# STRUCTURE AND PROPERTIES

# OF

# CLOSED CIRCULAR DNA

Thesis by

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In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1968

(Submitted September 15, 1967)

"I ca'n't believe that!" said Alice.

"Ca'n't you?" the Queen said in a pitying tone. "Try again: draw a long breath, and shut your eyes."

Alice laughed. "There's no use trying," she said: "One ca'n't believe impossible things."

"I daresay you haven't had much practice, "said the Queen. "When I was your age, I always did it for half-an-hour a day. Why, sometimes I've believed as many as six impossible things before breakfast. . . ."

from Through the Looking-Glass
by Lewis Carroll

# ACKNOWLEDGMENTS

My five years in the Caltech Biology Division have been a very eventful part of my life. I have learned a great deal not only about biology, but also about people. I am grateful to my advisor, Dr. Jerome Vinograd, for his constant interest in my work, his excellent advice, and his nearly infinite patience. The assistance and advice of Dr. Giuseppe Attardi and his staff are greatly appreciated. Mrs. LaVerne Wenzel helped a great deal with her knowledge of tissue culture methods.

Special thanks are due to Michael Fried for his help with polyoma DNA infectivity assays, Joel Huberman for his gifts of HeLa cells, and John Newbold for his kind assistance in the growth of $\phi$X174 virus. Further thanks go to Prof. I. R. Lehman for his gifts of E. coli phosphodiesterase, and to Don Robberson and Doug Mohr for their excellent instruction in the use of the electron microscope. I am greatly indebted to Dr. Jean Weigle and Bob Huskey for their help and advice in the growth of lambda virus.

Many thanks go to my colleagues in the sub-basement with whom I have spent a great deal of time the last several years. They have provided much help and many amusing moments.

Special thanks go to Don Robberson, Rebecca Kent, and Bruce Hudson for their constructive criticism and help in the preparation of this thesis.

I appreciate the financial support of the National Institutes of Health.

My colleague, William Bauer, has given much valuable advice and assistance.

Condolences go to William Scott who lost a small wager concerning the length of this thesis.

Finally, I wish to acknowledge the continual encouragement and help of my parents.

# ABSTRACT

This thesis is concerned with the structure and properties of closed circular DNA. Part I of the thesis reports on experiments performed in this laboratory by the author and others to determine the structure of polyoma DNA component II. The following results were obtained: (1) Polyoma II is a ring-shaped duplex DNA molecule. (2) It is generated by introducing one single strand scission in polyoma I by the action of pancreatic DNAase or chemical reducing agents. (3) The sedimentation coefficient of polyoma II is insensitive to several single strand scissions. (4) Polyoma II, when not excessively attacked by pancreatic DNAase or chemical reducing agents, is infective. A publication is enclosed.

The renaturation products of polynucleotide single strands obtained from nicked polyoma DNA were examined in Part II of the thesis. It was found that complementary, linear, single-stranded DNA can renature extensively. Preparations of single-stranded DNA containing more than 50% circular single strands renature to form two bands of DNA in CsCl buoyant density experiments. The buoyant density of one band is approximately that of native polyoma DNA and the buoyant density of the other is characteristic of denatured DNA. No evidence is found for any renaturation of complementary, circular, single-stranded DNA.

Part III of the thesis presents evidence indicating that non-nucleotide linkers are absent in both polyoma and $\phi$X174 DNA. This evidence was obtained by examining the exonuclease I digestion products of denatured, pancreatic DNAase treated polyoma and $\phi$X174 DNA in

alkaline CsCl velocity gradients.

Part IV of the thesis contains a publication describing a dye-buoyant density method for the isolation and detection of closed circular DNA. The method is based on the reduced binding of the intercalating dye, ethidium bromide, by closed circular DNA. In an application of the method, it was found that HeLa cells contain, in addition to closed circular mitochondrial DNA of mean length 4.81 microns, a heterogeneous group of smaller DNA molecules which vary in size from 0.2 to 3.5 microns, and a paucidisperse group of multiples of the mitochondrial length. In addition, methodological results and data not presented in the publication are presented and discussed.

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# GENERAL INTRODUCTION

# Polyoma Virus

## Discovery

The parotid tumor agent was discovered independently by Gross (1) and by Stewart (2, 3) during attempts to transmit AKR mouse* leukemia to newborn mice of another strain with filtrates of leukemia tissue homogenates. Both investigators found that the mouse leukemia filtrates, in addition to transmitting leukemia, were able to induce sarcomas in the parotid gland of mice inoculated shortly after birth. Soon after, it was discovered that the agent capable of inducing these parotid tumors and, indeed, many other types of neoplasms (4) in mice could be propagated in monkey kidney tissue cultures (5). The agent caused cytopathic changes in mouse embryo cell cultures (6).

## Independence of the tumor agent and the leukemic agent

Proof of the independence of the parotid tumor agent (PTA) from the mouse leukemic factor came with the development of a number of virological and immunological techniques useful for the detection and isolation of the PTA. One of these techniques was the hemagglutination assay of Eddy, Rowe, Hartley, Stewart and Huebner (7). Application of this method showed that antibody capable of inhibiting a hemagglutination reaction between hamster red blood

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* This is a strain of mice in which up to 90% of the mouse population develops spontaneous leukemia when approximately one year old.

cells and extracts of parotid tumor agent was present in several isolated mouse colonies. However, it was also shown there was no correlation between the hemagglutination-inhibiting antibody and the appearance of spontaneous or induced leukemia in mouse strains having a high natural incidence of leukemia (8). Further proof of the independence of the PTA was provided by (a) the different sensitivity of the two activities to ether (9), (b) the lack of leukemia producing activity in tissue culture preparations of the PTA (10) and (c) the lack of strain specificity of the PTA (11), a major characteristic of the AKR mouse leukemia.

The tumor agent is a virus

Several pieces of evidence directly supported the assumption that the parotid tumor agent was a virus. Probably the most convincing evidence was provided by electron microscopy, which not only showed the presence of virus particles in PTA concentrates (12) but also indicated these particles might have their origin in the nuclei of infected cells in cell culture (13). The nuclear origin of the particles was also demonstrated by Henle, Deinhardt, and Rodriguez (14) using fluorescent antibody staining techniques. Further work on the quantitation and detection of the tumor agent (15) and the development of techniques such as complement fixation (16), hemagglutination and hemagglutination-inhibition tests (7) and plaque assay methods (17, 18) provided direct and indirect proof that the tumor agent actually is a virus. Evidence for the singular nature of the virus was provided in experiments in which single isolated plaques were recultured and

extracts of these were inoculated into newborn mice. No differences were noted in the types of tumors which the mice developed (19). In addition, Winocour and Sachs (18) and Dulbecco and Freeman (17) demonstrated that the virus could be recovered from single isolated plaques.

## Natural history of polyoma virus

Polyoma virus, so named because of its ability to induce multiple types of neoplasms after inoculation into newborn mice, was found to induce sarcomas in hamsters (20) and rats (21) and fibromas in rabbits (22). However, it must be remembered that these are laboratory situations. In the natural situation, the effects of polyoma virus are much less obvious. In fact, the discovery of the virus was really quite fortuitous, for under natural conditions, this agent causes a latent or chronic infection in the mouse and only very rarely gives rise to neoplasms (23). The only non-laboratory animal in which antibody to polyoma virus has been found is the mouse.

## Biological properties

1. The cytolytic virus-cell interaction

a. The life cycle

Infection of mouse embryo cell cultures with polyoma virus results in a characteristic cytopathic effect (6). This effect, which may also be obtained with DNA isolated from the virus, has been studied in some detail. Such studies have helped to elucidate partially the life cycle of polyoma virus in cell cultures. The results

from the different investigations contain several discrepancies. Gershon and Sachs (25) outlined the following events as occurring during virus synthesis: at 8 to 9 hours after infection, early proteins appear in the infected cell; about 4 to 5 hours later, that is at 12 to 14 hours after infection, infectious viral DNA synthesis is initiated; about 2 hours later, proteins required for the production of virions appear, followed after an additional 7 to 9 hours by infectious virus particles (also 26). The results of Sheinin (27) do not completely agree and indicate that viral DNA is synthesized very late during the latent period, probably no more than 30 minutes prior to the formation of infectious virus. Gershon and Sachs attributed these differences to the nutritional conditions and the virus stocks.

b. Events occurring after infection

Further investigations into the events which occur after polyoma virus infection have focused on two observations which will be discussed in order: (a) There is an induction of cellular DNA synthesis after viral infection. (b) There is an induction in the activity of several cellular enzymes following infection.

Whether cellular and viral DNA or viral DNA alone is synthesized by polyoma virus infected cultures seems to be dependent upon the experimental conditions. For instance, mouse embryo cells infected in logarithmic phase at high multiplicity (about 1000 plaque forming units (pfu)/ml) undergo a productive infection in which all of the cells in the culture give rise to progeny virus. However, under these conditions, cellular DNA synthesis is inhibited (28, 29). If

mouse kidney cells in stationary phase are infected at lower multiplicities, fewer cells undergo productive infection and most of the DNA synthesized is cellular (30, 31, 32). The increased cellular DNA synthesis occurs only in the productively infected cells (33, 34). The viral DNA synthesis is semi-conservative (35). Using confluent mouse embryo cells, Ben-Porat, Coto, and Kaplan (36) further showed that cellular DNA is induced after infection at low multiplicities of virus. Their statements that this DNA is physically unstable with respect to time are not convincing. They did not, for example, adequately characterize the degraded DNA. From the preceeding discussion, it is evident that the relationship between virus infection and increased cellular DNA synthesis is by no means clear.

In addition to the increase in cellular DNA synthesis, infection of mouse cell cultures with polyoma virus effects an increase in the activity of several enzymes concerned with DNA synthesis. Sheinin (37) has shown that the activity of deoxythymidine kinase increases in cultures in which all of the cells are productively infected. Also, the infection of a high percentage of the cells in a culture has been shown to cause the increased activity of deoxythymidine kinase (38, 39), deoxycytidine-5′-monophosphate deaminase (38), DNA polymerase (38), deoxythymidylate synthetase (39), dihydrofolate reductase (40) and deoxythymidylate kinase (41). Further, the results of Hartwell, Vogt, and Dulbecco (42) indicate that it is an increase in the rate of synthesis of deoxycytidine-5′-monophosphate deaminase and thymidine kinase that is responsible for the increase in these enzyme activities.

Their experiments argue against the alternative that the increased enzyme activity in infected cells is due to the activation of pre-existing enzymes. Except for the work of Sheinin (37), it is still unknown whether these increased enzyme activities occur in the productively infected cells, the non-infected cells, or in all cells of the mouse cell cultures. Further, it is still unknown whether the induction of these enzyme activities is due to a derepression of host cell genes or is the result of the viral coded synthesis of new enzymes.

Gershon and Sachs (43) have shown that there is an early synthesis of viral specific RNA required for polyoma development and that this RNA is required not only for viral DNA synthesis but also for viral antigen and completed virion synthesis.

c. Electron microscopic observations

The process of infection has been studied by several investigators (44-48) with electron microscope techniques. These studies indicate the following course of events: (a) Cell cultures inoculated with virus respond in two general ways depending on the nature of the inoculum. Either individual virus particles are pinocytosed by the cell and wrapped in a layer of cell membrane or groups of particles are phagocytosed and wrapped in a membranous covering. (b) A process of reverse phagocytosis occurs at the nuclear membrane. Synthesis of new virus occurs in the nucleus and can be identified at an appropriate time after infection. This new virus is very often associated with bundles of filamentous structures whose diameter is that of the virus particle. It has been suggested that these structures

are developmental forms of the virus. (c) Completed virus particles move gradually into the cytoplasm where they appear to attach to cellular membranes. (d) Lysis of the cell eventually occurs with the release of virus. These observations depend upon the appearance of viral particles or virus related structures in the observed cells. The results provide no information concerning the events of early synthesis.

2. Cell transformation

a. Characteristics

In addition to a cytopathic effect polyoma virus is able to effect a transformation of mouse or hamster embryo cell cultures (49, 50, 51). This effect is, in a sense, the most interesting biological characteristic of the virus for it may provide insight into the cancer problem.

The frequency of transformation of primary embryonic cells with polyoma virus is usually less than 1%, although this is somewhat higher with hamster cells. The transformed cells are distinguishable from normal cells and cloning permits the establishment of transformed cell lines. These cell cultures have a morphology characterized by thick, multilayered cell sheets of an interwoven structure. These cells have lost the property of contact inhibition, one characteristic of a transformed cell line. Such cultures do not release virus but are able to produce tumors if inoculated into their respective hosts. Assays have been devised to determine the transformation activity of polyoma virus (52, 53) or infectious polyoma DNA (54, 55). These assays score for the number of cell clones that form in an

agar-containing medium. Most transformed cells can grow in such an agar suspension, but normal cells cannot.

b. Attempts to induce virus production in transformed cells

The finding that polyoma virus is capable of transforming embryonic tissue cultures led to a search for viral influence within the transformed cells. If this influence were not present in such cells, then it could be argued that the presence of the virus during infection had caused a heritable change in the cell but the presence of the virus was thereafter not needed. In attempts to induce viral constituents in transformed cells, methods successful in the induction of prophage in lysogenic bacteria were tried. Habel and Silverberg (56) were unable to induce viral production from a transplantable hamster tumor line either by in vivo treatment with X-ray, cortisone, or starvation or by X-ray and ultraviolet irradiation of tissue cultures made from the tumor. Vogt and Dulbecco (51), using a wider range of methods, were unable to induce any virus or infectious DNA in transformed mouse or hamster cell cultures. Their methods were sensitive enough to detect the presence of an average of 300 oncogenic but non-cytocidal particles per cell or one infectious DNA molecule per cell. Marker rescue experiments were also unsuccessful. After these results, it became more apparent that if viral influence were present it must be in a highly integrated and non-inducible state.

c. Transplantation antigen

Even though virus production could not be induced in transformed mouse cell cultures or in polyoma tumors, it soon became

obvious that viral influence was not altogether missing in these cells. Two investigators (57, 58) were able to show that polyoma tumor cells contain an antigen, distinct from the viral capsid antigen, which causes these cells to be rejected by mice immunized against polyoma virus. This is thought to occur as follows. If adult mice are infected with polyoma virus, an inapparent infection takes place. During this process, some normal cells are transformed to tumor cells containing the new tumor antigen. This antigen, however, is recognized by the immunologically competent adult mouse as foreign and is rejected. Thus, tumor development does not occur and the adult is sensitized to the tumor antigen. Attempts to transplant isologous polyoma tumors fail because of this sensitivity.

d. Complement fixing antigen

Habel (59) has presented evidence to show that one or more specific antigens are present in virus-free polyoma tumors, as demonstrated by their complement fixation reaction with serum from animals containing transplantable tumors. These do not seem to be related to the transplantation antigen and, of course, may not have any relation at all to the oncogenic properties of the cells containing them. However, the presence of both transplantation and complement fixing antigens in the transformed cells indicate that viral influence remains in the cell even though no infectious virus or DNA appears to be produced.

e. Hybridization

Nucleic acid hybridization experiments have suggested that at

least a part of the viral genome is incorporated into the genome of the transformed cell. Using the agar gel technique (60), Axelrod, Habel, and Bolton (61) found that a larger amount of polyoma viral DNA binds to the DNA extracted from polyoma induced mouse tumors than to the DNA extracted from normal mouse cells. A similar result was obtained with polyoma viral DNA and DNA extracted from polyoma induced hamster tumors. Their results also indicated there are regions of homology between polyoma viral DNA and normal mouse DNA. These latter experiments are not convincing in view of the results of Winocour (62). This investigator showed that only a small portion of the DNA extracted from polyoma virus is homologous with normal mouse synthetic RNA. The homologous DNA did not appear to have a viral origin. On the basis of these results, he proposed that a small amount of mouse cell DNA is encapsulated into virus particles during the maturation stage of development. This encapsulated DNA is thought to be polyoma DNA III which will be described later. Winocour (63) was not able to demonstrate a difference between the degrees of hybridization obtained with polyoma induced tumor cell DNA and normal mouse cell DNA with RNA prepared in vitro with unfractionated polyoma DNA as a primer. With suitable reconstruction experiments, Winocour was able to estimate that if polyoma DNA is present in transformed tumor cells, then there is less than the equivalent of 20 copies of the genome. In an alternate kind of hybridization experiment, Winocour further showed that there is no difference between the hybridizations of polyoma DNA with polyoma tumor

cell synthetic RNA and normal mouse synthetic RNA (64).

In another type of hybridization experiment, Benjamin (65) demonstrated that a small amount of rapidly-labeled RNA from virus-free polyoma transformed cells is capable of hybridizing with polyoma DNA. No hybridization is observed between this RNA and SV40 DNA or between the rapidly-labeled RNA from normal or spontaneous malignant cells and viral DNA. Again, this seems to provide evidence for the presence of at least a part of the viral genome in the transformed cell. However, the sensitivity of the technique was not adequate to determine the quantity of viral genome within the transformed cell nor the true nature of the integration.

## Physical properties

### 1. The virus particle

The polyoma virus is a small spherical particle with a diameter of 35 to 45 mμ (48, 66). There is a disagreement among several authors as to the number of capsomeres in the viral capsid; the reported numbers are 42 (66, 67, 68), 72 (69), or 92 (70). The value of 72, reported by Klug (69), was arrived at after careful consideration of both his electron micrographs and those of the other investigators involved. The particles have a standard sedimentation coefficient of 242 ± 1 svedbergs (71).

Preparations of the virus generally contain two types of particles, one type having a buoyant density in CsCl of 1.339 gm/ml, the other having a density of 1.297 gm/ml. The dense particle contains nucleic acid and is infective whereas the less dense one

contains no nucleic acid and is non-infective. From the difference in buoyant density of the two particles, the nucleic acid content of the dense particle is estimated to be 13.4% (71, 72).

2. The nucleic acid

a. Basic composition

Infectious nucleic acid was isolated from polyoma virus infected mouse embryo tissue cultures by DiMayorca, Eddy, Stewart, Hunter, Friend, and Bendich (24). They concluded that the nucleic acid was viral in origin because of its infectivity in mouse embryo tissue cultures and its sensitivity to DNAase. The DNA was extracted from purified viral preparations by Smith, Freeman, Vogt, and Dulbecco who showed that most of the DNA is made up of the four common bases (73).

The double-stranded nature of the DNA was demonstrated by Crawford (74) on the basis of melting curves which appeared similar to those of known double-stranded DNAs. He calculated from the melting curves that the guanine-cytosine (G-C) content was 47%. His preparations may have contained closed circular DNA (75), which does not heat denature like linear, double-stranded DNA (76). The melting temperature therefore may not be valid. Using a form of polyoma DNA that strand separates under denaturing conditions, Vinograd and Lebowitz obtained a melting temperature of 89°C (76). This corresponds to a G-C content of 48%. On the basis of buoyant density in CsCl, Crawford (74) and Weil (77) obtained G-C contents of 48 and 49%, respectively. However, the value of Weil is not thought

to be as reliable because the determination was made in the preparative ultracentrifuge and a wide, skewed band was obtained. Using dAT as a marker, this author (unpublished results) has obtained a value of $1.701_1$ gm/ml as the buoyant density of polyoma DNA in CsCl. This corresponds to a G-C content of 47%.

b. Nucleic acid molecular weight

By means of electron microscopic counting methods, Crawford (72) originally determined that polyoma virus contained a DNA molecule of molecular weight $7 \times 10^6$ daltons. Later, on the basis of the DNA band width in buoyant CsCl and sedimentation velocity experiments, he revised this value to $3.4 \times 10^6$ daltons (78). Weil and Vinograd (79) calculated the molecular weight of polyoma DNA from the length of the molecule as determined in electron micrographs and by the band width of the DNA band in buoyant alkaline CsCl. They obtained $3.0 \times 10^6$ and $2.4 \times 10^6$ daltons, respectively. With the help of Studier's equations (84), which relate molecular weight to sedimentation coefficient in neutral or alkaline solution, it is possible to calculate the molecular weight of the linear form of polyoma duplex DNA or of a single linear strand of the DNA. Such calculations give $2.5 \times 10^6$ and $1.6 \times 10^6$ daltons, respectively. These values are calculated using an $s^\circ_{20,w}$ of 14.5S at neutral pH for the duplex DNA and 15.7S for the single linear strands at high pH.

c. An unusual structure

Weil (77, 80) observed that DNA extracted from polyoma virus had several properties which were not observed with other double-

stranded DNAs. For instance, the DNA renatured extremely rapidly and heating at 100°C for 10 to 20 min followed by rapid cooling did not reduce the infective titer. An explanation of these results was given by Weil and Vinograd (79) and Dulbecco and Vogt (81), whose results revealed that preparations of polyoma DNA contained three sedimenting species, designated PY I, II, and III and having sedimentation coefficients, $s^{\circ}_{20,w}$, of 20.3S (20S), 15.8S (16S), and 14.4S (14S), respectively.

PY I was shown to be a circular molecule based on evidence from several types of experiments. First of all, the very rapid renaturation after heating to 100°C supported the idea that the two strands of the molecule were not separable. Secondly, the buoyant density of PY I in CsCl at pH 12.4, 1.778 gm/ml, indicated the molecular structure was not that of single-stranded DNA, for previous work (82) had shown that the buoyant density of the denatured single strands would be approximately 1.760 gm/ml. In addition, the band width of the molecule in pH 12.4 buoyant CsCl indicated the structure had twice the molecular weight of the denaturation products of PY II. Thirdly, the sedimentation velocity of PY I was increased from 20S to 53S upon increasing the pH to 12.5. These arguments, all based upon the resistance of the molecule to denaturing conditions, supported the hypothesis that PY I was a circular structure which became a compact denatured form at high pH. The inter-strand hydrogen bonding was destroyed but the strands were non-separable under such conditions. Finally, the electron micrographs prepared by Stoeckenius (79) clearly

showed the presence of circular DNA in Weil and Vinograd's preparations.

PY II, which usually comprised 0 to 20% of the preparations, was thought to be the linear form of PY I for several reasons. First, the buoyant density of II in neutral CsCl was the same as I. Upon thermal or alkaline denaturation, the buoyant density increase of II in neutral or alkaline CsCl, respectively, was the same as for known linear DNAs. Secondly, the lower sedimentation coefficient of PY II as compared to PY I became reasonable if PY II were a linear molecule, since a circular molecule of the same molecular weight would be expected to have a smaller frictional coefficient. Also, linear molecules were seen in electron micrographs. Finally, it was known that the action of pancreatic DNAase or exposure to concentrated CsCl was able to convert PY I to PY II. Dulbecco and Vogt believed the conversion using pancreatic DNAase was due to a double strand scission induced by the combination of an enzymatic single strand scission and the resulting ring strain.

PY III, which appeared in the viral DNA preparations in small variable quantities, was thought to be a contaminating DNA, presumably of mouse origin. PY III, in contrast to I and II, was found to be non-infective in a plaque assay on mouse embryo cell cultures (81). PY I and II are able to transform hamster embryo cells in the transformation assay (54), but III is inactive.

d. The twisted circular structure

Structural studies on polyoma viral DNA were continued with an

investigation of the conversion of PY I to PY II. A close study of the conversion product(s) by means of the analytical ultracentrifuge and the electron microscope resulted in a modification of the ideas concerning the structure of PY I and PY II. Vinograd, Lebowitz, Radloff, Watson, and Laipis (75) obtained results which proved (a) that PY II is a ring-shaped duplex molecule, (b) that it may be generated by introducing one single strand scission in PY I by the action of pancreatic DNAase or chemical reducing agents, (c) that it has a sedimentation coefficient which is insensitive to several single strand scissions, and (d) that PY II made by pancreatic DNAase treatment is infective when not excessively hit.

These findings, however, raised a new question. If both PY I and PY II are circular, why does the viral DNA, an intact duplex ring, sediment 25% faster than PY II, a circular molecule containing one or more single strand scissions? This question led to the proposal of a twisted circular structure for PY I. According to this proposal, PY I contains superhelices which are locked into the molecule by chemical forces. Two types of experiments suggested this structure.

(a) A study of the sedimentation velocity of the components of polyoma DNA as a function of pH revealed an interesting pH-melting curve for PY I. The sedimentation velocity of both I and II remained constant between pH 8.0 and approximately 11.5. At this pH, in accord with the results of Studier (84) and Freifelder and Davison (85) denaturation begins with a concommitant increase in the sedimentation coefficient. At a pH of approximately 11.8, strand separation

occurs and two components appear having lower sedimentation coefficients corresponding to the separated linear and circular single strands. The behavior of PY I, however, did not follow this pattern. At pH 11.5, the sedimentation coefficient first dropped and became the same as that of PY II from pH 11.6 to 11.8. As the pH was further increased, the sedimentation coefficient also increased with the formation of the double-stranded cyclic coil. The dip in the curve was initially quite unexplainable. However, if PY I contains right-handed twists in an interwound superhelical structure*, such behavior is not only plausible, but required. In the early stages of denaturation, some of the right-handed duplex turns unwind. So as to conserve the total winding number of the molecule, an equivalent number of right-handed superhelical turns must unwind, producing a molecule which should have, at some stage, a hydrodynamic structure equivalent to that of PY II. As the denaturation of the molecule continues, a left-handed twisting of the entire molecule should occur with the eventual formation of the double-stranded cyclic coil. It is required that this behavior be found under all denaturing conditions. That this may be true is seen from the results of Crawford and Black (86) who heated polyoma and SV40 in formaldehyde to various temperatures and obtained similar results.

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* This is the structure normally visualized in electron micrographs. It is equivalent to the toroidal model which contains left-handed, non-interwound, superhelical turns. The reversal of handedness is the result of a 90° rotation of the viewing axis.

(b) The twisted circular structure provides a satisfactory explanation for the configurational change that occurs when one single strand scission is introduced into PY I. Such a scission allows rotation to occur about the phosphodiester bond in the complementary strand opposite the break, thus relieving the topological restraint in the molecule which is responsible for the configurational characteristics of PY I.

Several of the stability characteristics of closed circular DNA are discussed in detail by Vinograd and Lebowitz and will not be discussed further here (76).

Part I of the thesis explains in detail work done by the author in the determination of the structure of PY II. A number of investigators collaborated on this work, so it will be necessary to integrate all of the results into a complete picture.

Part II of the thesis deals with an investigation of the renaturation properties of single-stranded polyoma DNA derived from polyoma II DNA containing one single strand scission.

Part III presents the results of a investigation of the possibility that polyoma DNA might contain a linker, that is some non-nucleotide structure linking its two ends together. The results indicate that polyoma DNA does not contain any non-nucleotide material within its structure.

## Closed Circular DNA

Covalently closed circular DNA has now been found in a large number of living organisms. These include the DNAs from the tumor viruses: polyoma (79, 81), SV40 (86), rabbit (87) and human papilloma (88); bacteriophage intracellular DNAs: the intracellular forms of $\phi$X174 (89, 90), M13 (91), $\lambda$ (92, 93) and P22 (94); the colicinogenic factor $E_1$, a bacterial plasmid (95); and the mitochondrial DNAs from unfertilized sea urchin eggs (96), beef heart (97), sheep heart (98), mouse liver (97), human leukocyte (99), and HeLa cells (100).

These DNAs have the same properties as polyoma DNA with respect to stability at high pH and temperature and reactions with chemical reducing agents and pancreatic DNAase (75, 76). Thus, there are several basic questions that need answering. (a) Why do these DNAs exist as twisted circular structures? Is the twisted circular structure present in the cell or virus or is it the result of the extraction procedure which requires a transition from a cellular or viral environment to some artificial environment? (b) What is the mode of replication of such molecules? (c) Since closed circular molecules with molecular weights from approximately $4\times10^5$ to $40\times10^6$ daltons have been found, may it be said that all of these molecular sizes contain the same number of superhelical turns?

The use of the dye ethidium bromide has allowed more detailed work to be initiated on the structure of closed circular DNAs. The binding of this intercalative dye has been shown to cause a partial unwinding of the duplex structure in such molecules (101-103).

This has the same effect on the configuration of the molecule as denaturation by heat or high pH. That is, as the unwinding of the duplex structure proceeds, the necessity for conserving the number of turns in the molecule forces the unwinding of right-handed interwound superhelical turns. At a critical pH, temperature or dye concentration, the hydrodynamic configuration of closed circular DNA should be the same as nicked circular DNA. By calculating the amount of dye bound at the equivalence point, Crawford (101) was able to determine that polyoma I DNA contains about 12 superhelical turns and Bauer and Vinograd (102) were able to determine that SV40 DNA contains $17.0 \pm 3.5$ turns.

The number of superhelical turns can also be estimated by the behavior of closed circular and nicked circular DNA in buoyant density gradients containing dye. Since closed circular SV40 DNA displays a greater affinity for dye than does the nicked form at low dye concentrations and a lower affinity at higher concentrations of dye, it was possible to find the number of superhelical turns knowing the buoyant density and the relations between the amount of dye bound and the buoyant density of the DNA-dye complex at the crossover point. This was found by Bauer and Vinograd (102) to be $14.2 \pm 0.9$ superhelical turns.

The binding of dye by closed circular DNA at high dye concentrations (100 $\mu$g/ml) is only approximately half that of nicked circular DNA. This fact provided the basis for the development of a method (100) for the isolation and detection of closed circular DNA in dye-buoyant density gradients in the preparative ultracentrifuge. In the

first application of the method, it was shown that various sizes of closed circular DNAs are present in HeLa cells. The details of the method are presented in Part IV of the thesis.

Several fundamental questions concerning the origin of the superhelices in closed circular DNA were raised earlier. At the present time, only speculative answers are possible. For instance, it might be hypothesized that the twisted circular structure assumes such a configuration only after extraction from a viral or cellular environment. In the cell, the viral DNA might have an average pitch which is slightly larger than that in the isolated DNA. Upon isolation a decrease in the pitch would cause the formation of superhelical turns. Alternatively, the closed DNA might be formed around a core or organizer and be superhelical in the cell. Upon isolation of the DNA, separation of the core from the DNA leaves the molecule in its previous superhelical state. Answers such as these are being actively investigated by a number of research groups.

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# PART I

# THE STRUCTURE OF POLYOMA II DNA

# INTRODUCTION

The conversion of polyoma I DNA to polyoma II was originally thought to represent a conversion from a circular to a linear molecule. From the results of Weil and Vinograd (1) and Vogt and Dulbecco (2) it was known that such a conversion could be effected by incubation of PY I DNA in concentrated CsCl or by reaction with pancreatic DNAase. Structural studies on PY I and II began with an investigation of these reactions. It was soon found that the conversions also occurred in the presence of mild reducing agents. These reagents were suggested by the observation that rigorous exclusion of impurities from the phenol, used in the isolation of the DNA, diminished the amount of PY II in the final DNA preparation.

An examination by electron microscopy of the products of a PY I to PY II conversion revealed that PY II is not linear but circular. Digestion of the denaturation products of PY II with exonuclease I and infectivity studies indicated (a) that PY II is a ring-shaped duplex molecule, (b) that it is generated by introducing one single strand scission in PY I by the action of pancreatic DNAase or chemical reducing agents, (c) that it has a sedimentation coefficient which is insensitive to several single strand scissions and (d) that PY II is infective when not excessively hit. The discussion of the implications of this structure are contained within the General Introduction.

A copy of the publication resulting from the work is found at the end of the Results section of this part of the thesis. The detailed

results and discussion will be limited to those aspects of the subject with which the author was directly concerned, since several investigators collaborated in this work.

# MATERIALS AND METHODS

## Materials

1) Medium

For all tissue culture work, reinforced Eagle's medium (REM) was used (4). This is Basal Eagle's containing four times the normal amount of vitamins and amino acids. In addition, 50 units/ml of potassium penicillin G, 1 mg/ml of streptomycin sulfate, and $2\times10^{-4}$ mg/ml of n-butyl-p-hydroxybenzoate were added as antibiotics. The REM was supplemented with 4 to 20% calf or horse serum and was used as a liquid medium or as a constituent of the agar overlay of cell monolayers.

2) Buffers

TD consists of 0.137 M NaCl, $7\times10^{-4}$ M $NaH_2PO_4$, 0.005 M KCl and 0.025 M Sigma 7-9, pH 7.4. In addition, 50 units/ml of potassium penicillin G and 1 mg/ml of streptomycin sulfate are added.

Tris-buffered saline (TBS) is TD buffer plus $4.9\times10^{-4}$ M $MgCl_2$ and $9.0\times10^{-4}$ M $CaCl_2$.

Ethylenediamine tetraacetate (disodium salt) will be referred to as EDTA. It was always used after titration to pH 8.0 with KOH.

SSC/10 containing 0.01 M tris, the standard DNA and virus buffer, contains 0.015 M NaCl, 0.0015 M $Na_3C_6H_5O_7$, and 0.01 M tris, pH 8.0. Tris was prepared by titrating Trizma Base (Sigma Chemical Co.) to pH 8.0 with HCl.

3) Enzymes

Receptor destroying enzyme (RDE), an enzyme which destroys

the affinity of receptor sites on the surface of erythrocytes and host cells for polyoma virus (5), was obtained as a lyophilized powder from Behringwerke A. G. The powder, supplied in serum bottles, was dissolved in 2. 5 ml of TBS.

Pronase (B grade) was obtained from Calbiochem and was dissolved in SSC/10, 0. 01 M tris, pH 8. 0 immediately before use.

Chymotrypsin (crystallized, salt free) was obtained from Worthington Biochemical Corp. and was dissolved in SSC/10 and 0. 01 M tris.

Pancreatic DNAase, 1 x crystallized, was obtained from Worthington Biochemicals Corp. Samples were made up at $7.2\times10^{-4}$ μg/ml in 0. 125 M NaCl, 0. 01 M tris, 0. 02 M $MgCl_2$ and 0. 01% BSA and were frozen at -20°C.

Trypsin 1-300 was obtained from Nutritional Biochemical Co.

Soybean trypsin inhibitor ( 2 x crystallized and lyophilized) was obtained from Worthington Biochemical Corp.

BSA, 30% bovine serum albumin, was obtained in a sterile form from Armour Pharmaceutical Co.

E. coli phosphodiesterase (exonuclease I) (3) was a gift from Professor I. R. Lehman. The original sample was 2000 units/ml but this was diluted to 250 units/ml by adding 75 μl of enzyme solution to 0. 525 ml of 0. 067 M glycine buffer, 0. 3% BSA, pH 10. 2.

4) Chemicals

Optical grade CsCl (Lots # 16-17) was obtained from Harshaw Chemical Co. Analar sucrose was purchased from The British Drug Houses, Ltd., Poole, England. All other chemicals were

reagent grade.

5) Virus

The polyoma virus was obtained from Professor R. Dulbecco, who originally obtained it from Dr. W. Rowe of the National Institutes of Health. The virus is the large plaque type. The properties of this strain have been described by Vogt and Dulbecco (6).

Methods

1) Mouse embryo secondary cultures

Primary cultures were made by removing embryos from 12 to 13 day pregnant, random bred Swiss mice, trypsinizing and dispersing the cells in REM containing 10% calf serum according to the method of Dulbecco and Freeman (7). Secondary cultures were made after confluent monolayers had formed, usually within 2 to 4 days. Such cultures became confluent in 1 to 3 days and were used for the assay of infectious DNA. These cultures were grown in 6.0 cm petri dishes.

2) Virus growth

The virus was grown according to the methods of Winocour (8). Uninfected mouse kidney monolayers were fluid changed with REM containing 10% horse serum 48 hours after they were made. After another 12 hours, the fluid was removed from the monolayers and 0.6 ml of a virus supension containing $5\times10^{7}$ to $5\times10^{8}$ plaque forming units (pfu)/ml were added. After 1 3/4 hours the monolayers were covered with REM containing 10% horse serum. Tritiated thymidine labeled virus was prepared by adding 20 to 30 $\mu$c of $H^{3}$

thymidine to each 10 cm petri dish at this time. After approximately 5 days, CPE (cytopathic effect) occurred and both the supernatant and remaining cell debris were collected from the plates. The cell debris was removed by low-speed centrifugation and sonicated for 2 to 4 min using the number 6 power setting on a Branson 125 S Sonifier. The large probe was not placed within the tube containing the cells but, instead, was held very near and outside the tube, both tube and probe being in a water reservoir. Both lysates and cellular debris were stored at -20°C.

3) Virus purification

A combination of two methods (8, 9) with various modifications was used for purifying the virus. Both cellular debris and lysates were used as starting material. (a) Concentration of virus present in crude lysates was accomplished by centrifugation in the Spinco 30 rotor for 2 hours at 27 K rpm and 10°C. The pellet was resuspended in a low salt buffer (SSC/10, 0.01 M tris or TD) and incubated with 0.025% trypsin for 30 min at 37°C. At this time, the preparation was sonicated for two 1-min periods by placing the microprobe in the sample (# 6 power level, 4°C). Sodium deoxycholate (9) was added to 0.5%. After another 20 min incubation period at 37° C, soybean trypsin inhibitor was added to 0.025%. Following a low speed spin (6 K rpm for 15 min in the Spinco 30 rotor), the virus was again pelleted and resuspended by sonication. As a further purification step, the virus preparation was banded in CsCl ($\rho$ = 1.33 gm/ml) in the Spinco SW 25.1 rotor for 48 hours at 23 K rpm. The band

(or bands) was removed and dialyzed against 3 or 4 8-hour changes of SSC/10, 0.01 M tris. If more than one band (in addition to the empty shell band) was found, they were used separately. Often, the trypsin and sonication steps were not used. An alternative method of concentrating the virus in crude lysates was by means of precipitation with absolute methanol (33% final volume) at -20° C. The methanol precipitate was then treated with 0.025% trypsin and the extraction proceeded as before. (b) If the virus was to be isolated from cellular debris, the debris was first incubated with RDE (0.5 ml/10 ml of sample) for 24 hours at 37° C. At the end of this time, the extraction proceeded as in (a) after the addition of trypsin. Because of the large amount of cellular debris present in this type of extraction, additional purification before banding in CsCl was necessary, either by means of differential centrifugation or a KBr gradient. In the latter case, 22 ml of preparation were layered over 8 ml of saturated KBr in 0.01 M EDTA and 0.01 M tris. After centrifuging for 3 hours at 23 K rpm in the Spinco SW 25.1 rotor, the virus bands separated from the cellular debris. These bands were collected after piercing the bottom of the tube, dialyzed against low salt buffer and rebanded in 1.33 gm/ml CsCl as before.

4) DNA extraction and purification

The DNA extraction procedure was essentially that of Weil (10). Appropriate salt solutions were added to the virus preparation (in SSC/10, 0.01 M tris, pH 8.0) so that the sample contained 0.27 M NaCl, 0.027 M $Na_3C_6H_5O_7$ , 0.01 M tris, and 0.01 M EDTA, pH 8.0. An equal volume of a solution consisting of 67% (V/V) redistilled phenol in

0. 038 M tris and 0. 17 M potassium trichloroacetate, pH 8. 0 to 8. 5, was added at room temperature. The mixture was shaken for a total of 15 min,alternately using a Vortex mixer and manual mixing. The emulsion was broken in the Sorvall SS-34 rotor at 7K for 10 min. The aqueous layer was extracted again with an equal volume of 80% phenol in 0. 046 M tris. The phenol and protein layer from the first phenol extraction was re-extracted using an equal volume of SSC. The resulting aqueous layer was used to re-extract the phenol layer of the second phenol extraction. Both this material and the aqueous layer of the second phenol extraction were dialyzed at 4°C against seven 8-hour changes of SSC/10, 0. 01 M tris, each change about 200 times the sample volume. The DNA samples were frozen at -20°C or -70°C. The specific activity of most labeled preparations was 10,000 to 20,000 cpm/$\mu$g.

5) Purification of the components of polyoma DNA

The components of polyoma DNA were separated by sedimentation velocity in a 5 to 20% linear sucrose gradient containing SSC/10 and 0. 01 M tris, pH 8. 0. In some experiments, the gradients also contained $10^{-3}$ M EDTA. The gradients were centrifuged for 17 to 18 hours in the Spinco SW 25. 1 rotor at 23 K rpm and 20°C. The centrifugation time was 9 hours at 30 K rpm and 4°C with the Spinco SW 39 rotor. The DNA was detected by absorbance at 260 m$\mu$ or by scintillation counting of fractions collected after puncturing the tube. The sucrose was removed by dialysis.

6) Analytical sucrose gradients

Samples which were to be analyzed for total counts and infectivity were centrifuged through 3 to 20% linear sucrose gradients containing SSC and 0.05 M tris, pH 8.0. The centrifugation was continued for 9 hours at 30 K rpm and 5 to 10°C. A Buchler (11) tube piercing device was used in the drop collection procedure. Drops were collected in an alternating sequence of 3 drops into 0.2 to 0.6 ml of 0.55 M NaCl and 0.05 M tris, pH 8.0, and 1 drop into 10 ml of Bray's solution (12).

7) DNA infectivity assay

These assays were done according to the method described by Weil (10). Secondary mouse embryo monolayers were used just after they had become confluent. The media was aspirated from the 6.0 cm plates and the monolayers were washed gently with 2.5 ml of 0.37 M NaCl, 0.05 M tris, pH 8.0, 29°C. After removing this buffer by aspiration, the monolayers were washed with 0.55 M NaCl, 0.05 M tris, pH 8.0, 29°C. Again, the wash was removed. The monolayers were now seeded with 0.1 ml of the DNA sample in 0.55 M NaCl and 0.05 M tris, pH 8.0. After incubating the plates by floating them in a 20°C water bath for 18 min, 4 ml of REM containing 20% calf serum were placed on each plate. After incubation at 37°C for approximately 10 hours, additional fresh mouse embryo cells were placed on the plates if the monolayers showed extensive damage due to the harsh ionic conditions. After the monolayers again became confluent, the media was removed from the plates and the monolayers were overlaid with 8 ml of agar containing REM and 4% horse serum.

After 7 to 10 days, 4 ml of 0.01% neutral red in agar containing REM and 4% calf serum were added. The following day, the number of plaques were counted. All 37°C incubations of infected and non-infected monolayers were done in well-humidified incubators having a $CO_2$ atmosphere appropriate for maintaining the pH of the cultures between 7.0 and 7.4.

8) Enzyme assays

a) Pronase and chymotrypsin

The relative activities of these enzymes were determined by measuring the acid soluble absorbance at 260 mμ produced by the action of the given enzyme in a solution of vitamin test casein after 20 min and 37°C.

b) Exonuclease I (3)

The activity of this enzyme was determined by measuring the acid insoluble absorbance at 260 mμ after incubating the enzyme with denatured and undenatured calf thymus DNA. The pH of the incubation sample was 10 to 10.2 and the reaction time was 30 min or 1 hour. It was found that the enzyme had almost no effect on the undenatured DNA.

9) Pancreatic DNAase and E. coli phosphodiesterase (exonuclease I) digestion

The conditions for the two sequential enzymatic reactions were essentially the same as described by Fiers and Sinsheimer (13). The initial reaction mixture contained 106 μg/ml pure PY I in 0.045 M NaCl, 0.0075 M $MgCl_2$, 0.01 M tris pH 8.0, 40 μg/ml BSA and

$2.7 \times 10^{-4}$ μg/ml of pancreatic DNAase. This was reacted for 20 min at 20°C. The reaction was stopped by the addition of 1/15 volume of 1 M glycine buffer, pH 9.8. The mixture was heated for 5 min at 100°C and cooled rapidly. One unit of exonuclease I was added for each μg of DNA present. The reaction mixture contained 70 μg/ml of DNA in 0.03 M NaCl, 0.005 M $MgCl_2$, 0.007 M tris, 0.067 M glycine buffer, 0.90 mg/ml BSA and 71 units/ml E. coli phosphodiesterase, pH = 9.8. One-tenth volume of 0.1 M EDTA was added after 90 min at 37°C.

10) Ultracentrifugation

a) Sedimentation velocity analyses were performed in a Spinco model E ultracentrifuge by band centrifugation (14, 15). Some experiments were done using the photoelectric scanning device. Films were traced with a Joyce-Loebl Mark III microdensitometer. Uncorrected sedimentation coefficients were calculated from the slope of the line formed by plotting the logarithm of the distance of the band maxima from the center of rotation versus time. $s^{Na}_{20,w}$ values were found by applying the corrections of Bruner and Vinograd (16). Buoyant density experiments were performed using the methods of Hearst and Vinograd (25). Band widths were determined from the slope of the straight line obtained when distance through a band was plotted versus fractional band height on Gaussian paper.

11) Radioactive counting procedures

As mentioned before, the radioactivity distribution in a gradient was determined by collecting drops directly into 10 ml of Bray's solution. Packard scintillation counters were used in all

experiments and the settings were determined to maximize the tritium counting efficiency.

12) Calculations

Formulas for the Poisson distribution are presented in Part III.

13) Dialysis

Dialysis tubing was prepared for use by boiling in 0.01 M EDTA for 15 min followed by a distilled $H_2O$ rinse.

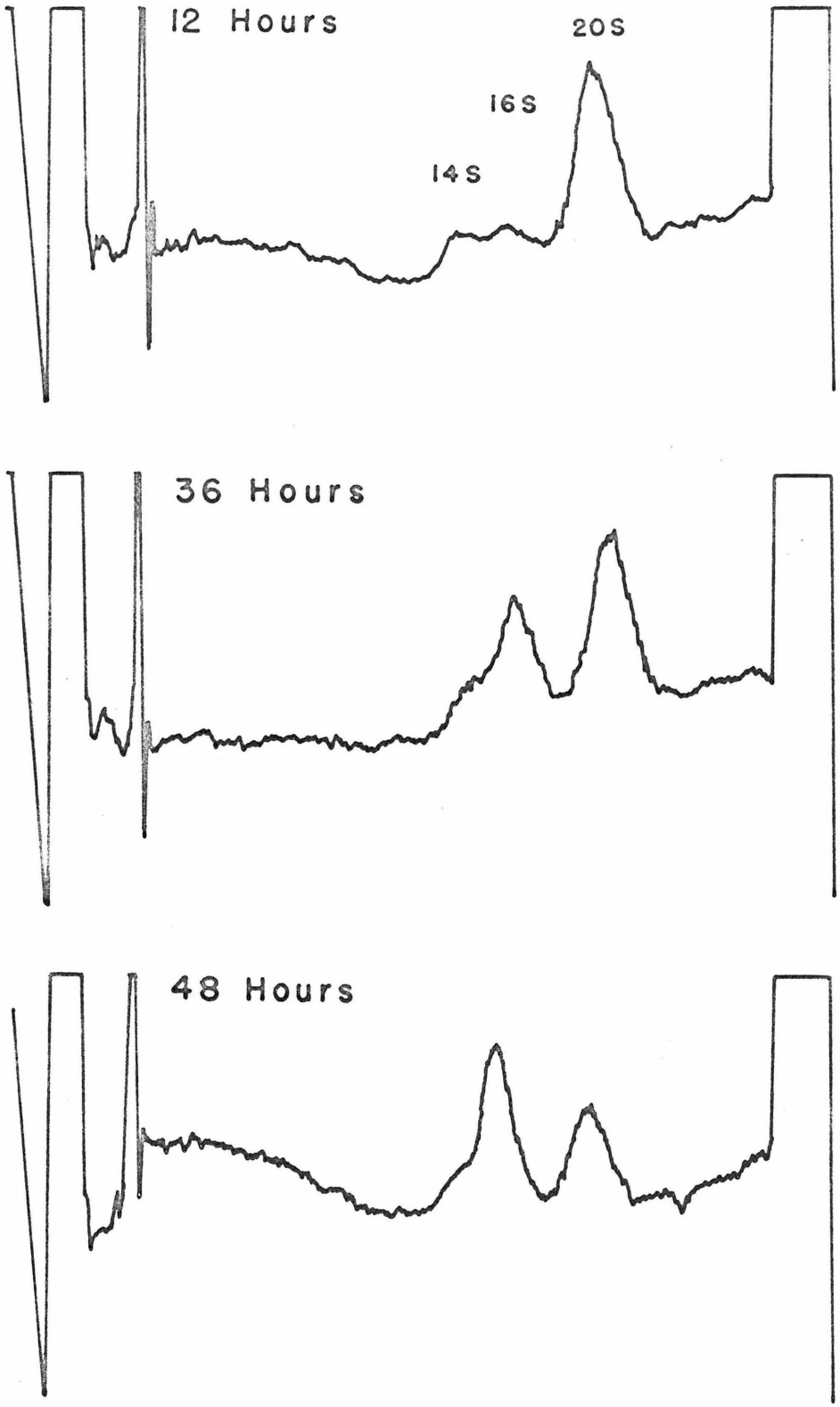
# RESULTS

## CsCl incubation

Weil and Vinograd observed a slow conversion of PY I to PY II during incubation in 1.72 gm/ml CsCl (1). At the time, these observations were thought to represent a conversion of a circular molecule to a linear molecule. It was decided to investigate this reaction in greater detail with the object of obtaining a deeper insight into the structure of PY I and II.

In the first experiments, the results of Weil and Vinograd (1) were corroborated. Solid CsCl was added to PY I DNA to a solution density of 1.72 gm/ml. Samples were removed, diluted and analyzed by band velocity sedimentation (14, 15, 16) in neutral 3 M CsCl after incubation times of 12, 24, 36 and 48 hours at room temperature. The results, as seen in Figure 1, indicate a slow conversion of PY I to PY II with a 50% conversion occurring in 40 to 44 hours. In addition, the conversion appears to proceed directly from one discrete component to another. No material sedimenting slower than PY II was present except for a constant amount of PY III. Since the conversion was thought to be caused by a double strand scission, thereby producing a linear molecule from a circular one, it was felt that such a conversion was not the result of a random attack which would have yielded a Gaussian distribution of products. After a 48 hour incubation, when the ratio of PY II to PY I is 1.3 to 1.5, 23 to 25% of the DNA present should have sedimented more slowly than PY II. This observation does not take into account the presence of PY III in the

Figure 1. Analytical band sedimentation velocity patterns of polyoma DNA incubated in CsCl, $\rho$ = 1.72 gm/ml and pH 8.0. The densitometer tracings represent the distribution of the components of polyoma DNA in samples incubated for 12, 36, or 48 hours in the presence of concentrated CsCl at room temperature. Approximately 1 $\mu$g of DNA was used for each band velocity experiment. The tracings represent the distribution of DNA in each analysis approximately 100 min after sedimentation began. The field is directed toward the right. The sedimentation solvent for the analytical centrifugation experiments was CsCl, $\rho$ = 1.35 gm/ml at pH 8.0. The conditions in the analytical ultracentrifuge were 44,770 rpm, 20°C.


12 Hours
20S
16S
14S
36 Hours
48 Hours

preparation. Thus, it was thought that the conversion of PY I to PY II was the result of breakage at one labile region in the molecule and that this region might be the only place where such breakage could occur.

This result seemed to be supported by the following experiment. Using the Spinco model E ultracentrifuge, PY I DNA was banded in CsCl for a total time of 120 hours. The band width was measured at intervals throughout this period and was found to be constant with a standard deviation of $\pm 1.5\%$. From the previous results, it could be predicted that if the incubation in CsCl were causing random double strand scissions, then after 120 hours, approximately 20% of the DNA originally present would have remained at the original molecular weight. The calculation shows that each molecule received, on the average, 3 hits. The remaining 80% would have been expected to increase the band width because of its smaller average molecular weight. The result supported the hypothesis that the conversion of PY I to PY II in 1.72 gm/ml CsCl, if indeed it were a conversion of a circular to a linear molecule, was due to a non-random breakage at a unique, labile region on the molecule.

Degradation of DNA in high molarity CsCl is not a process that has been noted before. For this reason it was felt that the particular sample of CsCl used was contaminated by some impurity. An analysis by the Geology Department of the California Institute of Technology revealed no elements which would form salts that obviously caused the degradation. Table 1 presents the spectrographic analysis.

When this series of experiments was again repeated, no

Table 1. Spectrographic Analysis

| Element or Compound | Amount Detected in ppm (a) Maywood | Harshaw 1 (b) | Harshaw 2 (c) | Sensitivity in ppm |
|---|---|---|---|---|
| Be | -- | -- | -- | 0.8 |
| Cu | -- | -- | -- | 1 |
| Ag | -- | -- | -- | 0.5 |
| Au | -- | -- | -- | 20 |
| Pt | -- | -- | -- | 50 |
| Mo | -- | -- | -- | 2 |
| B | -- | -- | -- | 10 |
| Ge | -- | -- | -- | 20 |
| Sn | -- | -- | -- | 5 |
| Pb | -- | -- | -- | 10 |
| As | -- | -- | -- | 200 |
| Sb | -- | -- | -- | 200 |
| Bi | -- | -- | -- | 10 |
| Zn | -- | -- | -- | 100 |
| Cd | -- | -- | -- | 20 |
| Ti | 20 | 10 | 10 | 1 |
| Mn | -- | -- | -- | 1 |
| Co | -- | -- | -- | 1 |
| Ni | -- | -- | -- | 1 |
| Ga | -- | -- | -- | 1 |
| In | -- | -- | -- | 10 |
| Cr | -- | -- | -- | 10 |
| V | 30 | 30 | 40 | 1 |
| Sc | -- | -- | -- | 5 |
| Y | -- | -- | -- | 10 |
| Yb | -- | -- | -- | 1 |
| La | -- | -- | -- | 100 |
| Zr | -- | -- | -- | 2 |
| Nb | -- | -- | -- | 20 |
| Ca | 1-1000 | 1-1000 | 2000 | 1 |
| Sr | -- | -- | -- | 1 |
| Ba | -- | -- | -- | 1 |
| Li | <20 | <20 | <20 | 20 |
| $K_2O$ | 10 | <10 | 10 | 10 |
| Rb | -- | 2100 | 4300 | 40 |
| Na | 70 | 100 | 90 | 40 |
| Fe | -- | -- | -- | Approx. 50 |

(a) Parts per million (ppm) is on a mass basis. A double dash means nothing was found within the sensitivity of the test.

(b) Harshaw 1 (Lots # 16-17) was the CsCl sample used as described in the text.

(c) Harshaw 2 was a sample of CsCl obtained in October 1962.

conversion occurred. This result indicates that it was some as yet unknown impurity in the original CsCl which caused the breakage.

Proteolytic enzymes

The suggestion that there might be some unique region in polyoma DNA susceptible to the action of certain reagents prompted the use of two proteolytic enzymes in an attempt to hydrolyze such a structure. Pronase, an enzyme with little specificity (17), and chymotrypsin, an enzyme which has a preferential specificity towards peptide linkages involving the carboxyl group of aromatic amino acids (18), were incubated with DNA in reaction systems previously tested with vitamin test casein. The results showed that neither chymotrypsin nor pronase was able to cause a transition of PY I to PY II. This indicated that the unique region, if it did exist, was either not a protein or was a protein in such a configuration that the enzymes could not attack it.

Reducing agents

Following attempts to produce PY II by the use of incubation in concentrated CsCl or proteolytic enzymes, it was found that treatment with mild reducing agents would efficiently effect this conversion. As stated in the Introduction to this part, this idea was suggested by the observation that impure phenol (containing hydroquinone) was able to cause a marked increased in the amount of PY II present in viral DNA preparations. Several reducing agents were studied in this laboratory, but only two will be discussed in detail here --

2-mercaptoethanol and $FeCl_2$.

A one hour incubation of PY I DNA with 0.007 M 2-mercaptoethanol (2-ME) at pH 8.0 caused a 12% conversion of PY I to II. However, if this reaction took place at pH 3.9 (established with 0.2 M acetic acid), a 67% conversion of PY I to PY II occurred. The conversion due to the low pH alone was 21%. As in the case of the CsCl conversion of PY I to PY II, these results appeared to indicate that the conversion reaction was producing a linear molecule from a circular molecule by means of a specific double strand scission.

The reducing agent $FeCl_2$ is also able to promote a conversion of PY I to PY II. Figure 2 shows the total $H^3$ count distributions and DNA infectivity distributions of $FeCl_2$ treated and untreated DNA sedimented through 3 to 20% sucrose velocity gradients. The $FeCl_2$ treated DNA sample had been incubated in $10^{-3}$ M $FeCl_2$ at pH 8.0 and room temperature for 1/2 hour previous to layering the samples on the surface of the gradients. PY I (20S), PY II (16S) and PY III (14S) are present in these gradients. PY I separated well from PY II, but the separation of II and III was incomplete. In this experiment, only a small amount of conversion occurred.

When polyoma DNA was incubated with $5 \times 10^{-3}$ M $FeCl_2$ for a period of 14 hours previous to the start of the sucrose velocity run, it was found that all of the PY I present was converted to PY II. Figure 3c and Figure 4c illustrate these conversions as compared to the controls in Figure 3a and Figure 4a. The absorption at the meniscus in Figure 3c is due to the oxidized $FeCl_2$. DNA infectivity assays were performed with samples obtained from the gradients of

Figure 2. Velocity sedimentation profiles of $H^3$-labeled polyoma DNA in 3 to 20% sucrose gradients. The field is directed toward the left. (a) $H^3$ count distribution of untreated polyoma DNA. (b) Samples were removed from the gradient in (a) and assayed for DNA infectivity as described in the Materials and Methods section of Part I. (c) $H^3$ count distribution of polyoma DNA incubated in $10^{-3}$ M $FeCl_2$ for 1/2 hour at room temperature, pH 8.0. (d) Samples were removed from the gradient in (c) and assayed for DNA infectivity. The gradients were centrifuged for 9 hours at 30K rpm and 5 to 10°C in a Spinco model L ultracentrifuge using an SW 39 rotor.

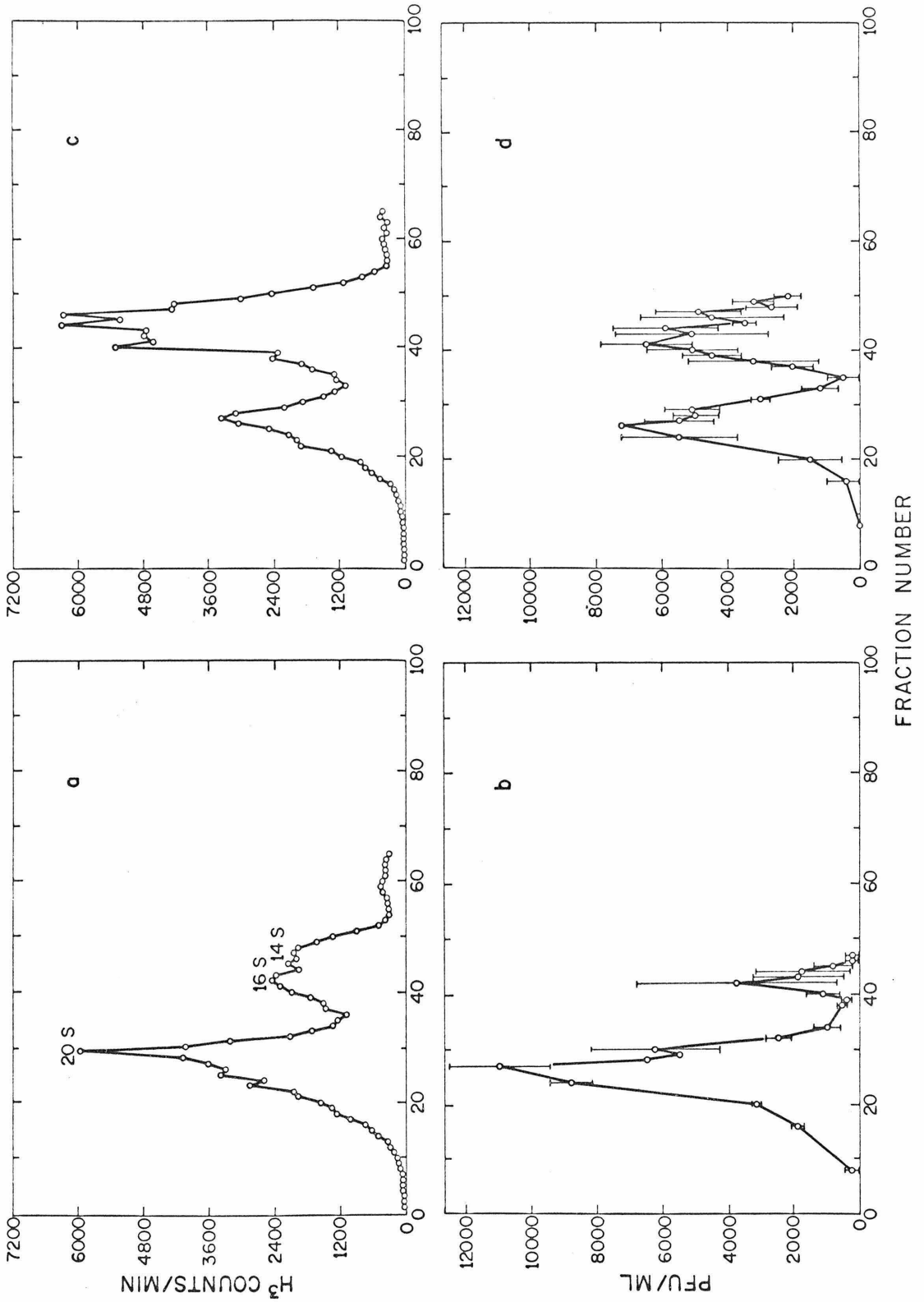

a
b
c
d
20 S
16 S
14 S
$H^3$ COUNTS/MIN
PFU/ML
FRACTION NUMBER

Figure 3. Analytical band sedimentation velocity patterns of polyoma DNA sedimented through CsCl, $\rho$ = 1.35 gm/ml. The densitometer tracings represent the distribution of DNA in each gradient. The field is directed toward the right. (a) Untreated DNA sedimented in CsCl at pH 8.0. The tracing shows the distribution 84 min after reaching speed. (b) Untreated DNA distribution at 77 min after reaching speed in a pH 12.5 CsCl gradient. The 53 S component had pelleted on the bottom of the centerpiece by this time. (c) Polyoma DNA incubated in $5 \times 10^{-3}$ M $FeCl_2$, pH 8.0, for 14 hours at room temperature. The centrifuge analysis was made at pH 8.0. This tracing represents the distribution of DNA 84 min after reaching speed. The background absorbance near the meniscus is due to oxidized $FeCl_2$. (d) Polyoma DNA incubated in $FeCl_2$ as in (c) but analyzed at pH 12.5. The tracing shows the distribution 71 min after reaching speed. The conditions for centrifugation were 44,770 rpm, 20°C.

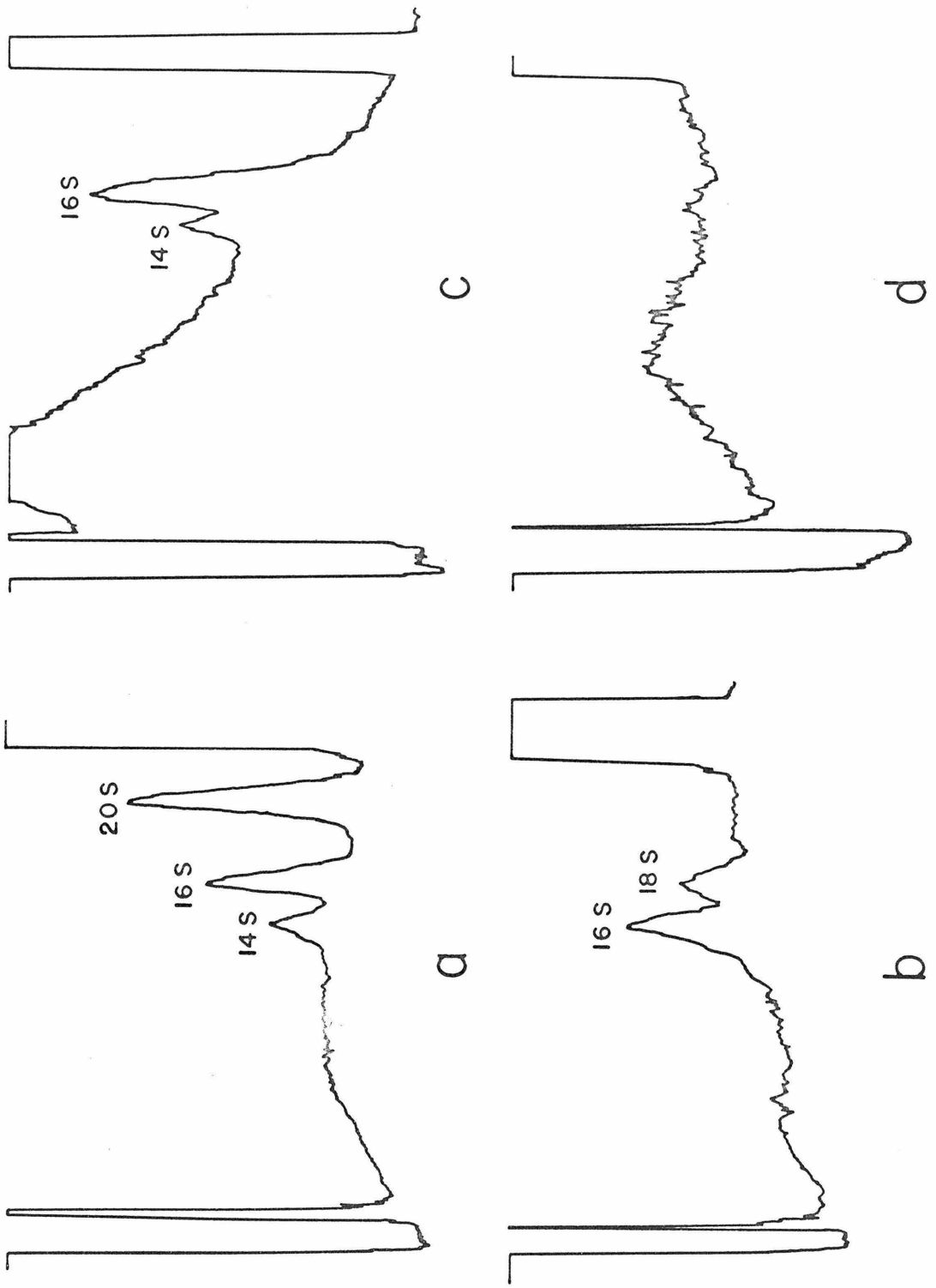

20S
16S
14S
a
16S
14S
c
16S
18S
b
d

Figure 4. Velocity sedimentation profiles of $H^3$-labeled polyoma DNA in 3 to 20% sucrose gradients. The field is directed toward the left. (a) $H^3$ count distribution of untreated polyoma DNA. (b) Samples were removed from the gradient in (a) and assayed for DNA infectivity as described in the Materials and Methods section of Part I. (c) $H^3$ count distribution of polyoma DNA incubated in $5 \times 10^{-3}$ M $FeCl_2$ for 14 hours at room temperature, pH 8.0 (d) Samples were removed from the gradient in (c) and assayed for DNA infectivity. The gradients were centrifuged for 9 hours at 30K rpm and 5 to 10°C in a Spinco model L ultracentrifuge using an SW 39 rotor.

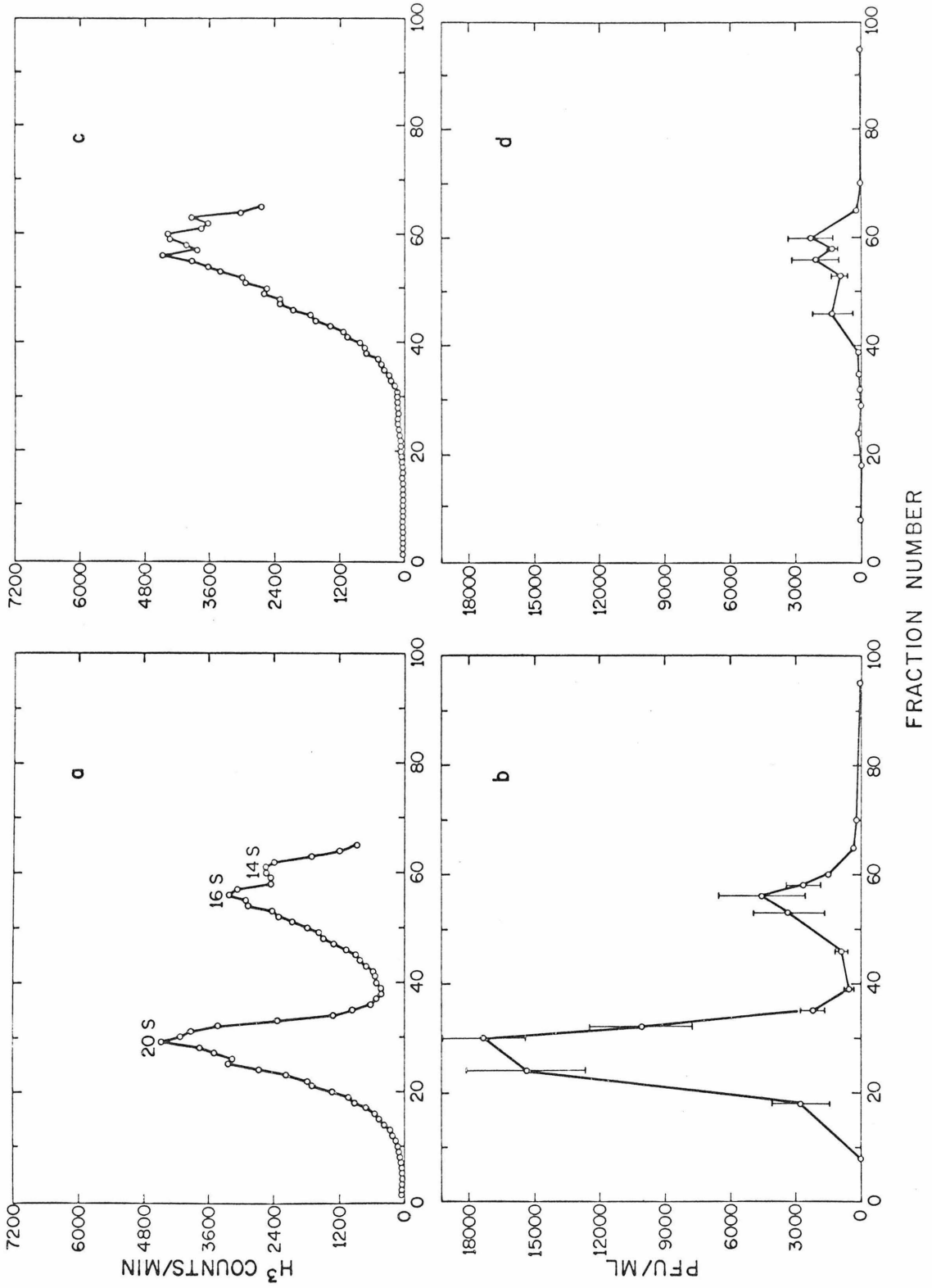

a
b
c
d
20 S
16 S
14 S
$H^3$ COUNTS/MIN
PFU/ML
FRACTION NUMBER

Figure 4a and Figure 4c. These are illustrated in Figure 4b and Figure 4d, respectively. The infectivity of the polyoma DNA produced as a result of extensive treatment with $FeCl_2$ is very low and is not proportional to the quantity of PY II present in the system. This result, coupled with the result that polyoma DNA extensively treated with $FeCl_2$ sedimented as a very heterogeneous population of short single strand molecules at pH 12.5 (Figure 3d), began to reveal that PY II might not be a discrete molecular species. A small constant amount of PY III was present in these preparations and was resolved only in the analytical band sedimentation velocity gradients (Figure 3).

PY II is circular

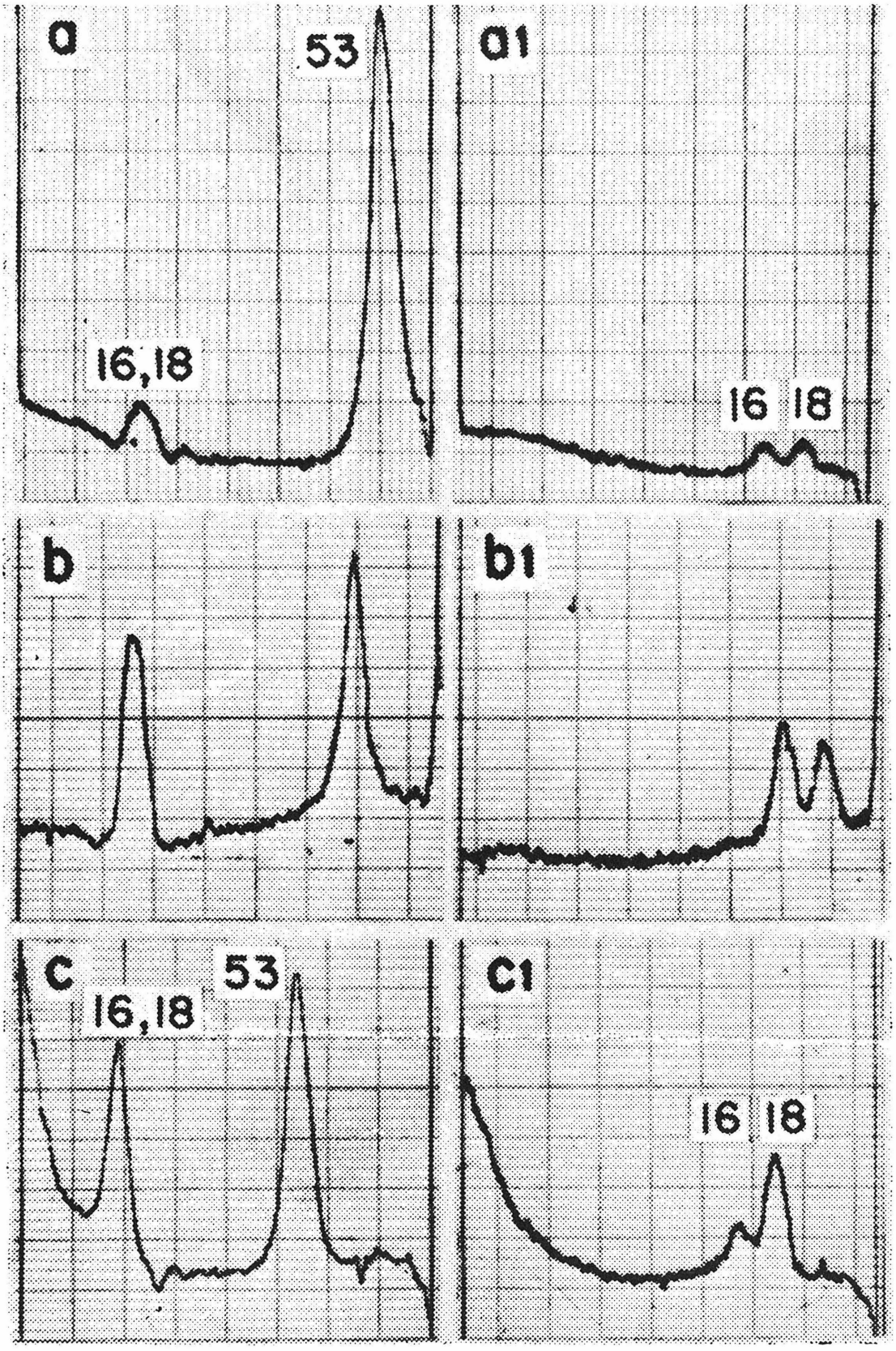
That PY II is circular was also shown by Philip Laipis in this laboratory. He found that the preparations which had undergone a 95% conversion of PY I to PY II contained exclusively circular molecules as determined by electron microscopy. This result led to the conclusion that pancreatic DNAase and reducing agents acted by causing single strand scissions and not double strand scissions. Therefore, PY II, although it appears to be a discrete molecular species when examined by means of neutral sedimentation velocity methods, is actually a population of molecules, each of which contains one or more single strand scissions.* This result is compatible with the previous experiments which demonstrated that the molecular

*It can be calculated that the circular form of the molecule should be retained until, on the average, 50 single strand scissions have been introduced (19).

weight of the hit molecules did not appear to decrease (as long as they were not denatured) but the biological activity of such molecules did decrease.

The proposition that pancreatic DNAase causes only single strand scissions in PY I, producing PY II, is supported by the following experiment, a modification of one carried out by Fiers and Sinsheimer (13). A preparation of nearly pure PY I DNA was lightly treated with pancreatic DNAase in order to produce a population of molecules containing on the average 0.6 hits per molecule. The hit molecules, upon sedimentation in pH 12.5 CsCl, should give rise mostly to single-stranded circular and single-stranded linear DNA. The results of Sinsheimer (20) indicated the single-stranded molecules should have different sedimentation velocities and the faster component of the two, the ring form, should not be susceptible to the action of E. coli phosphodiesterase. An example of such a result is given in Figure 5. The two single-stranded components, having $s^{Na}_{20,w}$ values of 18.4S ± 0.4 and 15.7S ± 0.3 for the alkaline Na DNA, are shown in Figure 5b in addition to the intact, double-stranded molecules sedimenting at 53S. An aliquot of the product of the pancreatic DNAase digestion (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, Figure 5c. Thus, PY II can contain a wholly intact circular strand. At this level of digestion, the

Figure 5. Band sedimentation velocity patterns of polyoma DNA in alkaline CsCl made with the photoelectric scanner on the Spinco model E ultracentrifuge. The left and right patterns are scans made approximately 30 min and 90 min, respectively, after sedimentation began. The field is directed toward the right. The bulk solution was CsCl, $\rho$ = 1.35 gm/ml and pH = 12.5, and the speed was 44,000 rpm. The leading band in (a), (b), and (c) is the 53 S component. The resolved slower bands in (a1), (b1), and(c1) are the 16 S and 18 S components. (a, a1) Control: Polyoma DNA component I, isolated in a sucrose gradient experiment, was treated identically as in (b) and (c) except for the absence of enzymes and BSA. (b, b1) Pancreatic DNAase treated polyoma component I: The reaction conditions are given in the Materials and Methods for Part I. (c, c1) Effect of heat denaturation followed by E. coli phosphodiesterase digestion: The products of (b) were heated for 5 min at 100°C and cooled rapidly. Exonuclease I was added as described in the Materials and Methods section.


a
53
16,18
a1
16 18
b
b1
c
16,18
53
c1
16 18

second strand in the molecule will contain only one or two breaks if the attack is statistical. Strand separation prior to adding the exonuclease was accomplished by thermal rather than alkaline denaturation.

The results of others in this laboratory, included in the accompanying publication, supported these ideas. (a) The rate of conversion of PY I to PY II was found to 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 PY I to II, a faster rate of conversion would be seen in alkali. (b) The infectivity of a polyoma DNA sample does not begin to decrease until each molecule has received an average of 1 1/2 to 2 hits. The infectivity may actually increase after each molecule has received an average of 0 to 1 hits (see Figure 3 of the enclosed publication). These results may be explained if the infectivity of PY II containing one circular strand is greater than the infectivity of PY I. Single-stranded circular polyoma DNA is infective (2). The initial rise in the infectivity may be due to the production of the more infective PY II containing one single, circular strand. If a hit in the second circular strand inactivates the molecule, the observed decrease in the infectivity would occur.

Reprinted from the PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES
Vol. 53, No. 5, pp. 1104–1111. May, 1965.

# *THE TWISTED CIRCULAR FORM OF POLYOMA VIRAL DNA**

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*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 renaturation[4] of this DNA and the failure of the strands to separate in strand-separating solvents.[1–3]

In previous studies[1–3] 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 method[4] 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 III[13] 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 junction[14] 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 |
| $Na_2SO_3$ ($1 \times 10^{-3}$ M $Cu_2SO_4$, 0.1 M $NH_4OH$)[d] | 0.01 | 10.8 | 60 | 45 |
| 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 μg/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: $0.5 \times 10^3$ pfu/μg DNA compared with $1.5 \times 10^3$ obtained for untreated DNA in the same assay.

[d] $Cu^{++}$ acts as a catalyst.[16] In the absence of $Cu^{++}$ or $NH_4OH$ 5% conversion was observed. In the absence of $SO_3^-$ no conversion occurred.

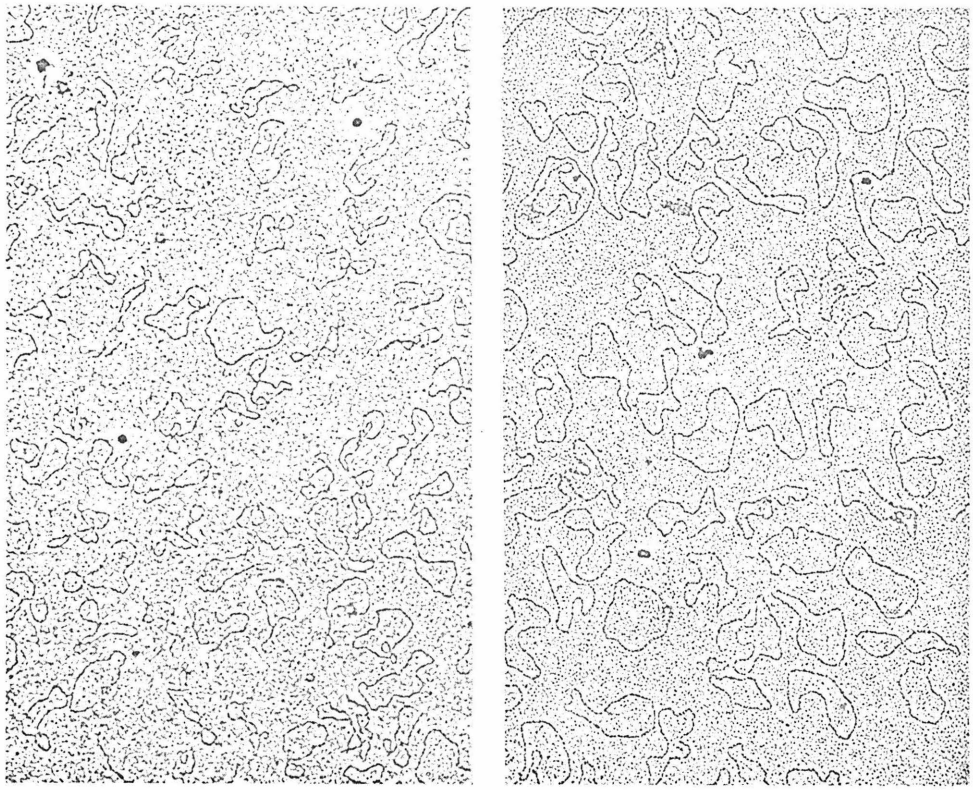
[e] Mercaptoethanol, cysteine, and glutathione. In experiments with acetic acid without thiols, 10% conversion occurred.

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 Vogt[1] 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 1*b* 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 1*b* 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 2*b*. These two components, $s^\circ_{w,20}$ = 18.4S ± 0.4, 15.7S ± 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 φX DNA by Fiers and Sinsheimer.[19] An aliquot of the product of the pancreatic DNAase digestion



(*a*) (*b*)

FIG. 1.—Electron micrographs of polyoma DNA × 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. 2*c*). 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 Vogt[1] 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 3*a* 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

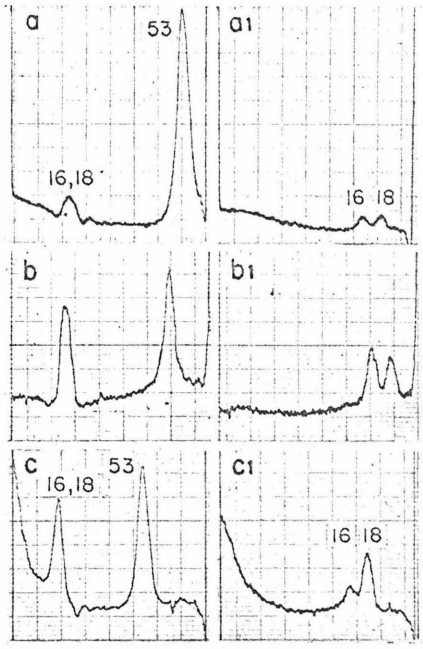



FIG. 2.—Sedimentation velocity patterns of polyoma DNA in alkaline CsCl. The *left* and *right* patterns are scans at about 30 min and 90 min after sedimentation begins. The field is directed toward the right. CsCl, $\rho$ = 1.35 gm cm$^{-3}$, pH 12.5, 44,000 rpm. (*a*, *a1*) *Control*: Component I isolated 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 $\mu$g/ml pure I in 0.048 *M* NaCl, 0.0075 *M* $MgCl_2$, 0.01 *M* Tris pH 8.0, 40 $\mu$g/ml BSA, and 2.7 $\times$ 10$^{-4}$ $\mu$g/ml enzyme, 20 min at 20°. Reaction stopped by $^1/_{15}$ 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 $\mu$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. 3*b*) 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

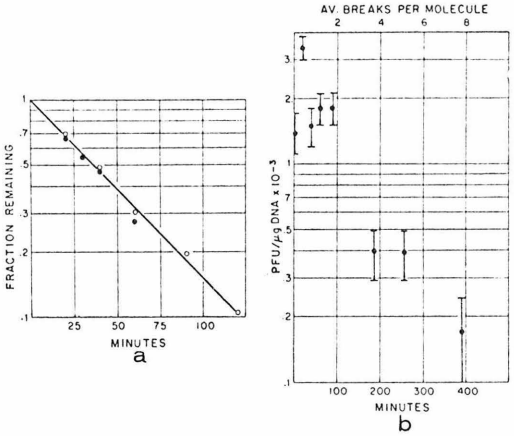



FIG. 3.—Chemical and biological effects of pancreatic DNAase treatment. (*a*) Analyses for single-stranded and double-stranded DNA. Extent of conversion was determined by band-sedimentation velocity experiments with photoelectric scanner. ⊙, (I)/(I + II) in neutral CsCl bulk solutions. ●, (53S)/(total) 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. 2*b*, except for enzyme concentration, 2.0 $\times$ 10$^{-4}$ $\mu$g/ml. 20-$\mu$l samples were withdrawn at the indicated times and added to 4 $\mu$l 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 reported[2] 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 II[2] make it unlikely that a nonlabeled, non-DNA mass is removed.

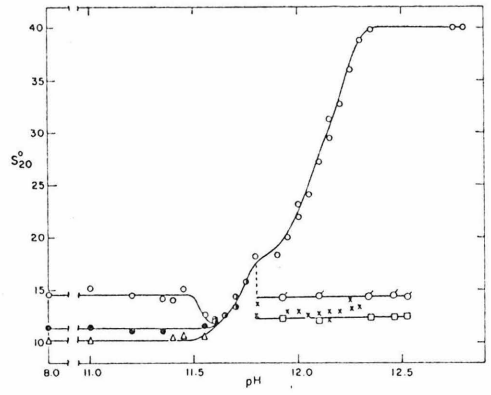



FIG. 4.—Sedimentation velocity-pH titration of the three components in polyoma DNA. I, ○; II, ●; III, △; ◑ only band present, cf. text; mixture of unresolved single strands in alkali, x; 29,500 rpm. Single linear, □, and single circular, ○, strands in alkali, 44,770 rpm. Sedimentation coefficients at 20° in CsCl, $\rho$ = 1.35 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.

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 contained[1] twisted circles to a variable extent (Fig. 1*a*). Grids prepared from polyoma II contained extended circles and no twisted configurations (Fig. 1*b*). The DNA samples used to prepare the grids for Figures 1*a* 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.

(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 coil[2] 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.

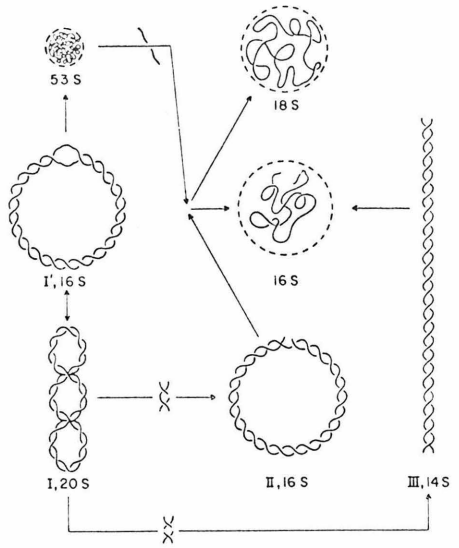



FIG. 5.—Diagrammatic representation of the several forms of polyoma DNA. The duplex segments shown contain 12 turns, about one fortieth of the total number. The twisted circular duplex shown contains one left-hand tertiary turn. 8% of the right-hand duplex turns in the model are unwound to form *I′*.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

*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's[21] 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

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 ± 0.5S predicted by Studier's relation[18] for a molecular weight[2] of 3.0 ± 0.3 × $10^6$. 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 reported[22] 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 DNA[24–26] 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 Black[3] 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 Sinsheimer[29] 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 Pouwels[30] 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.

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# DISCUSSION

The results obtained when reducing agents were used to effect the conversion of PY I to PY II were initially quite confusing. However, the discovery that PY II was circular gave rise to a correct interpretation of all the previous results. All the agents used for the conversion caused single strand and not double strand scissions in the molecule. A circular and not a linear molecule was being made. However, a linear form of polyoma DNA can be made by limited digestion of PY I with E. coli endonuclease I, an enzyme that introduces double strand scissions. The linear molecule has a sedimentation coefficient approximately 10% lower than that of the circular double-stranded molecule, PY II, and the same as naturally occurring PY III.

The mechanism of action of the reducing agents has not been completely clarified. McCarty (21) studied the effects of ascorbic acid and similar reducing agents on the transforming activity of pneumococcus and found that such reagents destroyed this activity. The effect could be prevented by excluding oxygen or by using catalase or sulfhydryl compounds in the reaction mixture. He concluded that inactivation of the transforming DNA was oxidative and was dependent upon the production of peroxides during ascorbic acid autoxidation. Bode (22), in a recent publication, incubated closed circular λ DNA (23) with dithiothreitol ( = DTT = 2,3-dihydroxy-1,4-dithiobutane), ascorbic acid, reduced diphosphopyridine nucleotide, and 2-mercaptoethanol. He found that all of these reducing agents were able to cause

single strand scissions in λ DNA. The reaction conditions for each reagent were somewhat different. The effect of DTT could be prevented if the incubations were carried out in a nitrogen atmosphere or if the radical scavenging agent, 2-aminoethylisothiouronium, were added to the reaction mixture. Presumably, the effect of reducing agents on DNA is indirect by acting in an oxygen-dependent reaction which produces radicals or peroxides. Such radicals, which can also be produced photochemically, are able to break phosphodiester bonds (24).

Examination of the $H^3$ count and infectivity profiles found in sucrose velocity gradients of polyoma DNA lightly or extensively treated with $FeCl_2$ (Figure 2 and Figure 4) supports the result of Vinograd, et al. (26). That is, PY I DNA lightly hit with a reducing agent does not lose its infectivity after the first hit, but upon more extensive treatment, the infectivity decreases. Similarly, McCarty (21) found that the transforming activity of pneumococcus decreased after treatment with reducing agent.

The exonuclease I digestion of linear, single-stranded DNA produced by the denaturation of PY II helped to elucidate the structure of this molecule. However, as can be seen in Figure 5c, not all of the linear, single-stranded DNA in the reaction mixture appears to be digested. This puzzling result can now be explained on the basis of the results of Part III of the thesis. (a) The pancreatic DNAase treated polyoma DNA was thermally denatured and quenched by rapid cooling. This is a very inefficient method, for the single strands of

small homogeneous DNAs renature very rapidly upon cooling and form structures which cannot be digested by the enzyme. However, in alkaline CsCl sedimentation velocity analyses, such renatured material again denatures, producing linear, single-stranded DNA. (b) Exonuclease I preparations often contain endonuclease activity. If excess amounts of exonuclease I are used, as was done in these experiments, endonuclease hits may be put into intact, undenatured PY I molecules during the exonuclease I digestion. The resulting single strands are detected only during the alkaline CsCl velocity analyses.

None of the results presented here can prove or disprove the presence of a linker in polyoma DNA, for all the reagents used caused random degradation. This topic is the subject of Part III.

The proposal for the structure of PY II led to the following question: If PY I is circular and PY II is circular and derived from PY I by one single strand scission, then why does PY I sediment 25% faster than PY II? Questions similar to this led to the proposal of a twisted circular structure for PY I. The structural features of this molecule are discussed in detail in the General Introduction.

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# PART II

# RENATURATION OF POLYNUCLEOTIDE SINGLE STRANDS OBTAINED FROM NICKED POLYOMA DNA

# INTRODUCTION

Part I of the thesis described studies of the structure of polyoma II DNA (PY II). This molecule is a circular, double-stranded DNA which contains one or more single strand scissions per molecule and fewer scissions than is required to produce a linear structure (1). Such a molecule can be produced by the limited treatment of polyoma I DNA (a twisted, closed circular DNA) with pancreatic DNAase or chemical reducing agents. If a sample of polyoma I DNA receives an average of one random hit per molecule, 37% of the final preparation will be polyoma II containing one single strand scission per molecule. Upon denaturation of this structure by heat or high pH, two polynucleotide single strands are obtained, one circular and the other linear. These can be separated by sedimentation velocity at high pH, both in the Spinco model E ultracentrifuge and in the SW 50 rotor of the Spinco model L ultracentrifuge. The difference in the frictional coefficients of the two structures results in a 10% difference in their sedimentation coefficients.

Part II of the thesis describes the method of isolation and an investigation of the properties of this single-stranded DNA. One of the primary questions asked was the following. Can two complementary, circular, single-stranded DNA molecules renature and if so, what is the structure of such a molecule? It is well known that two complementary, linear, single-stranded DNA molecules can, under the proper conditions, renature rapidly to form a right-handed, double-

stranded helical DNA. Crick and Watson (2) found by model building that it is possible to build a DNA model with only a right-handed sense. To construct left-handed helices, it was necessary to violate the permissible van der Waals' contacts. The formation of a left-handed helix must be less favorable energetically, but how much less cannot be tested experimentally with linear DNA. Two complementary, linear strands will simply choose to form a right-handed duplex. The topological winding number of two complementary, single-stranded circles is zero. It may be expected that two such complementary, circular strands will partially renature to form a right-handed duplex structure. The requirement for a constancy of the topological winding number would necessitate the formation of an equivalent number of left-handed duplex turns. A thermodynamically stable complex will form if the free energy of the complex is less than that of the two separate, circular single strands.

The separate single-stranded DNAs were isolated by means of alkaline CsCl velocity gradients. Following renaturation of either circular, single-stranded or linear, single-stranded DNA, the buoyant density of the products was determined in neutral CsCl. The buoyant density in CsCl can be used to determine the degree of renaturation since native, double-stranded DNA is approximately 0.015 gm/ml less dense than denatured DNA (3). The results of this study have indicated that two complementary, circular, single-stranded DNA molecules cannot completely renature with one another, at least under the conditions employed here. The analytical methods were not sensitive enough to determine whether very short regions of the

two molecules were renatured. Under the same conditions, it was found that linear, single-stranded polyoma DNA can renature nearly completely.

The renaturation of the single strands of polyoma DNA was studied for the primary purpose of examining the reaction products rather than the reaction kinetics. The renaturation reaction has been studied quite extensively the past several years by several authors (4-10). Their results can be summarized briefly:

(a) The renaturation reaction is second order in DNA phosphate concentration.

(b) As the native molecular weight (and genetic complexity) increases within a series of different DNA species, the rate of renaturation of the DNA within this series decreases.

(c) As the molecular size of a given DNA species is decreased by degradative procedures, the rate of renaturation decreases.

(d) The optimum temperature for renaturation is found approximately 25°C below the melting temperature.

(e) The rate of renaturation is a function of the solvent in which the reaction occurs. Below 0.4 M sodium ion, the renaturation rate is highly salt dependent. Above 0.4 M sodium ion, the rate is not very sensitive to changes in salt concentration. In addition, an increase in the viscosity of the solvent decreases the rate of renaturation.

The experiments which follow are concerned only with the renaturation of the single strands of polyoma DNA. The variables in these experiments are temperature and solvent composition.

# MATERIALS AND METHODS

1) Isolation and purification of polyoma DNA

The methods used for the isolation and purification of polyoma DNA are given in Part I of the thesis.

2) Alkaline CsCl gradients

Preformed alkaline CsCl density gradients were used for the analysis of polyoma DNA samples and for the preparation of circular and linear, single-stranded DNA. These were made as follows: A linear 4.75 ml gradient was established by pumping the products of a linear gradient making apparatus through a capillary to the bottom of a Spinco polyallomer tube. The density range was 1.30 to 1.40 gm/ml. After the gradient was established, 0.25 ml of a CsCl solution having a density of 1.75 gm/ml was pumped into the bottom of the tube. This concentrated CsCl provided a dense "pad" which slowed down the fastest sedimenting material. The two solutions used in the gradient maker were 0.1 M $K_2HPO_4$ in CsCl, $\rho$ = 1.30 gm/ml and $\rho$ = 1.40 gm/ml, and the dense "pad" solution was 0.1 M $K_2HPO_4$ in CsCl, $\rho$ = 1.75 gm/ml. The pH of the two less dense solutions was adjusted to the desired value at 20°C by adding 0.1 M $K_2HPO_4$ and 1 M KOH in CsCl, $\rho$ = 1.30 and 1.40 gm/ml, respectively. The pH of the dense solution was adjusted at 20°C by adding 5 M KOH. The pH of the alkaline CsCl velocity gradients was usually 11.85. This pH was high enough to cause denaturation and strand separation of nicked molecules and was low enough to avoid pelleting the denatured, double-stranded molecules at the bottom of the tube. The sedimentation velocity of the intact, double-stranded molecules is extremely pH dependent between

pH 11.6 and 12.3. All titrations were done immediately before making the gradients. During the titrations and manufacture of the gradients, the solutions were kept under an atmosphere of argon. A Beckman Research model pH meter, a general purpose probe glass electrode, and a calomel reference electrode modified with a ground glass junction (11) were used in the titrations. Samples to be centrifuged through the gradient were brought to the pH of the experiment and 0.25 ml or less was layered on the gradient surface. The sedimentation velocity experiments were performed at 20 ± 1°C and 45,000 ± 500 rpm. The total centrifugation time was 4 hours, including the five minutes needed for acceleration. The deceleration time (approximately 15 min) is not included in this 4 hours.

3) Collection of the gradients

Seven and one-half μl drops were collected from the gradients using a piercing device from Buchler Instruments. Alternate 5 drop fractions were collected into 0.1 ml SSC/10, 0.02 M tris and 1 drop fractions were collected on Whatman GF/A filter papers (2.4 cm diameter) in Packard scintillation vials. The filter papers were dried by placing the bottles on a hot plate. Ten ml of toluene-PPO-POPOP scintillation fluid [4 gm PPO (Packard Instruments) and 0.05 gm POPOP in 1 liter of toluene] were added and the tritium counts were determined in a Packard Tri-Carb scintillation counter. The contents of the vials containing the single-stranded DNA were frozen at -20°C.

4) Renaturation methods

The renaturation methods are presented in the text and Table 1

of this part of the thesis.

5) Buoyant density experiments and calculations

Buoyant density experiments were done as described by Vinograd and Hearst (12). In most experiments, 2° or 4° 12 mm Kel-F centerpieces were used. Centrifugation was carried out for approximately 24 hours at 44,770 rpm and 44,000 rpm in Spinco Model E ultracentrifuges. The results were recorded either on photographic film (using exposures where the film response is linear) or with an ultraviolet photoelectric scanner. The Joyce-Loebl microdensitometer was used to measure the film optical densities. All experiments were conducted using crab dAT as a marker. This material was a gift from Dr. Jack Widholm and was found to have a buoyant density of $1.669_5$ gm/ml.

6) Electron microscopy

DNA specimens were prepared for electron microscopy by the method of Kleinschmidt and Zahn (13). DNA samples taken directly from the gradient contained approximately 1.1 M CsCl and 0.1 M tris. Sufficient cytochrome c at 1 mg/ml and 1.875 M ammonium acetate were added to produce a sample containing 0.1 mg/ml of cytochrome c and 1.5 M ammonium acetate. From 25 to 100 $\mu$l of this sample was allowed to flow down an inclined clean glass slide, one end of which was immersed in 0.15 M ammonium acetate. The sample formed a film on the surface of the ammonium acetate, the film area being delimited by talcum powder. After waiting for approximately 30 sec, the surface of a copper grid (200 mesh, 2 mm, Ladd Industries)

covered with a Parlodion (cellulose nitrate) film was touched to the cytochrome c-DNA film. The film was fixed to the grid by immersing it for 30 sec in 95% ethanol containing 0.08 mg/ml uranyl acetate (9). The grid was next dipped into isopentane for 10 sec. A Philips EM200 electron microscope was used to examine the grids.

# RESULTS

## Isolation of linear and circular, single-stranded DNA

The denaturation of a sample of polyoma I DNA lightly hit with pancreatic DNAase produces four classes of components upon sedimentation in an alkaline (pH 11.85) CsCl density gradient: twisted-circular duplex DNA sedimenting at approximately 20 S; circular, single-stranded DNA sedimenting at approximately 18 S; linear, single-stranded DNA sedimenting at approximately 16 S and single-stranded DNA sedimenting less than 16 S. Because of the 10% difference in sedimentation coefficient between circular, single-stranded DNA and linear, single-stranded DNA, it was felt that the isolation of such DNA was feasible.

The preparation of the alkaline CsCl density gradients used for this purpose is described in the Materials and Methods section. Briefly, the gradients contained 0.1 M $K_2HPO_4$ in CsCl. The density range in each gradient was 1.30 gm/ml to 1.40 gm/ml with an additional 0.25 ml of CsCl at 1.75 gm/ml placed on the bottom. The pH of each experiment was usually 11.85. The separation of components attained in these preparative gradients was nearly the same as that found in analytical band velocity sedimentation experiments (14). Alkaline sucrose velocity gradients were not used in these experiments because of the lower tritium counting efficiencies found in such a system.

Figure 3a (page 124, Part III of the thesis) illustrates a typical alkaline velocity gradient containing the four components mentioned earlier. The 18 S and 16 S components were isolated from the fractionated

gradients. Only the leading half of the 18 S band and the trailing half of the 16 S band were used in order to minimize cross contamination.

Renaturation methods

Because of the somewhat exploratory nature of this series of experiments, no one method of renaturation was employed. However, most experiments were performed in one of the two following ways.
(a) The renaturation solvent had an ionic strength of 1.27 M due mostly to CsCl and the DNA was incubated for at least 1 hour at 50°C.
(b) The renaturation solvent had an ionic strength of 0.31 or 0.45 M due mostly to sodium salts. The DNA was incubated for 10 to 15 min in this solvent at 95°C followed by an extended cooling period during which the temperature of the reaction was dropped from its maximum to 50°C or less. The details are given in Table 1. Several experiments were attempted in which the Gilford recording spectrophotometer was used to monitor the results, but due to the low concentrations of DNA (2 to 4 μg/ml) and small volumes of material available, these results were neither consistent nor meaningful.

The half-time necessary for the renaturation of the complementary strands in a homogeneous sample of DNA having the duplex molecular weight of polyoma viral DNA can be calculated from the results of Wetmur (9, 10). Such calculations show that the renaturation half-time should be 36 to 48 sec if renaturation conditions (a) are used, and 63 to 147 sec if conditions (b) are used. These calculations were made with the assumption that the DNA concentrations were 2 to 4 μg/ml. Since the renaturation times employed were longer than these values, all

Table 1

Renaturation Experiments

| Exp. No. | Composition of Starting Material | | Buoyant Density Before Renaturation[b] | | Renaturation Conditions | Buoyant Density After Renaturation | |
|---|---|---|---|---|---|---|---|
| 1 | 16 S | | $1.702_9$<br>$1.714_8$ | 20 ± 1%<br>80 ± 2% | 5 2/3 hr at 50°C in 1.27 M ionic strength. | $1.705_6$<br>Dense shoulder | 95 ± 3%<br>5 ± 3% |
| 2 | 16 S<br>18 S | 81 ± 2%[a]<br>19 ± 1% | Light shoulder<br>$1.715_8$ | ~ 5%<br>~ 95% | 3 hr at 50°C. Same solvent as exp. 1. | $1.706_0$ | 100% |
| 3 | 16 S<br>18 S | 32 ± 2%[a]<br>68 ± 2% | $1.701_4$<br>$1.717_8$ | 22 ± 2%<br>78 ± 2% | 1 hr at 50°C with a 2 hr slow cooling period. Final temp. = 25°C. Same solvent as exp. 1. | $1.703_0$<br>$1.715_1$ | 49 ± 2%<br>51 ± 2% |
| 4 | 16 S<br>18 S | 50 ± 4%[a]<br>50 ± 4% | — | | 2.2 hr linear heating to 94°C. To 50°C in 16 min. Slow cooling to 12°C in 19.5 hr. Solvent is 0.48 M ionic strength. | $1.699_9$<br>$1.707_5$ | 81 ± 2%<br>19 ± 2% |
| 5 | 16 S<br>18 S | 37 ± 7%[c]<br>63 ± 7% | — | | 12 ± 3 min at 95°C. Cool non-linearly to 15°C in about 1 hr. (To 50°C in about 20 min). Same solvent as exp. 4. | $1.700_2$<br>$1.713_9$ | 80 ± 5%<br>20 ± 5% |
| 6 | 16 S | 100%[c] | — | | 12 ± 3 min at 95°C. Cool non-linearly to 50°C in 20 min. Same solvent as exp. 4. | $1.703_1$ | 100% |
| 7 | — | | $1.701_5$<br>$1.716_2$ | 19 ± 7%<br>81 ± 7% | 12 ± 3 min at 95°C. Cool non-linearly to 27°C in 63 min. (To 50°C in about 20 min). Solvent ionic strength = 0.31 M. | $1.701_7$ | 100% |

(a) These percentages were determined after renaturation by finding the relative amount of each component present in an alkaline CsCl velocity gradient as performed in the Spinco model L.

(b) These percentages were determined by finding the relative areas under the respective peaks in the optical density profiles of the buoyant density experiments. Those areas representing denatured DNA were corrected for the increased absorbance of the denatured DNA.

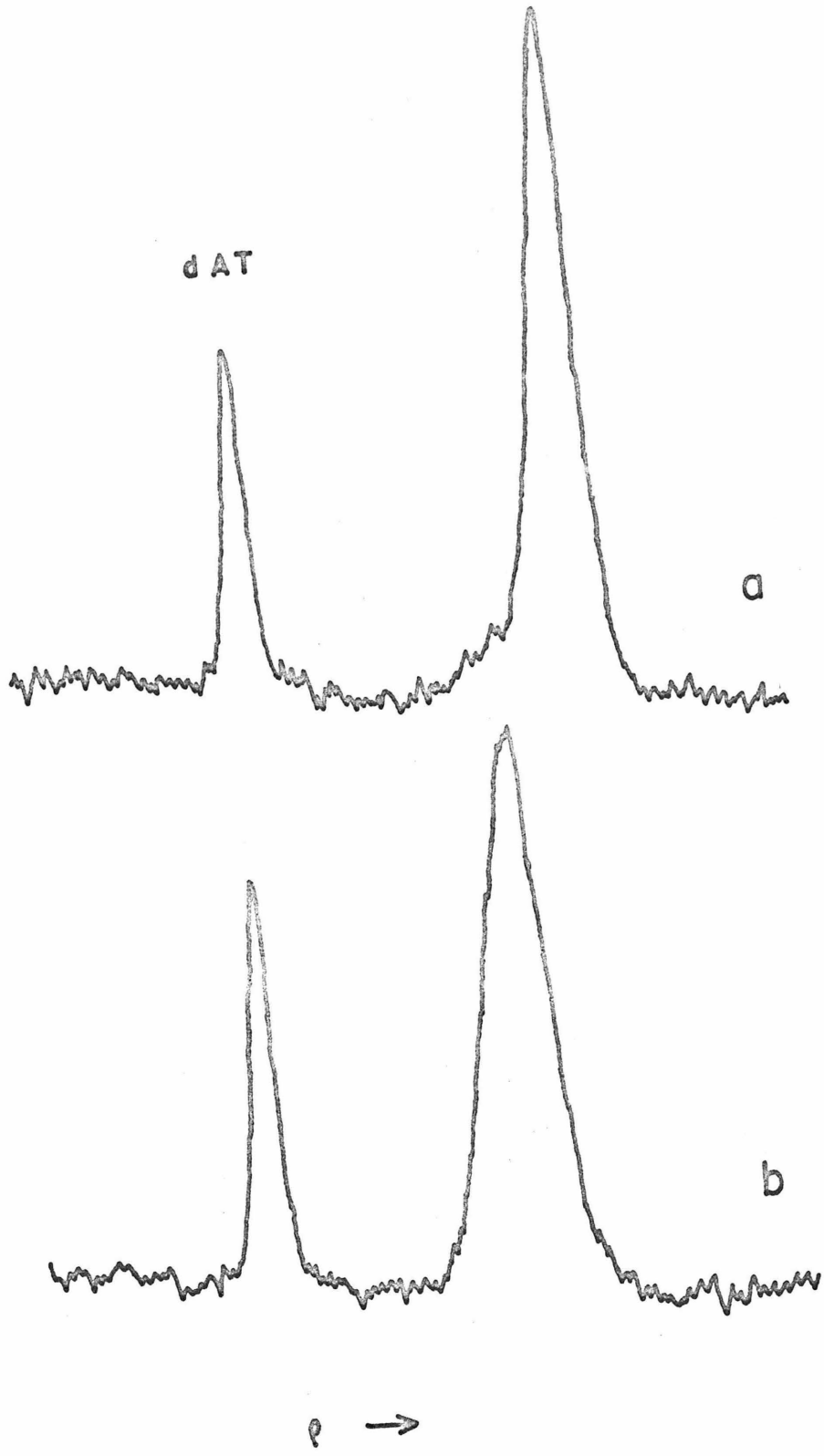
(c) These percentages were determined before renaturation by finding the relative areas under the respective peaks on the photoelectric scanner records of band alkaline sedimentation velocity experiments in the Spinco model E (11).

samples should have had adequate time for renaturation.

Renaturation of 16 S linear, single-stranded DNA

These experiments are outlined in Table 1 as 1, 2, 6 and 7. The ionic strength in the first two experiments was 1.27 M and in the remaining two 0.48 M and 0.31 M. The results can be summarized as follows. A sample of mostly 16 S linear, single-stranded DNA can be renatured to form one band of material which is buoyant in CsCl at a density near that of the native DNA, $1.701_1$ gm/ml. However, the buoyant density seems to be dependent upon the renaturing solvent, for, as can be seen in Table 1, the buoyant density of the DNA renatured in 1.27 M ionic strength (Exps. 1 and 2) is higher than that renatured in the lower ionic strength salt (Exps. 6 and 7). Thus, a more complete reaction may occur at the lower salt level. This may be due to the occurrence of a larger amount of non-specific base pairing in the higher salt leading to the formation of structures containing more than two strands. Such a structure might not be completely double-stranded (5, 6). All of the starting solutions contained a small amount of renatured material. This renaturation may have occurred at room temperature during the handling of the DNA samples (5, 6). Figure 1 shows the buoyant density profiles of linear, single-stranded 16 S DNA before and after renaturation as described in Experiment 2. The marker in all these experiments is dAT, which bands in CsCl at a density of $1.669_5$ gm/ml. This number is the average of many determinations made in this laboratory.

Figure 1. CsCl buoyant density analyses of (a) denatured and (b) renatured 16 S linear, single-stranded polyoma DNA. The renaturation conditions and composition of the starting material are given in Exp. 2 of Table 1. The centrifugation conditions were 24 hours at 44,770 rpm and 25°C. The starting solutions consisted of renatured or denatured polyoma DNA, 0.5 $\mu$g dAT, and CsCl, $\rho$ = 1.695 gm/ml and pH 8.0. The figure was made from microdensitometer tracings of the ultraviolet film records.

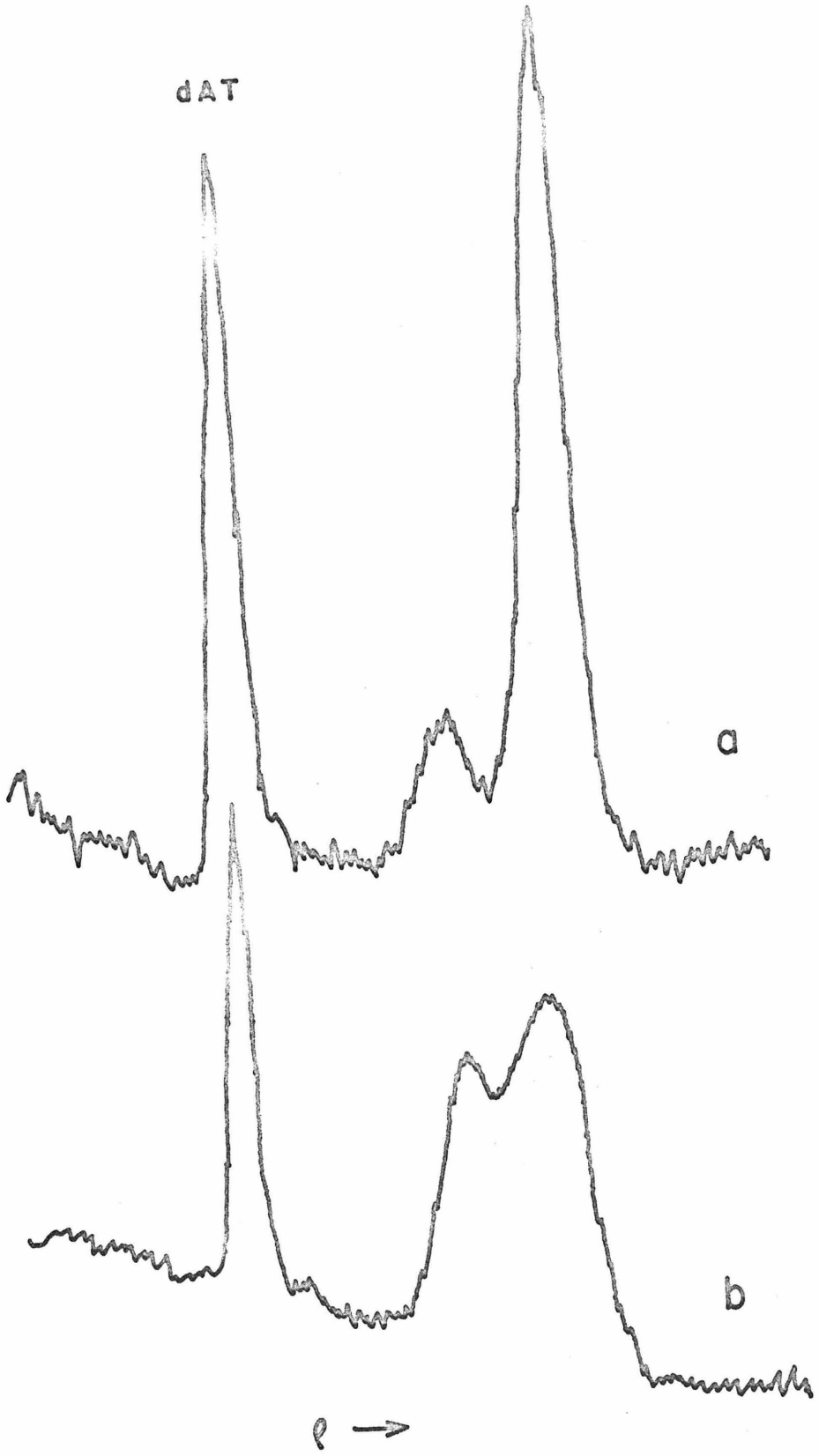

d A T
a
b
ρ →

Renaturation of circular, single-stranded 18 S DNA

These experiments are outlined in Table 1 as 3, 4, and 5. In Experiment 3, a preparation of single-stranded DNA was renatured by heating for 1 hour at 50°C in 1.27 M ionic strength solvent. The composition of the renatured material, as determined in an alkaline CsCl velocity gradient after renaturation, was calculated to be 68 ± 2% circular, single-stranded and 32 ± 2% linear, single-stranded DNA. Analysis in a buoyant density gradient revealed that 49 ± 2% of the renatured sample is found near the buoyant density of native duplex DNA while the remainder, 51 ± 2%, is found at a buoyant density characteristic of denatured DNA. If we assume that complementary, circular, single-stranded DNA will not renature, predictions can be made of the amounts of renatured and non-renatured DNA present in the buoyant density gradients. A maximum amount of renatured DNA will be found when linear single strands renature exclusively with circular single strands. A minimum amount of renatured DNA will be found when linear single strands renature exclusively with other linear single strands. All of the circular single strands then remain non-renatured. In Experiment 3, the maximum amount of DNA that can be renatured is 64%. The minimum amount is 36%. The experimental result of 49% renatured DNA indicates that linear single strands renatured with both linear and circular, single-stranded DNA, leaving 51% of the single-stranded circles unable to renature. Figure 2 presents the buoyant density profiles of the DNA preparations in Experiment 3 before and after renaturation.

The renaturation method of Experiment 5 was different. A DNA

Figure 2. CsCl buoyant density analyses of a preparation of polyoma DNA containing 32 ± 2% linear, single-stranded DNA (16 S) and 68 ± 2% circular, single-stranded DNA (18 S). (a) Buoyant density profile before renaturation. (b) Buoyant density profile after renaturation as described in Exp. 3 of Table 1. The centrifugation conditions were the same as those described in the legend of Figure 1.


dAT
a
b
ρ →

preparation containing 63 ± 7% circular, single-stranded 18 S and 37 ± 7% linear, single-stranded 16 S DNA was renatured by heating for 12 ± 3 min at 95°C and cooling to 50°C in about 20 min. The ionic strength of the renaturation solvent was 0.48 M. The maximum amount of DNA that can be renatured in this system is 74 ± 7%, and the minimum amount is 37 ± 7%. The experimental result obtained, 80 ± 5% renatured DNA, is approximately the maximum plus the error involved. The analysis of the starting material was made before the renaturation experiment and the unknown amount of hydrolytic breakage which occurred during the thermal treatment would increase the 74 ± 7% figure. The latter value is therefore a minimum value. The experimental result indicates that approximately 20% of the circular, single-stranded DNA failed to renature. It is not clear why the amount of DNA renatured in Experiment 5 is so much closer to the maximum value than the amount of DNA renatured in Experiment 3.

The results of Experiment 4 are not clear. If we assume that complementary, circular, single-stranded DNA cannot renature, the maximum amount of renaturation would be 100% and the minimum amount would be 25%. It was found that 81% of the sample banded at a buoyant density characteristic of duplex DNA and the remaining 19% banded at a buoyant density between fully denatured DNA and native duplex DNA. The latter DNA could represent either partially renatured, circular, single-stranded DNA or partially renatured complexes consisting of linear and circular, single-stranded DNA as discussed below.

By means of the Kleinschmidt technique (13) and the staining procedure of Wetmur (9), electron micrographs were made of the

renatured DNA as described in Experiment 3. Figure 3 shows a number of typical molecules. A small number of complete, renatured, duplex DNA molecules are seen. These have the same length, 1.5 $\mu$, as native polyoma DNA. A larger number of smaller, circular molecules are found with one puddled region on the perimeter of each molecule. The formation of a "hairpin" structure in a circular, single-stranded DNA might lead to a small circular duplex molecule containing a puddled region. The "hairpin" would allow only the partial renaturation of the circular strand with a complementary linear, single-stranded DNA. Both strands would contain regions not able to renature completely. Figure 4 is an electron micrograph of a mixture of 80% linear and 20% circular, single-stranded DNA renatured in 0.48 M ionic strength solvent. This particular field contains a number of completely renatured, circular molecules with a length of 1.5 $\mu$. Few molecules contain puddled regions. It is possible that some of the circles result from the renaturation of linear, single-stranded DNA. Since the nucleotide sequence of linear, single-stranded DNA, produced by the random attack of pancreatic DNAase on polyoma DNA, is circularly permuted, these strands are capable of renaturing to form circular DNA (15).

Figure 3. Electronmicrographs of single-stranded polyoma DNA renatured as described in Exp. 3 of Table 1. The length of the molecule in the upper left hand corner is 1.5 $\mu$. Total magnification is 46,240.

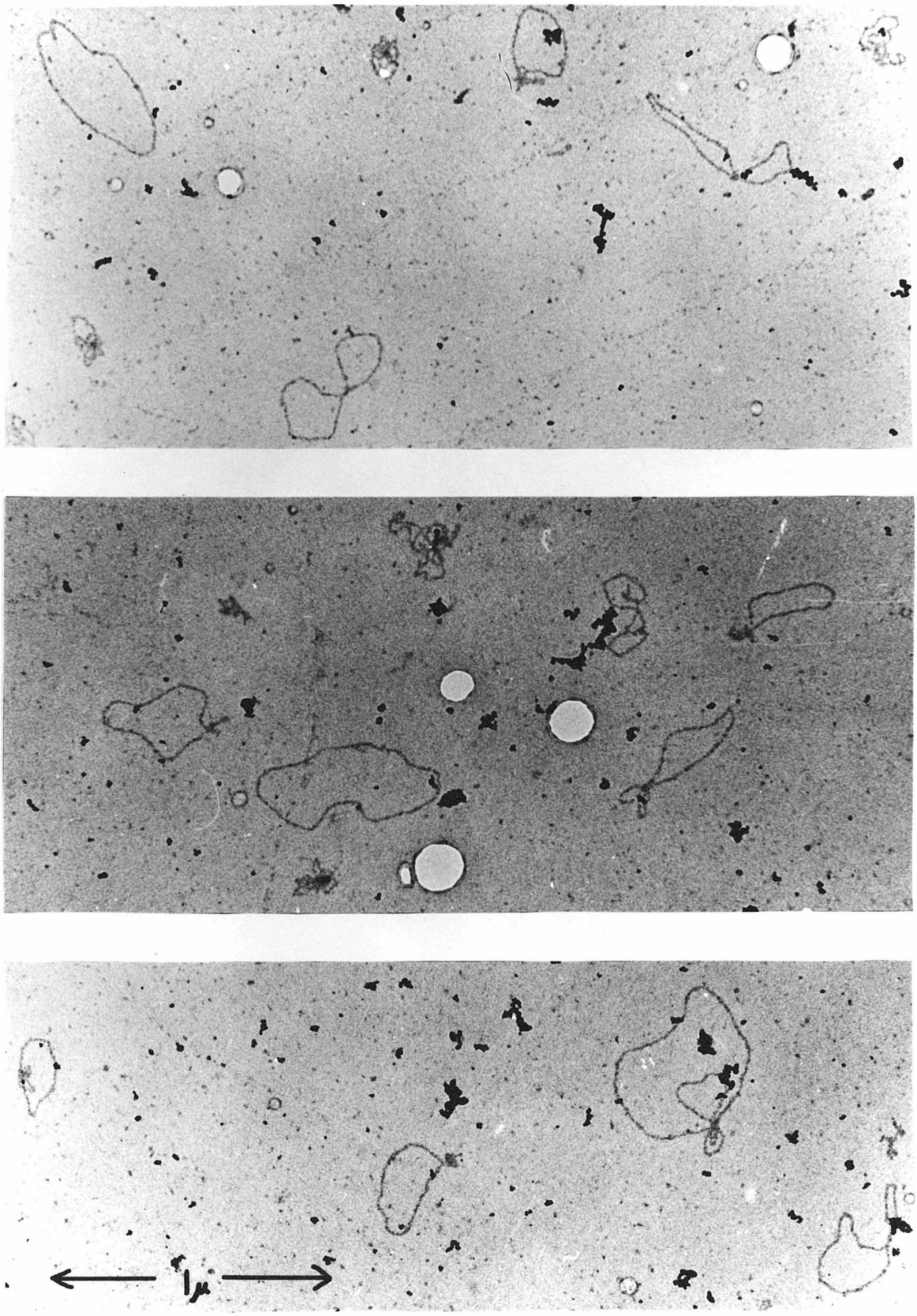
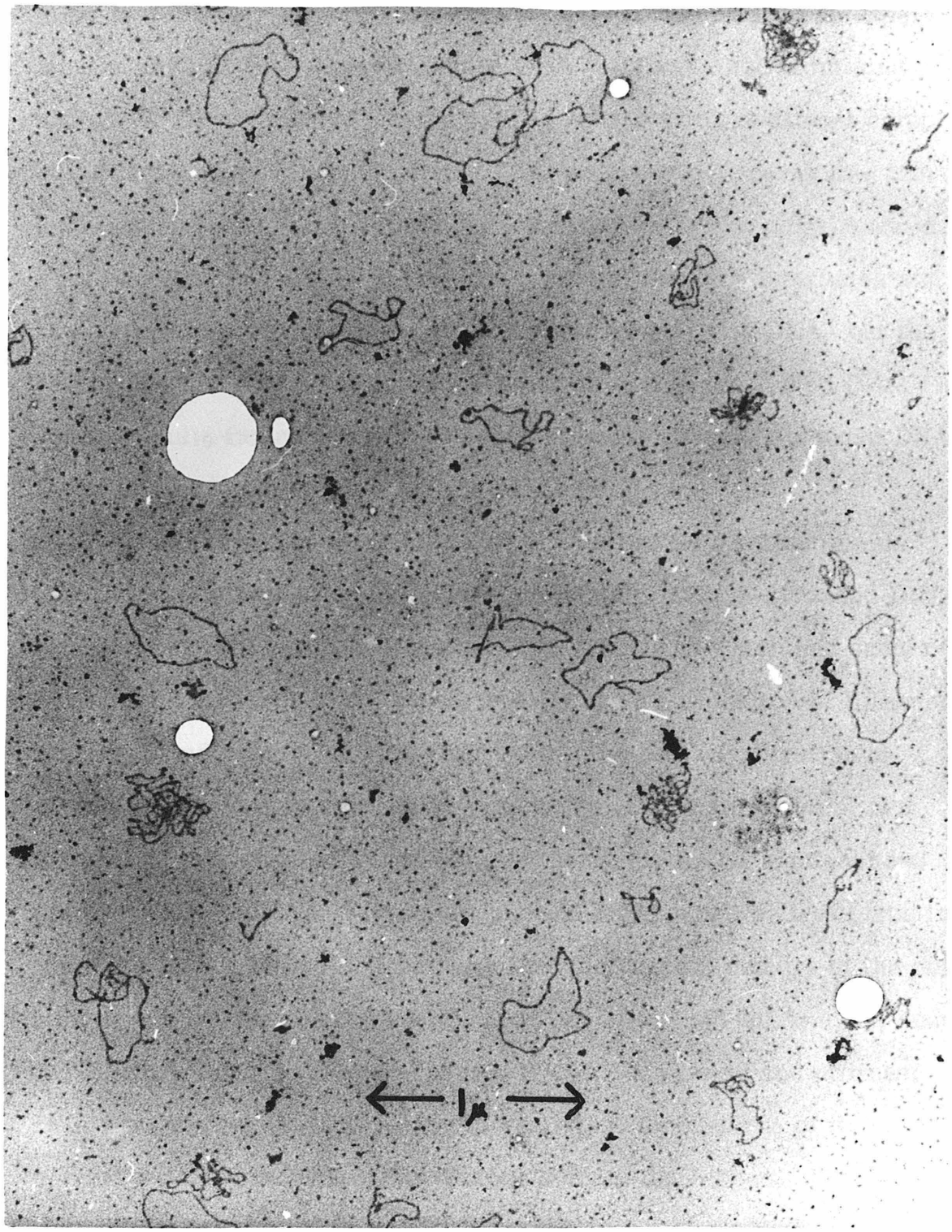

1μ

Figure 4. Electronmicrograph of single-stranded polyoma DNA renatured in 0.48 M ionic strength solvent. The unrenatured sample contained approximately 80% linear, single-stranded DNA (16 S) and 20% circular, single-stranded DNA (18 S). Renaturation occurred upon cooling the samples from 95°C to 50°C in approximately 20 min. The molecule directly above the 1 $\mu$ marker has a length of 1.5 $\mu$. Total magnification is 34,000.


1μ

# DISCUSSION

In summary, the following can be said concerning these experiments: (a) Linear, single-stranded DNA (16 S) obtained from singly nicked PY I renatures extensively. The renaturation is almost complete in the lower ionic strength solvent. (b) Preparations containing more than 50% circular, single-stranded DNA obtained from singly nicked PY I renature in a different manner. Part of the DNA renatures and has a buoyant density similar to that of native polyoma DNA, but the remainder has a buoyant density similar to that of denatured DNA. These results indicate that the excess circular, single-stranded DNA in such preparations is not able to renature.

The assumption was made during this series of experiments that the buoyant density of renatured, circular, single-stranded DNA would reflect the quantity of duplex DNA formation which had occurred during the renaturation. This is true for the renaturation of linear, single-stranded DNA but may not be true for the renaturation of circular, single-stranded DNA. The complete renaturation of two circular single strands would require the formation of right-handed helical turns in one-half the molecule and left-handed helical turns in the other half. Although it is clear that the buoyant density of the one half would be that of native DNA, it is not clear that the buoyant density of the other half would be the same. When a single strand of linear DNA renatures with another single strand, it loses some of its close contact with the water and its buoyant density decreases. If such a strand were to renature with another single strand to form a left-handed helix, unknown changes in the water structure might occur.

The buoyant density of a complex containing one-half right-handed helical turns and one-half left-handed helical turns could conceivably be that of denatured DNA.

The renaturation of the 16 S linear, single-stranded DNA in either high (1.27 M) or low (0.31 or 0.48 M) ionic strength solvents was nearly complete as determined by buoyant density. A more complete reaction appears to occur in the lower ionic strength solvents. This result may be due to the increased non-specific renaturation which occurs in higher molarity salts (5, 6). This interrupts the winding process which normally takes place very rapidly after the nucleation reaction. The unreacted regions then renature with other complementary strands, forming higher molecular weight complexes containing non-renatured regions. The renaturation of the 16 S linear, single-stranded DNA negates the possibility that pancreatic DNAase attacks at one unique region on polyoma I DNA. An attack of this nature would allow the isolation (as described above) of only identical, linear, single-stranded DNA and identical, circular, single-stranded DNA. Within each population, no renaturation would be possible.

The renaturation of samples containing more than 50% circular, single-stranded DNA may occur in the following manner. Linear, single-stranded DNA can renature either with complementary, linear or circular, single-stranded DNA. As soon as the less populous linear molecules are depleted, no further renaturation occurs. The remaining circular strands either do not renature at all or form structures not identifiable by the method of buoyant density centrifugation.

The electron micrographs (Figures 3 and 4) reveal that circular, double-stranded DNA is formed as a result of the renaturation of preparations containing linear and circular, single-stranded DNA. The circular DNA formed in the 1.27 M ionic strength buffer appears to consist mainly of shortened circles having puddled regions on their perimeters (Figure 3). As was explained earlier, such molecules might arise after the formation of a "hairpin" region in a circular single strand. The electron micrograph of Figure 4 illustrates molecules which appear to be more fully renatured. Many of them lack a puddled region.

It does not seem likely that extreme variations in the renaturation methods would allow the renaturation of circular, single-stranded DNA. The renaturation of linear DNA is most favorable at 20 to 25°C below the melting temperature and in a solvent of about 0.3 M ionic strength. These conditions would most likely be the best conditions for the renaturation of circular single strands because this process would necessarily be the simultaneous formation of right-handed helices and left-handed turns of some type.

The experiments contained within this part of the thesis were done approximately 1 1/2 years ago. At that time, the experimental work suffered from the lack of pure linear and circular, single-stranded DNA. Such difficulties could now be overcome with the proper use of exonuclease I (as described in Part III of the thesis) and long column sedimentation velocity gradients.

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# PART III

# THE ABSENCE OF A NON-NUCLEOTIDE LINKER IN POLYOMA AND $\phi$X174 DNA

# INTRODUCTION

The presence of a small quantity of amino acids in hydrolyzed preparations of highly purified DNA has suggested that DNA may contain amino acid linkages within its structure. Bendich and Rosenkranz (1) proposed a structure containing serine (or a seryl-peptide) in the main polynucleotide chain of DNA. The serine (or serylpeptide) would be involved in an amino acid-ester linkage to the 3′-carbon of a nucleotide. At one time, Dulbecco believed a linker of some type to be present in the structure of polyoma viral DNA (2). His reasoning was based on an earlier incorrect understanding of the structure of this molecule and its degradation products. Part III of the thesis reports on experiments performed to determine whether polyoma DNA contains a linker within its structure. In the course of this work, the DNA of the virus $\phi$X174 has been used for comparison purposes. It has been reported that this single-stranded DNA contains a block to the action of E. coli phosphodiesterase (exonuclease I) (3). The work to be reported here indicates that neither $\phi$X174 nor polyoma DNA contain any non-nucleotide blocks to the action of exonuclease I.

# MATERIALS AND METHODS

## Materials

1) Growth medium for $\phi$X174

One-half gm of NaCl, 8.0 gm KCl, 1.1 gm $NH_4Cl$, 0.2 gm $MgCl_2 \cdot 6H_2O$, 0.023 gm $KH_2PO_4$, 0.8 gm sodium pyruvate, 11.44 gm Trizma HCl, 3.32 gm Trizma Base (Sigma Chemical Co.), 1.0 ml 1 M $CaCl_2$ and 1.0 ml 0.16 M $Na_2SO_4$ were dissolved in 250 ml distilled $H_2O$. 2.7 gm of amino acid mixture (special 20 natural L-amino acid mixture, Nutritional Biochemicals Corp.) were dissolved in 500 ml of $H_2O$. The two solutions were mixed, the volume was brought to 1 liter and the resulting preparation was autoclaved. Twenty ml of sterile 10% glucose were added. Five $\mu$g/ml of thymidine were added for thymidine-requiring strains of bacteria.

2) E. coli strain

The bacterial strain used was E. coli HF4704. This is a thymine (or thymidine) requiring strain isolated by Dr. T. Komano from E. coli HF4701, which, in turn, is a strain developed by Dr. P. Howard-Flanders. E. coli HF4701 is a hybrid formed by recombination between E. coli K12 and E. coli C and is uvr-A and $\phi$X174 sensitive. The bacteria were a gift of Mr. C. A. Hutchison, III.

3) $\phi$X174 virus

$\phi$X174 am3, a lysis defective mutant, was a gift of Mr. C. A. Hutchison, III.

4) Enzymes

Lysozyme, 2x crystallized, was obtained from Worthington Biochemical Corp.

Pancreatic DNAase, 1x crystallized, was obtained from Worthington Biochemical Corp. Small volumes of the enzyme in 0.06% BSA, 0.125 M NaCl, 0.01 M $MgCl_2$, and 0.01 M tris, pH 8.0, were frozen in vials at -20 or -70°C.

E. coli phosphodiesterase (exonuclease I) (4), a gift from Prof. I. R. Lehman, was divided into 20 $\mu$l aliquots and frozen at -70°C. Each aliquot was sampled only once.

5) Chemicals

Bovine serum albumin (BSA) was obtained from Armour Pharmaceutical Co.

Harshaw optical grade CsCl was used.

Calf thymus DNA was obtained from Nutritional Biochemicals Corp., Cleveland.

Phenol redistilled under nitrogen was used in all DNA extractions.

All other chemicals were reagent grade.

6) Scintillation counting solution

One liter of counting solution was prepared by dissolving 4 gm of PPO and 0.05 gm of dimethyl POPOP or POPOP (Packard Instrument Co.) in 1 liter of toluene.

Methods

1) Polyoma virus

The growth and isolation of polyoma virus and the extraction and purification of the viral DNA has been described in Part I of the thesis.

2) $\phi$X174 virus

The $\phi$X174 virus was grown and purified using the method of Hutchison and Sinsheimer (5) and Hutchison and Edgell (6). The E. coli bacteria were grown at 37°C to a concentration of $2 \times 10^8$ /ml, spun down, and resuspended in one volume of fresh media containing 0.3 $\mu$c/ml $H^3$-thymidine. At this time, the cells were infected with $\phi$X174 am3 at a multiplicity of infection of 5. After 3 hours, the unlysed cells were spun down, washed with 0.05 M borate buffer (pH 9), resedimented, and resuspended in a small volume of 0.05 M borate buffer. 0.5 mg/ml of lysozyme was added. After 10 min at room temperature, EDTA was added to 0.01 M (7). The suspension was allowed to incubate at room temperature for 30 min, after which it was sonicated for 5 one-min periods at 4°C using the #6 power setting and the microprobe of the Branson 125 S sonifier (Branson Instruments, Inc., Danbury, Conn.). After the sedimentation of the bacterial debris, CsCl was added to the supernatant to obtain a solution density of 1.40 gm/ml. These preparations were centrifuged at 40K rpm for 24 hours at 9°C in the Spinco SW50 rotor. The virus band was withdrawn from the top of the tube by means of a pasteur pipet and propipet. Because of the large amount of bacterial debris in the first gradient, the virus were rebanded. The virus were collected from the

second gradient using a drop collecting device. The purified preparation was dialyzed against 0.05 M borate buffer and $10^{-3}$ M EDTA to remove the CsCl.

3) $\phi$X174 DNA extraction

The DNA of $\phi$X174 was isolated by the method of Guthrie and Sinsheimer (8). Solid sodium tetraborate and 0.2 M EDTA were added to the virus preparation to increase the concentrations to 0.1 M and 0.02 M, respectively. One volume of hot (70°C) redistilled phenol saturated with 0.05 M borate buffer was added to the virus sample. This mixture was shaken on a vortex mixer for 20 to 30 sec, rewarmed to 70°C, shaken, rewarmed, etc. for a total period of 5 min. The emulsion was broken by centrifugation at 7000 rpm in the Sorvall SS-34 rotor for 10 min. The aqueous layer was removed and extracted twice more in a similar manner using room temperature phenol. The phenol was removed by 6 eight-hour dialyses against 500 volumes of SSC/10, 0.01 M tris, pH 8.0. As analyzed by band velocity sedimentation (9), the sample contained 90 to 95% circular material. The specific activity was 4700 cpm/$\mu$g.

4) Ultracentrifugation

Sedimentation velocity analyses were performed using preformed alkaline CsCl gradients in the SW 50 rotor in the Spinco model L ultracentrifuge. The gradients were made as follows: A linear 4.75 ml gradient was established by pumping the products of a linear gradient making apparatus through a capillary to the bottom of a Spinco polyallomer tube. The density range was 1.30 to 1.40 gm/ml.

After the gradient had been prepared, 0.25 ml of a CsCl solution having a density of 1.75 gm/ml was pumped to the bottom of the tube. This concentrated CsCl provided a dense pad which slowed down the fastest sedimenting material. The two less dense solutions used in the gradient maker were 0.1 M $K_2HPO_4$ in CsCl, $\rho$ = 1.30 and $\rho$ = 1.40 gm/ml. The dense solution was 0.1 M $K_2HPO_4$ in CsCl, $\rho$ = 1.75 gm/ml. The pH of the two less dense solutions was adjusted to the desired pH at 20° C by adding 1.0 N KOH in 0.1 M $K_2HPO_4$ and CsCl, $\rho$ = 1.30 and 1.40 gm/ml, respectively. The pH of the dense solution was adjusted at 20° C by adding 5 M KOH. The pH of the alkaline CsCl velocity gradients was usually 11.85. This pH was high enough to cause denaturation and strand separation of nicked molecules and was low enough to avoid pelleting the denatured, double-stranded molecules at the bottom of the tube. The sedimentation velocity of the intact double-stranded molecules is extremely pH dependent between pH 11.6 and 12.3. All titrations were done immediately before making the gradients. During the titrations and manufacture of the gradients, the solutions were under an atmosphere of argon. A Beckman Research model pH meter, a general purpose probe glass electrode and a calomel reference electrode modified with a ground glass junction (10) were used in the titrations. Sample preparations were brought to the pH of the experiment and 0.25 ml or less were layered gently onto the surface of the gradient. The sedimentation velocity experiments in the Spinco model L were performed at 20 ± 1° C and 45,000 ± 500 rpm. The total centrifugation time for each experiment was 4 hours. This

time included the 5 min needed for acceleration but did not include the approximately 15 min of non-brake deceleration time.

5) Pancreatic DNAase treatment of $\phi$X174 DNA (3, 11)

The reaction mixture contained 90 $\mu$g/ml $H^3$-labeled $\phi$X174 circular DNA in 0.043 M NaCl, 0.01 M $MgCl_2$, 0.01 M tris, 41 $\mu$g/ml BSA and 0.014 $\mu$g/ml of pancreatic DNAase, pH 8.0. The total reaction time was 30 min and the temperature was 20°C. Because of the inability to determine enzyme activities at such low concentrations with normal procedures, the reaction conditions had to be determined empirically. The reaction was allowed to proceed for a period of time, an aliquot of the mixture was removed and the remainder of the reaction mixture was quickly frozen. 0.2 M EDTA was then added to the aliquot to 0.05 M and the number of hits was determined by alkaline band sedimentation velocity (12). If the reaction had not gone far enough, the reaction mixture was thawed and incubated for an additional period of time until the desired distribution of products was obtained. At this point, the reaction was stopped temporarily either by freezing or by adding EDTA.

6) E. coli phosphodiesterase treatment of $\phi$X174 DNA (3)

The product of the reaction outlined in 5) was thawed and 1 M glycine buffer, pH 10.2, was added to a concentration of 0.07 M to prevent further pancreatic DNAase activity. After the addition of 22 units of exonuclease I, the reaction volume became 62 $\mu$l and contained 70 $\mu$g/ml $\phi$X174 DNA in 0.034 M NaCl, 0.0078 M $MgCl_2$, 0.0078 M tris, 0.059 M glycine, 32 $\mu$g/ml BSA and 22 units of exonuclease I, pH 9.8.

The reaction time was 2 hours and the temperature was 37°C. The reaction was stopped by the addition of 1/6 volume of 0.2 M EDTA.

7) Pancreatic DNAase treatment of polyoma DNA (11)

The reaction mixture contained 9.6 μg/ml, 100% $H^3$-labeled PY I DNA in 0.04 M NaCl, 0.0066 M $MgCl_2$, 0.01 M tris, 33 μg/ml BSA and $2.4 \times 10^{-4}$ μg/ml enzyme, pH 8.0. The reaction time was 7 hours at 20°C. As before, the extent of the reaction was determined by alkaline band velocity sedimentation. The reaction was terminated by the addition of 0.2 M EDTA to a concentration of 0.01 M.

8) E. coli phosphodiesterase treatment of pancreatic DNAase treated polyoma DNA (3)

BSA, 30 mg/ml, was added to the pancreatic DNAase treated polyoma DNA. The final BSA concentration was 60 μg/ml. Fifty-five μl of 0.2 N KOH were added and rapidly mixed with 250 μl of the DNA preparation. Both solutions were at 4°C. The resulting solution was warmed to room temperature and incubated for 5 min. After recooling the sample to 4°C, 12.5 μl of 1 M glycine buffer, pH 9.0, were added as quickly as possible. 8.5 μl of 0.2 M $MgCl_2$ were then added. Three μl of exonuclease I (0.6 units) were added to one-half the sample, and 3 μl of SSC/10, 0.01 M tris were added to the other half. Both samples were incubated for 30 min at 37°C. The pH of the reaction mixture was 9.8 to 10.0. The latter value was determined in pilot experiments with larger volumes of the reagents. The reaction was terminated by adding a one-tenth volume of 0.2 M EDTA.

9) Analysis of the alkaline CsCl gradients

Seven and one-half $\mu$l drops were collected from the gradients using a piercing device from Buchler Instruments. Alternate 4 drop fractions were collected into 0.5 ml of SSC/10, 0.01 M tris and $5 \times 10^{-3}$ M EDTA containing 35 $\mu$g of calf thymus DNA, and 2 drop fractions were collected on Whatman GF/A filter papers (2.4 cm diameter) in Packard scintillation vials. The filter papers were dried by placing the bottles on a hot plate. Ten ml of toluene-PPO-POPOP scintillation fluid were added and the tritium counts were determined in a Packard Tri-Carb scintillation counter. The contents of the vials containing the buffer-calf thymus DNA solution were analyzed for acid precipitable counts. One-half ml of 10% trichloroacetic acid (TCA) at 4°C was added to each vial. Usually after 10 to 15 min, and never after more than 60 min, the precipitated samples were filtered through a 2% TCA-wetted Whatman GF/A filter using a vacuum apparatus. The vial was washed with 5 ml of cold 2% TCA and the filter apparatus was washed with 10 ml. The filters were dried and counted as before.

The background level in the scintillation counting system was determined with 4 scintillation vials, each containing 10 ml of scintillation fluid and a Whatman GF/A filter paper upon which 15 $\mu$l of 0.1 M $K_2HPO_4$ in CsCl, $\rho$ = 1.30 gm/ml and pH 11.9 had been dried. The test vials were counted for a large number of 10 min counting periods. The average background was found to be $16.35 \pm 1.42$ cpm for the polyoma DNA experiments. The background level used for the $\phi$X174 DNA experiments was 16.0 cpm.

In the radioactivity counting systems used, the change in quenching through the gradient was found to be negligible.

10) Calculations

The standard deviations which are given with each percentage value in the tables and text represent the statistical deviations in the radioactive counting procedure.

The fraction of a given component present in either a neutral or alkaline sedimentation velocity gradient analysis after a random attack on a population of double-stranded or single-stranded circular DNA can be calculated with the Poisson relation, $f(x) = e^{-\mu} \mu^x/x!$. $f(x)$ is the fraction of DNA remaining having x hits per molecule after a random attack of $\mu$ hits/molecule, on the average. The distribution in Figure 1a is useful in the analysis of pancreatic DNAase treated closed circular, double-stranded DNAs. It may also be used to describe the product distribution for circular, single-stranded DNAs after pancreatic DNAase attack. Figure 1b presents the product distribution that would be obtained in an alkaline (pH > 11.8) analysis of circular, double-stranded DNA. The formulas of Table 1 were used in constructing Figure 1. The figure contains summation signs with an upper limit of n. Strictly speaking, the upper limit should be $\infty$ in order to apply Poisson statistics. Of course, both polyoma and $\phi$X174 DNA contain a finite number of phosphodiester bonds, 10,000 and 5000, respectively. These numbers are large enough to insure the validity of Poisson statistics when each molecule has received only a small number of hits.

Table 1

Analysis of Hit and Intact Closed Circular DNA

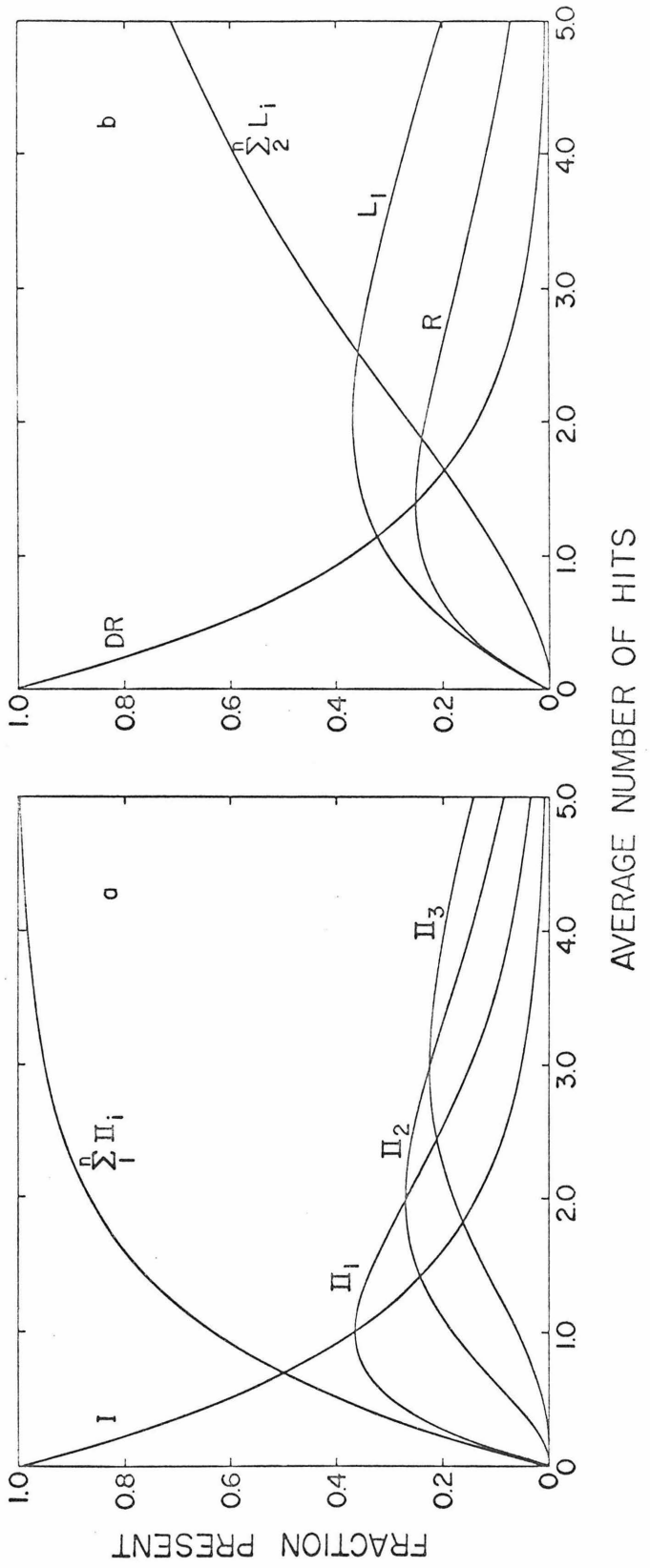
Neutral analysis

| Component | | Fraction of original DNA |
|---|---|---|
| I | No hits/molecule | $e^{-\mu}$ |
| $II_1$ | One hit/molecule | $\mu e^{-\mu}$ |
| $II_2$ | Two hits/molecule | $\mu^2 e^{-\mu}/2$ |
| $II_3$ | Three hits/molecule | $\mu^3 e^{-\mu}/6$ |
| $\sum_1^n II_i$ | Sum of all hit molecules [Includes linear molecules produced after an excessive number of hits on circular molecules (13, 14)] | $1-e^{-\mu}$ |

Alkaline analysis

| Component | | Fraction of original DNA |
|---|---|---|
| DR | Double-stranded ring | $e^{-\mu}$ |
| R | Single-stranded ring (18S for polyoma DNA) | $\sum_{m=1}^{\infty} \frac{e^{-\mu}\mu^m}{m!\,2^m}$ |
| $L_1$ | Linear, single-stranded DNA produced by one break in either DR or R (16S for polyoma DNA) | $\sum_{m=1}^{\infty} \frac{e^{-\mu}\mu^m}{(m-1)!\,2^m}$ |
| $\sum_2^n L_i$ | Single-stranded DNA other than $L_1$ | $1-e^{-\mu}-\sum_{m=1}^{\infty} \frac{e^{-\mu}\mu^m}{2^m}\left[\frac{1}{m!}+\frac{1}{(m-1)!}\right]$ |

Figure 1. Analysis of hit and intact closed circular DNA. This figure was constructed with the formulas given in Table 1. (a) The Poisson distribution in Figure 1a is useful in the analysis of nicked circular, duplex DNA or nicked circular, single-stranded DNA. I represents the fraction of intact closed circular DNA. $II_i$ is nicked circular DNA containing i hits/molecule. (b) Figure 1b is useful in the alkaline analysis of nicked circular DNA. The symbols used are defined in Table 1. The formulas used for the construction of 1b can by derived by applying the Poisson relation to a sample of denatured, hit, closed circular DNA. The respective fraction of linear, single-stranded DNA (16 S) or circular, single-stranded DNA (18 S) can be derived by the addition of the respective components present in a population of denatured, closed circular DNA molecules containing 1, 2 or more hits per molecule.


a
b
I
$\sum_1^n \Pi_i$
$\Pi_1$
$\Pi_2$
$\Pi_3$
DR
$\sum_2^n L_i$
$L_1$
R
FRACTION PRESENT
AVERAGE NUMBER OF HITS
0
0.2
0.4
0.6
0.8
1.0
1.0
2.0
3.0
4.0
5.0

# RESULTS

Exonuclease I degrades linear, single-stranded DNA from the 3'-OH end of the molecule (4, 15). If exonuclease I is added to a sample of closed circular polyoma DNA I after successive treatment with pancreatic DNAase (1 to 2 hits/molecule), heat denaturation and quick cooling, the resulting single-stranded circles (18 S) and double-stranded closed circles should be resistant to the exonuclease. The linear, single-stranded DNA (16 S and less) should be completely digestible if no block to enzyme activity is present in the molecular structure.

The results of Vinograd et al. (13) showed that most of the linear, single-stranded DNA (16 S and less) produced by the denaturation of pancreatic DNAase treated polyoma I DNA was digested by exonuclease I (see Part I of the thesis). There was, however, a small amount of DNA left at the 16 S position in the alkaline CsCl velocity gradients. This appeared to indicate that some linear, single-stranded DNA was not digestible with exonuclease I. Further experiments with preformed, alkaline CsCl velocity gradients in the Spinco model L ultracentrifuge supported this observation. Initially this was thought to indicate that there might be a block to the action of the enzyme in the molecule. However, it became apparent that the explanation was not completely satisfactory for the following reasons. (a) The amount of single-stranded DNA sedimenting at 16 S in the alkaline CsCl gradient was variable depending upon the denaturation method used previous to the exonuclease I incubation. (b) The distribution of the degradation

products in the gradient was not that expected after digestion of linear, single-stranded DNA containing a block to exonuclease I. Most of the digested linear, single-stranded DNA either had a very low sedimentation coefficient characteristic of the expected mononucleotides or sedimented at 16 S. Very little partially digested single-stranded DNA was found with intermediate sedimentation coefficients. If the block were located at a random position in 1/2 * of the molecules within a population of 16 S linear, single-stranded polyoma DNA, we would expect that 3/4 of the original 16 S DNA would be in the form of acid soluble mononucleotides and dinucleotides after exonuclease I digestion. The remaining 1/4 would be found uniformly distributed with respect to molecular length between the top of the gradient and the 16 S position. No peak would be expected at the 16 S position. A criterion for the presence of a block in one strand of polyoma DNA can be derived from the above statements. The conversion by exonuclease I of more than 3/4 of the original 16 S linear, single-stranded DNA to acid soluble material provides evidence for the absence of a block to the action of the enzyme in either strand of polyoma DNA.

Experiments with $\phi$X174 DNA

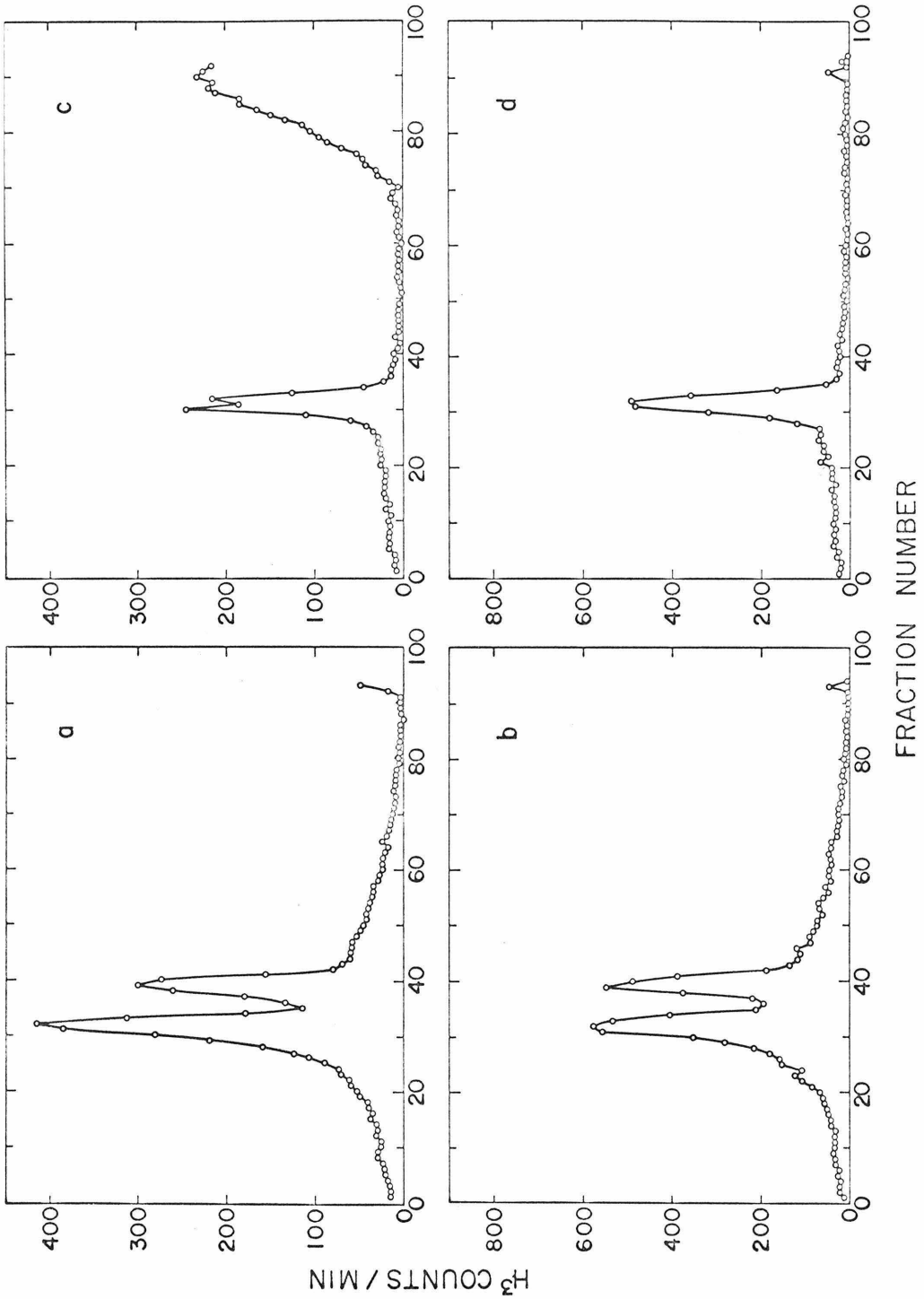
Fiers and Sinsheimer (3) had previously indicated that $\phi$X174, a circular, single-stranded DNA, has a block to the action of exonuclease I. It was felt that experiments with $\phi$X174 DNA would provide a control on

* Polyoma DNA might have a block in only one of the two strands of the duplex molecule. This supposition is more difficult to establish than one in which polyoma DNA contains a block in each strand.

experiments with polyoma DNA. ϕX174 DNA containing more than 95% circular molecules was treated with pancreatic DNAase in a limited reaction. The distribution of the products of this reaction after sedimentation in a pH 12 CsCl preformed density gradient is shown in Figure 2a and 2b. Both total $H^3$ count (Fig. 2a) and acid insoluble $H^3$ count (Fig. 2b)analyses were made upon the material in this gradient. Both circular (18 S) and linear (16 S), single-stranded DNAs are present. There is also a small amount of DNA sedimenting slower than 16 S. In this particular experiment the total count profile and the acid precipitable count profile were not completely superimposable. A repeat, in part, of this experiment and results from the analytical ultracentrifuge indicate that the ratio of 18 S to 16 S is slightly high in the total count profile and slightly low in the acid insoluble count profile. It is estimated from the Poisson distribution (Fig. 1a) that the preparation received 0.6 to 0.8 hits/molecule from the pancreatic DNAase.

After subjecting the product of the pancreatic DNAase treated ϕX174 to exonuclease I digestion, the sample was analyzed as before in a pH 12.0 CsCl sedimentation velocity gradient. Figure 2c and 2d present the total count and acid precipitable count profiles, respectively. From Figure 2c, it was calculated that 34.7 ± 1.2% of the total counts in the gradient are found between fraction 36 and the bottom of the gradient. This represents the 18 S circular, single-stranded DNA which cannot be digested by exonuclease I. The linear DNA products in Figure 2c have to be distinguished from the mononucleotides and

Figure 2. Sedimentation velocity profiles of $H^3$-labeled $\phi$X174 DNA in preformed pH 12.0 CsCl density gradients. The preparation of the gradients and details of the enzyme experiments are described in the Materials and Methods section of Part III. (a) Total count distribution of pancreatic DNAase treated $\phi$X174 DNA. (b) Acid precipitable count analysis of the gradient shown in (a). (c) Total count distribution of pancreatic DNAase treated $\phi$X174 DNA after digestion with exonuclease I. (d) Acid precipitable count analysis of the gradient shown in (c). (a) and (b) represent two methods of analysis for the same gradient. The same is true for (c) and (d). The gradients were centrifuged for 4 hours at 45,000 rpm, 20°C, in a Spinco model L ultracentrifuge with an SW 50 rotor. The direction of sedimentation is toward the left.


a
b
c
d
$H^3$ COUNTS / MIN
FRACTION NUMBER

dinucleotides * formed in the reaction which are found between fraction 70 and the top of the gradient. The results of Figure 2d were normalized so that the height of the peak in 2d corresponded to the height of the peak in 2c. The curves were superimposed and the curve for 2d was used in the linear DNA analysis of 2c from the top of the gradient down to the point of intersection at fraction 70. A very small amount of mononucleotides or dinucleotides that may have diffused past fraction 70 were regarded as linear DNA resistant to exonuclease. The mononucleotides or dinucleotides found between fraction 70 and the top of the gradient in 2c were determined by subtraction of the linear DNA products in this region. The calculations show that 59.0 ± 1.6% of the counts in the gradient are acid soluble mononucleotides and dinucleotides and 6.3 ± 0.7% of the counts represent single-stranded, linear DNA found between fraction 36 and the top of the gradient. From the fraction of circular, single-stranded DNA remaining in Figure 2c, it was calculated with the Poisson distribution that the preparation received approximately 1.07 ± 0.04 hits/molecule due to endonuclease action before and during the exonuclease I digestion. Therefore, the preparation received approximately 0.2 to 0.4 endonuclease hits/molecule during the exonuclease reaction.

Theoretically, after receiving 1.07 ± 0.04 hits, 65.5 ± 1.2% of the DNA should be acid soluble if there is no block in the molecule.

* It is presumed that the material between fraction 70 and the top of the gradient in Figure 2c is in the form of mononucleotides and dinucleotides, the normal products of exonuclease I digestion. However, the presence of a small quantity of acid soluble polynucleotides in this region cannot be ruled out.

Although a portion of the endonuclease action occurred during the exonuclease digestion, the calculations were based on the premise that all the endonuclease action preceeded the exonuclease digestion. The theoretical value of 65.5 ± 1.2% is therefore too large because less linear DNA is being exposed to the exonuclease activity than would be calculated with the value of 1.07 hits/molecule. This explanation can account for the presence of the small fraction of linear DNA found in the gradient (6.3 ± 0.7%) and for the low fraction of mononucleotides and dinucleotides found (59.0 ± 1.6%).

If there is a block to the action of the enzyme in φX174 DNA, the fraction of linear DNA remaining after the exonuclease I digestion can be predicted by the application of an equation from Fiers and Sinsheimer (3)*. This calculation shows that 27.0 ± 1.5% of the DNA in Figure 2c should be linear after the exonuclease digestion. The calculation is performed on the premise that 1.07 ± 0.04 endonuclease hits/molecule occurred before the exonuclease I digestion. Experimentally, 6.3 ± 0.7% of the product was linear. This result indicates that there is no block in φX174 to the action of exonuclease I.

The fraction of 16 S linear DNA which would have been found in the φX174 DNA preparation just prior to the exonuclease digestion was calculated to be 36 ± 2%. This calculation is made on the premise that all the endonuclease activity (1.07 ± 0.04 hits/molecule) occurred prior to the exonuclease reaction. Because a hypothetical block would

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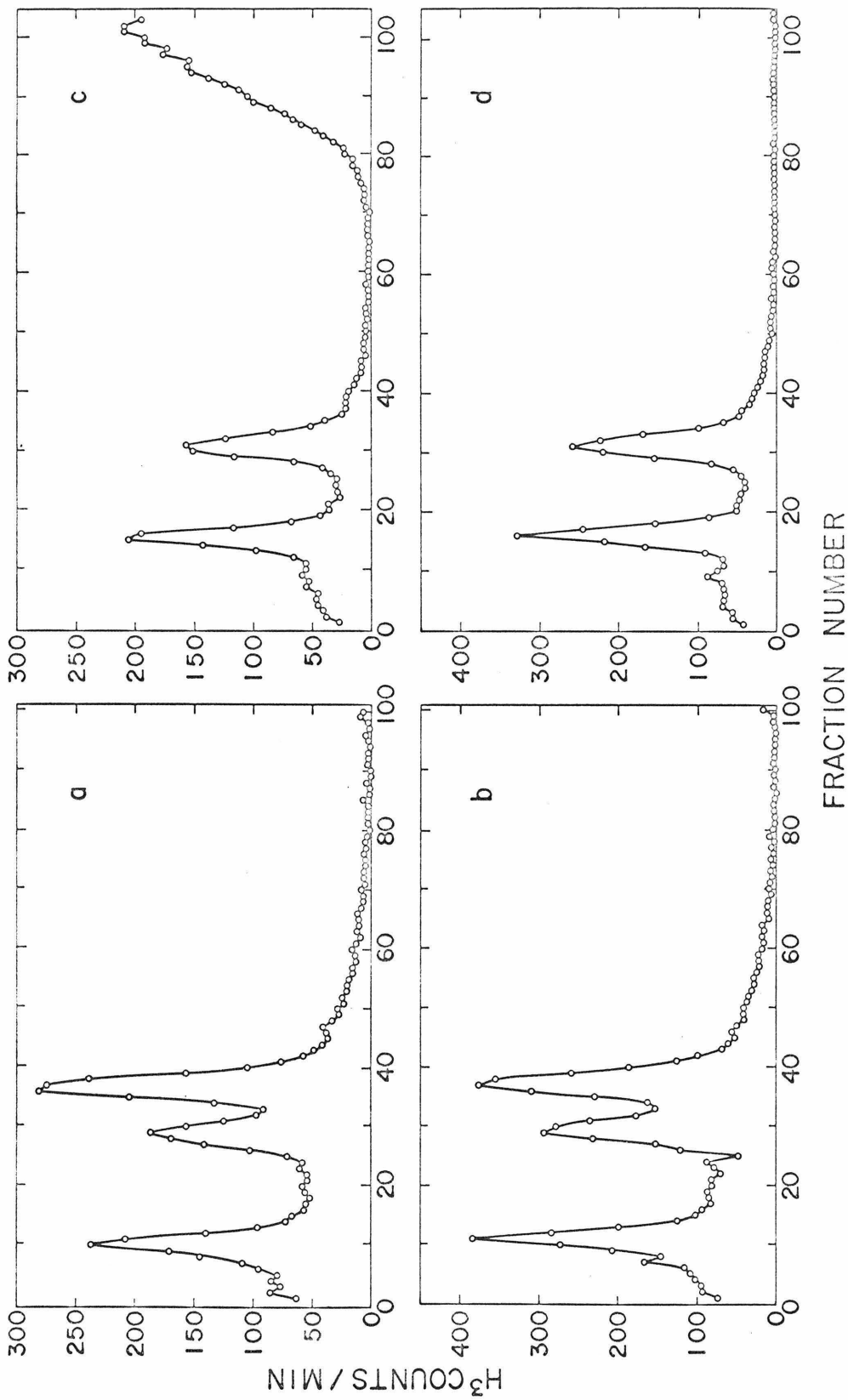
*If one block were present, the fraction of acid soluble DNA after exonuclease digestion would be $(\mu + e^{-\mu} - 1)/\mu$, the fraction of circular DNA would be $e^{-\mu}$, and the fraction of linear DNA would be $1 - e^{-\mu} - (\mu + e^{-\mu} - 1)/\mu$. $\mu$ is the average number of hits/molecule.

be found at a random position in a population of 16 S linear ϕX174 DNA, only 1/2 of the DNA is potentially digestible with exonuclease I. In the present experimental situation, (36 ± 2%)/2 of the 16 S DNA should remain linear and non-digestible by exonuclease I if a block is present. This is a minimum value because the calculation was performed on the premise that all of the 1.07 hits/molecule occurred before the exonuclease I reaction. The value of 6.3 ± 0.7% is much less than (36 ± 2%)/2. Again, this result indicates that there is no non-nucleotide block in ϕX174 to the action of exonuclease I. The same results were obtained when this series of experiments was repeated.

Experiments with polyoma DNA

A preparation of polyoma DNA consisting of 100% PY I was treated with pancreatic DNAase. The distribution of the products of this reaction after sedimentation in a pH 11.85 CsCl preformed density gradient is illustrated in Figures 3a and 3b, which present the total count distribution and the corresponding acid precipitable count distribution, respectively. There are four classes of components: double-stranded cylic coil DNA; circular, single-stranded DNA (18 S); linear, single-stranded DNA (16 S); and linear, single-stranded DNA slower than 16 S. If exonuclease I were now added to the pancreatic DNAase hit polyoma DNA no reaction would occur because this enzyme acts only on linear, single-stranded DNA. Such single strands can be produced either by thermal denaturation followed by quick cooling or by alkaline denaturation and quick neutralization. However, if the digestion of the resulting single-stranded DNA is to occur efficiently,

Figure 3. Sedimentation velocity distributions of $H^3$-labeled polyoma DNA in preformed pH 11.85 CsCl density gradients. The preparation of the gradients and details of the enzyme experiments are described in the Materials and Methods section of Part III. (a) Total count distribution of pancreatic DNAase treated polyoma DNA. (b) Acid precipitable count analysis of the gradient shown in (a). (c) Total count distribution of pancreatic DNAase treated polyoma DNA after denaturation and digestion with exonuclease I. (d) Acid precipitable count analysis of the gradient presented in (c). (a) and (b) represent two methods of analysis for the same gradient. The same is true for (c) and (d). The gradients were centrifuged for 4 hours at 45,000 rpm, 20°C, in a Spinco model L ultracentrifuge with an SW 50 rotor. The direction of sedimentation is toward the left.


a
b
c
d
$H^3$ COUNTS / MIN
FRACTION NUMBER

the denaturation of the nicked molecules must be complete and no renaturation can be allowed to occur. If some renaturation does occur, the renatured regions might act as blocks to the action of the enzyme. For this reason alkaline denaturation followed by quick neutralization was the method chosen for producing linear, single-stranded polyoma DNA prior to exonuclease I digestion. However, careful control of the magnesium concentration was necessary because of the precipitation of DNA which occurs in the presence of magnesium at high pH. The details of the precipitation reaction are not understood, although it is well known that denatured DNA binds $Mg^{++}$ quite strongly at pH 8.0 (16) and that $Mg(OH)_2$ is very insoluble at high pH.

Following the denaturation of the pancreatic DNAase treated polyoma DNA and the digestion of the preparation with exonuclease I, a product distribution analysis was performed in a pH 11.85 CsCl sedimentation velocity gradient. The total $H^3$ count profile and the acid precipitable $H^3$ count profile for this single gradient are shown in Figures 3c and 3d, respectively. It appears that most of the linear, single-stranded DNA (16 S) has been digested to acid soluble nucleotides. The intact, duplex DNA and circular, single-stranded DNA (18 S) are resistant to the action of the enzyme.

The fraction of each component in each of the four gradient patterns in Figure 3 was determined by initially assuming symmetric distributions for each of the discrete peaks. Small departures from symmetry were sometimes required so that all of the counts in each gradient were conserved. Because of the arbitrary nature of this procedure, it was necessary to construct alternative resolution

patterns to gain some idea of the change in the fraction of a given component with a change in the resolution pattern. The demonstration that less than 1/4 of the original 16 S material is resistant to exonuclease I depends upon the correct calculation and analysis of both the 16 S linear, single-stranded DNA present before the exonuclease I digestion and the linear, single-stranded DNA present after the exonuclease I digestion.

In order to determine in Figure 3c the fraction of linear DNA resistant to exonuclease I which is derived from the 5' (left) side of the randomly located block in a 16 S linear molecule, it was necessary to solve two problems. (i) The linear DNA products (Figure 3c) of the reaction have to be distinguished from the mononucleotides and dinucleotides produced from the digestion of the DNA on the 3' (right) side of the block. (ii) The larger linear DNA (16 S and less) must be distinguished from the circular, single-stranded DNA from which it does not adequately resolve.

(i) The results of Figure 3d for the acid insoluble DNA were normalized so that the two peak heights in 3d corresponded to the two peak heights in 3c. The curves were superimposed and the curve for 3d was used in the analysis of 3c from the top of the gradient down to the point of intersection at fraction 70. A very small amount of mononucleotides or dinucleotides that may have diffused past fraction 70 was regarded as linear DNA resistant to exonuclease.

(ii) Figure 4 presents the critical part of 3 alternative patterns which indicate possible resolutions of the 18 S circular, single-stranded DNA from the linear, single-stranded DNA of 16 S and less

Figure 4. Alternative patterns indicating possible resolution of the circular, single-stranded DNA of 18 S from the linear, single-stranded DNA of 16 S and slower in Figure 3c. The region between fractions 20 and 44 of Figure 3c is presented in Figure 4. The arrows represent the 18 S and 16 S positions in the gradient as determined by examination of Figure 3a. Alternative 1, —·—· ; alternative 2, – – – –; and alternative 3, ·····.

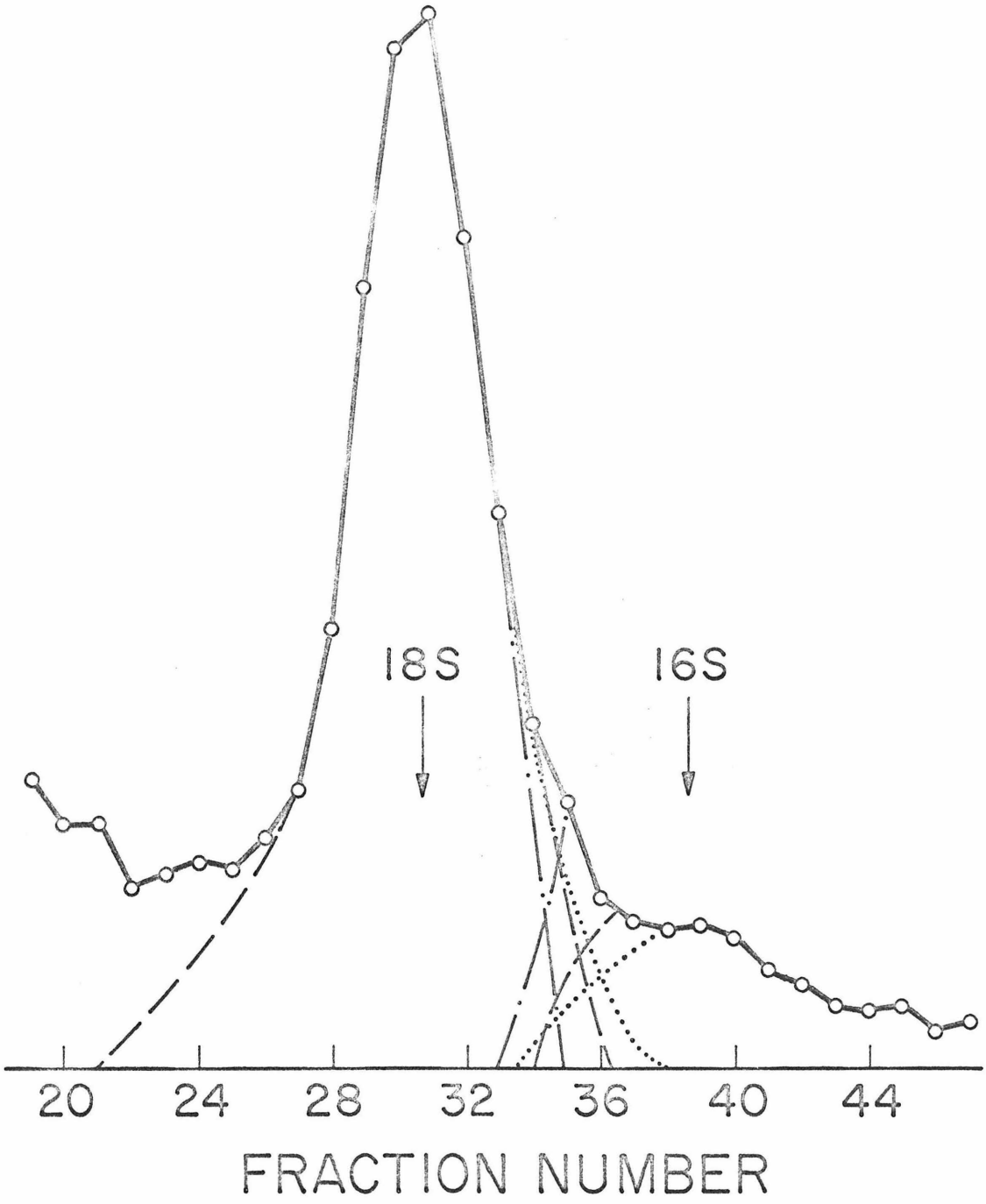

18S
16S
20
24
28
32
36
40
44
FRACTION NUMBER

Table 2

Alternative Resolution Patterns Constructed from Figure 3c

| | Percent linear DNA |
|---|---|
| Alternative 1 | 6.5 ± 0.7% |
| Alternative 2 | 5.5 ± 0.7% |
| Alternative 3 | 5.2 ± 0.7% |

in Figure 3c. Arrows have been placed on the figure representing the 16 S and 18 S positions, determined by examination of Figure 3a. Table 2 presents the fraction of linear DNA, 16 S and less, present in 3c depending upon whether alternative 1, 2 or 3 is chosen to be the most likely resolution pattern. The results of 3d are used in making this calculation as is discussed in (i). Examination of Figure 4 reveals that alternative 1 is unreasonable in view of the position of the 16 S material in the gradient. Alternative 3, $5.2 \pm 0.7\%$, is close to the correct resolution pattern, but alternative 2, $5.5 \pm 0.7\%$, was adopted to increase the difficulty of satisfying the criterion for the absence of a block.

The pattern in Figure 3d was analyzed as in the profile for 3c. To express the amount of each component as a percentage of the total DNA applied to the gradient, it would have been necessary to know the total number of acid soluble counts in addition to the known number of acid insoluble counts. Because this was not experimentally feasible, an alternative procedure was used. The fraction of counts remaining in Figure 3c after subtraction of the mononucleotide and dinucleotide counts was multiplied by the fraction of counts present as 16 S and smaller linear DNA present in 3d. This product represents an alternative determination of the quantity of 16 S and slower DNA present in the sample applied to the gradient represented by Figure 3c and 3d. This number is $6.1 \pm 0.5\%$. It is averaged with the value obtained from Figure 3c, $5.5 \pm 0.4\%$. The demonstration that less than 1/4 of the original 16 S material is resistant to exonuclease I depends on the

magnitude of this number, which represents the fraction of acid insoluble linear DNA resistant to exonuclease I. This value will be compared with the fraction of acid insoluble, linear 16 S DNA found in the preparation before exonuclease I treatment as explained below.

Figure 5 illustrates the critical part of three alternative diagrams which indicate possible resolutions of the 16 S DNA from the 18 S DNA and the remainder of the linear DNA in Figure 3a. Table 3 presents the percentage of 16 S DNA determined in each of the three cases. Alternative 1 appears to be unreasonable because its shape is unlike the isolated bands seen in either preparative or analytical sedimentation velocity experiments. Alternatives 2 and 3 appear to be more reasonable, but again alternative 2, $19.5 \pm 0.9\%$, was adopted to increase the difficulty of satisfying the criterion. Figure 3b was resolved in a similar way. The value obtained for 16 S DNA, $27.5 \pm 0.7\%$, was averaged with the value obtained in Figure 3a to give a mean of $28.3 \pm 0.6\%$.

The experimentally determined values for the distribution of components in the pancreatic DNAase treated polyoma DNA (Figure 3a and 3b) and in the exonuclease treated polyoma DNA (Figure 3c and 3d) are presented in Table 4. These results are found in columns 1 and 3, respectively, and include those values the determination of which was discussed in detail in the last several paragraphs. If the original preparation of polyoma DNA had received 1.02 hits/molecule, the distribution shown in column 2 of Table 4 would be found. The values in this column are similar to those found in column 1, indicating that

Figure 5. Alternative patterns indicating possible resolution of the 16 S linear, single-stranded DNA from the 18 S circular, single-stranded DNA and from the linear DNA slower than 16 S in Figure 3a. The region between fractions 24 and 48 is presented in Figure 5. Alternative 1, —·—·; alternative 2, – – – –; alternative 3, ·····.

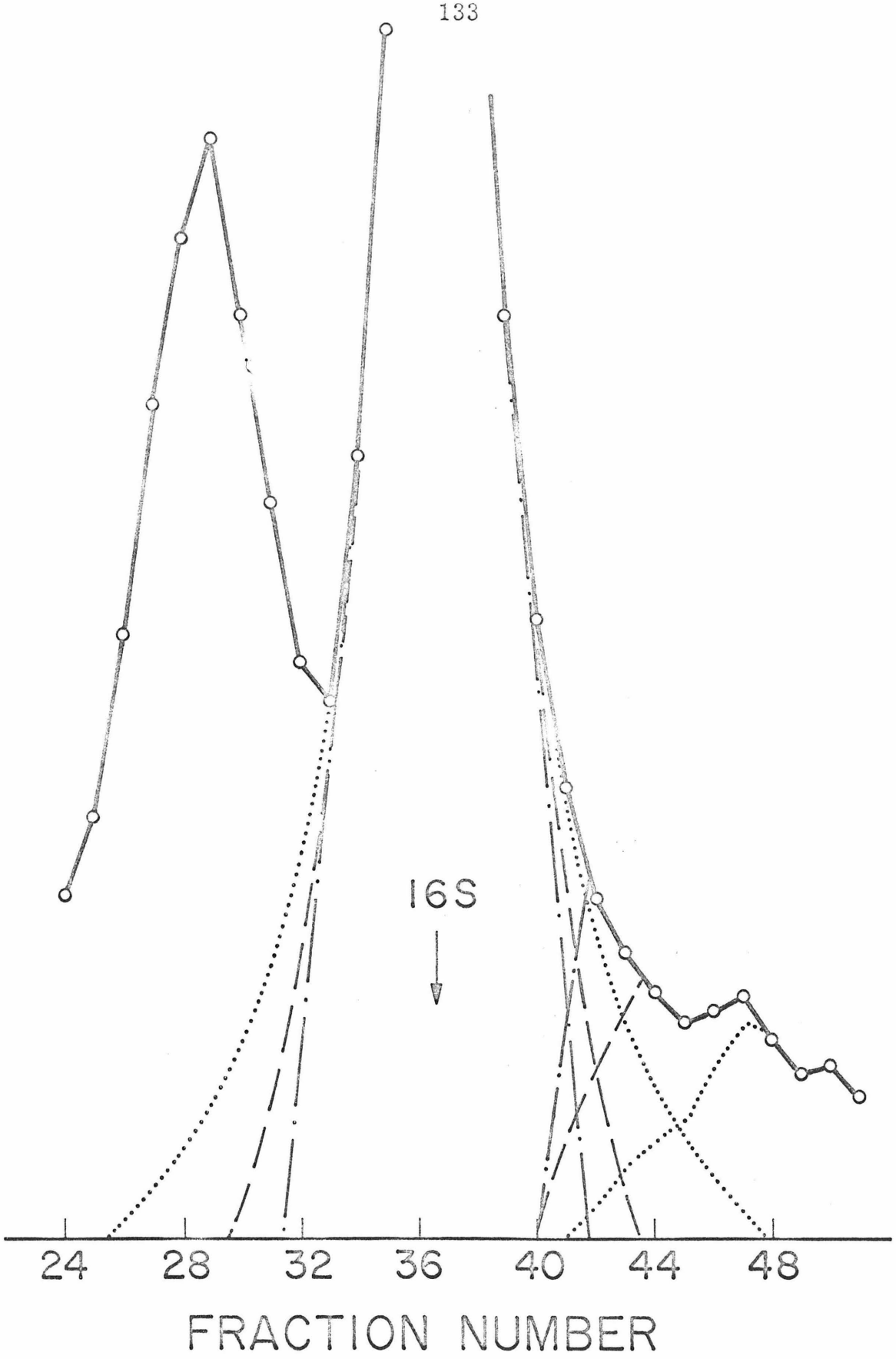

16S
24
28
32
36
40
44
48
FRACTION NUMBER

Table 3

Alternative Resolution Patterns Constructed

from Figure 3a

| | Percent 16 S linear DNA |
|---|---|
| Alternative 1 | 27.8 ± 0.9% |
| Alternative 2 | 29.5 ± 0.9% |
| Alternative 3 | 34.0 ± 1.0% |

Table 4

Enzymatically Treated Polyoma DNA*

| | Pancreatic DNAase treatment | | Pancreatic DNAase treatment followed by exonuclease I digestion |
|---|---|---|---|
| | Experimental | Theoretical (at 1.02 hits/duplex) | Experimental |
| Intact duplex DNA | 36.1 ± 0.7% | 36.1% | 27.3 ± 0.7% |
| Single-stranded 18 S circles | 22.6 ± 0.5% | 24.0% | 16.4 ± 0.5% |
| Single-stranded 16 S linears ($L_1$) | 28.3 ± 0.6% | 30.6% | |
| Single-stranded DNA < 16 S ($\sum_{2}^{n} L_i$) | 13.4 ± 0.5% | 9.3% | |
| All linear DNA | | | 5.9 ± 0.4% |
| Mononucleotides | | | 50.6 ± 1.2% |

* Analyses in alkaline CsCl velocity gradients

pancreatic DNAase attacked the polyoma DNA in a random fashion. The experimentally determined distribution shown in column 3 shows that the fraction of intact duplex DNA decreased during the exonuclease I digestion. As was noted in the experiments with $\phi$X174, the exonuclease I used in the experiments contained some endonuclease activity. This endonuclease is able to introduce single strand scissions into $\phi$X174 DNA and presumably into the single strands of polyoma DNA during the exonuclease I digestion. It is not known if the endonuclease activity introduces single or double strand scissions into intact double-stranded polyoma DNA during the exonuclease digestion. The effect of the endonuclease on the results can be evaluated by calculating the effect of the endonuclease on each component. The fraction of linear DNA remaining after exonuclease I digestion, $5.9 \pm 0.4\%$, cannot be compared with the fraction of 16 S DNA present before exonuclease I, $28.3 \pm 0.5\%$, until the effect of the endonuclease on the system is evaluated.

We now assume that the endonuclease activity introduced single strand scissions in both the double- and single-stranded DNA and that the pancreatic DNAase treated polyoma DNA sample contained 100 mass units. The effect of the endonuclease upon the results when it introduces double strand scissions into the intact duplex DNA and single strand scissions into single-stranded DNA will be discussed later.

(A) Prior to exonuclease I digestion, $36.1 \pm 0.7$ mass units of intact duplex DNA were present (see Table 4). After exonuclease I digestion, $27.3 \pm 0.7$ mass units of duplex DNA were present, with $8.8 \pm 1.0$ mass units having received one or more scissions. This

degradation corresponds to 0.28 ± 0.05 endolytic hits/molecule which would produce 4.4 ± 0.6 mass units of linear, single-stranded DNA (16 S), 4.0 ± 0.6 mass units of circular, single-stranded DNA (18 S), and 0.3 ± 0.1 mass units of DNA slower than 16 S during the exonuclease I digestion. The assumption is made that none of the endolytically hit double-stranded molecules are available for exonuclease I digestion because they remain double-stranded throughout the exonuclease I reaction.

(B) The original 22.6 ± 0.5 mass units of circular, single strands (18 S) in Figure 3a are seen to decrease to 16.4 ± 0.5 mass units after exonuclease I digestion. Since 4.0 ± 0.6 mass units have been added due to the endolytic attack on the intact double strands, we conclude that 10.2 ± 0.9 mass units of the original 22.6 ± 0.5 mass units have been converted to linear molecules during the exonuclease I digestion. From this one can calculate that the intact, circular, single strands received 0.61 ± 0.07 endolytic hits/molecule during the exonuclease reaction. Of the 10.2 ± 0.9 mass units converted to linear molecules, 7.4 ± 0.4 have received only one hit, converting them to linear molecules of 16 S. We now make the assumption that all the linear, single-stranded DNA produced by endolytic activity during the exonuclease I digestion was available to the exonuclease at the beginning of the digestion period.

(C) It was calculated, assuming 0.61 ± 0.07 endolytic hits per 16 S linear DNA, that 15.5 ± 1.2 mass units of the original 16 S linear, single-stranded DNA (28.3 ± 0.6 mass units) would remain intact

after the endonuclease action. The remainder of the 28.3 ± 0.5 mass units received one or more hits.

(D) The total number of intact 16 S linear DNA available to exonuclease I digestion, with the stringent assumption that all endonuclease action preceeded the exonuclease I digestion, is 7.4 ± 0.4 plus 15.5 ± 1.2 mass units. We now restate our criterion that less than 1/4 of the available, 16 S linear, single-stranded DNA should remain as linear DNA between the 16 S position and the meniscus in the gradient if the non-existence of blocks in each strand is to be established.

(E) As calculated earlier, 5.9 ± 0.4 mass units of linear DNA were found between the 16 S position and the top of the gradient. We remember that 4.4 ± 0.6 mass units of 16 S linear, single-stranded DNA plus 0.3 ± 0.1 mass units of linear DNA slower than 16 S were produced by endonuclease action on the duplex DNA. At no time was this DNA available to the exonuclease I action as single-stranded DNA. The linear DNA must be present between the 16 S position and the top of the gradient in Figure 3c. If the total of 4.7 ± 0.6 mass units of 16 S and slower single-stranded DNA is subtracted from 5.9 ± 0.4 mass units of 16 S and slower, single-stranded DNA, we obtain 1.2 ± 0.7 mass units. This represents the number of mass units of linear DNA not digestible by exonuclease I. This is less than 1/4 of the intact, 16 S single-stranded DNA, 22.9 ± 1.3, obtained in (D). We conclude that there is no non-nucleotide linker in either strand of polyoma DNA.

At several places in the above calculations, we have made simplifying assumptions which have reduced the amount of intact 16 S

single-stranded DNA by significant, although difficult to evaluate amounts. The effect of these assumptions is to have made the criterion for the absence of a block more stringent.

We now assume that the endolytic activity introduced double strand scissions into the double-stranded DNA and single strand scissions into the single-stranded DNA during the exonuclease I digestion. As before, it is assumed that all the linear, single-stranded DNA produced by endolytic activity during the exonuclease I digestion was available to the exonuclease at the beginning of the digestion. An analysis similar to the previous one showed that a total of $25.9 \pm 1.0$ mass units of 16 S linear, single-stranded DNA were available to exonuclease I digestion. The amount of linear DNA present in Figure 3c is calculated to be $5.9 \pm 0.4$ mass units. The action of the endonuclease on the intact duplex DNA would produce $8.8 \pm 1.0$ mass units of 16 S and slower linear DNA not available to exonuclease at any time during the digestion period. The value obtained by subtracting $8.8 \pm 1.0$ mass units from $5.9 \pm 0.4$ mass units, $-2.9 \pm 1.1$ mass units, represents the number of linear, single-stranded DNA mass units which are not digestible with exonuclease I. Although this number is negative, the error values involved make it close to zero. We again conclude that there is no non-nucleotide linker in polyoma DNA.

Chemical reducing agents also produce single strand scissions in polyoma closed circular DNA (13). The action of exonuclease I on polyoma DNA randomly hit by an unknown chemical reducing agent during storage produced results similar to those shown here. These results indicate that the unknown reducing agent introduced phospho-

diester scissions leaving 3'-OH groups. If this were not the case, the digestion of these denatured molecules by exonuclease I would not have occurred.

The endonuclease problem was much more serious when the exonuclease I sample was used in great excess. In this case, the exonuclease digested all the linear, single-stranded DNA present in the reaction. The products of the endonuclease-hit double-stranded DNA produced, upon alkaline sedimentation, a peak at the 16 S position and a large reduction in the amount of intact double-stranded DNA remaining. These results demonstrate conclusively that the endonuclease activity is not just pancreatic DNAase activity remaining from the original pancreatic DNAase treatment.

# DISCUSSION

The evidence presented indicates that neither polyoma DNA nor ϕX174 DNA contains any non-nucleotide blocks to the action of exonuclease I. This enzyme was kindly supplied to us by Prof. I. R. Lehman who has described its isolation and properties (4). Further studies on the specificity have been reported by Lehman and Nussbaum (15). The interpretation of our results depends entirely on the specificity of this enzyme. The enzyme digests single-stranded DNA starting at a free 3'-OH end. If the enzyme were to start at both ends of the linear, single-stranded DNA, the results would be meaningless. However, exonuclease I does have a definite specificity, and no exonuclease of E. coli is known that starts at the 5'-$PO_4$ end of single-stranded DNA.

The results obtained for the quantity of linear, single-stranded DNA remaining in either the ϕX174 or the polyoma DNA preparations after the exonuclease I digestion depend critically upon the subtraction of an accurately determined background in the scintillation counter. In the analysis, we had to sum the results of a large number of low activity samples. Four samples, analogous to those counted in the experiment but containing no DNA, were prepared and counted in four sample vials. The background subtraction was then determined with an accuracy of about 10%. Component resolution is difficult with the graphical representation of the count distributions. This resolution, which involves subjective procedures, was carried out as described earlier so as to err in a direction which would make the

test for a linker more stringent. The standard deviations in the tables and text represent deviations due only to the statistical deviations in the radioactive counting.

It may be argued that if pancreatic DNAase attacks either polyoma or ϕX174 DNA in a non-random fashion, for example directly on the 5' or left side of the linker, the exonuclease would be able to digest all the linear DNA. It seems likely that this enzyme is acting in a random manner (14, 17). If the enzyme were preferentially introducing a single strand break at only one point in each polyoma duplex DNA molecule, the distribution of products would not correspond to that predicted with the Poisson relation. It was found, however, that the product distribution did correspond to the result calculated with the Poisson. For a duplex molecule with only one sensitive site, equal amounts of single-stranded circles (18 S) and single-stranded linears (16 S) and no linears slower than 16 S would occur. In an analogous way, no short (< 16 S) DNA would be found in ϕX174 DNA preparations. Similar arguments of this type can be made if more than one special susceptible attacking site on either polyoma or ϕX174 DNA is postulated.

It has been reported that purine-3'-P-5'-pyrimidine linkages have a higher probability of being attacked than other linkages (18, 19, 20). It is felt that such linkages will be randomly found throughout the molecule so that the mode of action of the pancreatic DNAase can be characterized as essentially random with respect to position of the sites in the molecule.

The results of Mitra et al. (21) provide no support for the presence

of a linker in M13, a phage related to $\phi$X174, although, as they state, their evidence does not eliminate the possibility.

It is difficult to assess the results of Fiers and Sinsheimer (3) because their experimental conditions were somewhat different from those reported here. The DNA concentration in their exonuclease I mixture was twice that used in the present experiments and, in addition, a smaller amount of enzyme was used. As suggested by Dr. Sinsheimer, the higher DNA concentration might allow some type of intermolecular concentration dependent binding to occur between two complementary regions in the two non-complementary molecules. Such a duplex region would constitute a block.

On the basis of some inconsistencies found in structural and compositional studies of DNA, Bendich and Rosenkranz have proposed that DNA (at least in some species) contains approximately one serine residue for every 500 to1000 nucleotides. This serine residue might be inserted in the main polynucleotide chain of DNA, presumably by an amino acid ester linkage to the 3' carbon of a nucleotide (1). This would mean that polyoma DNA might contain 10 to 20 serine residues and $\phi$X174 DNA might contain 10 serine residues. If these were acting as blocks to the action of exonuclease I, very little DNA would be digestible after a limited pancreatic DNAase attack. It is unlikely that exonuclease I would proceed through these residues if they were in the main polynucleotide chain, for it is known that the presence of an acetyl group at the 3'-OH terminus of a deoxyribo-oligonucleotide blocks the action of the enzyme on this particular substrate (15).

In conclusion, it seems unlikely that polyoma DNA and $\phi$X174 DNA contain any non-nucleotide linkers.

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# PART IV

# A DYE-BUOYANT DENSITY METHOD FOR THE ISOLATION OF CLOSED CIRCULAR DNA; THE CLOSED CIRCULAR DNA IN HELA CELLS

# INTRODUCTION

A buoyant density method for the isolation and detection of closed circular DNA has been developed. The method is based on the reduced binding of the intercalating dye, ethidium bromide, by closed circular DNA. An investigation utilizing this method has shown that HeLa cells contain (a) closed circular mitochondrial DNA of mean length 4.81 microns, (b) a heterogeneous group of smaller DNA molecules which vary in circumference from 0.2 to 3.5 microns, and (c) a paucidisperse group of multiples of the mitochondrial length.

The following publication has been inserted into the thesis in its published form. Due to space limitations in the publishing journal, several methodological and experimental results had to be omitted in the final form of the paper. These will be presented and discussed following the publication.

Reprinted from the PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES
Vol. 57, No. 5, pp. 1514–1521. May, 1967.

# *A DYE-BUOYANT-DENSITY METHOD FOR THE DETECTION AND ISOLATION OF CLOSED CIRCULAR DUPLEX DNA: THE CLOSED CIRCULAR DNA IN HELA CELLS**

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*Communicated by James Bonner, March 2, 1967*

Covalently closed circular duplex DNA's are now known to be widespread among living organisms. This DNA structure, originally identified in polyoma viral DNA,[1, 2] has been assigned to the mitochondrial DNA's in ox[3] and sheep heart,[4] in mouse and chicken liver,[3] and in unfertilized sea urchin egg.[5] The animal viral DNA's—polyoma, SV40,[6] rabbit[7] and human[8] papilloma—the intracellular forms of the bacterial viral DNA's—$\phi$X174,[9, 10] lambda,[11, 12] M13,[13] and P22[14]—and a bacterial plasmid DNA, the colicinogenic factor $E_2$,[15] have all been shown to exist as closed circular duplexes. Other mitochondrial DNA's[16, 17] and a portion of the DNA from boar sperm[18] have been reported to be circular, but as yet have not been shown to be covalently closed.

The physicochemical properties of closed circular DNA differ in several respects from those of linear DNA or of circular DNA containing one or more single-strand scissions.[19] The resistance to denaturation,[2, 20] the sedimentation velocity in neutral and alkaline solution, and the buoyant density in alkaline solution are all enhanced in the closed circular molecules. These three effects are a direct consequence of the topological requirement that the number of interstrand cross-overs must remain constant in the closed molecule.

The principal methods currently used for the detection and the isolation of closed circular DNA are based on the first two general properties. In this communication we describe a method based on the buoyant behavior of closed circular DNA in the presence of intercalating dyes.

The binding of intercalative dyes has recently been shown to cause a partial unwinding of the duplex structure in closed circular DNA.[22–24] In such molecules any unwinding of the duplex causes a change in the number of superhelical turns, so that the total number of turns in the molecule remains constant. A small and critical amount of dye-binding reduces the number of superhelical turns to zero. Further dye-binding results in the formation of superhelices of the opposite sign or handedness. The creation of these new superhelices introduces mechanical stresses into the duplex and a more ordered conformation into the molecule. These effects increase the free energy of formation of the DNA-dye complex. The maximum amount of dye that can be bound by the closed molecule is therefore *smaller* than by the linear or nicked circular molecule. Correspondingly, since the buoyant density of the DNA-dye complex[23, 25] is inversely related to the amount of dye bound, the buoyant density of the closed circular DNA-dye complex at saturation is *greater* than that of the linear or nicked circular DNA-dye complex.[23] Bauer and Vinograd have shown that the above effect results in a buoyant density difference of approximately 0.04 gm/ml in CsCl containing saturating amounts of ethidium bromide, an intercalating dye extensively studied by Waring[26] and Le Pecq.[27]

The method has been tested with known mixtures of nicked and closed circular viral DNA's, and has been used to isolate closed circular DNA from the mitochondrial fraction of HeLa cells and from extracts of whole HeLa cells.

*Materials and Methods.—Preparation of HeLa cell extracts:* HeLa S3 cells were grown on Petri dishes in Eagle's medium containing 10% calf serum. $H^3$-thymidine, 18 c/mmole, was obtained from the New England Nuclear Corporation. Ten $\mu$c per ml of medium were added to each plate 20 hr before the cells were harvested. After washing with isotonic buffer and decanting, the cells were treated by the method described by Hirt[28] for the separation of polyoma DNA from nuclear DNA. Approximately 2 ml of 0.6% sodium dodecylsulphate (SDS), in 0.01 *M* ethylenediaminetetraacetate (EDTA), 0.01 *M* tris, pH 8, were added to the plates. After 30 min at room temperature, the viscous extracts were gently scraped from the plates with a rubber policeman and transferred with a widemouth pipet to a centrifuge tube. Either 5 *M* NaCl or 7 *M* CsCl was then added with gentle mixing to a final salt concentration of 1 *M*. The resulting solution was cooled to 4° and centrifuged for 15 min at 17,000 × *g* in a Servall preparative ultracentrifuge. The supernatant solution was dialyzed at 4° overnight against two changes of 0.01 *M* EDTA, 0.01 *M* tris, pH 8 buffer in order to remove $H^3$-thymidine. The mitochondria from HeLa cells were isolated by differential centrifugation of an homogenate followed by banding in a sucrose gradient.

*Preparation of viral DNA:* Polyoma viral DNA was prepared as described previously.[19] The intracellular lambda DNA was kindly supplied by John Kiger and E. T. Young, II, of the Biology Division.

*Chemicals:* The ethidium bromide was obtained as a gift from Boots Pure Drug Co., Ltd., Nottingham, England. Harshaw optical grade cesium was used. The SDS was obtained from Matheson Company. All other chemicals were of reagent grade.

*Preparative ultracentrifugation:* The experiments were performed in SW50 rotors in a Beckman Spinco model L preparative ultracentrifuge at 20°C. The CsCl solutions, in either cellulose nitrate or polyallomer tubes, were overlaid with light mineral oil. After centrifugation, the tubes were fractionated with a drop-collection unit obtained from Buchler Instruments. The drops were collected in small vials or on Whatman glass-fiber GF/A filters. The dried filters were immersed in 10 ml of toluene-PPO-POPOP, and the samples were counted in a Packard Tri-Carb scintillation counter.

*Electron microscopy:* Specimens for electron microscopy were prepared by the method of Kleinschmidt and Zahn[29] and were examined in a Phillips EM200 electron microscope. All electron micrographs were made at a magnification of ×5054. The magnification factor was checked with a grating replica. Cytochrome *c* was obtained from the California Biochemical Corporation.

*Fluorescence:* Prior to drop collection, the centrifuge tubes were examined in a darkened room with 365 m$\mu$ light from a Mineral Light Lamp or preferably from a "Transilluminator" supplied by Ultraviolet Products, Inc., San Gabriel, California. The tubes were photographed on Polaroid type-146L film through a "contrast filter" from Ultraviolet Products, Inc. The fluorescence measurements were performed with a double-monochromator apparatus constructed in this laboratory by W. Galley and N. Davidson. The instrument was calibrated with solutions of ethidium bromide ($5 \times 10^{-3}$ to 1.0 $\mu$g/ml) in calf thymus DNA, 20 $\mu$g/ml, 1 *M* CsCl. The intensity of fluorescence was measured at 590 m$\mu$ with an exciting wavelength of 548 m$\mu$.[30]

*Results.—Selection of initial dye concentration, cesium chloride concentration, and centrifugation variables:* The conditions for the experiments in the swinging-bucket rotor in the model L ultracentrifuge were selected with the object of obtaining separations similar to those obtained at equilibrium within a reasonable period of time. At equilibrium, the separation between components is approximately constant at initial dye concentrations between 50 and 100 $\mu$g/ml.[23] At these dye levels the buoyant densities are between 1.57 and 1.62 gm/ml. Figure 1 presents the dye and density distributions in CsCl solutions centrifuged for 24 and 48 hours. The initial dye concentration in both cases was found at a distance from the meniscus corresponding to four-tenths the length of the liquid column. In

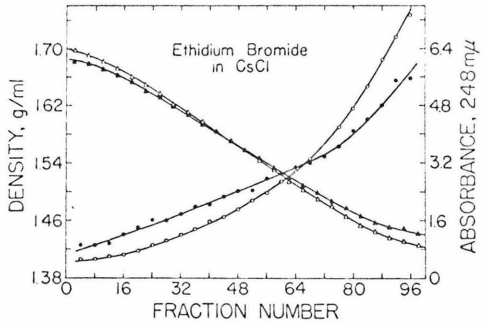


FIG. 1.—Density and dye distributions in CsCl density gradient columns, 3.00 ml 1.550 gm/ml CsCl, 100 μg/ml ethidium bromide, 24 (●, ▲) and 48 (○, △) hr at 43 krpm in a SW50 rotor at 20°.

this region of the cell, the time dependence of the dye concentration is minimal, and the constant density gradient is equal to approximately 0.10 gm/cm⁴.

*Results with purified mixtures of nicked and closed circular DNA:* Figure 2 presents the results obtained with 1.5 μg of tritiated polyoma DNA. Substantially complete resolution was obtained with this DNA which has a molecular weight of three million daltons. Since the fractional amount of closed circular material corresponded to the fraction obtained in analytical sedimentation velocity analyses,[21] it is concluded that single-strand scissions did not occur during the course of the experiments. An experiment in which the DNA was contained in a thin lamella at the top of the liquid column was performed to test the effect of the initial DNA distribution. The results were substantially the same as those obtained when the DNA was uniformly distributed in the CsCl solution.

The results obtained with a mixture of approximately 60 μg of covalently closed and nicked circular lambda DNA, mol wt = $3 \times 10^7$, are presented in Figure 3.

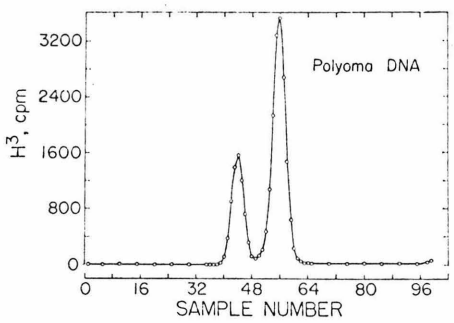


FIG. 2.—A mixture of purified polyoma DNA I and II 1.5 μg in buoyant CsCl, 3.00 ml 1.566 gm/ml CsCl, 100 μg/ml ethidium bromide, 24 hr at 43 krpm, 20°. The band maxima were separated by 12 fractions (four 7.5-μl drops per fraction). The buoyant densities of I and II are 1.588 and 1.553 gm/ml, respectively. The sample contains 30% I as indicated above compared with 32% by analytical band centrifugation.[21]

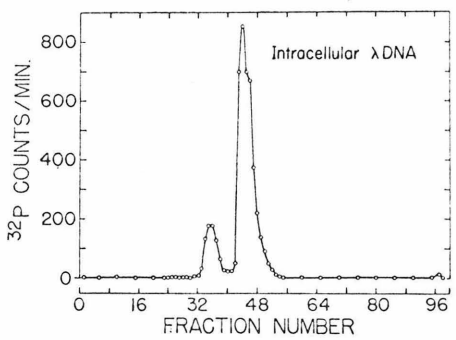


FIG. 3.—A mixture of purified intracellular lambda DNA I and II, 3.00 ml 1.55 gm/ml CsCl, 100 μg/ml ethidium bromide, 24 hr at 43 krpm, 20°. The centroids are separated by 9.5 fractions and 0.31 ml. Component I accounts for 16% of the total counts.

In this experiment, the nicked circular DNA formed a band of relatively high viscosity that may have distorted and broadened the light band during drop collection. An experiment to determine the effect of ethidium bromide on the protoplast assay for lambda DNA[32] was performed in collaboration with J. Kiger and E. T. Young, II. It was found that linear lambda had a normal titer when diluted

by a factor of 1000 from a solution containing ethidium bromide and CsCl at the concentrations which occur at band center.

*Detection of DNA by absorbance, fluorescence, and scintillation counting:* The data in Figures 2 and 3 were obtained by scintillation counting of dried filter papers containing dye, cesium chloride, and labeled DNA. To examine the effect of the presence of the dye upon counting efficiency, a series of sixteen 50-$\mu$l samples of $H^3$-thymidine in a 1.55 gm/ml CsCl solution containing ethidium bromide in concentrations varying from 0 to 286 $\mu$g/ml were counted. The relative counting efficiency decreased linearly with a least-squares slope of $8.6 \times 10^{-4}$ ml/$\mu$g. The dye gradient thus caused a difference of 1 per cent in relative counting efficiency between the two bands in Figure 2, while the dye depressed the relative counting efficiency by 8 per cent.

Red bands containing about 5 $\mu$g of DNA can be observed visually in the centrifuge tube. If an adequate amount of DNA is present, the fractionated gradient may be assayed spectrophotometrically at 260 m$\mu$. At saturation, the increase in absorbance caused by dye-binding is about 40 per cent with linear DNA and about 20 per cent with closed circular DNA.

The photograph in Figure 4 shows the fluorescent emission from two DNA bands. The fluorescence from DNA in amounts as low as 0.5 $\mu$g per band are detectable visually. Since the separation between the two bands corresponds reliably to 0.30–0.36 ml, it was possible to use the fluorescent, less dense band as a marker to locate closed circular DNA present in amounts below the limit of detectability by spectroscopic or radioactive procedures. The method is thus especially suitable for examining nonradioactive preparations from tissues of higher animals.

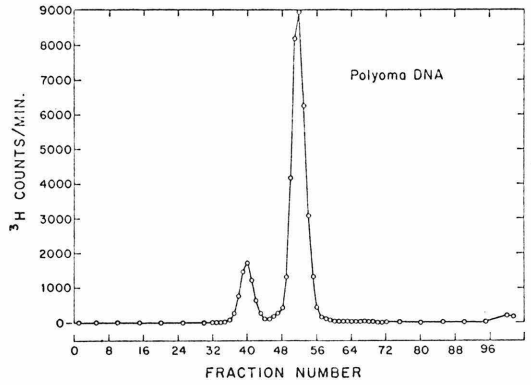


FIG. 4.—A mixture of purified polyoma DNA I and II, 3.00 ml, 1.558 gm/ml CsCl, 100 $\mu$g/ml ethidium bromide, 48 hr at 43 krpm, 20°. (*a*) The band maxima are separated by 12 fractions and 0.36 ml. The calculated buoyant densities are 1.592 and 1.556 gm/ml. (*b*) A photograph of the centrifuge tube prior to drop collection. The tube contains a total of 4 $\mu$g of DNA, and 0.64 $\mu$g of component I.

*Removal of ethidium bromide from DNA solutions:* It is often desirable to remove the dye quantitatively from a DNA sample. This was accomplished in a single passage of 1.0 ml of polyoma I DNA (40 $\mu$g/ml DNA, 100 $\mu$g/ml dye, 1 *M* CsCl) through a 0.8 × 4.5-cm column of analytical grade Dowex-50 resin. The fractions containing DNA were consolidated and were found to contain less than $1 \times 10^{-2}$ $\mu$g dye in a fluorometric analysis.

*Isolation of closed circular DNA from $H^3$-thymidine-labeled HeLa cells:* Hela cell monolayers in Petri dishes containing $10^7$ cells were treated with SDS by the pro-

cedure described by Hirt.[28] The results obtained when the extract was purposely sheared and when the shear was minimized (Figs. 5*a* and *b*) show that the relative amount of material in the light band was decreased when shear was minimized. The dense band in Figure 5*a* is clearly contaminated by material from the light band and reprocessing the dense band in a second CsCl-dye gradient would be necessary to obtain resolved bands.

*Electron-microscope examination of the circular DNA in the dense band of HeLa cell extracts:* Examination of electron micrographs of specimens prepared from fractions 33–36 in the dense band of HeLa cell extracts (Fig. 5*c*) showed less than 0.1

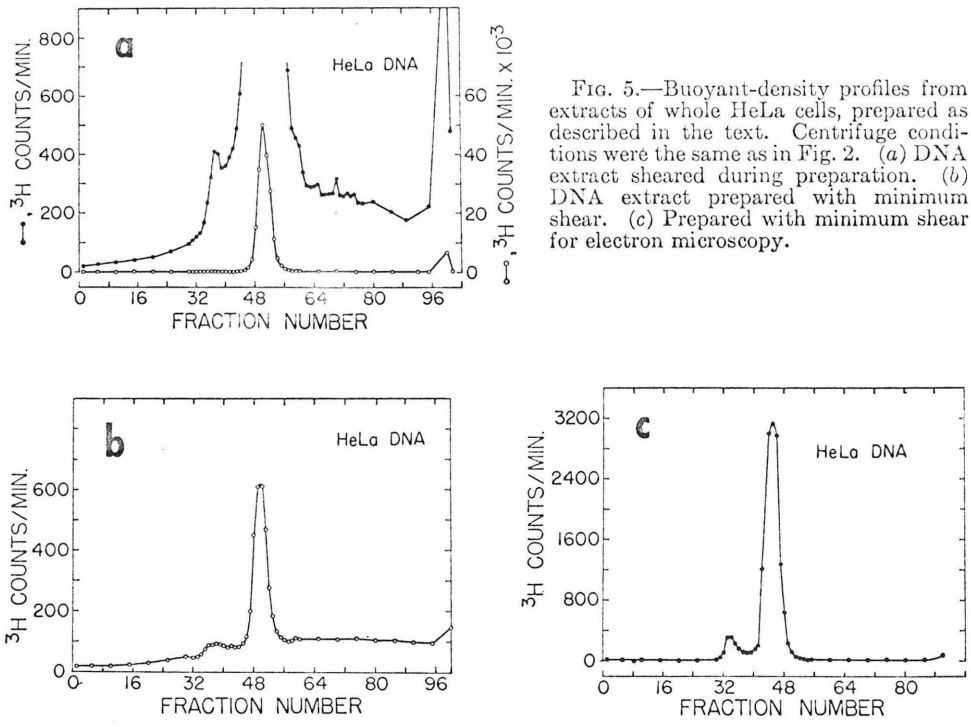


FIG. 5.—Buoyant-density profiles from extracts of whole HeLa cells, prepared as described in the text. Centrifuge conditions were the same as in Fig. 2. (*a*) DNA extract sheared during preparation. (*b*) DNA extract prepared with minimum shear. (*c*) Prepared with minimum shear for electron microscopy.

per cent of distinguishably linear DNA. The light band contained long linear DNA. The dense band contained an array of circles in three size groups: a homogeneous group of molecules with a mean length of 4.81 ± SE 0.24 microns; a heterogeneous population with lengths from 0.2 to 3.5 microns; and a paucidisperse set 2–4 times the length of the DNA in the homogeneous group. Figure 6 presents micrographs selected to illustrate the three size classes, and Figure 7 shows the frequency distribution among the first two groups. A survey of several hundred molecules on sparsely populated specimen grids revealed that the frequencies of the small and the large size classes were each about one-tenth the frequency of the homogeneous size class. In the large size class, the dimers were observed more frequently than the trimers or tetramers. The DNA from mitochondria isolated from HeLa cells consisted principally of molecules in the homogeneous size group. We conclude, therefore, that the homogeneous size class in Figure 7 is of mito

chondrial origin. Small circular DNA's were not observed in significant amounts in the preparation of DNA from the isolated mitochondria.

*Discussion.*—The method described in this paper has proved to be a simple and direct procedure for obtaining closed circular DNA from extracts of whole cells and cell fractions. The method employs chemically mild conditions; single-strand scissions are not introduced by interaction with the dye, nor is there any permanent rearrangement of the DNA structure. All the steps of the separation

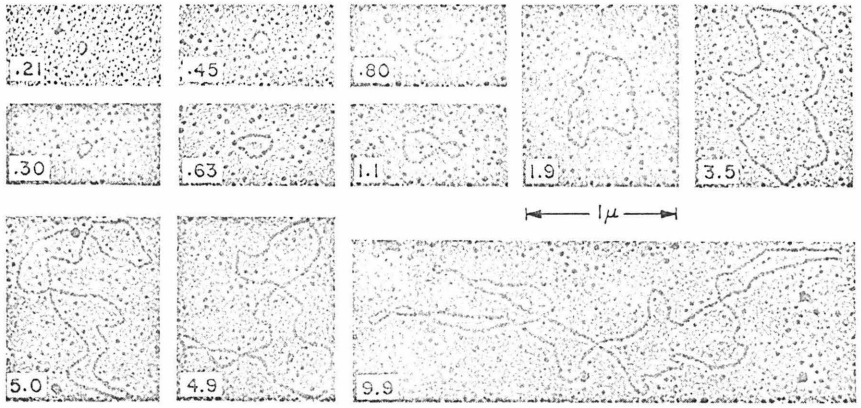


FIG. 6.—Electron micrographs of circular DNA from HeLa cells. Fractions 33–36 from the dense band in Fig. 5*c* were pooled and used in the specimen preparations. The top photographs present selected molecules of the small size range. The number in each insert gives the length in microns of the molecule. The first two molecules in the second row are of mitochondrial size; the third is twice the mitochondrial length.

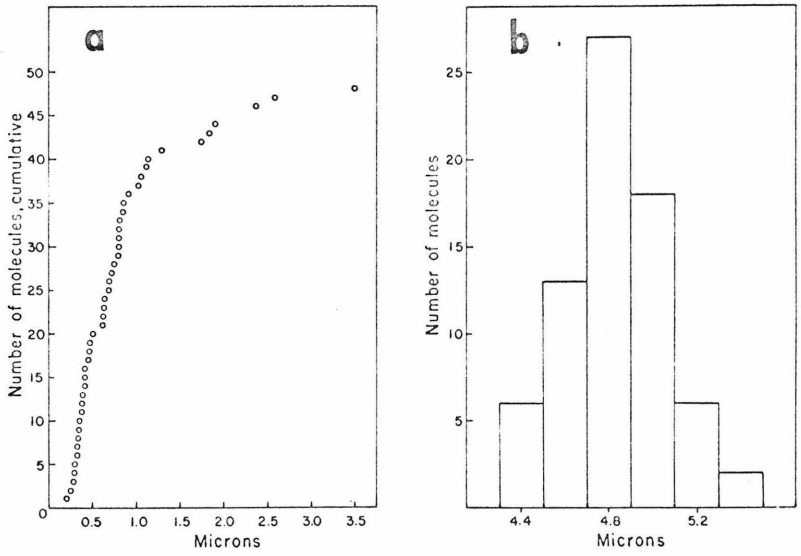


FIG. 7.—Frequency distribution of lengths of circular DNA isolated from the HeLa cell band referred to in the legend for Fig. 6. (*a*) A cumulative frequency distribution of lengths of molecules in the submitochondrial size class. (*b*) A histogram of the distribution of lengths of molecules in the mitochondrial size class.

are carried out at room temperature, or below, and at neutral pH. In the experiments performed so far, the maximum amount of DNA introduced in a single tube was 125 $\mu$g of polyoma DNA.

It has become clear, in the course of this initial study of the closed circular DNA in malignant human cells, that a portion of the mitochondrial DNA in HeLa cells is in the form of closed duplexes. These circular molecules are similar in form, size, and homogeneity to the mitochondrial DNA's from invertebrate and vertebrate species previously investigated.[3-5, 16, 17]

The heterogeneous group of small molecules, 0.2–3.5 microns, are to be compared with the size range, 0.5–16.8 microns, reported by Hotta and Bassel[18] to be present in preparations of unfractionated boar sperm DNA. The conclusion that the small circles, seen in our electron micrographs, represent DNA molecules rests on three observations. (1) The small circles were found at the same level in a CsCl-dye gradient as were the closed circular mitochondrial DNA molecules. (2) The grain pattern of the shadowed metal on the small circles viewed at a magnification of $1 \times 10^5$ was indistinguishable from the pattern on mitochondrial DNA molecules in the same field. (3) The small circles were seen only very infrequently in DNA preparations from isolated HeLa cell mitochondria. It is thus unlikely that the small circles arise from an artifact in the electron-microscope procedure. A more definitive characterization of these small DNA molecules, which can code for only 200–3500 amino acid residues, requires the preparation of larger quantities of material. The frequency of occurrence of the small molecules in the SDS extracts we have studied does not necessarily represent the frequency of occurrence in the HeLa cell. It is emphasized here that the dye-buoyant method segregates only *closed* circular duplexes. Molecules that contain even one single-strand scission, for whatever reason, find their way to the less dense band and, in whole cell extracts, intermingle with the large excess of linear DNA.

The larger-size circles, which were also seen at a frequency of about 10 per cent relative to mitochondrial DNA in the dense band, are clearly multiples of the mitochondrial length. The mean lengths from measurements of 43 double-length circles and a smaller number of larger multiples were $9.56 \pm 0.42$, $14.1 \pm 0.4$, and $19.8 \pm 1.1$ microns. We have so far not found a fully extended large circle. Nor have we found, in the several large circles which contain infrequent crossovers, any one which could not have arisen from a pairing of two or more mitochondrial molecules. An investigation of the significance of these multiple-size circles in HeLa DNA is in progress. Nass[17] has reported measurements of multiple lengths of mitochondrial molecules liberated from mitochondria by osmotic shock during specimen preparation. She attributed the multiples to the superposition of DNA molecules which originated from single mitochondria.

*Summary.*—A buoyant-density method for the isolation and detection of closed circular DNA is described. The method is based on the reduced binding of the intercalating dye, ethidium bromide, by closed circular DNA. In an application of this method we have found that HeLa cells contain, in addition to closed circular mitochondrial DNA of mean length, 4.81 microns, a heterogeneous group of smaller DNA molecules which vary in size from 0.2 to 3.5 microns and a paucidisperse group of multiples of the mitochondrial length.

*Note added in proof:* That double-length closed mitochondrial DNA molecules do occur has been shown by D. Clayton in this laboratory with preparations from leucocytes obtained from the blood of a donor with chronic granulocytic leukemia. Circular DNA molecules free of cross-overs and of twice the mitochondrial length were observed in electron micrographs.

It is a pleasure to thank J. Huberman for providing us with the labeled HeLa cells; J. Kiger and E. T. Young, II, for their gift of closed circular lambda DNA and for allowing us to quote their unpublished analysis; G. Attardi for advice and assistance in the preparation of HeLa mitochondria; L. Wenzel and J. Eden for their assistance in the culture of the polyoma virus; R. Watson for his several technical contributions; and R. Kent for assistance in the preparation of the manuscript.

* This work was supported in part by grants HE 03394 and CA 08014 from the U.S. Public Health Service and by fellowships from the U.S. Public Health Service and the National Science Foundation.

† Contribution 3490 from the Laboratories of Chemistry.

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# Erratum

The following change should be made on the top of page 1516 of the Proceedings of the National Academy of Sciences U. S. :

. . ., and the constant density gradient is equal to approximately 0.10 [ not 0.010 ] $gm/cm^4$. . . .

# ADDITIONAL METHODS

1) The use of sarcosyl NL 30

Sarcosyl NL 30 was tested as a possible alternative to sodium dodecyl sulfate (SDS) in the nucleic acid extraction procedure described in the publication. The solubility of this detergent in high molarity CsCl was thought to be a useful characteristic. It was found, however, that the presence of either SDS or sarcosyl in the dye-CsCl gradients was not tolerable since the micelles bind a large amount of dye. Cesium-SDS is relatively insoluble and forms a skin at the meniscus of the liquid column. Sarcosyl NL 30 was obtained from the Geigy Chemical Company.

2) Isolation of mitochondria (1)

HeLa S 3 cells were grown in spinner cultures in a modified Eagle's medium and 5% calf serum. The concentration of cells upon harvesting varied from 1 to $4 \times 10^5$ cells/ml. The cells were sedimented in the International PR 2 at 250 g for 6 min. Subsequently, the samples were kept at 4°C. The cells were resuspended in 20 to 30 volumes of TD buffer (0.137 M NaCl, $7 \times 10^{-4}$ M $NaH_2PO_4$, $5 \times 10^{-3}$ M KCl and 0.025 M tris, pH 7.4) and resedimented. This washing procedure was repeated once again. The cell pellet (approximately $10^8$ cells/ml of packed cells) was resuspended in 6 volumes of LIS (low ionic strength) buffer consisting of 0.01 M tris, 0.01 M KCl, and 0.005 M EDTA, pH 7.4 (at 4°C). After 1 min, the swollen cells were homogenized in a Potter-Elvehjem glass homogenizer equipped

with a motor driven teflon pestle. It was found that 15 strokes of the homogenizer rotating at 500 rpm were adequate to break open about 1/2 of the cells. After homogenization, LIS buffer containing 2 M sucrose was added to obtain a concentration of 0.25 M sucrose. The nuclei, large debris and unbroken cells were removed from the solution by sedimentation at 1400 g for 3 min . The supernatant was spun at 11,000 g for 15 min in the Sorvall RC2-B. The pelleted mitochondria were resuspended in LIS buffer containing 0.25 M sucrose. The final 2 ml of the suspension contained the mitochondria from $5\times10^{7}$ broken cells. Two ml of the suspension were layered onto the surface of a 1 to 2 M linear sucrose gradient in LIS buffer and centrifuged for 10 to 12 hours at 24 K rpm in an SW 25.1 rotor using a Spinco model L ultracentrifuge. A definite band of turbidity had formed at this time and was removed by collecting drops from the bottom of the pierced tube. The collected sample was diluted to 0.88 M in sucrose by the addition of LIS. At this point, the mitochondria were pelleted and the nucleic acids extracted by means of the Hirt procedure.

3) Electron microscopy

DNA specimens were prepared for electron microscopy by the method of Kleinschmidt and Zahn (2). DNA samples taken directly from the collected gradient fractions contained approximately 4.5 M CsCl and 100 $\mu$g/ml of ethidium bromide. Sufficient cytochrome c at 1 mg/ml and $10^{-3}$ M EDTA were added to produce a sample containing

0.1 mg/ml of cytochrome c and 1.5 M CsCl. From 25 to 100 $\mu$l of this sample were allowed to flow down an inclined, clean glass microscope slide one end of which was immersed in 0.15 M ammonium acetate. The sample formed a film on the surface of the ammonium acetate, the film area being delimited by talcum powder. After waiting for 30 sec to several min , the surface of a copper grid (200 mesh, 2 mm, Ladd Industries) covered with Parlodion (cellulose nitrate) was touched to the cytochrome c-DNA film. The film was fixed to the grid by dipping the grid first into 90 to 95% ethanol containing a 50-fold dilution of a uranyl acetate stain ($5 \times 10^{-3}$ M uranyl acetate, $5 \times 10^{-2}$ M HCl in 95% ethanol) (10). After the 30 sec staining period, the grid was dipped into isopentane for a 10 sec period. The Parlodion covered grids were made by allowing a film of Parlodion floating on water to drop onto the grids as the supporting water was drained. The Parlodion film was made by placing 1 drop of 3% Parlodion in n-amyl acetate on the water surface.

The grids were shadowed in an evacuated chamber with a platinum-palladium alloy. This metal, as a foil, was mounted on a tungsten wire 8 horizontal cm away from the sample and 1 to 2 cm above it. The grids were rotated during the shadowing process.

# ADDITIONAL RESULTS

## Resolution of bands

The resolution of closed circular and linear duplex DNA in a 3.00 ml dye-CsCl gradient at 43 K rpm in an SW 50 rotor was examined as a function of centrifugation time. The resolution parameter Y, defined as $d/(\sigma_1 + \sigma_2)$ where d is the peak to peak separation and $\sigma_1$ and $\sigma_2$ are the band widths of the two bands, was used to compare the resolution attained in gradients run under different conditions. In experiments with a mixture of PY I and PY II DNA in a dye-CsCl gradient, the parameter Y increased from 3.6 to 4.1 as the time of centrifugation was increased from 24 to 48 hours. In a practical sense, this increase in resolution is not a necessity, since the resolution after 24 hours with 3.00 ml gradients is adequate in most cases for good physical separation. However, it is desirable to centrifuge for a longer period of time if longer liquid columns are used or if the proportion of closed circular DNA is small. With preparations containing 5 to 10% closed circular DNA, 36 to 48 hours have been found to be necessary to obtain maximum resolution of the bands.

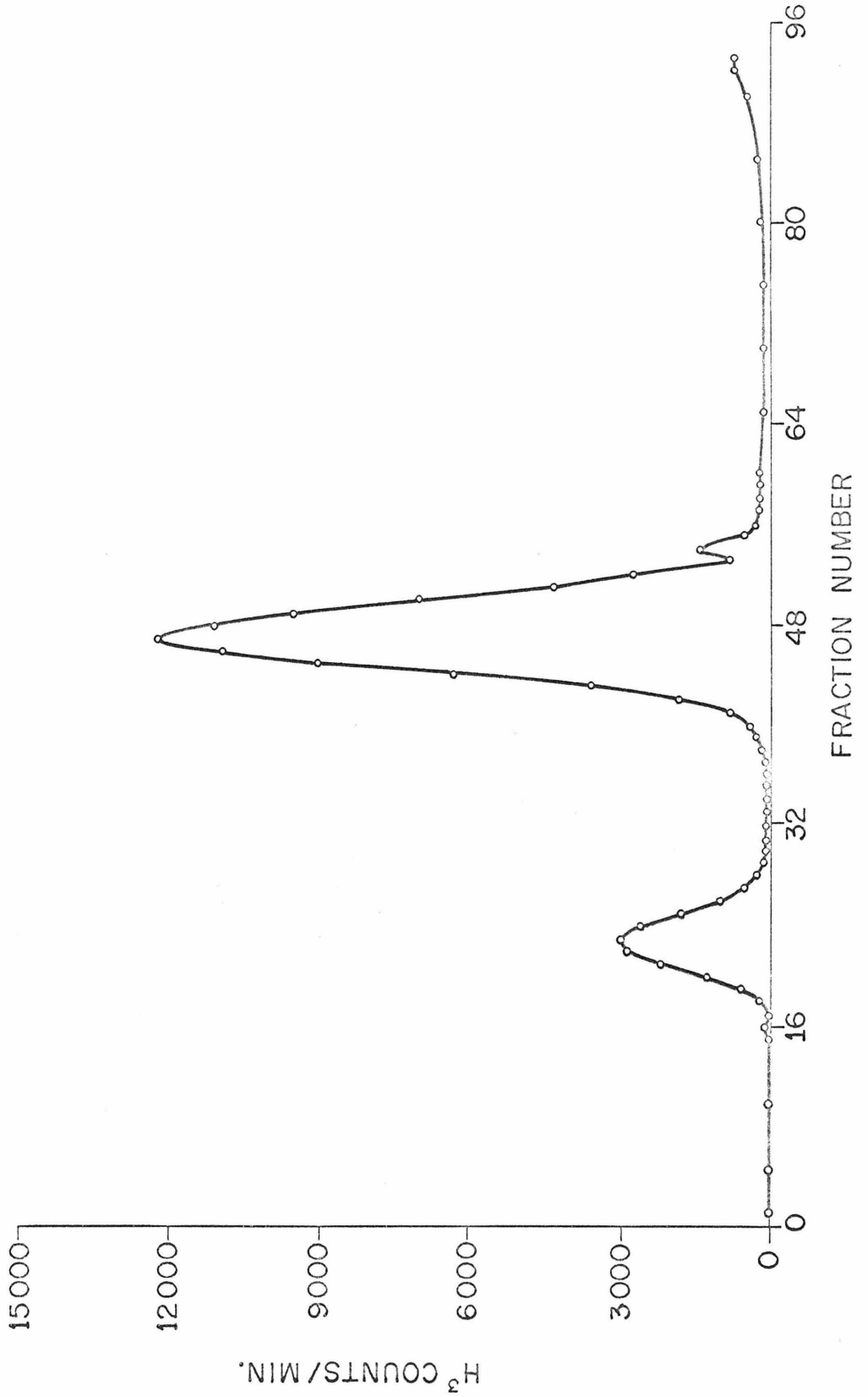
## Procedures for separating larger amounts of DNA

Physical studies of closed circular DNA usually require large scale preparative procedures because such DNA is present in low concentrations in most of the systems examined to date. The enclosed publication describes in detail the use of 3.00 ml dye-CsCl gradients, but these usually are not adequate for large scale preparations.

There are two alternatives which appear to be useful. (a) 5.00 ml gradients ($\rho$ = 1.55 gm/ml, 100 $\mu$g/ml of dye) can be used in Spinco SW 50, SW 41 or SW 65 rotors as opposed to 3.00 ml gradients. Such gradients are usually run for a total of 48 hours. (b) Angle head rotors, as described by Flamm (3), have been tried in this laboratory with good results. We have used the Spinco Titanium 50 angle head rotor, 4.5 ml per tube and 12 tubes per rotor. This is approximately 3 times the capacity of the SW 50 rotor (with 3.0 ml gradients). In addition, the resolution is greater in the fixed angle rotors. A resolution parameter of 4.9 was obtained for a mixture of PY I and PY II in a 4.5 ml dye-CsCl gradient (100 $\mu$g/ml of dye, $\rho$ = 1.55 gm/ml) centrifuged in the Spinco Titanium 50 rotor for 24 hours at 43 K rpm. This result is illustrated in Figure 8.

There are disadvantages that must be considered before using the angle head rotors. (i) Relatively clean starting solutions must be used as an insoluble layer will form at any gradient meniscus if too much Cs-SDS or precipitated protein is present. This poses no real problem with swinging bucket rotors, for all bands of nucleic acid and the insoluble layers always remain perpendicular to the axis of the polyallomer or cellulose nitrate tubes. However, when fixed angle rotors are used, the orientation of the insoluble surface layers and bands, with respect to the axis of the tube, changes during rotor deceleration. The insoluble layers usually stick to the sides of the tubes and the drop collecting procedure is hampered. (ii) The true length of a 4.5 ml gradient during centrifugation in a Spinco Titanium

Figure 8. A buoyant density profile of a mixture of purified $H^3$ polyoma DNA I and II. The gradient contains 5 $\mu$g of DNA in 4.5 ml of 1.55 gm/ml CsCl and 100 $\mu$g/ml of ethidium bromide. Each tube was overlaid with mineral oil. The gradient was centrifuged for 24 hours at 43 K rpm and 20°C in a Spinco Titanium 50 fixed angle rotor. The band maxima are separated by 24 fractions and 0.9 ml. The sample contains 16% closed circular DNA.


15000
12000
9000
6000
3000
0
$H^3$ COUNTS/MIN.
0
16
32
48
64
80
96
FRACTION NUMBER

50 fixed angle rotor is shorter than a 3.0 ml gradient in a Spinco SW 50 swinging bucket rotor. If the former rotor is used, it is imperative that the investigator adjust his initial solution densities properly. A small error in density will lead to a much larger error in band position in the fixed angle gradient than in the swinging bucket gradient.

Dye removal

The removal of ethidium bromide from DNA is described in the enclosed publication. It was found that the integrity of closed circular PY I DNA originally in 100 μg/ml of dye was not changed as a result of passing the DNA through a dye-removing Dowex 50 column. However, the analysis of the dye-free DNA was made in a neutral CsCl velocity gradient in the Spinco model E and the argument can be made that hypothetical dye-induced changes, such as depurination, might not be evident in this type of analysis. Dr. J. Jordan, in a purification of SV40 viral DNA, analyzed for the presence of dye-induced changes using both neutral and alkaline CsCl band velocity gradients. The same quantity of closed circular DNA was found in both analyses (4). By qualitative observation, it is known that solutions of dye in CsCl do change color upon long exposure to room light. The nature of this reaction is not yet known nor is it known whether DNA would be damaged in such a reaction.

Electron microscopy of closed circular DNA

A large fraction of the closed circular HeLa DNA examined by the Kleinschmidt technique was found to be twisted -- most molecules were measurable, but others were not. The degree of twisting varied considerably in different grid preparations. The variation was probably due to unknown variables in the grid making procedure. In order to maximize the number of untwisted circles in the preparations, hydroquinone was added to obtain a final concentration of $2\times10^{-4}$ M. This should have caused, in a 30 minute incubation period, a 100% conversion of closed circular DNA to nicked circular DNA (5). This preparation contained, however, highly twisted molecules. When hydroquinone was added to $5\times10^{-3}$ M and the incubation times were increased to 1 or 2 hours, the results were still quite variable. The action of this reducing agent may be quite different in the high salt conditions used here from those used previously. Ethidium bromide was not removed from the DNA samples before electron microscopy. It is possible that the dye inhibits the action of the reducing agent hydroquinone by acting as a free radical trap (see Part I of the thesis).

Formaldehyde was added to some of the samples and to the hypophase in attempts to prevent twisting of the molecules on the grids. This experiment again produced quite variable results. In one case, however, where formaldehyde was added only to the sample and not to the hypophase, the 15 open mitochondrial size molecules measured had an average length of $5.14\pm0.20$ $\mu$ compared to the

4.81 ± 0.24 $\mu$ found previously. The former value indicates that the combination of open molecules and the spreading forces due to the improper use of the formaldehyde produced somewhat stretched molecules.

From the same preparation, the circumference of 30 small molecules was measured. These measurements were not included in Figure 7a in the inserted publication because of the different experimental conditions. However, Figure 9, a cumulative number distribution, includes these 30 lengths in addition to those previously measured. No correction was made to compensate for the possibility of stretched molecules. The cumulative distribution shows that most of the small molecules have lengths between 0.2 and 1.5 $\mu$.

Multiples of the mitochondrial size molecules

In the inserted publication, it was noted that extracts of HeLa cells contained DNA molecules which appeared to be multiples of the mitochondrial length. Examples of these multiples are shown in Figures 10, 11, 12, and 13. As was also stated in the publication, no fully extended circles were found, even though the methods mentioned in the last section were employed in efforts to untwist the molecules. Therefore, it is not possible to say that these multiples are single molecules, for they could have arisen as a result of non-random combinations of 2, 3, or 4 mitochondrial size molecules. Since the number of molecules in these samples was quite low, it is unlikely that all of the multiples could have arisen by the random overlapping of 2, 3, or 4 mitochondrial size molecules.

Figure 9. Cumulative frequency distribution. This distribution contains lengths of molecules in the submitochondrial size class. The open circles represent the lengths presented in Figure 7 of the enclosed publication. The closed circles represent additional molecules from other experiments.

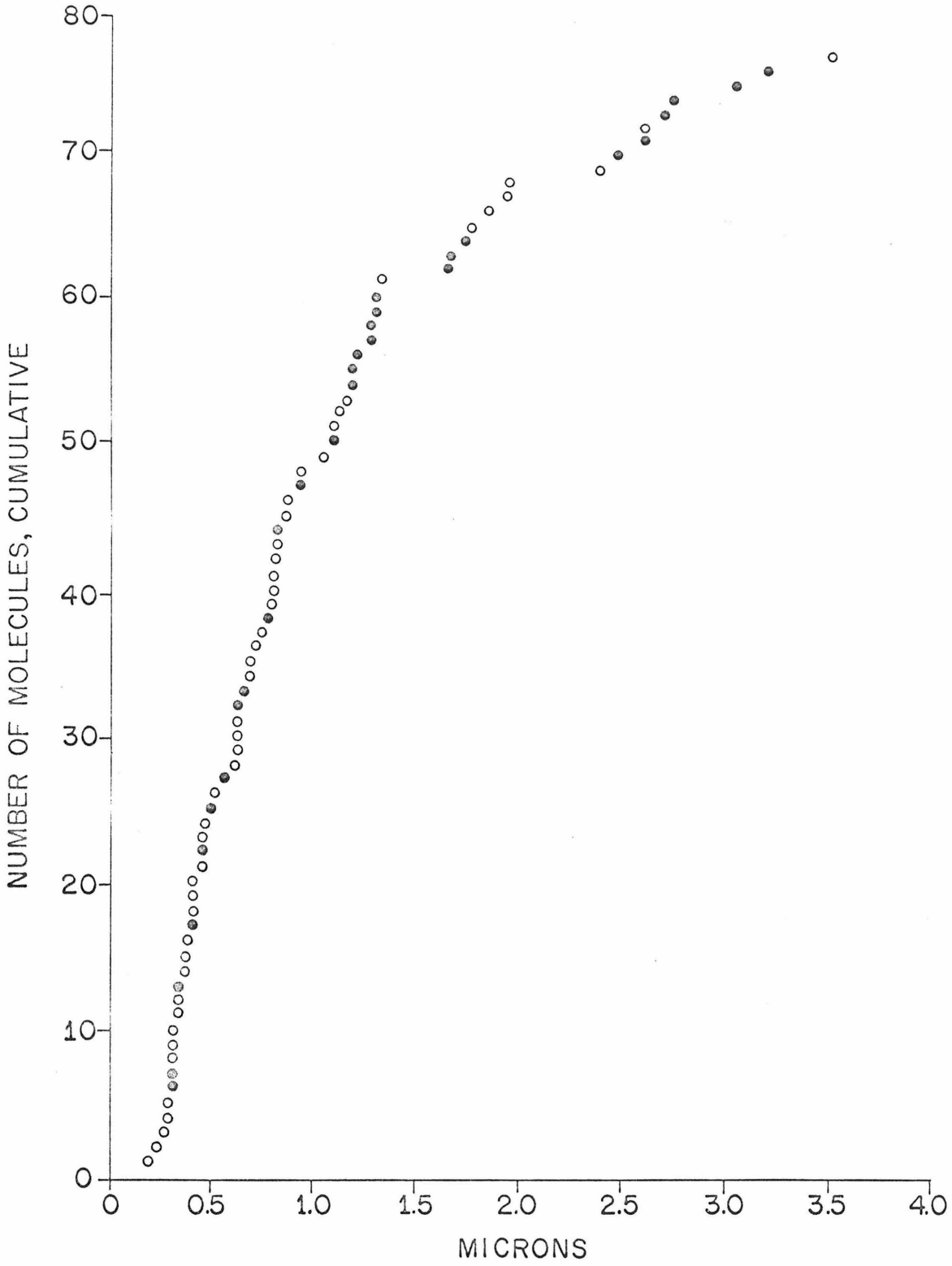

NUMBER OF MOLECULES, CUMULATIVE
0
10
20
30
40
50
60
70
80
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
MICRONS

Figure 10. Electronmicrograph of circular DNA from HeLa cells. Fractions 33 to 36 from the dense band in Figure 5c of the enclosed publication were pooled and used in the specimen preparation. The total magnification is 34,000. The length of the structure shown here is 19.9 $\mu$.

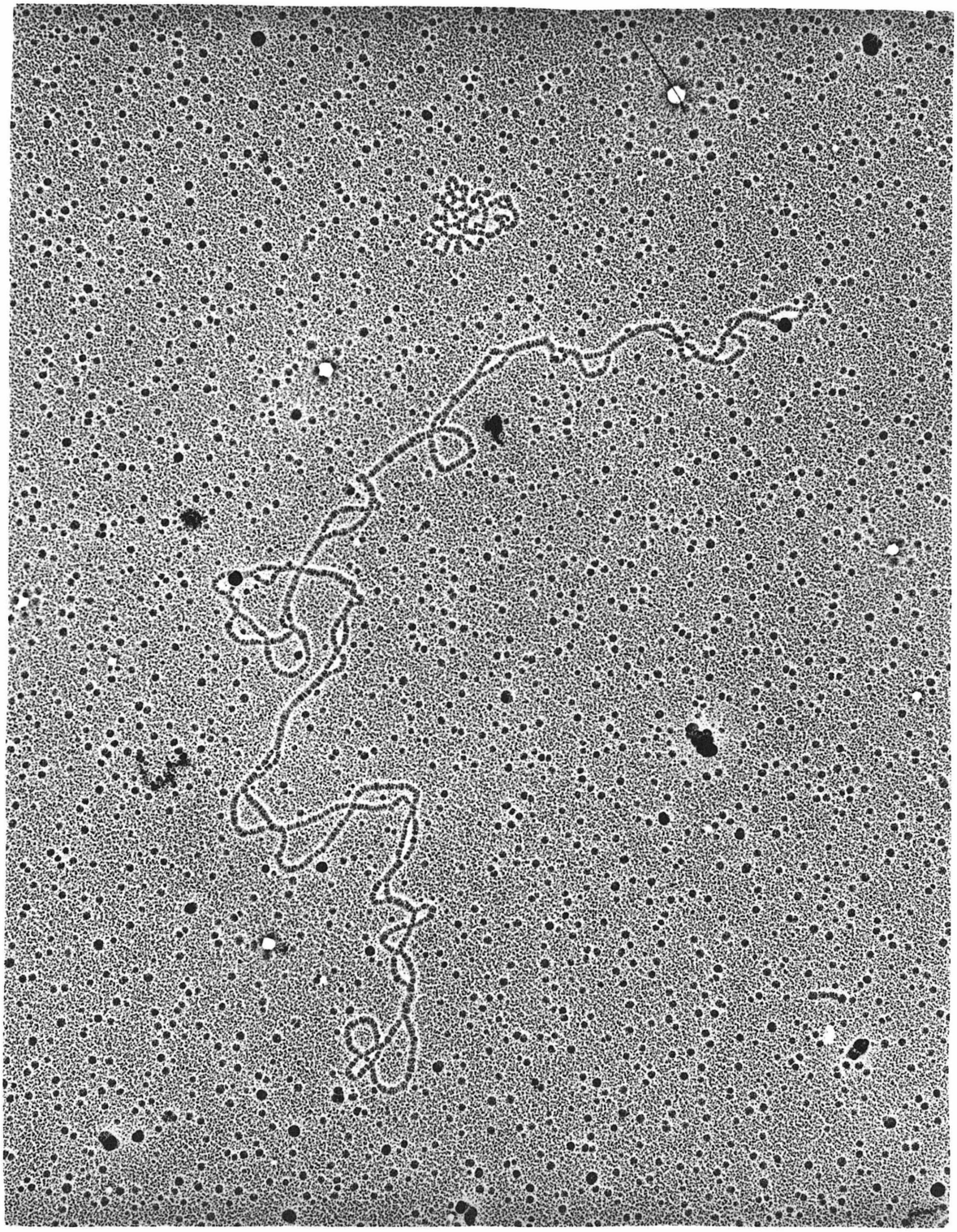


Figure 11. Circular DNA from HeLa cells. The conditions are the same as for Figure 10. The length of the longer structure is 14.2 $\mu$ and the length of the shorter is 5.1 $\mu$. The magnification is 34,000.

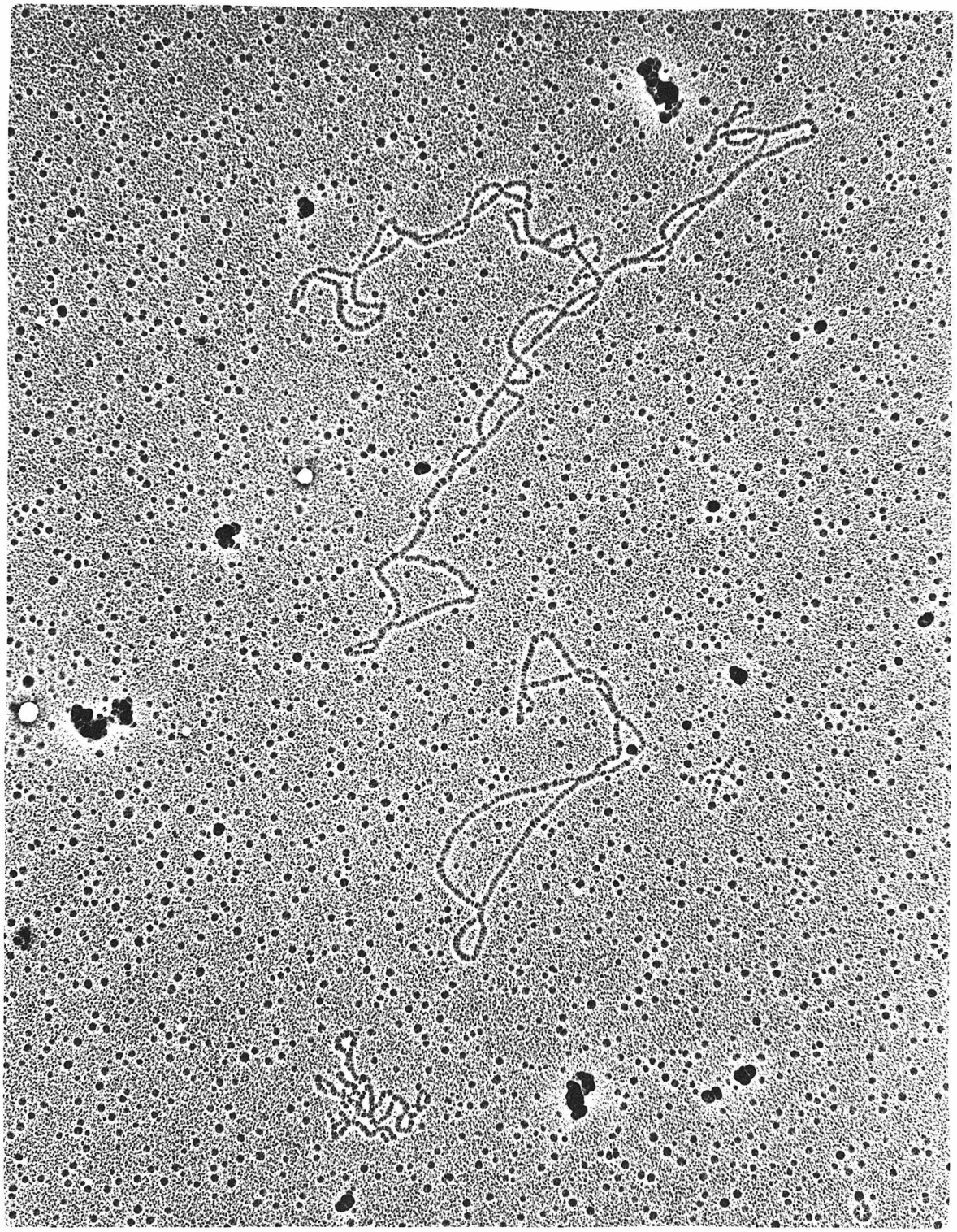


Figure 12. Circular DNA from HeLa cells. The conditions are the same as for Figure 10. The length of the molecule shown here is 9.4 $\mu$. The total magnification is 34, 000.

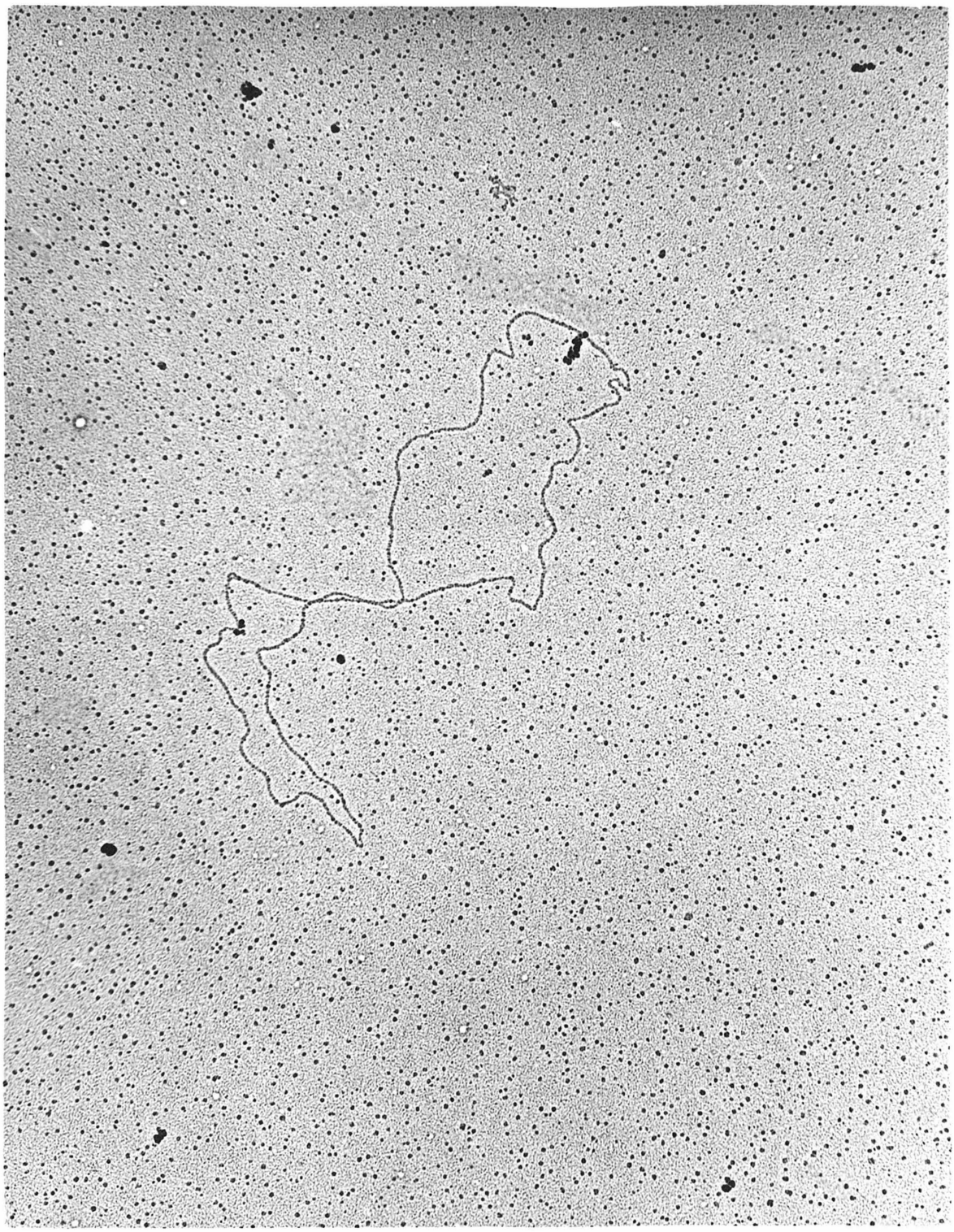
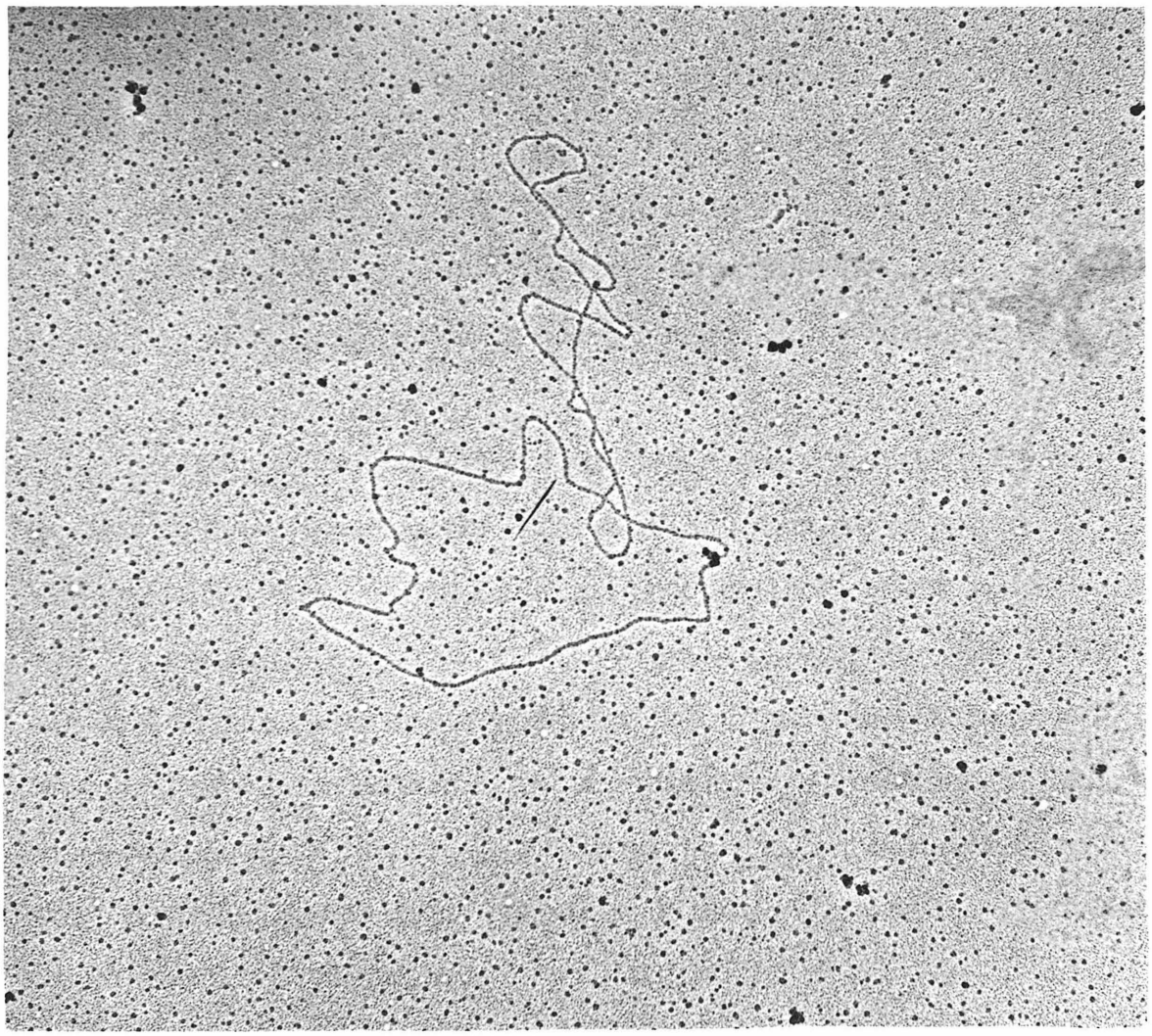


Figure 13. Circular DNA from HeLa cells. The conditions are the same as for Figure 10. The length of the open molecule is 4.8 $\mu$ and the length of the more twisted molecule is 4.9 $\mu$. The total magnification is 34,000.



# DISCUSSION

The results presented in the Additional Results section are concerned essentially with extending the usefulness of the dye-buoyant density method. Further studies have been started in this laboratory in attempts to answer some of the many questions raised in the enclosed publication.

The dye-buoyant density method has to this date been mainly analytical in nature. However, since it will become desirable to obtain larger amounts of material appropriate for physical studies, large scale preparative procedures will have to be developed. The beginnings of such methodology are presented in the Additional Results section.

Electron microscopy proved to be a rather inconsistent method as used in these experiments. These difficulties can be separated into two categories. In the first category are those problems concerned with the technical details of grid making and shadowing. Such difficulties can be overcome with skill and experience. The second category of problems is concerned with the visualization of closed circular DNA. Preparations often contain very twisted molecules making detailed study difficult. Procedures need to be developed to enable investigators to differentiate between closed circular and nicked circular DNA with the electron microscope and to transform, by chemical means, the former to the latter with a minimum of difficulty. At the present time, there is no way to distinguish completely between closed circular DNA molecules containing locked-in superhelices and nicked circular molecules inwhich there are a number of internal overlaps. In the hands of this

investigator, molecules labeled as closed circular DNA would sometimes be very twisted and at other times would be quite open. It seems likely that there are several unknown variables in the grid making procedure whose results are unknown. For example, some recent work in this laboratory has indicated that aging of the cytochrome c-DNA films for 5 to 10 min , as opposed to 1 or 2 min, produces a higher proportion of open circular molecules.

Nothing is known of the function or even the location of the small closed circular DNA found in HeLa cells. There is a very large range of sizes for DNA smaller than the mitochondrial DNA. Small circles have been found in yeast (6), boar sperm (7), mouse embryo cells (4) and in human leukocytes obtained from the blood of a donor with chronic granulocytic leukemia (8). Is the presence of this small DNA essential to the cell or is it an undesirable and non-essential relationship?

The mitochondrial DNA is, of course, localized in the mitochondria. Its true function has not yet been completely clarified. The multiples of the mitochondrial size are very unusual. If they are single molecules, why are they multiples of the mitochondrial size? If they are not single molecules, what is the relationship of the components? Are the multiples artifactual with respect to the HeLa cell system or are they meaningful complexes related in some way to the replicative process?

Multiples of the mitochondrial size have also been found in human leukocytes obtained from the blood of a donor with chronic

granulocytic leukemia (8). In contrast to the DNA from the HeLa cell system, these multiples are open. It has been determined that preparations of bacterial plasmid DNA from the colicinogenic factor $E_1$ (isolated from a strain of Proteus mirabilis) contain not only a 2.3 $\mu$ circular molecule, but also 10 to 20% of a molecule whose length is 4.7 $\mu$ (9).

The large variation in sizes of circular DNA found in HeLa cell DNA preparations does not appear to be a phenomenon specific to this cell line, but seems to be widespread in nature. Studies on the function and properties of such DNA should promote a great deal of interesting and basic research.

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