA. A part of the DNA from rabbit papilloma virus,²³ SV40 virus,³ and the replicating form of ϕX DNA²⁴⁻²⁶ have all been shown to be circular duplex molecules which do not strand-separate in alkali or after heating in formaldehyde. Two sedimentation velocity components differing by 20-30 per cent have been reported for the above DNA's.²⁷ Crawford and Black³ observed sedimentation velocity-denaturation curves that are similar to our pH-melting curve upon heating SV40 DNA and polyoma DNA in formaldehyde solutions to various temperatures. No explanation was offered for this behavior, which we interpret as indicating the presence of a left-handed, twisted circular structure.²⁸

Burton and Sinsheimer²⁹ have shown that the slow component II in RF- ϕX DNA dissociates in alkali to form linear and circular single-stranded molecules and have concluded that both of the undenatured forms of the DNA are circular. While this communication was in preparation, Jansz and Pouwels³⁰ reported that the pancreatic DNAase-induced conversion of I to II in RF- ϕX DNA represents a conversion between circular duplex molecules. No explanation for the change in the sedimentation coefficient was offered. In view of the results described here, it is likely that RF- ϕX DNA is in the twisted circular form. A common mechanism for the incorporation of the tertiary turns during replication is a strong possibility, and allows us to predict that the tertiary turns in the RF- ϕX DNA will be found to be left-handed.

Summary.—The results of this study show that circular duplex polyoma DNA may be converted to a less compact circular duplex by introducing a single-strand scission. The viral form contains tertiary turns which appear to have been locked in during replication.

It is a pleasure to thank I. R. Lehman for the generous gift of the *E. coli* enzymes, R. L. Sinsheimer, J. Petruska, and M. Fried for helpful discussions, R. Dulbecco for allowing us to quote his unpublished results, and T. Benjamin, L. Wenzel, and A. Drew for advice and assistance in the culture of the virus and the assay of the DNA. This work was supported in part by grants HE 03394 and CA 08014 from the U.S. Public Health Service.

* This work was reported in part at the 9th Annual Meeting of the Biophysical Society, February 24, 1965. Contribution 3227 of Gates and Crellin Laboratories of Chemistry.

- ¹ Dulbecco, R., and M. Vogt, these PROCEEDINGS, 50, 236 (1963).
- ² Weil, R., and J. Vinograd, these PROCEEDINGS, 50, 730 (1963).
- ³ Crawford, L. V., and P. H. Black, *Virology*, 24, 388 (1964).
- ⁴ Weil, R., these PROCEEDINGS, 49, 480 (1963).
- ⁵ Crawford, L. V., R. Dulbecco, M. Fried, L. Montagnier, and M. Stoker, these PROCEEDINGS, 52, 148 (1964).
- ⁶ Winocour, E., *Virology*, 19, 158 (1963).
- ⁷ Murikami, W., *Science*, 142, 56 (1963).
- ⁸ Vinograd, J., R. Bruner, R. Kent, and J. Weigle, these PROCEEDINGS, 49, 902 (1963).
- ⁹ Beckman Instruments Co., Spinco Division, Palo Alto, Calif.
- ¹⁰ Hanlon, S., K. Lamers, G. Lauterbach, R. Johnson, and H. K. Schachman, *Arch. Biochem. Biophys.*, 99, 157 (1962).
- ¹¹ Lehman, I. R., G. G. Roussos, and E. A. Pratt, *J. Biol. Chem.*, 237, 819 (1962).
- ¹² Lehman, I. R., *J. Biol. Chem.*, 235, 1479 (1960).
- ¹³ Vinograd, J., R. Radloff, and R. Bruner, *Biopolymers*, in press.
- ¹⁴ Lebowitz, J., and M. Laskowski, Jr., *Biochemistry*, 1, 1044 (1962).
- ¹⁵ Kleinschmidt, A. K., and R. K. Zahn, *Z. Naturforsch.*, 14b, 770 (1959).
- ¹⁶ Swan, J. M., *Nature*, 180, 643 (1957).
- ¹⁷ Thomas, C. A., Jr., *J. Am. Chem. Soc.*, 78, 1861 (1956). The estimate of 50 was obtained for the case of a 10% lowering of the weight average molecular weight.
- ¹⁸ Studier, F. Wm., *J. Mol. Biol.*, 11, 373 (1965).
- ¹⁹ Fiers, W., and R. L. Sinsheimer, *J. Mol. Biol.*, 5, 408 (1962).
- ²⁰ Davidson, P. F., and D. Freifelder, *J. Mol. Biol.*, 5, 643 (1962).
- ²¹ Dulbecco, R., private communication.
- ²² Hershey, A. D., E. Burgi, and L. Ingraham, these PROCEEDINGS, 49, 748 (1963).
- ²³ Crawford, L. V., *J. Mol. Biol.*, 8, 489 (1964).
- ²⁴ Kleinschmidt, A., A. Burton, and R. L. Sinsheimer, *Science*, 142, 961 (1963).
- ²⁵ Burton, A., and R. L. Sinsheimer, *Science*, 142, 962 (1963).
- ²⁶ Burton, A., and R. L. Sinsheimer, *Abstracts*, 8th Annual Meeting of the Biophysical Society, 1964.
- ²⁷ R. Weil has informed us that the slow component II in rabbit papilloma DNA is circular as seen in the electron microscope.
- ²⁸ Human papilloma DNA appears to be similar in configuration to SV40 DNA and polyoma DNA (Crawford, L. V., manuscript submitted for publication).
- ²⁹ Burton, A., and R. L. Sinsheimer, private communication.
- ³⁰ Jansz, H. S., and P. H. Pouwels, *Biochem. Biophys. Res. Commun.*, 18, 589 (1965).

REFERENCES

- 1 Kleinschmidt, A.K., and R.K. Zahn, Z. Naturforsch., 146:770 (1959)
- 2 Weil, R. and J. Vinograd, Proc.Natl.Acad.Sci.U.S., 50:730 (1963)
- 3 Vinograd, J., J. Lebowitz, R. Radloff, R. Watson, and P. Laipis, Proc.Natl.Acad.Sci.U.S., 53:1104 (1965)
- 4 Lehman, I.R., G.G. Roussos, and E.R. Pratt, J. Biol. Chem., 237:819 (1962)
- 5 Lebowitz, J., R. Watson, and J. Vinograd, in preparation
- 6 Melnick, J.L., Science, 135:1128 (1962)
- 7 Sweet, B.H., and M.R. Hilleman, Proc. Soc. Exptl. Biol. Med., 105:420 (1960)
- 8 Eddy, B.E., G.S. Borman, G.E. Grubbs, and R.D. Young, Virology, 17:65 (1962)
- 9 Rabson, A.S., G.T. O'Connor, R.L. Kirschstein, and W.J. Branigan, J. Natl. Cancer Inst., 29:765 (1962)
- 10 Crawford, L.V., Virology, 19:279 (1963)
- 11 Dulbecco, R., Proc. Natl. Acad. Sci. U.S., 38:747 (1952)
- 12 Vinograd, J., R. Bruner, R. Kent, and J. Weigle, Proc.Natl. Acad.Sci.U.S., 49:902 (1963)
- 13 Studier, F.W., J. Mol. Biol., 11:373 (1965)
- 14 Crawford, L.V., and P.H. Back, Virology, 24:388 (1964)
- 15 Schildkraut, C.L., J. Marmur, and P. Doty, J. Mol. Biol., 4:430 (1962)
- 16 Zimmer, C., and H. Reinert, Anal. Bioch., 14:1 (1966)