I STUDIES ON BACTERIOPHAGE DNA'S AND MINICIRCULAR DNA'S IN *M. LYSODEIKTICUS* AND *E. COLI* 15

II FLOW DICHROISM OF DNA SOLUTIONS

Thesis by

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Dedicated to the memory of my mother

Acknowledgments

Acknowledgments are given at the end of each chapter; these are not repeated here.

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I have learned from Toto Olivera and Jim Wang the quick albeit sometimes sloppy way of performing experiments and getting results. All the folks in the ND group during my stay, directly or indirectly, helped me and are collectively acknowledged here.

Ray transformed my Korean-like English to his American-like English in part of this thesis.

Preface

Most of the work in this thesis has been published and is in publication. For convenience in writing, each paper is presented as a separate chapter. Therefore, each chapter has its own references, figure and plate numbers.

Because of the diversity of the research projects undertaken it seemed appropriate to give a general introduction and overview. It will, I believe, help for the reader to choose what to read according to his interest; the understanding of early chapters is not necessary for understanding later parts. In some respects, this thesis resembles a symposia report.

Chapter 1 on physicochemical studies on N phage DNA's has been accepted for publication in Virology. Chapters 2 and 3 on covalently closed circular intracellular N1 phage DNA and minicircular DNA in M. lysodeikticus are reproduced here with written permission from Academic Press, Inc. Chapter 4 on coliphage 15 DNA has been submitted to the Journal of Molecular Biology. Part II of this thesis on the flow dichroism of DNA is reproduced here with written permission from John Wiley and Sons, Inc.

Abstracts

Part I

Chapter 1. . . . . A physicochemical study of the DNA molecules from the three bacteriophages, N1, N5, and N6, which infect the bacterium, M. lysodeikticus, has been made. The molecular weights, as measured by both electron microscopy and sedimentation velocity, are 23 X 10^6 for N5 DNA and 31 X 10^6 for N1 and N6 DNA's. All three DNA's are capable of thermally reversible cyclization. N1 and N6 DNA's have identical or very similar base sequences as judged by membrane filter hybridization and by electron microscope heteroduplex studies. They have identical or similar cohesive ends. These results are in accord with the close biological relation between N1 and N6 phages. N5 DNA is not closely related to N1 or N6 DNA. The denaturation T_m of all three DNA's is the same and corresponds to a (GC) content of 70%. However, the buoyant densities in CsCl of N1 and N6 DNA's are lower than expected, corresponding to predicted GC contents of 64 and 67%. The buoyant densities in Cs_2SO_4 are also somewhat anomalous. The buoyant density anomalies are probably due to the presence of odd bases. However, direct base composition analysis of N1 DNA by anion exchange chromatography confirms a GC content of 70%, and, in the elution system used, no peaks due to odd bases are present.

Chapter 2. . . . . A covalently closed circular DNA form has been observed as an intracellular form during both productive and abortive infection processes in M. lysodeikticus. This species has been isolated by the method of CsCl-ethidium bromide centrifugation and examined with

an electron microscope.

Chapter 3. . . . . A minute circular DNA has been discovered as a homogeneous population in M. lysodeikticus. Its length and molecular weight as determined by electron microscopy are 0.445 μ and 0.88 X 10^6 daltons respectively. There is about one minicircle per bacterium.

Chapter 4. . . . . Several strains of E. coli 15 harbor a prophage. Viral growth can be induced by exposing the host to mitomycin C or to uv irradiation. The coliphage 15 particles from E. coli 15 and E. coli 15 T^- appear as normal phage with head and tail structure; the particles from E. coli 15 TAU are tailless. The complete particles exert a colicinogenic activity on E. coli 15 and 15 T^-, the tailless particles do not. No host for a productive viral infection has been found and the phage may be defective. The properties of the DNA of the virus have been studied, mainly by electron microscopy. After induction but before lysis, a closed circular DNA with a contour length of about 11.9 μ is found in the bacterium; the mature phage DNA is a linear duplex and 7.5% longer than the intracellular circular form. This suggests the hypothesis that the mature phage DNA is terminally repetitious and circularly permuted. The hypothesis was confirmed by observing that denaturation and renaturation of the mature phage DNA produce circular duplexes with two single-stranded branches corresponding to the terminal repetition. The contour length of the mature phage DNA was measured relative to φX RFII DNA and λ DNA; the calculated molecular weight is 27 X 10^6. The length of the single-stranded terminal repetition was compared to the length of φX 174 DNA under conditions where single-stranded

DNA is seen in an extended form in electron micrographs. The length of the terminal repetition is found to be 7.4% of the length of the non-repetitious part of the coliphage 15 DNA. The number of base pairs in the terminal repetition is variable in different molecules, with a fractional standard deviation of 0.18 of the average number in the terminal repetition. A new phenomenon termed "branch migration" has been discovered in renatured circular molecules; it results in forked branches, with two emerging single strands, at the position of the terminal repetition. The distribution of branch separations between the two terminal repetitions in the population of renatured circular molecules was studied. The observed distribution suggests that there is an excluded volume effect in the renaturation of a population of circularly permuted molecules such that strands with close beginning points preferentially renature with each other. This selective renaturation and the phenomenon of branch migration both affect the distribution of branch separations; the observed distribution does not contradict the hypothesis of a random distribution of beginning points around the chromosome.

Chapter 5. . . . . Some physicochemical studies on the minicircular DNA species in E. coli 15 (0.670 μ, 1.47 X 10^6 daltons) have been made. Electron microscopic observations showed multimeric forms of the minicircle which amount to 5% of total DNA species and also showed presumably replicating forms of the minicircle. A renaturation kinetic study showed that the minicircle is a unique DNA species in its size and base sequence. A study on the minicircle replication has been made under

condition in which host DNA synthesis is synchronized. Despite experimental uncertainties involved, it seems that the minicircle replication is random and the number of the minicircles increases continuously throughout a generation of the host, regardless of host DNA synchronization.

Part II

The flow dichroism of dilute DNA solutions ($A_{260} \approx 0.1$) has been studied in a Couette-type apparatus with the outer cylinder rotating and with the light path parallel to the cylinder axis. Shear gradients in the range of 5-160 $sec.^{-1}$ were studied. The DNA samples were whole, "half," and "quarter" molecules of T4 bacteriophage DNA, and linear and circular $\lambda b_2 b_5 c$ DNA. For the linear molecules, the fractional flow dichroism is a linear function of molecular weight. The dichroism for linear λ DNA is about 1.8 that of the circular molecule. For a given DNA, the dichroism is an approximately linear function of shear gradient, but with a slight upward curvature at low values of G, and some trend toward saturation at larger values of G. The fractional dichroism increases as the supporting electrolyte concentration decreases.

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Introduction and Overview

This thesis consists of several sections which are not closely related in their biological significance. The principal unifying theme is the application of physicochemical methods for the study of deoxyribonucleic acids.

In the course of the work, several previously unstudied DNA's were encountered and characterized. By applying a variety of physicochemical methods, it is possible to learn a great deal about a DNA. Indeed, in each of the several instances studied here, more is now known about the physicochemical properties of the DNA then about its biological function and significance. It is anticipated that such studies as performed here will provide some background for further understanding of their biological functions.

The properties that can be measured and the methods used are listed below. In the instances marked by an asterisk, significant advances in methodology were developed here.

	Property	Method
(a)	molecular weight	sedimentation velocity
		electron microscopy
		flow dichroism*
(b)	base composition	chemical analysis
		buoyant density
		thermal denaturation
(c)	occurrence of rare or modified bases, or unusual sequence effect	discrepancies between the three methods in (b)

(d)	genome size (complexity)	renaturation kinetics*
(e)	topological properties (closed circular, linear, linear with cohesive ends)	sedimentation velocity buoyant density in CsCl-ethidium bromide electron microscopy
(f)	occurrence of terminal repetitions and circular permutations in a linear phage DNA; length heterogeneity of the therminal repetition	electron microscope heteroduplex methods*
(g)	sequence homology between related DNA's	membrane filter hybridization electron microscope heteroduplex methods*
(h)	intracellular forms and replicating forms	electron microscopy

The physicochemical techniques referred to above have been applied in this thesis for the investigations of the DNA's from several microorganisms. The motives, results and conclusions of these studies will be briefly described below.

Preliminary study in this laboratory has shown that one of DNA's from N phages in M. lysodeikticus, N1 DNA, has cohesive ends and is capable of thermally reversible cyclization (Wetmur, Davidson and Scaletti, 1966). This was the only DNA known to have cohesive ends, isolated from a phage in a bacterial family other than enterobacteriaceae. Dr. J. V. Scaletti provided us two more different N phages,

N5 and N6. The DNA's from these phages in addition to phage N1 have been characterized here physicochemically.

Earlier studies on this N phage system had shown that N1 and N6 phages are serologically very similar and that they are capable of genetic recombination (Naylor and Burgi, 1956). By these criteria, N5 phage is not related to the above two phages. N5 phage is lysogenic to a certain strain of M. lysodeikticus, whereas lysogenic hosts of N1 and N6 phages have not been found (Field and Naylor, 1962). Both N1 and N6 phages infect a strain of M. lysodeikticus, ML 1, and produce infective phage particles (productive infection). However, when N1 phage infects another strain of M. lysodeikticus, ML 53-20, which was derived from ML 1 by uv mutagenesis, the infected cells lyse but do not produce infective phage particles (abortive infection). On the other hand, N6 phage is productively infective to ML 53-20. In a mixed infection of N1 and N6 phages to this host, however, both infective N1, N6 and their recombinants are produced (Naylor and Burgi, 1956).

It is shown in this thesis that the properties of the DNA molecules are generally consistent with the above observations on the phages. The molecular weights, as determined by both sedimentation velocity and electron microscopic contour length, are 31×10^6 daltons for N1 and N6 DNA and 23×10^6 daltons for N5 DNA. All three DNA's have cohesive ends so that they undergo a thermally reversible cyclization. Furthermore, N1 and N6 DNA's share common cohesive ends for mutual dimerization and higher aggregations. A membrane filter hybridization study shows that N1 and N6 DNA's have more than 90% homology in their

base sequences, whereas there is only 1.7% homology between N1 and N5 DNA's. Further electron microscopic hybridization shows a complete homology between N1 and N6 DNA within the accuracy of this method. The Tm's of all three DNA's are the same and correspond to a (GC) content of 70%. These results are in accord with the close biological relations between N1 and N6 phages.

However, the buoyant densities in CsCl of N1 and N6 phages and DNA's differ by 0.002 and 0.004 g/ml, respectively. Moreover, the buoyant densities of these two DNA's are lower than expected from their Tm, corresponding to (GC) contents of 64 and 67% for N1 and N6 DNA's respectively. A direct base composition analysis of N1 DNA by column chromatography confirms a (GC) content of 70%. A minor peak presumably due to 5-methylcytosine is observed in the elution system used. However, the amount is not sufficient to explain the unusual lowering of the buoyant density. The explanation of this anomaly is not clear.

A covalently closed circular DNA form of N1 phage DNA is observed as an intracellular form during both productive and abortive infection processes. Such an intracellular form after phage infection has been found earlier in a coliphage λ and in a Salmonella phage P22 (Young and Sinsheimer, 1964; Rhoades and Thomas, 1968).

While studying the intracellular form of N1 DNA, a minute circular DNA was discovered in M. lysodeikticus cells. There is about one minicircle per bacterium. Its molecular weight as determined by electron microscopy is 0.88 (± 0.04) X 10^6 daltons. This size is considerably smaller than a plasmid DNA with a molecular weight of 1.47 X 10^6

daltons found in E. coli 15 (Cozzarelli, Kelly and Kornberg, 1968). A small circular DNA with a size similar to that of M. lysodeikticus minicircle has recently been observed in the kinetoplasts of Trypanosoma cruzi (Riou and Delain, 1969).

The biological significance of the small circular DNA molecules above is not known. The size of the minicircle in M. lysodeikticus is so small that it can code for only 450 amino acids, at most two genes. A physicochemical study of a minicircular DNA might provide some insights into its biological function. The small amount of the minicircle present in M. lysodeikticus cells made such a physicochemical study impracticable. Therefore, we chose to study the minicircle in E. coli 15, since there are about 15 copies per chromosome.

An episomal or a plasmid DNA normally persists as one copy per chromosome. Multiple copies of a drug resistance factor have been found only when this factor was transferred from a normal host E. coli to P. mirabilis (Rownd, Nakaya and Nakamura, 1966). Thus, one may ask whether all the copies of the minicircle in E. coli 15 have the same unique base sequences. The same question can also be raised from a speculation that the presence of multiple copies might be a result of an amplification of some gene(s) in the host chromosome. A ribosomal RNA gene amplification has been observed in oocytes (Brown and Dawid, 1968).

The replication of a plasmid must, in some respects, be autonomous, since it is physically separated from the host chromosome. However, its replication is not entirely independent from the host since the number of determinants per chromosome remains constant for generations. There may be several explanations for this phenomenon. One of them

is that the host chromosome and the plasmid replicate at some specific sites on the cell membrane and the number of these sites is limited (Jacob, Brenner, and Cuzin, 1963). Another possibility is that the replication of the host chromosome provides some sort of signal for the replication of the plasmid. These possibilities can be studied under the conditions which the host DNA replication or the cell division is synchronized. For instance, do all the copies of the minicircles replicate all at once at a certain stage of host replication or cell division?

Studies on these matters have been undertaken. A result shows that the kinetic complexity of the minicircle in terms of daltons is the same as the molecular weight as determined by contour length measurements. It is concluded that the minicircle is a unique DNA species in its size and base sequence.

A study on the replication of the minicircle has been made under conditions in which the host DNA synthesis is synchronized. A method based on the synchronization of cell division was not used since such a method is effectively applicable only at low concentrations of cells. We attempted to synchronize replication of the host chromosome by the method of amino acid starvation and successive thymine starvation of E. coli 15 TAU-bar (Maaløe and Hanawalt, 1961; Hanawalt and Wax, 1964; Cerdá-Olmedo, Hanawalt and Guerola, 1968). Because of some uncertainty in the synchronization method as will be presented in detail in Chapter 5, a firm conclusion can not be drawn from this study. However, a tentative conclusion is that the number of the minicircles per chromosome

is fairly constant during a synchronized growth, as has been observed in a normal exponential growth. This suggests that the minicircles do not replicate all at once at a certain stage of host DNA synthesis but at a constant rate during the whole period of replication of the chromosomal DNA.

Electron microscopic evidences show that there exist two forms of presumed replicating minicircles; One is a circular structure with a long protruded linear double-stranded branch and the other a circular molecule with two forks, presumably one corresponding to the origin of replication and the other corresponding to replicating point. These two forms have been observed in replicating λ DNA molecules (Okawa, Tomizawa and Fuke, 1968; Weissbach, Bartl and Salzman, 1968).

In addition to the existence of the minicircle, a distinctive characteristic of E. coli 15 from other E. coli strains is that some of E. coli 15 strains produce "colicin 15" particles by common inducing agents. This colicin differs from others in that it kills its own host and that it has a morphology of phage. We, therefore, prefer to call it "coliphage 15". Even though the coliphage 15 kills its own host (colicinogenic activity), no productive infection is observed. A plaque assay on various strains of E. coli has been negative. Within this criterion, this phage may be called a "defective phage".

Since E. coli 15 strains have the two unique properties of harboring the prophage of colicinogenic defective phage and of harboring the minicircles, we sought to ascertain whether there was any relation between the minicircle and coliphage 15 DNA. No obvious relation has

been found. However, the DNA from coliphage 15 has very interesting properties. In this thesis, the coliphage 15 DNA has been studied almost exclusively by electron microscopy. This study, we believe, is a good example of the use of the electron microscope for characterization of a phage DNA.

DNA molecules from mature phage are linear with a contour length of 12.8 μ corresponding to a molecular weight of 27×10^6. This DNA does not have cohesive ends. However, there exists a covalently closed circular DNA form *in vivo* only during the induction process. The length of this *in vivo* circular DNA is 11.9 μ, which is 0.9 μ shorter than the mature linear DNA. This difference is significant and beyond the experimental error. The length difference immediately suggested to us the possibility of the presence of terminal repetition and circular permutation in this phage DNA. After denaturation and renaturation of mature phage DNA, it is indeed observed that *in vitro* circular molecules are formed with two single-stranded branches corresponding to the terminally repetitious portions. A direct contour length measurement of single-stranded repetitious branches shows that the size of the terminal repetition is 7.4% of the length of the nonrepetitious genome which is in exact agreement with the value deduced from the length difference shown above. Furthermore, the length of the terminal repetition is rather heterogeneous, suggesting that there is no accurate mechanism for packaging the DNA into the phage coat. This is in accordance with the "headful" mechanism proposed previously (Streisinger, Emrich and Stahl, 1967).

It is further observed, in the electron micrographs of the in vitro circular molecules, that in some cases the protruding repetitious branch consists of one single-strand with one free end and in other cases the branch has forked into two sub-branches. The sum of the lengths of two sub-branches corresponds to the size of the terminal repetition. This is explained by branch migration, that is, that a repetitious single-strand branch is not fixed at a point but migrates within the distance of a branch length, resulting in two sub-branches, as an equilibrium process.

The distribution of branch separations in the circular molecule is of interest. The distribution is not uniform. Instead, there occur more renatured molecules as the separation decreases from 0.5 to 0.15 of the circumference and then the number of molecules with a branch separation close to zero falls to a very low value (Fig. 4 in Chap. 4). This nonuniform distribution is attributed to an excluded volume effect and to branch migration. The excluded volume effect says that a point near the topological end of a strand is, on the average, closer to the physical outside of the random coil and is more available for the initiation of a renaturation reaction than is a point close to the topological center of the strand. Thus, the observed distribution does not contradict the hypothesis of a random distribution of the branch separation around the circular molecule.

Another possibility is suggested for the explanation of the non-uniform distribution. Assume that a moderately long concatenate is synthesized starting at a specific origin of replication in a bacterium.

The concatenates are then cut to a mature size in accordance with the headful mechanism at approximately constant lengths of one genome plus one terminal repetition. The result will be that more molecules have beginning base sequences close to the origin of replication, and the most probable beginning points will be spaced by units of a terminal repetition.

A characteristic of the B form of the Watson-Crick double helix is that the planes of bases are perpendicular to the helix axis. The ultraviolet absorption in DNA is in accord with transition moments of chromophores (bases in DNA) so that a light polarized along the helix axis will be less absorbed and a light polarized perpendicular to the helix axis will be more absorbed in an oriented DNA molecule. The orientation of DNA molecules in solution can be achieved either by a flow field or by an electric field.

Flow dichroism has been applied for the studies of the orientation of bound dyes in DNA, the studies of the organization of DNA in phage and the studies of the dissociation of nucleohistone (Lerman, 1963; Gellert, Smith, Neville and Felsenfeld, 1965; Gellert and Davis, 1964; Ohba, 1966). However, few basic studies on DNA solutions have been done especially at low shear gradients where the effects of various parameters are larger. Therefore, it was desirable to construct a Couette-type apparatus for basic studies such as molecular weight dependence, shear gradient dependence and ionic strength dependence on the flow dichroism. The range of shear gradients in this apparatus is 5-160 sec^{-1}.

The main results are that the dichroism is a linear function of molecular weights at a range of shear gradient of 100-160 sec^{-1}, that the dichroism of linear λ DNA is 1.8 that of the circular molecule, and that the effect of the ionic strength on the DNA configuration in solution is more drastically revealed than other physical methods. From a rough comparison to the theory of a random coil polymer, it is concluded that DNA molecules behave roughly as a flexible random coil at moderately high ionic strength (> 0.01 M $[Na^+]$) and at relatively low shear gradient (< 20 sec^{-1}). That is, the theory is applicable only at small distortions and extensions of macromolecules.

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Part I

Studies on Bacteriophage DNA's and Minicircular DNA's in *M. lysodeikticus* and *E. coli* 15

Chapter 1

Studies on the Deoxyribonucleic Acids from Bacteriophages of *Micrococcus lysodeikticus*[1,2]

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Summary

A physicochemical study of the DNA molecules from the three bacteriophages, N1, N5, and N6, which infect the bacterium, M. lysodeikticus, has been made. The molecular weights, as measured by both electron microscopy and sedimentation velocity, are 23 X 10^6 for N5 DNA and 31 X 10^6 for N1 and N6 DNA's. All three DNA's are capable of thermally reversible cyclization. N1 and N6 DNA's have identical or very similar base sequences as judged by membrane filter hybridization and by electron microscope heteroduplex studies. They have identical or similar cohesive ends. These results are in accord with the close biological relation between N1 and N6 phages. N5 DNA is not closely related to N1 or N6 DNA. The denaturation T_m of all three DNA's is the same and corresponds to a (GC) content of 70%. However, the buoyant densities in CsCl of N1 and N6 DNA's are lower than expected, corresponding to predicted GC contents of 64 and 67%. The buoyant densities in Cs_2SO_4 are also somewhat anomalous. The buoyant density anomalies are probably due to the presence of odd bases. However, direct base composition analysis of N1 DNA by anion exchange chromatography confirms a GC content of 70%, and, in the elution system used, no peaks due to odd bases are present.

1. Introduction

Several distinct bacteriophages, denoted the N phages, of the bacterium Micrococcus lysodeikticus were isolated from sewage by Naylor and Burgi (1956). In the present communication we report a general physicochemical study of the DNA of three particular phages, N1, N5, and N6. These DNA's are like λ DNA in that they have molecular weights of about 30×10^6 and they are capable of reversible cyclization. They have base compositions of about 70% GC, which is close to that of the host bacterium (72%). The viruses, N1 and N6, were already known to be closely related serologically and to be capable of genetic recombination (Naylor and Burgi, 1956; Scaletti,* 1967). One of the points of interest in the present investigation was the degree of homology between the several DNA's and the relation, if any, between their cohesive ends.

Preliminary reports on the properties of the DNA of phage N1 (Wetmur, Davidson, and Scaletti, 1966) and on the formation of covalently closed, twisted, circular DNA of N1 as an intracellular form after infection of the host (Lee, Davidson, and Scaletti, 1968) have already appeared.

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*Personal communication.

2. Materials and Methods

(a) Solutions and buffers

ML broth contains 1.5% Bacto typtone, 0.5% Bacto yeast extract, 0.5% glucose, and 0.5% NaCl. EDTA buffers in the pH range 7 to 8 were prepared by adding NaOH to Na_2 EDTA solutions. Tris buffers were prepared from tris-OH with HCl. SSC contains 0.15 M NaCl, 0.015 M Na_3-citrate.

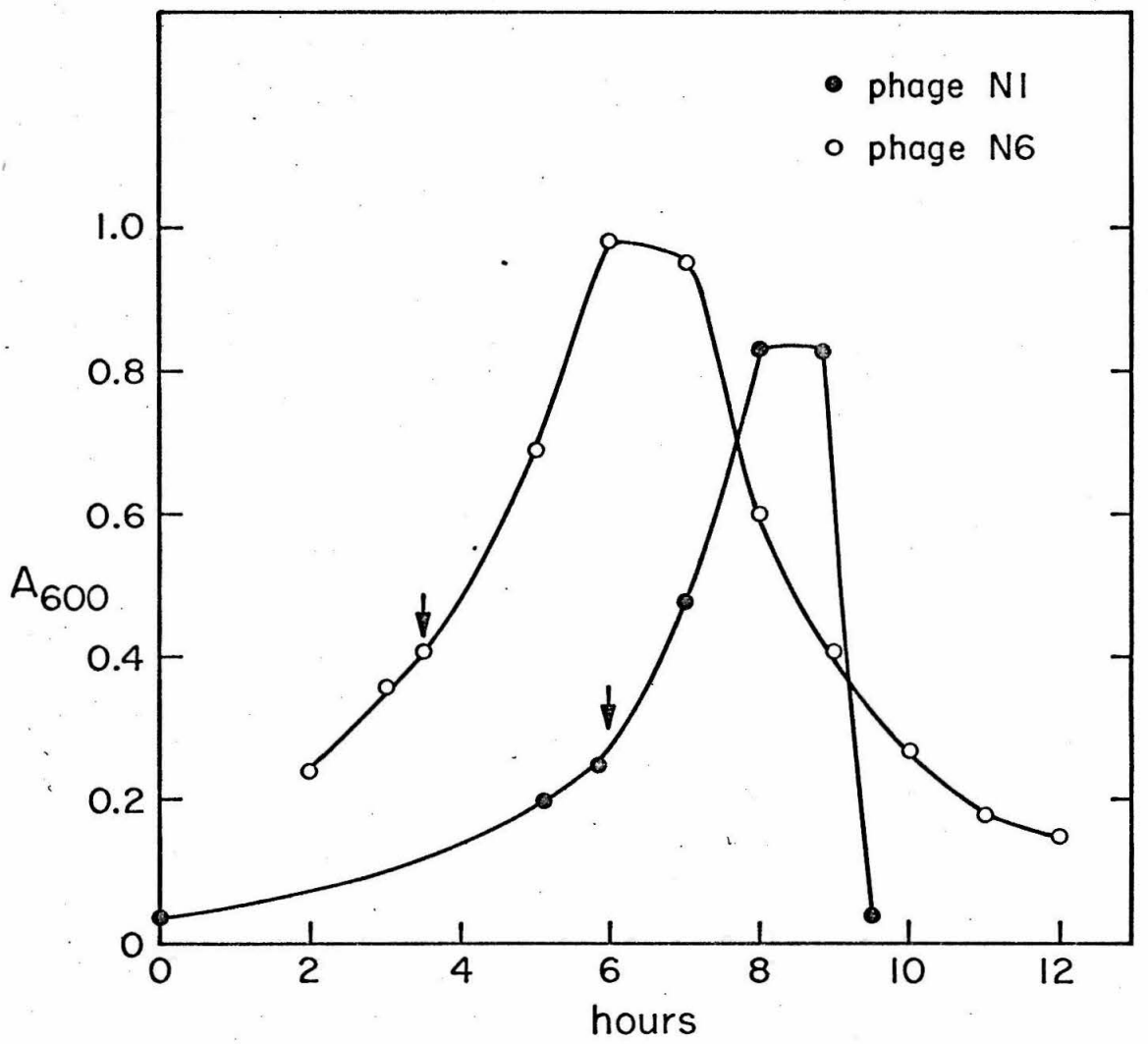
(b) Growth and purification of phages

The strains, ML1 and ML 53-20 of Micrococcus lysodeikticus, and the phages N1, N5, and N6 were a generous gift by Dr. J. V. Scaletti. Bacterial strains were stored at 5°C in agar slants containing 1.5% Bacto agar in ML broth. One ml of saturated culture of ML bacteria was added to 1.5 liter of fresh ML broth, and the cells were grown at 32°C with vigorous aeration. When A_{600} = 0.3 - 0.4, the cells were infected with phage with a multiplicity of infection (m.o.i.) of 0.1. Some typical growth curves of bacteria infected with phages N1 and N6 are shown in Fig. 1. At the end of lysis, 20 ml of chloroform were added and aeration was continued for an additional 15 min.

Bacterial debris was removed from the lysate by centrifugation in a model L ultracentrifuge at 7,000 rpm for 15 min with an L-19 rotor. The phages were then pelleted by centrifugation at 17,000 rpm for 5 hrs with an L-19 rotor, resuspended in 0.01 M tris, 0.01 M $MgSO_4$ (pH 8) and CsCl was added to give a density of 1.5 g/ml, and the phages were banded by centrifugation at 35,000 rpm for 24 hrs with an SW 50 L rotor. The bluish-white phage band was collected by puncturing the

Fig. 1. Growth of phages N1 and N6 on bacterial strain ML 1. The arrows indicate where the cells were infected with phages.

Fig. 1



centrifuge tube at the side, and dialyzed vs. 0.01 M tris-$MgSO_4$ medium for storage.

For the growth of P^{32}-labeled phage N1, 2-3 mc of carrier-free P^{32}-phosphoric acid per liter were added 20 min before the phage infection. The cells were grown in the low phosphate Neopeptone medium previously described (Lee and Davidson, 1968a).

The yield of unlabeled phages was usually 3-10 X 10^{10} phage/ml. The specific activity of P^{32}-labeled N1 DNA was about 10^5 cpm/μg DNA. Phages N1 and N6 were plated on ML 1 and ML 53-20 bacteria, respectively. The top agar contains 0.6% agar in the ML broth. (N1 grows on ML 1 only, whereas N6 grows on both ML 1 and ML 53-20.)

(c) Extraction of DNA from phages and bacteria

Phage DNA was isolated by phenol extraction as previously described (Lee and Davidson, 1968b) and dialyzed vs. a buffer of 1 M NaCl, 0.01 M tris, 0.001 M EDTA (pH 8). The DNA solution was stored at 5°C over a few drops of chloroform.

DNA was extracted from bacteria by a modification of procedures previously described (Marmur, 1961; Jensen, 1965).

ML 1 was grown in 2 liters of ML broth to saturation and the Neopeptone medium was used for P^{32}-labeling of bacterial DNA. Two to five grams of cells were suspended in 50 ml of 0.01 M tris, 0.01 M EDTA (pH 7). Lysozyme (crystalline, Worthington) was added to give 0.4 mg/ml and the cell suspension was incubated at 37°C for 15-30 min. After the addition of SDS and $NaClO_4$ to give concentrations of 5% and 1 M, respectively, the viscous suspension was stirred slowly at room

temperature for 20 min. When it looked emulsified, an equal volume of chloroform ($CHCl_3$:i-amyl alcohol = 24:1 by volume) was added and the stirring continued for 15 min. This very viscous suspension was centrifuged at 15,000 rpm for 20 min in a Spinco model L with a SW 25.1 rotor. The aqueous phase was carefully drawn out with a disposable pipette. This $CHCl_3$ extraction was repeated three times or more until there was very little material left at the interphase. The DNA solution was slowly poured into twice its volume of i-propanol and the DNA precipitate was wound up with a glass rod. It was washed with 95% ethanol, dried, and dissolved in 10 ml of 0.01 M tris (pH 8) by gentle shaking at 5°C.

To this DNA solution, RNAse A (phosphate free, Worthington), which had been pre-incubated at 80°C for 10 min, was added to give 40 μg/ml and the mixture was incubated at 37°C for 1.5 hour. The DNA solution was then phenol-extracted three times or more. The dissolved phenol in the DNA solution was removed by three ether extractions. The DNA was precipitated as before and dissolved in a desired buffer.

The preparation of covalently closed circular N1 DNA by ethidium bromide-CsCl centrifugation has been reported previously (Lee, et al., 1968).

(d) Cyclization of N phage DNA's

N phage DNA's in a buffer of 1.0 M or higher NaCl, 0.01 M EDTA (pH 8) were heated at 75°C for 10 min followed by incubation at 50°C for one hour or longer (Wang and Davidson, 1966a; Wetmur, et al.,1966). Under these conditions, more than 50% of the DNA was cyclized.

(e) Ultracentrifugation

Analytical band velocity sedimentation was done as reported previously (Lee and Davidson, 1968b). Buoyant density centrifugations were done with 12 mm centerpieces in a Spinco model E ultracentrifuge. The bottom windows were either flat or side-wedge windows, and -1^{o} or -2^{o} windows for CsCl or Cs_2SO_4 medium, respectively, were on top. The DNA concentration was adjusted to give 0.5 to 1 μg per band. The buoyant density of a DNA was calculated from that of a marker DNA in the same cell as prescribed by Vinograd and Hearst (1962) and by Erikson and Szybalski (1964) for CsCl and Cs_2SO_4, respectively.

(f) Electron microscopy

Bacteriophage were stained with either 2% phosphotungstic acid or 1% uranyl acetate. DNA samples for electron microscopy were mounted in cytochrome films by the detailed procedures reported previously (Wetmur, et al.,1966; Davis and Davidson, 1968; Lee, Davidson and Scaletti, 1968).

(g) Denaturation of DNA

The object of these experiments was to accurately compare the thermal denaturation behavior of several DNA's under identical conditions. DNA's to be compared were simultaneously and exhaustively dialyzed vs. a common solution of 0.01 M NaCl, 0.0005 M EDTA (pH 7.3) for two days. The presence of EDTA was necessary for the reproducibility of T_m. Optical melting curves were observed in a Gilford apparatus. Microcells (width 2.5 mm) or ordinary cells (width 10 mm) with an optical path of 10 mm were used depending on the available amount of DNA. The DNA

solutions in the cells were degassed by suction for 10 min prior to running the heating curves. The thermal denaturation behavior of three DNA solutions could be simultaneously observed with this apparatus.

(h) DNA-DNA Hybridization

Denatured DNA was adsorbed on membrane filters essentially as described by Gillespie and Spiegelman (1965). For purposes of estimating the DNA retention on the membrane filter, a small amount (1%) of H^3-labeled ascites tumor DNA (gift of Dr. M. Dahmus) was mixed with the cold DNA (N1, N5, N6, M. lysodeikticus or ascites tumor DNA) in 0.01 X SSC. This mixture was denatured by heating at 100° for 2 min and diluted to give a DNA concentration of 10 µg/ml in 6 X SSC for loading on the filter. Schleicher and Schuell type B6 membrane filters, diameter 11 mm, were presoaked in 6 X SSC, washed with 2 ml of 6 X SSC and then loaded with 0.4 ml of the denatured DNA solution. The filters were washed twice with 10 ml of 6 X SSC by slow suction, dried at room temperature overnight and then in an 80°C vacuum oven for 2 hours.

Three ml of P^{32}-labeled N1 DNA at a concentration of 250 µg/ml and a specific activity of 1.56 X 10^5 cpm/µg DNA in 1.25 X SSC was sonicated with a Bronson sonicator at power level 2 for 2 min. Sonicated DNA was then denatured by heating at 100°C for 2 min. DNA-DNA membrane filter hybridization was carried out with the above samples by the method of Warnaar and Cohen (1966). Five-ml vials each containing 0.5 ml of sonicated DNA solution at a different concentration, two cold DNA filters, and one blank filter in between, were maintained at 60°C for 24 hours with occasional gentle shaking. After incubation, the filters were

soaked in 3 X 10^{-3} M tris (pH 9.4) for about two min and washed on each side twice with 10 ml of the same buffer. They were dried completely at room temperature before the radioactivity counting.

The radioactivity on the filter papers was counted in a Beckman Liquid Scintillation System (model LS-200B) with a gain set 300, P^{32} with H^3 in channel A and H^3 in channel B. The scintillation fluid used here was made as follows: Toluene (Reagent Grade) 1 ℓ, PPO (2, 5-diphenyl oxazole, scintillation grade, Packard Instruments Co.) 7g, and POPOP { 1, 4-bis-[2-(5-phenyloxazolyl)]-benzene, scintillation grade, Packard Instruments Co. } 0.2 g.

In order to evaluate the exact amount of hybridization, the following corrections were made. The amount of P^{32} overlap in H^3 channel was 1.94%; therefore the counts in H^3 channel had to be reduced by 1.94%. DNA retention on the filter was estimated by mixing a small known amount of H^3-ascites tumor DNA with cold phage DNA's. Furthermore, since there was appreciable non-specific P^{32}-DNA binding to a blank filter, it was necessary to use a DNA completely non-homologous to the N phage DNA's as the control. Ascites tumor DNA satisfied this requirement. The P^{32} counts of ascites tumor DNA filter were about half of those of M. lyso DNA filter.

(i) Electron microscope heteroduplex studies

In order to investigate further the sequence homology between N1 and N6 DNA's, heteroduplex molecules, prepared by renaturing mixtures of denatured (fully strand-dissociated) N1 and N6 DNA's were examined in the electron microscope according to the general method of Davis

and Davidson (1968). In further experiments, mixtures of denatured sheared N6 DNA and unsheared N1 DNA were allowed to renature essentially according to the methods of Wulff, Jamieson, and Davidson (1969).*

Alkaline denatured DNA solutions were prepared from bacteriophage stock solutions as follows: A quantity of 0.05 ml of 0.2 M EDTA (pH 8), 0.35 ml of H_2O, an appropriate small amount of phage stock solution to give a final DNA solution of A_{260}=0.2, and 0.05 ml of 1 M NaOH were mixed. The solution was allowed to stand at room temperature for 30 min. When desired, the N6 DNA was sheared by forcing the alkaline solution out of a 0.5 ml disposable syringe through a 27 gauge needle with maximum thumb pressure 30 times. This treatment resulted in sheared single-strand DNA with an average size of 1/7th that of the whole DNA strand. The mixtures were chilled to 4°C and neutralized by adding 1/10 volume of 1.8 M tris-HCl, 0.2 M tris-hydroxide. Renaturation of a mixture of two DNA's was carried out with 0.05 ml of N1 DNA plus 0.05 ml of N6 DNA (sheared or unsheared), and 0.10 ml of formamide. This mixture was allowed to stand for 1/2 hour at room temperature. Electron microscope grids were prepared from this solution. As a control, only unsheared N1 DNA was used. Molecules of the several types of interest were scored by visual examination on the fluorescent screen of the electron microscope.

(j) Base composition analysis by column chromatography

DNA was hydrolyzed to 5'-mononucleotides as described by Ray and

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*Unpublished results.

Hanawalt (1964) and Wu and Kaiser (1967). Ninety μg of P^{32} -N1 DNA (specific activity 8, 920 cpm/μg DNA) and 1.05 mg of calf thymus DNA as a carrier in a 1.5 ml volume were precipitated with an equal volume of 7% $HClO_4$ and allowed to stand in the cold for 15 min. The DNA precipitate was collected by centrifugation and washed twice with 80% ethanol and once with ether. After evaporation of the ether, the DNA precipitate was dissolved in 2 ml of 0.01 M tris, 0.005 M $MgCl_2$ (pH 8).

One hundred μl of DNAse stock solution containing 1 mg/ml each of pancreatic DNAse I (Worthington) and bovine serum albumin in 0.01 M tris (pH 8) was added to the above solution. After incubating at 37°C for 3 hours the pH was adjusted by addition of 200 μl of 0.3 M glycine buffer (pH 8.9). Then 400 μl of a stock solution containing 1 mg/ml of snake venom phosphodiesterase (Worthington) and 1.5 mg/ml of bovine serum albumin were added. The digestion was continued at 37°C for 3 hours. The pH at this point was 8.5 and was adjusted to 4.0 by the addition of 2 N HAc for chromatography.

Anion exchange column chromatography was carried out as described by Hurst, Little, and Butler (1951) and by Hurst, Marko, and Butler (1953). Ten g of Dowex-1 (X8, 200-400 mesh, ionic form Cl^-, Baker Chemical) were washed five times with 250 ml of water by decantation and then successively with 200-ml portions of 1 N NaOH, H_2O, 1 N HAc, and H_2O in the order given. A column (25 cm X 0.18 cm^2) was loaded with the resin and washed with 100 ml of 2 N HAc followed by H_2O until the eluate showed no acidity to litmus paper.

The enzyme hydrolysate was loaded on top of the column and the column was then washed with 20 ml of H_2O. The elution was done stepwise with the eluants, 0.05 N HAc, 0.5 N HAc, 1 N HAc with 0.01 N NaAc, 1 N HAc with 0.1 N NaAc, 1 N HAc with 0.5 N NaAc, and finally 2 N HAc with 1 N NaAc in the order given. The flow rate was 0.2 - 0.3 ml/min.

Fifty drop fractions were collected with an LKB fraction collector. The absorbance of the eluate was continuously monitored with a Beckman DU spectrophotometer at 271 mμ and radioactivity was counted from 100 μl portions in a gas flow counter. The total amount of each nucleotide was determined by pooling all the fractions in one peak and counting an aliquot.

3. Results and Discussion

(a) Physicochemical parameters of N phages and their DNA's.

The morphologies of the N phages are generally similar to phage λ, in that they show a regular hexagonal head and a flexible tail as illustrated in Plate I. The dimensions and buoyant densities of the several phages are shown in Table 1.

The DNA's of all three phages, N1, N5, and N6, are capable of reversible cyclization under the experimental conditions described in Methods. Cyclization has been observed by band velocity sedimentation and by electron microscopy (Plate II).

A summary of the physical properties of the DNA's is given in Table 2. Length histograms of the N phage circular DNA's are shown in Fig. 2. From the measured lengths, the molecular weights were calculated to be 30.8 (± 1.0) X 10^6, 30.5 (± 1.0) X 10^6, and 23.2 (± 1.0) X 10^6 for N1, N6, and N5 DNA's, respectively. The molecular weight standard for the electron microscope measurements was λc_{26} DNA with a molecular weight taken as 31 X 10^6 and a contour length of 14.2 (± 0.3) μ under the conditions used. The sedimentation coefficients ($\underline{S}^{o}_{20,w}$) of linear N1, N6, and N5 DNA's were measured as 35.7 S, 35.8 S, and 32.8 S, respectively. Molecular weights calculated from the sedimentation velocities of the linear molecules according to Crothers and Zimm (1965) are 32.4 X 10^6, 32.4 X 10^6, and 25.8 X 10^6, in agreement with the values calculated from the electron microscope lengths.

The ratio of sedimentation velocities for the hydrogen bonded circular molecules to the linear molecules varies from 1.12 to 1.15, as expected.

Plate I Electron micrographs of N phages. a, phage N1; b, phage N6; c, phage N5; all were stained with 2% phosphotungstic acid.

Plate I

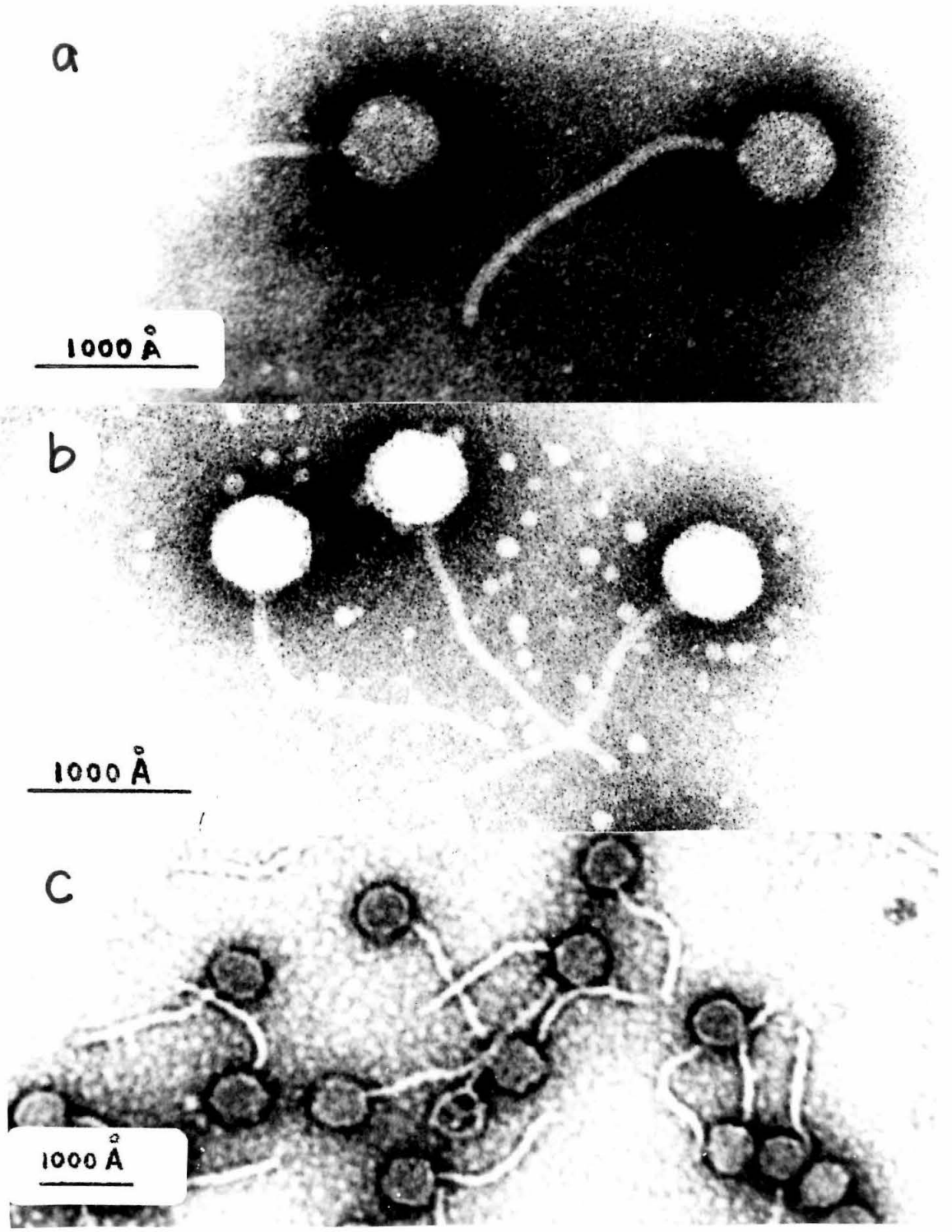


TABLE 1

Physical Parameters of N Phages

Phage	Diameter of head (A)		Length of tail (A)		Buoyant density (g/ml)
	a	b	a	b	
N1	580(±24)	573(±15)	2140(±48)	2200(±77)	1.490
N6	647(±12)	588(±12)	2320(±74)	2360(±71)	1.492
N5	528(±12)	460(±15)	1500(±27)	1620(±86)	1.499

[a]Phosphotungstic acid stain.

[b]Uranyl acetate stain.

Buoyant density centrifugation was done at 44,147 rpm for 18 hours (20°C) in a Spinco model E.

Plate II Electron micrographs of circular molecules of N phage DNA's. a, N1 DNA; b, N6 DNA; c, N5 DNA. DNA's were rotary-shadowed after staining with a uranyl salt (see text).

Plate II

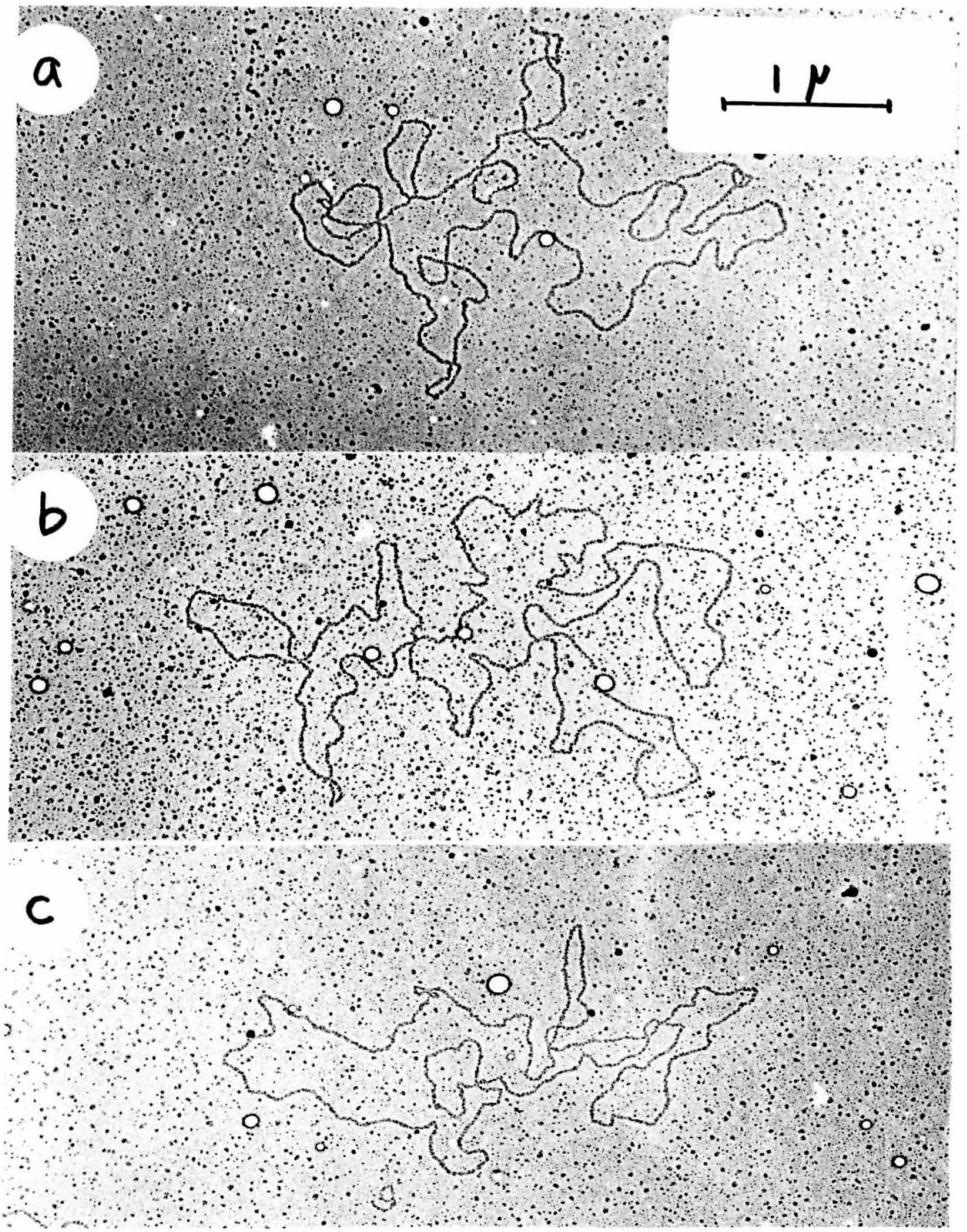


Fig. 2. Length histograms of N1, N6, and N5 DNA's. Circular molecules were chosen for the length measurements. The reference DNA for the length measurements was λ c_{26} DNA with a length of 14.2 ($\pm$ 0.3) μ, when mounted under the same conditions.

Fig. 2

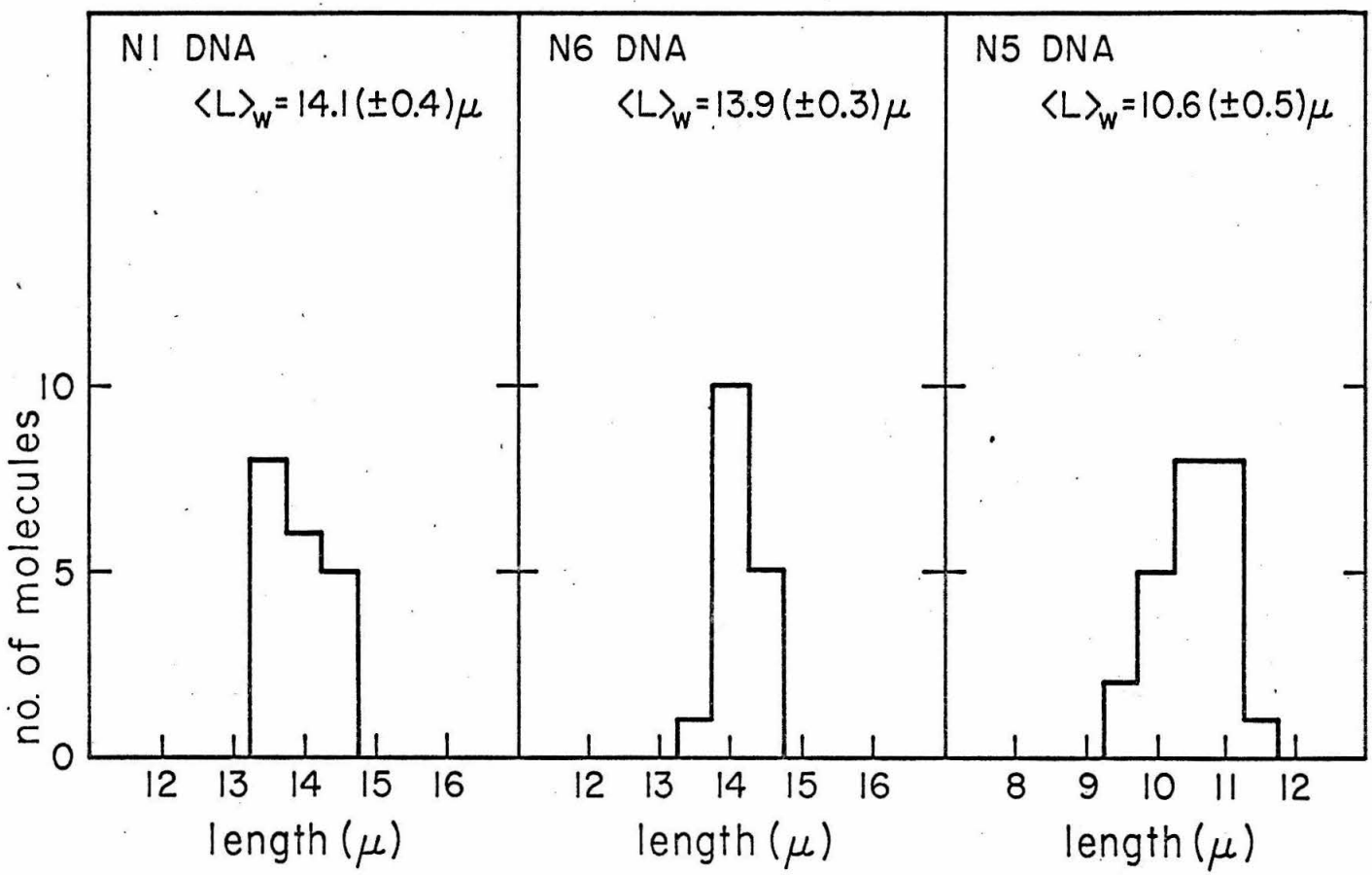

N1 DNA
$\langle L \rangle_w = 14.1 (\pm 0.4) \mu$
N6 DNA
$\langle L \rangle_w = 13.9 (\pm 0.3) \mu$
N5 DNA
$\langle L \rangle_w = 10.6 (\pm 0.5) \mu$
no. of molecules
0
5
10
12 13 14 15 16
length (μ)
12 13 14 15 16
length (μ)
8 9 10 11 12
length (μ)

TABLE 2

Physical Parameters of N Phage DNA's

	N1	N6	N5	M. lysodeikticus
Contour lengths (μ)	14.1(±0.4)	13.9(±0.3)	10.6(±0.5)	
$S^{o}_{20,w}$ (Svedberg unit)[a]	35.7(40.1)	35.8(41.3)	32.8(37.6)	
Buoyant density in CsCl (g/ml) (pH 7)[b]	1.7181	1.7220	1.7250	1.7265
Buoyant density in CsCl (g/ml) (pH 14)[c]	1.7806	1.7858		
Buoyant density in Cs_2SO_4 (g/ml)[d]	1.4353	1.4344	1.4400	1.4362
Tm in $[Na^+]$ = 0.0115 M (°C)	79.5	79.7	79.5	80.7
Cohesive ends	present	present	present	

[a]The values in parentheses are for circular molecules.

[b]Buoyant density of marker λ b_2b_5c DNA taken as 1.7041 g/ml.

[c]Buoyant density of marker *E. coli* DNA taken as 1.7660 (Vinograd, *et al.*, 1962).

[d]Buoyant density of marker *E. coli* DNA taken as 1.4260 (Erikson and Szybalski, 1964).

Buoyant density profiles for the several DNA's at pH 7 in CsCl and Cs_2SO_4 are shown in Figs. 3 and 4 and the buoyant densities are summarized in Table 2. Buoyant densities measured in CsCl at pH 14 are also shown for N1 and N6 DNA. No significant physical separation of the strands into two bands was observed for either DNA at this pH. No strand separation was achieved by buoyant banding in the presence of poly-IG (Kubinski, Opara-Kubinska and Szybalski, 1966).

The melting temperatures of the several DNA's are also presented in the table. The optical absorbance melting profiles are shown in Fig. 5a. The GC content of the reference DNA's, as reported by other authors, are plotted vs. the $\underline{T}_m$'s determined in these experiments (Fig. 5b). The slope $d\underline{T}_m/d\underline{X}_{GC}$ is 43°C in satisfactory agreement with the value of 41° reported by Marmur and Doty (1962). As will be discussed later, there is a discrepancy between the base compositions deduced from the buoyant densities and those deduced from the melting behavior.

(b) Sequence homology between N phage DNA's.

(i) DNA-DNA hybridization.

Sequence similarities between the several DNA's were investigated by hybridizing sheared P^{32}-labeled N1 DNA in solution with N1, N6, N5, and M. lysodeikticus DNA's on membrane filters. In Fig. 6a the amount of N1 DNA hybridized is plotted as a function of the ratio of sheared DNA in solution to DNA on the filter. It was not practicable to add enough N1 DNA in solution to saturate the N1 and N6 DNA's on the filters, so reciprocal plots were used to estimate saturation values (Fig. 6b). From the intercepts the saturation values for the per cent hybridization

Fig. 3. Buoyant density profiles of N phage DNA's in CsCl. λ b_2b_5c DNA (θ = 1.7041 g/ml) was used as an internal marker. Centrifugation was at 44,147 rpm for 20 hours (20°C) in a Spinco model E.

Fig. 3

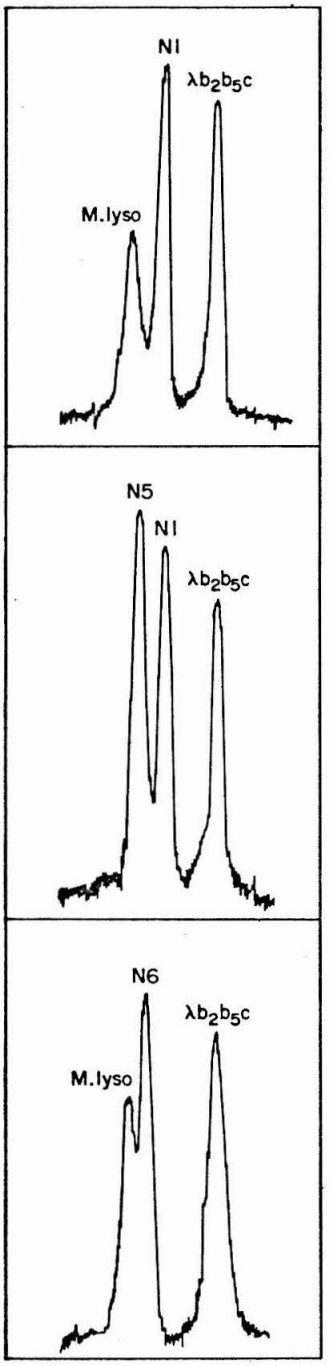


Fig. 4. Buoyant density profiles of N phage DNA's in Cs_2SO_4. *E. coli* DNA (θ = 1.4260) was used as an internal marker. Centrifugation was at 40,000 rpm for 24 hours (20°C).

Fig. 4

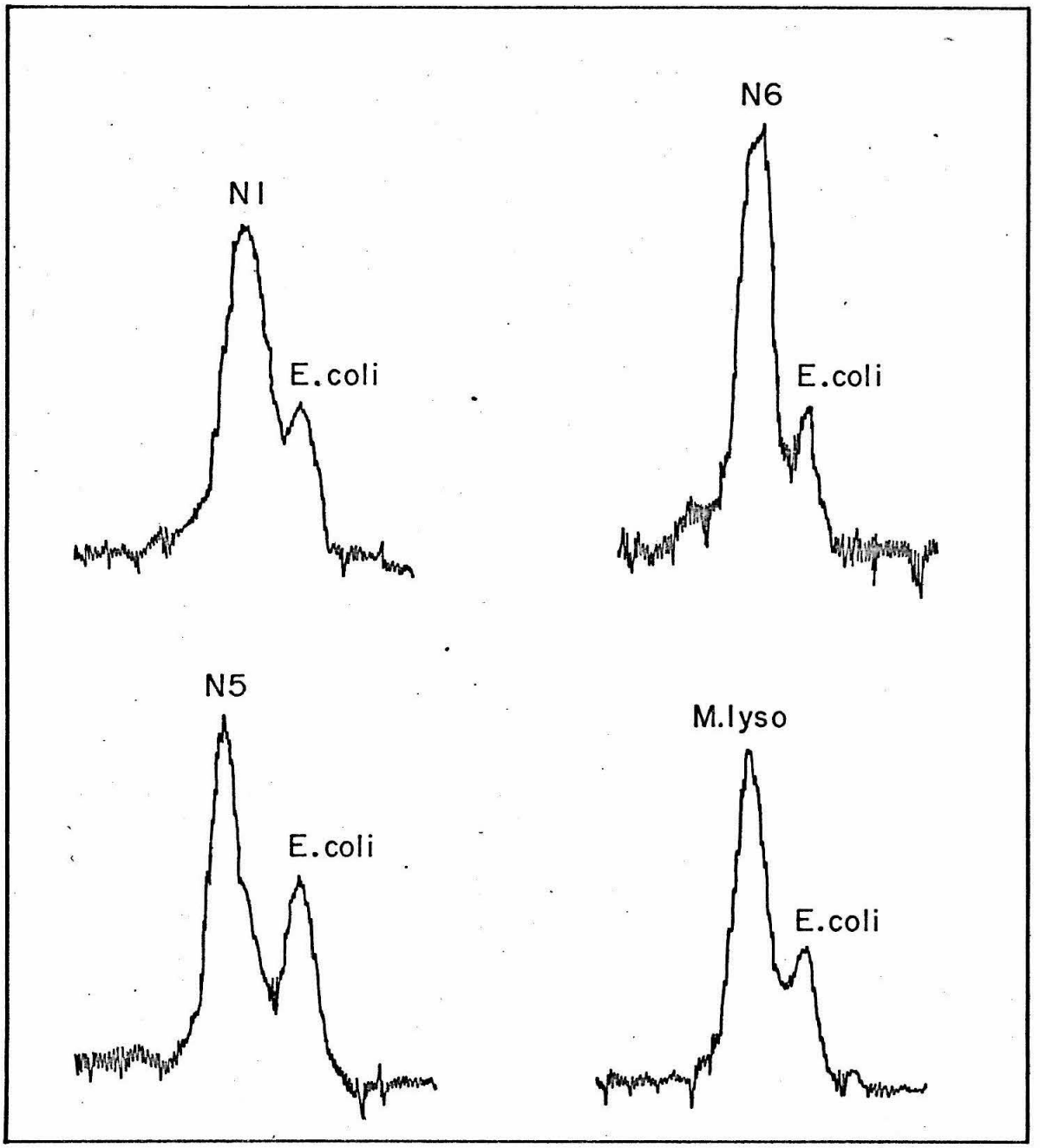


Fig. 5a. Thermal denaturation profiles of several DNA's. N6 DNA is not shown because it overlaps the N1 and N5 curves. $[Na^+]$ = 0.0115 M.

5b. GC content vs. T_m. The base composition of the reference DNA's were taken as and the T_m measured as: T4, 34% GC, 64.3°C; *E. coli*, 50% GC, 71.1°C; *M. lysodeikticus*, 72% GC, 80.7°C. Base compositions for N1, N5, and N6 DNA's, which all have the same T_m, were calculated by plotting this T_m on the line (●).

Fig. 5

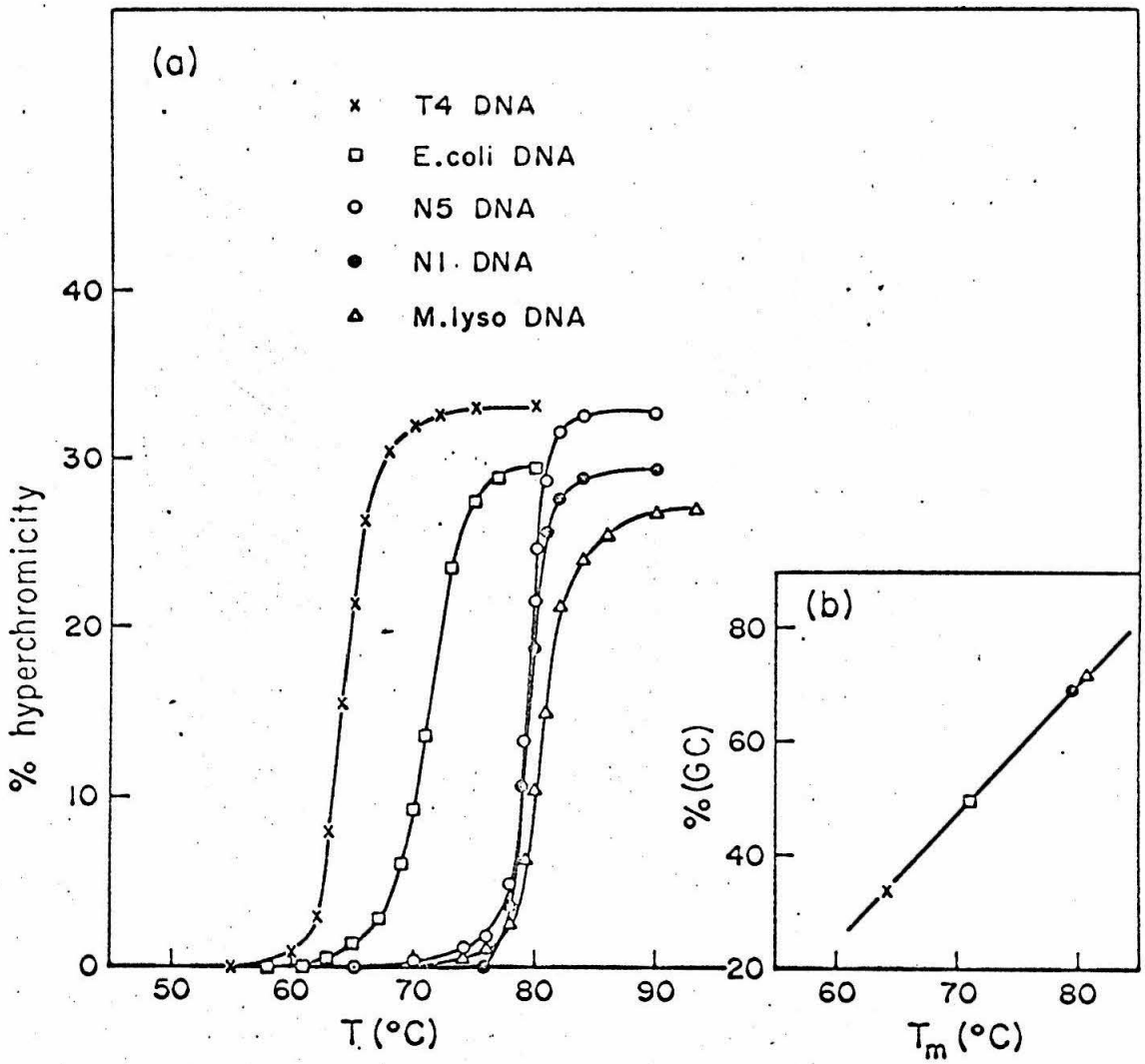
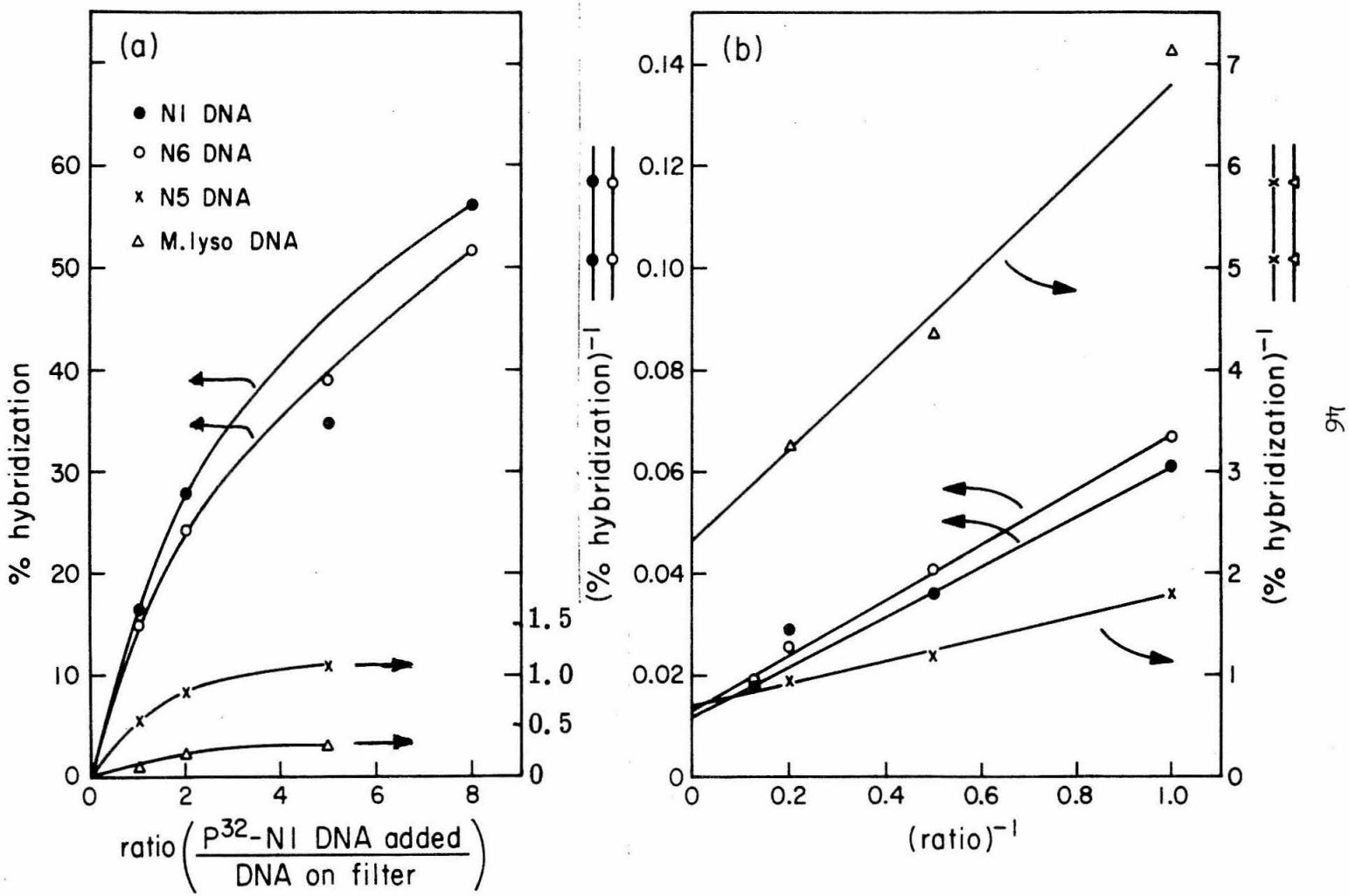

(a)
T4 DNA
E.coli DNA
N5 DNA
N1 DNA
M.lyso DNA
% hyperchromicity
T (°C)
(b)
% (GC)
T_m (°C)

Fig. 6a. Hybridization of P^{32}-N1 DNA to N1, N6, and N5 and M. lysodeikticus DNA's. The ordinate gives the weight ratios of P^{32}-N1 DNA in the incubation medium to cold DNA (4 μg) adsorbed on the filter.

6b. Reciprocal plot of data from Fig. 6a. The symbols are given in Fig. 6a.

Fig. 6


(a)
N1 DNA
N6 DNA
N5 DNA
M.lyso DNA
% hybridization
(% hybridization)⁻¹
ratio (P32-N1 DNA added / DNA on filter)
(b)
(ratio)⁻¹

are calculated as 83.3, 77.0, 1.43, and 0.44% for N1, N6, N5, and M. lysodeikticus DNA's respectively. By normalizing to 100% homology with N1 DNA, homologies of N6, N5, and M. lysodeikticus DNA's to N1 DNA are calculated as 92.5, 1.72, and 0.52%, respectively. Thus, there is almost complete homology between N1 and N6 DNA's. We believe that the measured small degree of homology of 1.7% between N1 and N5 DNA's is real, and not experimental error.

(ii) EM heteroduplex studies.

The principle of these experiments is as follows: If a mixture of denatured N1 and N6 DNA's is renatured and if there is sufficient sequence homology between the two DNA's to allow cross renaturation, the resulting mixture should contain some still denatured single strands, some fully renatured N1 molecules, some fully renatured N6 molecules, and some heteroduplexes with one strand from N1 and one strand from N6. In these heteroduplexes, significantly long stretches of non-homology between the two DNA's will appear as "bushes" in the micrographs when mounted under the conditions used here (Davis and Davidson, 1968).

When this experiment was done, on careful examination of the grids, only perfect double-stranded molecules were found. There are two possible interpretations of this observation:

(α) Despite the expectation from the membrane filter hybridization studies, N1 and N6 DNA's do not renature to each other at all and the perfect double-stranded molecules seen were all either N1 or N6 DNA, but not heteroduplexes.

(β) Heteroduplexes are formed, and the sequence similarity

between the two DNA's is so great that there are no recognizable single-strand regions. The general experience in this laboratory is that a bush of length of about 1% of these DNA's (~ 500 nucleotides) is clearly recognizable. If the two DNA's differ by having a number of non-homology regions which are substantially shorter than this, for example, if they differ only by a set of point mutations, the heteroduplex molecules would appear to be perfect double strands.

It seemed to us desirable to further confirm the expectation from the membrane filter hybridization experiments that N1 and N6 DNA's are capable of hybridizing with each other. The most elegant way to investigate this question would be to separate the complementary strands of both DNA's, renature a mixture of one strand from N1 and one strand from N6, and examine the preparation for renatured molecules in the electron microscope. This experiment was not done since, as already remarked, the standard methods of strand separation were not effective for these DNA's. An alternative procedure to show that N1 and N6 DNA's can hybridize under the conditions of renaturation used here is to shear N6 and renature a mixture of sheared N6 and unsheared N1 DNA's. In such an experiment, heteroduplex formation between N6 and N1 would result in the formation of a short double-stranded DNA region with single-stranded bushes on one or both sides due to the unreacted segments of the whole N1 strand (Wulff, et al, 1969*). In a typical experiment of this type with N6 DNA sheared to an average size of 0.14 full strand size, the following major molecular species were observed by electron microscopy: (A) long, linear double-stranded DNA's of whole molecule

*Unpublished results.

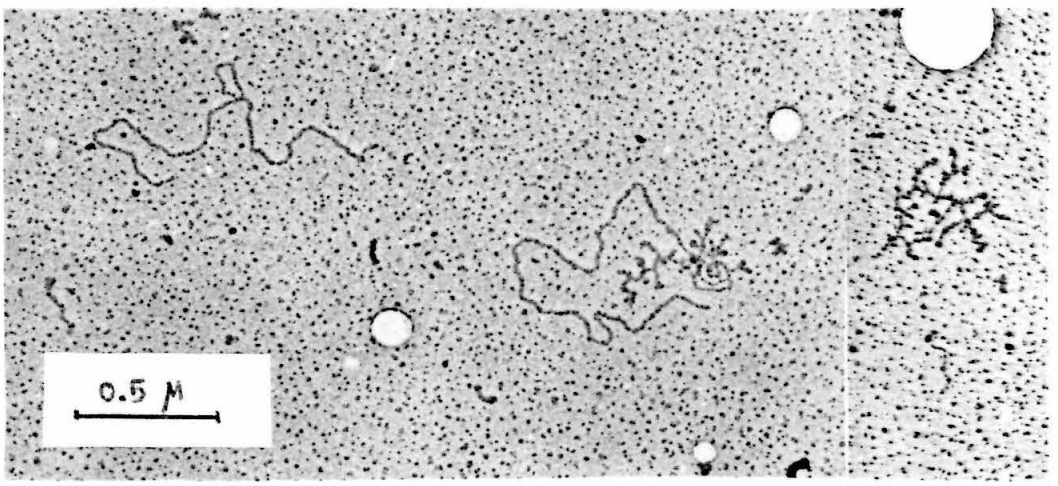
size: (B) large bushes due to unsheared, unrenatured, single-stranded DNA: (C) heteroduplexes of N1 DNA and sheared N6 DNA with a short double-stranded region and large end bushes: (D) short, double-stranded DNA's with very small end bushes due to renaturation of N6 segments with each other: and (E) small single-stranded bushes. Species A, B, and C were counted on several grids; 113, 88, and 22 molecules, respectively, of the three kinds were observed. A typical heteroduplex between whole N1 DNA and sheared N6 DNA (species C), an example of species B, an example of D, and several examples of E are shown in Plate III. In a control experiment with unsheared N1 DNA only, the corresponding populations were 128 whole double-stranded molecules, 100 large, single-stranded bushes, and 1 molecule with a short, double-stranded section and large bushes. Thus, the occurrence of many molecules with a short, double-stranded region and large end bushes in the renatured mixture of sheared N6 and unsheared N1 DNA's, shows that N1 and N6 DNA's are capable of renaturation. We conclude that the explanation in paragraph (β) immediately above applies.

(iii) Sequence homologies between the cohesive ends.

N1, N6, and N5 DNA's are all capable of reversible cyclization. By slow cooling of the solution from 50°C of any sodium ion concentration between 0.1 and 2.0 M, preparations which are over 50% circular, as observed by electron microscopy or by sedimentation, are obtained. Only incidental observations on the kinetics and equilibrium conditions for the cohesion reaction have been made. All of the DNA's are completely converted to the linear form by heating to 75° in 1 M NaCl solution.

Plate III Electron micrograph of a preparation of heteroduplexes. Several molecular species described in the text are shown.

Plate III



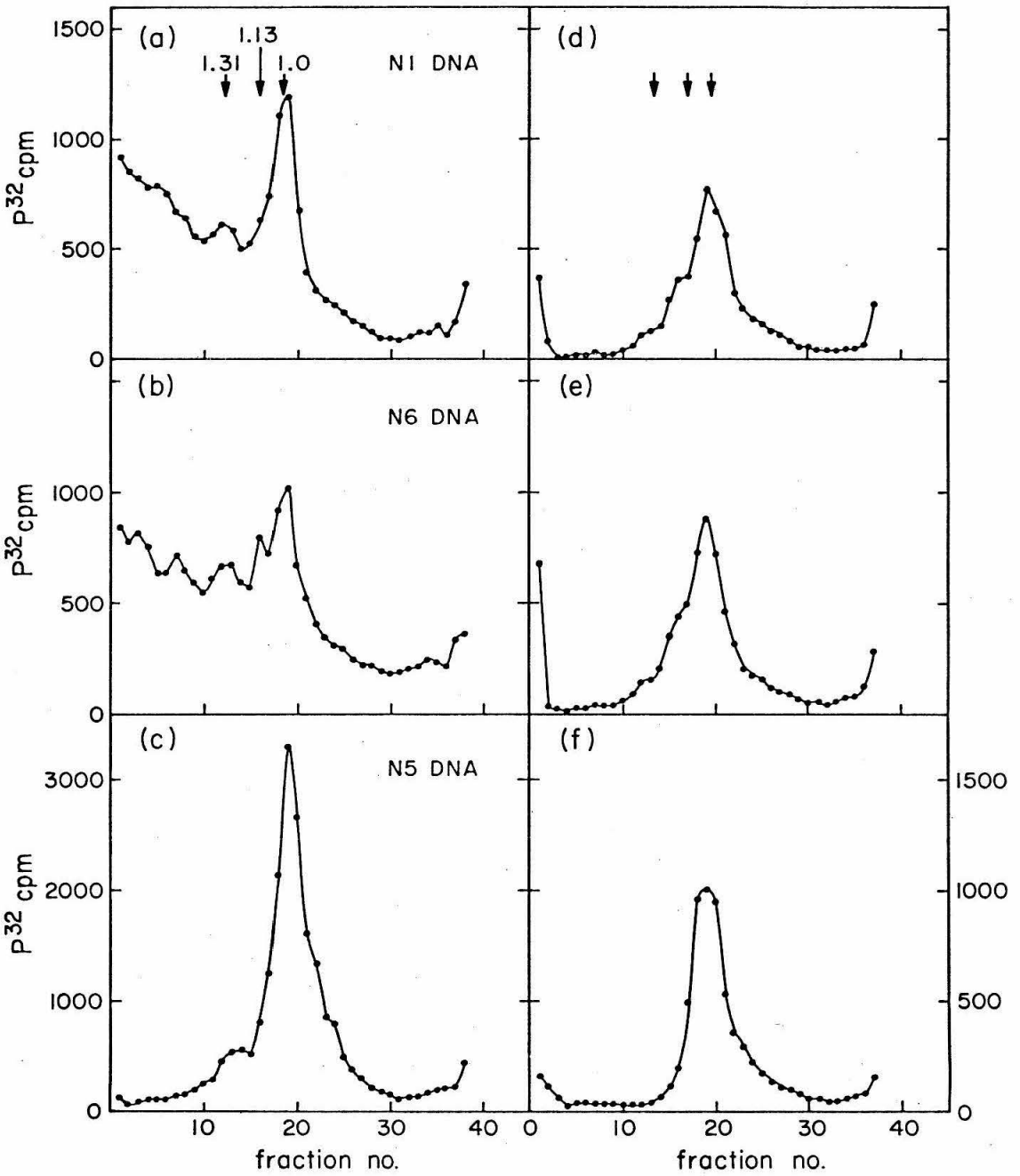
The T_m for cyclization of N1 DNA in 0.12 M Na^+ is between 40.6 and 44.7° (Wetmur, 1967). (Under these conditions the T_m for λ cyclization is 50°C.) N1 DNA cyclizes in a few hours in 2.0 M NaCl at 25° (Wetmur, et al.,1966) which is somewhat faster than the rate of cyclization of λ under the same conditions. A single observation of the rate of cyclization of N5 DNA at 40°C in 1.5 M NaCl solution gives a rate constant for cyclization of 7×10^{-3} min^{-1}, which is about 1/3 the rate for λ under the same conditions.

An experiment was performed to ascertain whether the cohesive ends of N1 are sufficiently sequence homologous to the cohesive ends of N6 and/or N5 DNA's so that they can mutually cohere. The technique employed was adapted from that of Yamagishi, Nakamura, and Ozeki (1965). The principle of the method is as follows: If molecule A, which has cohesive ends, is annealed at low concentration, it will cyclize. If it is annealed at a somewhat higher concentration, it will also form linear and cyclic multimers, which sediment more rapidly (Hershey, Burgi, and Ingraham,1963; Wang and Davidson, 1966b). If a mixture of a low concentration of molecule A and a high concentration of molecule B is annealed, B will form some rapidly sedimenting multimers. If the ends of A are not capable of cohering to the ends of B, A will form only cyclic monomers. If the ends of A are capable of cohering to the ends of B, some A molecules will be bound in the rapidly sedimenting multimers.

The results of experiments of this type with annealed mixtures containing low concentrations of P^{32}-labeled N1 DNA with a tenfold excess of cold N1, N6, or N5 DNA are shown in Fig. 7. It may be

Fig. 7. Zone centrifugation analyses of cohesive end homologies. 1.43 μg of P^{32}-N1 DNA and 14.3 μg of cold N1, N6, or N5 DNA's were mixed in 315 μl of 1 M NaCl, 0.01 M EDTA (pH 7) and incubated at 50°C for two hours after heating at 75°C for 10 min. The resulting DNA concentration was 50 μg/ml under which condition the formation of dimers or higher aggregates is favored (Wang and Davidson, 1966b). Zone centrifugation in a sucrose gradient was done at 30,000 rpm for 3.5 hours in a Spinco model L ultracentrifuge with an SW 50 L rotor. A linear sucrose gradient was obtained by mixing 5 and 20% (w/v) sucrose solutions containing 1 M NaCl and 0.02 M tris (pH 8) with a Buchler pump. One hundred μl of DNA solution was layered on top of the sucrose gradient. 7a, a mixture of P^{32}-N1 DNA and N1 DNA; 7b, a mixture of P^{32}-N1 DNA and N6 DNA; 7c, a mixture of P^{32}-N1 DNA and N5 DNA. For control experiments, the mixtures were heated at 75°C for 10 min to dissociate the cohered sticky ends. 7d, 7e, and 7f are for each heated mixture described above. The arrows indicate the probable positions of linear monomers, circular monomers, and linear dimers with relative ratios of sedimentation coefficients of 1.0, 1.13, and 1.31, respectively, as shown in 7a.

Fig. 7


(a)
1.13
1.31
1.0
N1 DNA
(d)
(b)
N6 DNA
(e)
(c)
N5 DNA
(f)
P32 cpm
fraction no.
0
500
1000
1500
2000
3000
10
20
30
40

seen that some labeled N1 DNA cosediments with the N1, and the N6 DNA multimers, but not with N5. In particular, the linear dimer peaks are well resolved in Figs. 7a and 7b. Figures 7d, 7e, and 7f show the results of the sedimentation experiments after heating and quenching the mixtures to dissociate the cohered multimers. Small amounts of circular molecules and linear dimers can be seen in Figs. 7d and 7e due to a finite cohesion rate after quenching. However, the main point is that the multimers are dissociated by a treatment which dissociates the cohered ends, as expected. Thus, the results clearly show that N1 and N6 DNA's are capable of mutual cohesion, but that N1 and N5 DNA's are not. This result is, of course, expected by analogy with the lambdoid phages (Baldwin, et al., 1966) in view of the genetic recombination between N1 and N6.

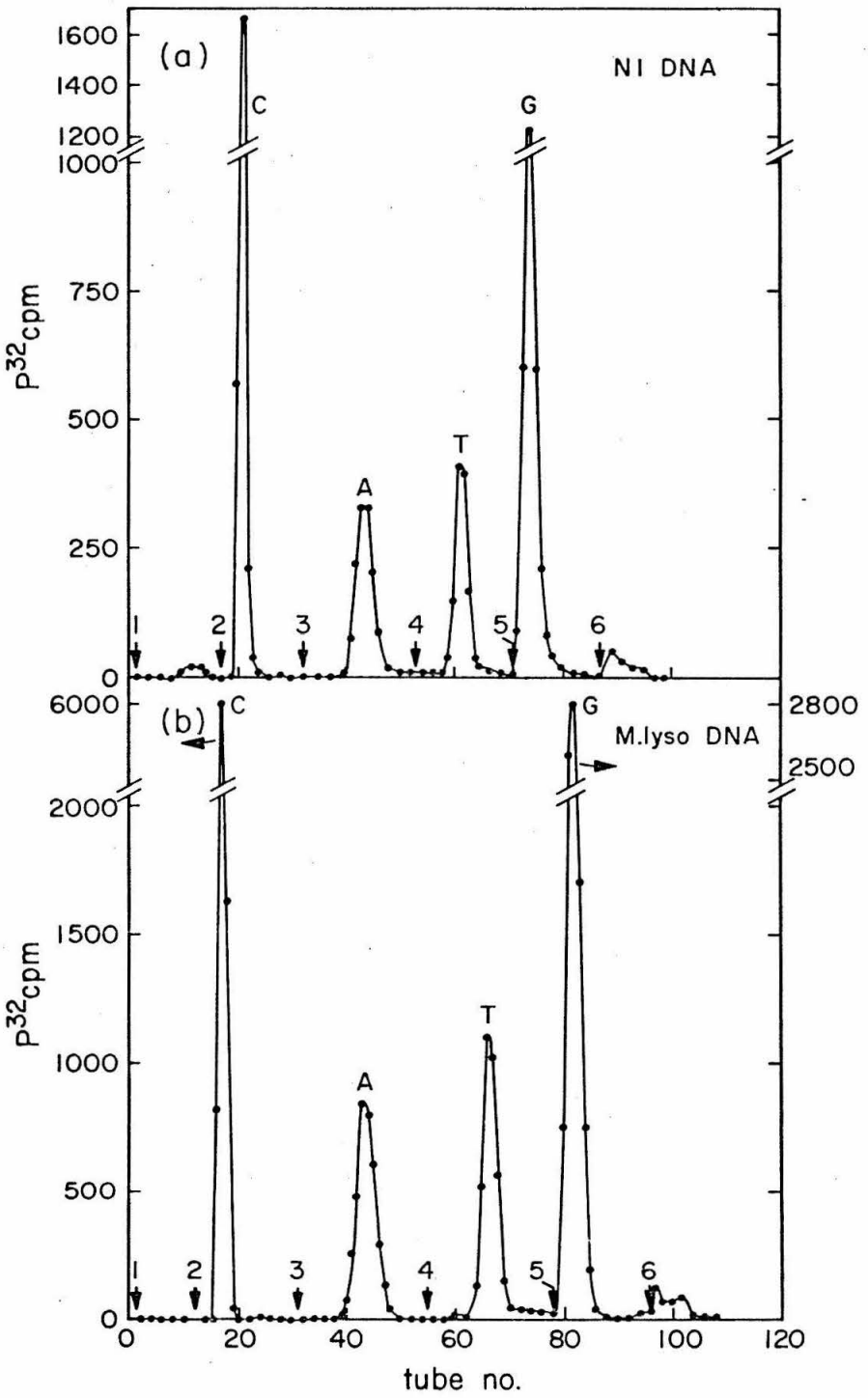
(c) Base composition of N1 DNA.

As will be discussed later, there is a discrepancy between the base composition of N1 DNA as deduced from its buoyant density and as deduced from its melting behavior. The possibility exists that this discrepancy is due to the presence of unusual bases such as 5-methylcytosine. It accordingly seemed worthwhile to measure the base composition and look for odd bases by chromatographic analysis. Base composition analysis of N1 and N6 DNA's by paper chromatography after acid hydrolysis has been carried out some time ago (Scaletti and Naylor, 1959). The methods used, however, were not highly accurate and the measured base composition, A = 18, T = 13, C = 37, G = 32%, did not conform to the expected equalities, A = T, G = C. In the present experiments, anion

exchange chromatography on Dowex-1 was used. This method gives a clean separation of several minor bases including 5-methylcytosine and is accurate for quantitative determinations of the main constituents. The elution profiles for the enzyme hydrolysates of N1 and M. lysodeikticus DNA's are shown in Fig. 8. From this figure the base composition of M. lysodeikticus DNA is evaluated as C = 37.1%, G = 35.4%, A = 13.6%, and T = 13.9%. The calculated GC content is 72.5 (± 2.0)%, in good agreement with the value of 72% reported by Lee, Wahl and Barbu (1956). For N1 DNA, we find C = 35.5%, G = 34.6%, A = 15.4% and T = 14.3%, corresponding to a GC content of 70.2 (± 2)%. There exists a minor amount of a mononucleotide which is eluted with 0.05 N HAc (Fig. 8a). This amounts to 0.37% of the total amount of nucleotides. 5-Methylcytosine is reported to be eluted at this point (Hurst, et al.,1953) and it is therefore possible that this peak is, in fact, due to 5-methylcytosine. The material in the eluates with 2 N HAc and 1 N NaAc for N1 and M. lyso DNA's is probably due to incomplete hydrolysis. It amounts to less than 2% of the total nucleotides.

Fig. 8. Elution profiles of enzymatic digests of N1 DNA and M. lysodeikticus DNA. The procedure is described in the text. The eluting solutions are: 1, 0.05 N HAc; 2, 0.5 N HAc; 3, 1 N HAc and 0.01 N NaAc; 4, 1 N HAc and 0.1 N NaAc; 5, 1 N HAc and 0.5 N NaAc; 6, 2 N HAc and 1 N NaAc. For N1 DNA, 5-MC = 708 cpm (0.37%), C = 6.81 X 10^4 cpm (35.5%), A = 2.95 X 10^4 cpm (15.4%), T = 2.72 X 10^4cpm (14.3%) and G = 6.58 X 10^4 cpm (34.6%) ((GC) = 70.2%). For M. lysodeikticus DNA, C = 2.13 X 10^5 cpm (37.1%), A = 7.78 X 10^4 cpm (13.6%), T = 8.0 X 10^4 cpm (13.9%) and G = 2.03 X 10^5 cpm (35.4%) ((GC) = 72.5%).

Fig. 8


(a)
NI DNA
C
G
T
A
1
2
3
4
5
6
1600
1400
1200
1000
750
500
250
0
P32cpm
(b)
M.lyso DNA
6000
2000
1500
1000
500
2800
2500
0
20
40
60
80
100
120
tube no.

4. Further Discussion

(a) Nature of the cohesive ends.

N1, N5, and N6 DNA's have cohesive ends leading to reversible cyclization or linear aggregation. We assume that the cohesive ends are mutually complementary, single-strand segments just as for the lambdoid phages (Kaiser and Wu, 1968). The rates of joining of the cohesive ends of the lambdoid phages and of phage 186 differ from the rate of renaturation of other single-strand DNA segments in that there is a rather high temperature coefficient for the cohesion reaction corresponding to an activation energy of about 25 kcal, which is much larger than the temperature coefficient and activation energy for ordinary renaturation. It has been suggested that the high activation energy for cohesion (which serves to make the linear DNA metastable at low temperatures) is due to a small amount of secondary structure in one or both of the single strand ends (Wang and Davidson, 1968). As reported in the Results section, the temperature dependence of the rate and equilibrium constants for the cyclization of the N phage DNA's are qualitatively similar to those for λ. It seems likely that whatever structural features are responsible for the high activation energy for the cohesion process in λ apply also to the N phages.

(b) Relations between N phages.

N1 and N6 phages are closely related but not identical, serologically (Naylor and Burgi, 1956). They are capable of genetic recombination. The buoyant densities of the phages are slightly different (Table 1). The data concerning the properties of the DNA molecules are consistent

with these properties of the phages. The buoyant densities of the DNA's differ by 0.004 g/ml. The molecular weights are the same. The cohesive ends are capable of mutual cohesion. The base sequences in the two DNA's are closely homologous. (We do not know whether the observed degree of homology of 92.5% as measured by membrane filter hybridization differs from 100% by more than the experimental uncertainty of the method.) The electron microscope renaturation studies show that there is no completely non-homologous region in the two DNA's of length greater than 1% of the genome.

There is a slight sequence homology between N1 and N5 DNA's (1.7%). The cohesive ends do not cohere with each other. The biological significance of the sequence homology is not known.

(c) Buoyant densities, melting temperatures and base compositions.

In the ionic medium used, the T_m's of N1, N5, and N6 DNA's are all the same within the experimental error of $\pm$ 0.2° and are 2.0° below that of M. lysodeikticus DNA. This corresponds to a base composition difference of 2.6% between M. lysodeikticus DNA and any one of the N phage DNA's, in reasonably good agreement with the direct chemical analyses for M. lysodeikticus and N1 DNA's, which give base compositions of 72.5% GC and 70.2% GC, respectively.

There are discrepancies between the base compositions inferred from the denaturation T_m's and from the buoyant densities. For the sake of discussion, we will assume that the T_m's are correct and that N1, N6, and N5 DNA's all have a GC content about 2.5% below that of M. lysodeikticus DNA.

The buoyant densities in CsCl of the several DNA's (Table 2) in conjunction with the relation $d\theta/dX_{GC} = 0.10$ (Schildkraut, Marmur, and Doty, 1962) imply that the GC contents are 64%, 67%, and 70%, for N1, N6, and N5, respectively (with M. lysodeikticus taken as 72%). Thus, the buoyant densities in CsCl are anomalous for N1 and N6 DNA's; this suggests that some modified bases or nucleosides are present, either in N1 and N6 DNA or (conceivably) in M. lysodeikticus DNA. (By analogy with other cases, it is assumed that these odd bases engage in the same hydrogen bonding patterns as the normal bases and do not markedly affect the denaturation T_m.)

By comparison with M. lysodeikticus, the buoyant density of N1 DNA in Cs_2SO_4 is as expected for its GC content, the buoyant density of N6 DNA is slightly (0.0006) too low, and that of N5 is 0.0047 higher than expected.

Our chemical analyses of N1 DNA and of M. lysodeikticus DNA have not succeeded in revealing significant amounts of odd nucleosides. The buoyant density of wheat germ DNA, which contains 6% 5-methylcytosine (5-MC) (Chargaff, 1955) is 0.003 g/ml less than that expected for a DNA with only cytosine (Schildkraut, Marmur, and Doty, 1962). The observed anomaly of 0.006 g/ml of N1 DNA would then imply 12% 5-MC. The chromatographic peak in Fig. 8a, which may be due to 5-MC, contains only 0.4% of the total nucleosides. The chromatographic elution profile of N1 DNA is in other respects normal; if odd nucleosides are present, they must chromatograph with one of the common nucleosides.

The anomalous densities cannot be due to hydroxymethylcytosine, to glucosylated hydroxymethylcytosine, or to hydroxymethyluracil, because these constituents all increase the buoyant density (Erikson and Szybalski, 1964).

We conclude that the causes of the density anomalies, whether they be odd nucleosides or some unusual sequence effects, have not been revealed in our experiments.

Acknowledgments

We are deeply indebted to Prof. J. V. Scaletti who first interested us in the N phages and who has generously provided us with information, bacteria, and phages. The investigations of the N phages in this laboratory were initiated by Dr. J. G. Wetmur. We thank Dr. M. Dahmus for advice on the DNA-DNA hybridization experiments. The patient and generous guidance we have received from Dr. Jean Weigle in phage techniques was both invaluable and unforgettable.

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Chapter 2

COVALENTLY CLOSED CIRCULAR DNA FROM MICROCOCCUS LYSODEIKTICUS CELLS INFECTED WITH PHAGE N1

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The DNA of the Micrococcus lysodeikticus bacteriophage, N1, contains cohesive ends so that it is possible to convert the linear molecule to a circular form in vitro by annealing, and to convert the circular molecule back to the linear form by heating and quenching (Wetmur, Davidson, and Scaletti, 1966). The DNA of coliphage λ and the DNA of certain other temperate coliphages also contain cohesive ends. When the linear λ DNA molecules are injected into a bacterium upon infection, they are converted to a covalently closed twisted circular form (Young and Sinsheimer, 1964; Bode and Kaiser, 1965; Ogawa and Tomizawa, 1967; Bode and MacHattie, 1968). For λ it is also known that additional covalently closed twisted circles are made after infection (Young and Sinsheimer, 1967). Covalently closed twisted circles also occur as an intracellular form of the DNA of the Salmonella phage, P22. This DNA in its mature form is linear, terminally redundant, circularly permuted, but without cohesive ends (Rhoades and Thomas, 1968). In the present communication we report that covalently closed twisted circles of N1 DNA occur as an intracellular form after N1 infection also.

† Contribution No. 3727

Experimental. A strain of M. lysodeikticus, ML 1, and its bacteriophage, N1, were used. ML broth contains 1.5% Bactotryptone (Difco), 0.5% yeast extract (Difco), 0.5% glucose, and 0.5% NaCl (pH 7.4 before autoclaving) (Scaletti and Naylor, 1959). TE buffer is 0.01 M Tris OH, 0.01 M Na_2H_2 EDTA, pH 7.0. SDS solution contains 5% sodium dodecyl sulfate in TE buffer.

A 160-ml bacterial culture was grown in ML broth at 32° with vigorous aeration. One generation time is about 40 min. At $A_{600} = 0.6 (4 \times 10^8$ cells/ml), 10^{12} pfu of N1 phage were added, giving a multiplicity of infection of about 16. Aliquots (40 ml) were withdrawn at 15, 30, 60, and 90 min after infection. The bacterial cells were pelleted at 7,000 rpm for 15 min in a SW 25.1 rotor, resuspended in 5 ml of TE buffer, and incubated with 2 mg of lysozyme for 15 min at 37°. After addition of 0.5 ml of SDS solution, and 0.2 ml of 2 mg/ml preincubated pronase solution, the solution was maintained at 37° for 1 hr. Cell lysis occurs and the solution becomes clear and very viscous. Bacterial debris and high molecular weight DNA are pelleted by centrifugation in a type 40 Spinco rotor at 29,000 rpm for 15 min (Kiger, Young, and Sinsheimer, 1968).

The procedure of Radloff, Bauer, and Vinograd (1967) for the separation of closed circular DNA from linear or nicked circular DNA by buoyant banding in CsCl with added ethidium bromide was followed. The CsCl and ethidium bromide were added directly to the above solution. Centrifugation of the four samples was done at 42,000 rpm for 24 hours in a SW 50 Spinco rotor. No fluorescent band due to closed circular DNA below the main band could be detected by visual examination under uv light after centrifugation (Fig. 1a). Therefore the regions below the main band in the four tubes were pooled and recentrifuged. A closed circular band was then evident (Fig. 1b). Basic protein film samples for electron microscopy were prepared from the several DNA bands by spreading a DNA-cytochrome c solution in 0.5 M salt (CsCl or a mixture of NH_4Ac and CsCl)

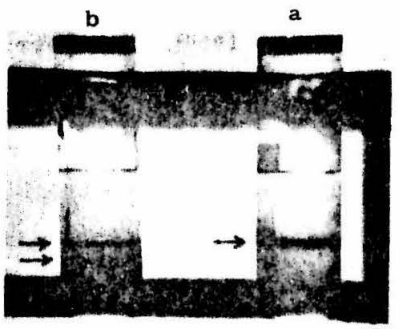

Fig. 1. Photographs under uv light of the fluorescent DNA bands (arrows) after CsCl, ethidium bromide centrifugation. (a) First centrifugation; (b) after pooling the appropriate region below the main band in (a) and recentrifuging. The lower fluorescent band contains the covalently closed circles.

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onto 0.25 M NH_4Ac. The grids were uranyl stained and/or rotary shadowed with Pt-Pd, followed, sometimes, by shadowing in one direction (Wetmur, Davidson, and Scaletti, 1967; Davis and Davidson, 1968).

Results and Discussion. Fig. 1b shows that there was sufficient closed circular DNA in the pooled, recentrifuged sample to give a visible band in the expected position. Linear or open circular N1 DNA and M. lyso DNA have buoyant densities in CsCl (without added ethidium bromide) of 1.718 and 1.726 and form a single band in the present CsCl-ethidium bromide procedure. Electron micrographs from the lower band showed twisted circular structures of the expected size for N1 DNA, as shown in Figs. 2a, 2d, and 2e. The lighter band contained open circular N1 DNA (Figs. 2b and 2c) and linear DNA.

N1 phages give an abortive infection in the mutant bacterium ML 53-20, derived from ML 1 (Naylor and Burgi, 1956). Excess DNA is synthesized and the cells lyse, but no phage are formed. A faint band of closed

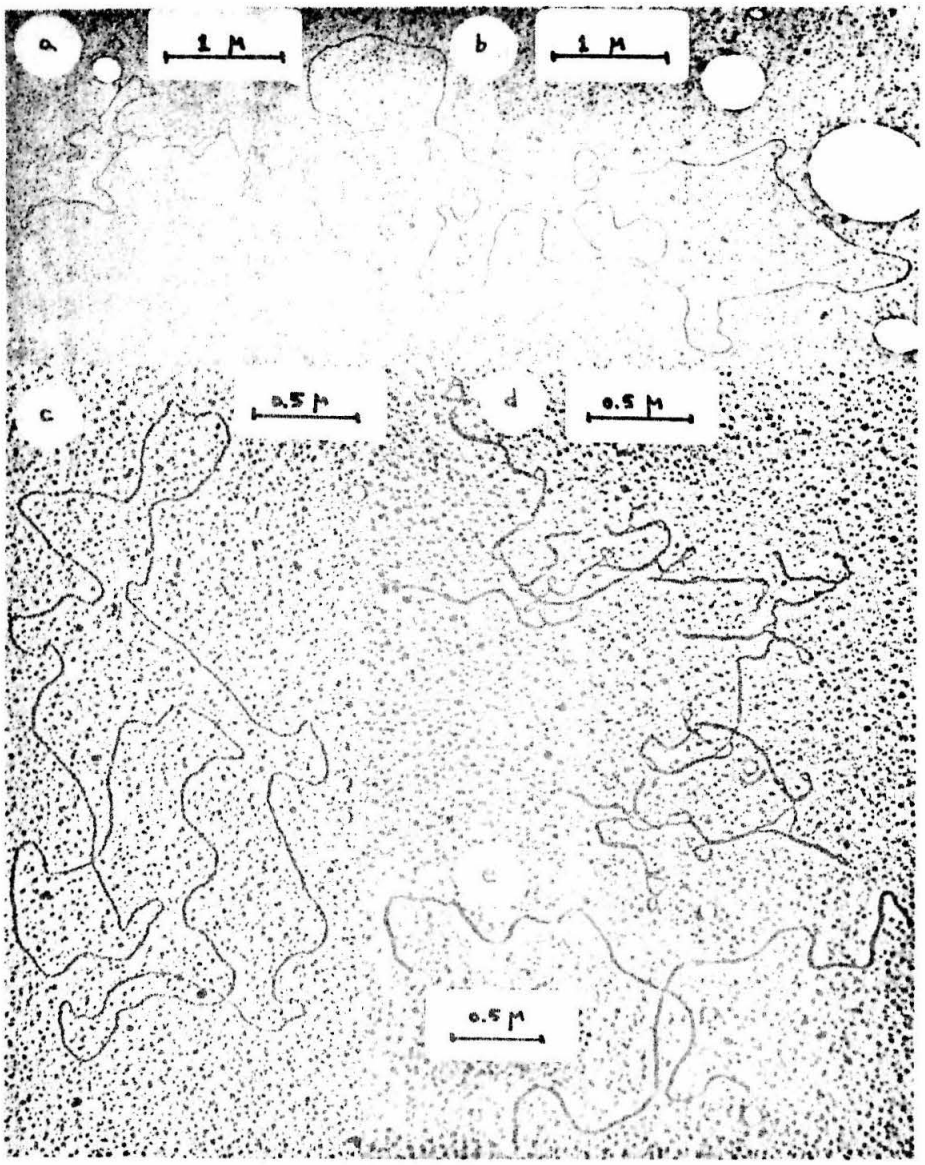

Fig. 2. Electron micrographs of twisted circles (a, d, e) and open circles (b, c) of N1 DNA from the heavy and light bands respectively. (a) and (b), stained only; (c), (d), and (e), stained and shadowed.

circular DNA was obtained after premature lysis in such an infection too.

The results thus show that a twisted closed circular form of N1 DNA is formed intracellularly after infection. It is not known whether this is newly synthesized DNA or the infecting DNA. It thus appears that a closing enzyme (ligase) exists in M. lyso cells. This is in accord with the high uv resistance of the bacterium and possibly with the findings of Elder and Beers (1965) dealing with the repair capacity of M. lyso extracts for uv damaged transforming DNA.

The result confirms the expectation that the formation of intracellular covalently closed supertwisted circles after phage infection is a rather common phenomenon. Closed circular N1 DNA has been made in vitro (Wang, Baumgarten, and Olivera, 1967). Thus, physical studies on in vivo and in vitro closed circular molecules with a high GC content (70%) are now possible.

Further studies on the time course of formation of the closed circular DNA after infection and of its significance in the infection process are now being made.

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Chapter 3

COVALENTLY CLOSED MINICIRCULAR DNA IN MICROCOCCUS LYSODEIKTICUS

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During the search for an intracellular form of N1 phage DNA in infected M. lysodeikticus cells described in the preceding communication (Lee, Davidson, and Scaletti, 1968), we have discovered the existence of small closed circular DNA molecules (which we call minicircles) in uninfected as well as infected cells. We estimate about one minicircle per bacterium. The minicircles have a uniform contour length of 0.445 (±0.024)μ, corresponding to a molecular weight of 0.88×10^6. Very recently, Cozzarelli, Kelly, and Kornberg (1968) have found minute circular DNA molecules with a contour length of 0.96 μ in E. coli strain 15. The biological significance of these minicircles is not known.

Experimental. Some of the experimental details are described in the preceding paper (Lee, Davidson, and Scaletti, 1968). Neopeptone medium, which contains 1% Difco Neopeptone, 0.5% NaCl, 0.5% glucose, 0.002 M $MgSO_4$, is a modification of the low phosphate medium used by Kellenberger, Zichichi, and Weigle (1961). For general purposes, ML 1 bacteria were grown in 500 ml of ML broth at 32° C to $A_{600} = 0.4$ (~ 3×10^8 cells/ml). For P^{32} labeling, bacteria were grown in 500 ml of Neopeptone medium with 2-3 millicuries of carrier free P^{32} phosphoric acid. The DNA specific activities so obtained are about 4×10^4 cpm/μg M. lyso DNA and 5×10^5 cpm/μg N1 DNA. The cells were pelleted, resuspended, and gently lysed as

† Contribution No. 3728

described in the preceding paper. Bacterial debris and high molecular weight pieces of bacterial DNA were sedimented at 35,000 rpm for 1 hour in an SW 50L rotor. Cesium chloride and ethidium bromide were added directly to the supernatant solution. Buoyant density centrifugation in CsCl, with or without ethidium bromide, was done at 40,000 rpm for 64 hours or longer.

Before banding the P^{32} DNA, a broad background due to low molecular weight P^{32}-labeled components was partially removed by hydroxy-apatite fractionation. The lysed supernatant, after pelleting the bacterial debris, was dialyzed against 0.1 M phosphate buffer (pH 6.8). DNA was absorbed by passage through about 1 gram of hydroxy apatite on a column. Elutable counts were removed with 0.1 M phosphate washes, and the DNA eluted in a volume of 6 ml by washing with 0.3 M phosphate solution.

Because of the small amount of minicircular DNA, electron microscopy was used for detection and assay. Generally speaking, the techniques described by Davis and Davidson (1968) were used. A grid was scanned as thoroughly as possible at 2300 X (on the 35-mm film) and the minicircles were photographed at 9500 X. Contour lengths (about 20 cm for a minicircle) were measured on tracings made on Nikon shadowgraph at 50 X. A diffraction grating with 21,600 lines/cm was used in each experiment to calibrate the electron microscope.

The purity of the bacterial strains, ML 1 and ML 53-20, was in part checked by plating with phage N1. ML 1, which is sensitive to N1, gave clear plaques. No resistant bacterial colonies were present. There were no plaques at all with ML 53-20 bacteria, a uv induced mutant of ML 1, which give an abortive infection with N1 (Naylor and Burgi, 1956).

Results and Discussion. The minicircles were first observed in the fractions just below the main DNA band in a CsCl, ethidium bromide centrifugation during the search for intracellular closed circular N1 DNA described in the preceding paper. They were then observed in the same

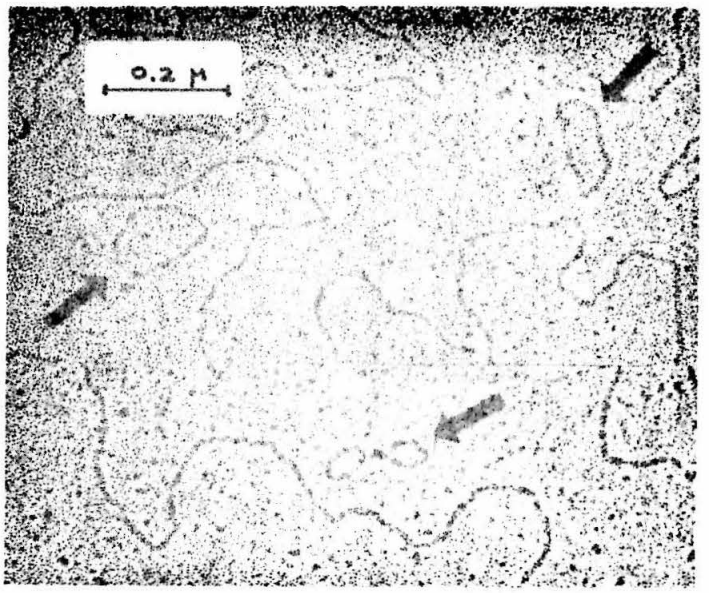

Fig. 1. Some examples of minicircles (arrows). This sample was taken from the DNA peak in Fig. 3. The specimen was stained with uranyl salt and shadowed with Pt/Pd during rotation.

fractions of DNA preparations of uninfected bacteria. They were not present in a sufficiently high concentration to be visible as a fluorescent band in the centrifuge tube. Several typical molecules are shown in Fig 1.

A histogram of the contour length distribution for measurements on 63 molecules is shown in Fig. 2. The weight average contour length is 0.445 (± 0.024)μ. By comparison with the contour length of N1 DNA which has a molecular weight of 33×10^6 (Wetmur, Davidson, and Scaletti, 1966), the molecular weight of the minicircles is calculated as $0.88(\pm 0.05) \times 10^6$.

The buoyant density of the minicircular DNA was measured by banding a P^{32} labeled M. lyso lysate in CsCl in the absence of ethidium bromide,

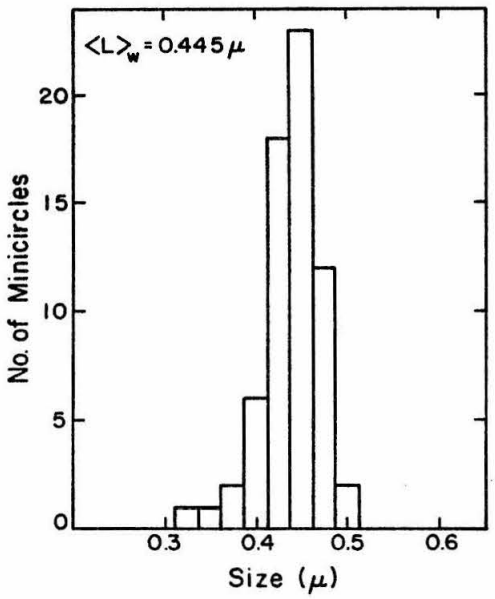

Fig. 2. The contour length distribution of minicircles (total 63 molecules). The weight average contour length is 0.445 (±0.024)μ which corresponds to the molecular weight of 0.88 (±0.05) × 10^6.

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and assaying aliquots of the same fractions for minicircles by electron microscopy. The result (Fig. 3) is that minicircular DNA has the same density as the rest of the bacterial DNA to less than the density difference between fractions (0.005 g/cc).

In the electron microscope, most of the minicircles seen had an open structure, but a few had one or two tertiary turns visible.

An approximate estimation indicated that there is about one minicircle per bacterium. The number of minicircles in a preparation was estimated from the number (usually 15–20) seen in a single square of an electron microscope grid by assuming that the efficiency of attachment to a cytochrome film is the same for the minicircles as for N1 DNA which was mounted under identical conditions from a solution with a known DNA concentration. Cozzarelli, Kelly, and Kornberg (1968) report an average of 15 minicircles

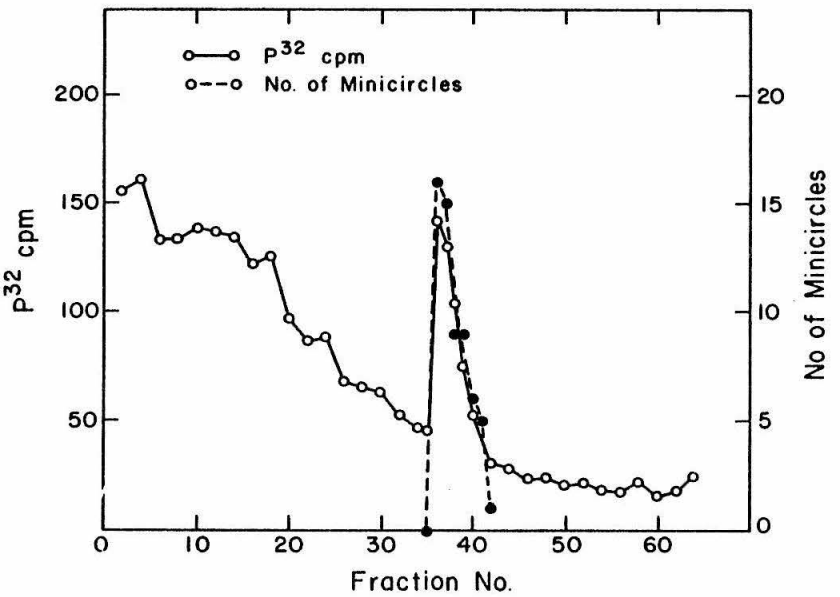

Fig. 3. The identity of the buoyant densities between minicircles and M. lysodeikticus DNA. P^{32} counts of total DNA (o ——— o) and the counts of minicircles in a square of an electron microscope grid (● - - - ●) are plotted vs. fraction number.

per cell in E. coli 15. Minicircles were found during exponential growth and in stationary cultures of ML 1. They are also found in ML53-20.

The biological significance of these minicircles, which can code for only 400 amino acids, is unknown. It should be recalled that Radloff, Bauer, and Vinograd (1967) observed circular DNA with lengths ranging down to 0.2 μ in HeLa cells, but these minute circles were not a homogeneous fraction of uniform length.

We are grateful to Professor J. V. Scaletti for M. lysodeikticus strains and phage N1, and for his advice and interest. We thank Professor A. Kornberg and collaborators for communicating their results before publication. The guidance and help of Mr. Ron Davis in matters electron microscopical has been invaluable. This research has been supported by grant GM 10991.

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CHAPTER 4

A Physical Study by Electron Microscopy of the Terminally Repetitious, Circularly Permuted DNA from the Phage Particles of E. coli 15

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Running title: Coliphage 15 DNA

Summary

Several strains of E. coli 15 harbor a prophage. Viral growth can be induced by exposing the host to mitomycin C or to uv irradiation. The coliphage 15 particles from E. coli 15 and E. coli 15 T^- appear as normal phage with head and tail structure; the particles from E. coli 15 TAU are tailless. The complete particles exert a colicinogenic activity on E. coli 15 and 15 T^-, the tailless particles do not. No host for a productive viral infection has been found and the phage may be defective. The properties of the DNA of the virus have been studied, mainly by electron microscopy. After induction but before lysis, a closed circular DNA with a contour length of about 11.9 μ is found in the bacterium; the mature phage DNA is a linear duplex and 7.5% longer than the intracellular circular form. This suggests the hypothesis that the mature phage DNA is terminally repetitious and circularly permuted. The hypothesis was confirmed by observing that denaturation and renaturation of the mature phage DNA produce circular duplexes with two single-stranded branches corresponding to the terminal repetition. The contour length of the mature phage DNA was measured relative to φX RFII DNA and λ DNA; the calculated molecular weight is 27×10^6. The length of the single-stranded terminal repetition was compared to the length of φX 174 DNA under conditions where single-stranded DNA is seen in an extended form in electron micrographs. The length of the terminal repetition is found to be 7.4% of the length of the nonrepetitious part of the coliphage 15

DNA. The number of base pairs in the terminal repetition is variable in different molecules, with a fractional standard deviation of 0.18 of the average number in the terminal repetition. A new phenomenon termed "branch migration" has been discovered in renatured circular molecules; it results in forked branches, with two emerging single strands, at the position of the terminal repetition. The distribution of branch separations between the two terminal repetitions in the population of renatured circular molecules was studied. The observed distribution suggests that there is an excluded volume effect in the renaturation of a population of circularly permuted molecules such that strands with close beginning points preferentially renature with each other. This selective renaturation and the phenomenon of branch migration both affect the distribution of branch separations; the observed distribution does not contradict the hypothesis of a random distribution of beginning points around the chromosome.

1. Introduction

Certain strains of E. coli derived from strain 15 lyse when treated with common inducing agents (Ryan, Fried, and Mukai, 1955; Mukai, 1960). The lysis was originally attributed to production of a colicin, but later studies showed that this lysis was due to the induction and release of bacteriophage-like particles (Sandoval, Reilly, and Tandler, 1965; Endo, Ayabe, Amako, and Takeya, 1965; Mennigmann, 1965). These particles may be defective phages; they kill certain E. coli 15 strains, but efforts to obtain a productive infection of a host have not been successful. Of the several names which have been given to these particles--colicin 15 particles, phage Ψ (Mennigmann, 1965) and coliphage 15 (Frampton and Mandel, 1968), we prefer the latter.

The morphology and colicinogenic activity of coliphage 15 have been described in the references given above. The buoyant density and melting temperature (Frampton and Mandel, 1968) of the DNA, and its degree of homology with host DNA (Cowie and Szafranski, 1965) have been reported.

The present work was initiated during a study of the small (0.67 μ) plasmid DNA molecules in E. coli 15 strain (Cozzarelli, Kelly and Kornberg, 1968) when we sought to determine whether there was any relation between the small plasmid DNA and the colicin particles. No relation has been discovered, but it was observed that after induction with mitomycin or uv light, an additional intracellular closed circular DNA of contour length 11.9 μ was formed. Later, lysis occurred and

mature phage particles were released.

This intracellular DNA is presumably a precursor of the mature linear phage DNA, but its contour length is 0.9 μ shorter. This discrepancy suggested the hypothesis that the mature DNA is terminally repetitious.

We have found that coliphage 15DNA is indeed terminally repetitious and circularly permuted. The present investigation deals primarily with properties of the DNA related to its terminal repetition and circular permutation. In part, this work is a physico-chemical study of the renaturation of complementary, circularly permuted, terminally repetitious DNA strands.

Electron microscopy has been the crucial physical tool for obtaining the results reported here. Technical advances in the methodology for the study of terminal repetitions and circular permutations by electron microscopy will be described.

2. Materials and Methods

(a) Bacterial strains

E. coli 15 wild type (WT) and *E. coli* 15 T^- were gifts from Professor S. S. Cohen. *E. coli* 15 TAU and *E. coli* 15 TAU-bar were gifts from Dr. F. Funk of Professor R. L. Sinsheimer's laboratory. A gift of *E. coli* 15 TAU-bar was also generously made by Professor P. C. Hanawalt. The *E. coli* strains S/6, B/5, W3110, and C600 were from stocks of Professor R. S. Edgar and the late Dr. J. J. Weigle.

(b) Growth media

E. coli 15 WT was grown in M9 buffer supplemented with 0.4% glucose, 1 m*M* $MgSO_4$, and 0.1 m*M* $CaCl_2$. M9 buffer contains 7 g of Na_2HPO_4, 3 g of KH_2PO_4, 1 g of NH_4Cl, and 0.5 g of NaCl per liter.

E. coli 15 T^-, TAU, and TAU-bar were grown in the above medium supplemented with 20 μg/ml thymine, 30 μg/ml uracil and cytosine, and 1/20 volume of an amino acid solution which contains 7.6 mg/ml arginine, 6 mg/ml of methionine, 2.8 mg/ml of tryptophan, and 7.5 mg/ml of proline.

Other strains of *E. coli* were usually grown in tryptone broth.

(c) Procedures for induction by mitomycin C and ultraviolet light, and purification of the phage

Mitomycin C induction was carried out essentially as described by Endo, *et al.* (1965) and Okamoto, *et al.* (1968). Two hundred ml of a bacterial culture were grown at 37° with vigorous aeration in one of

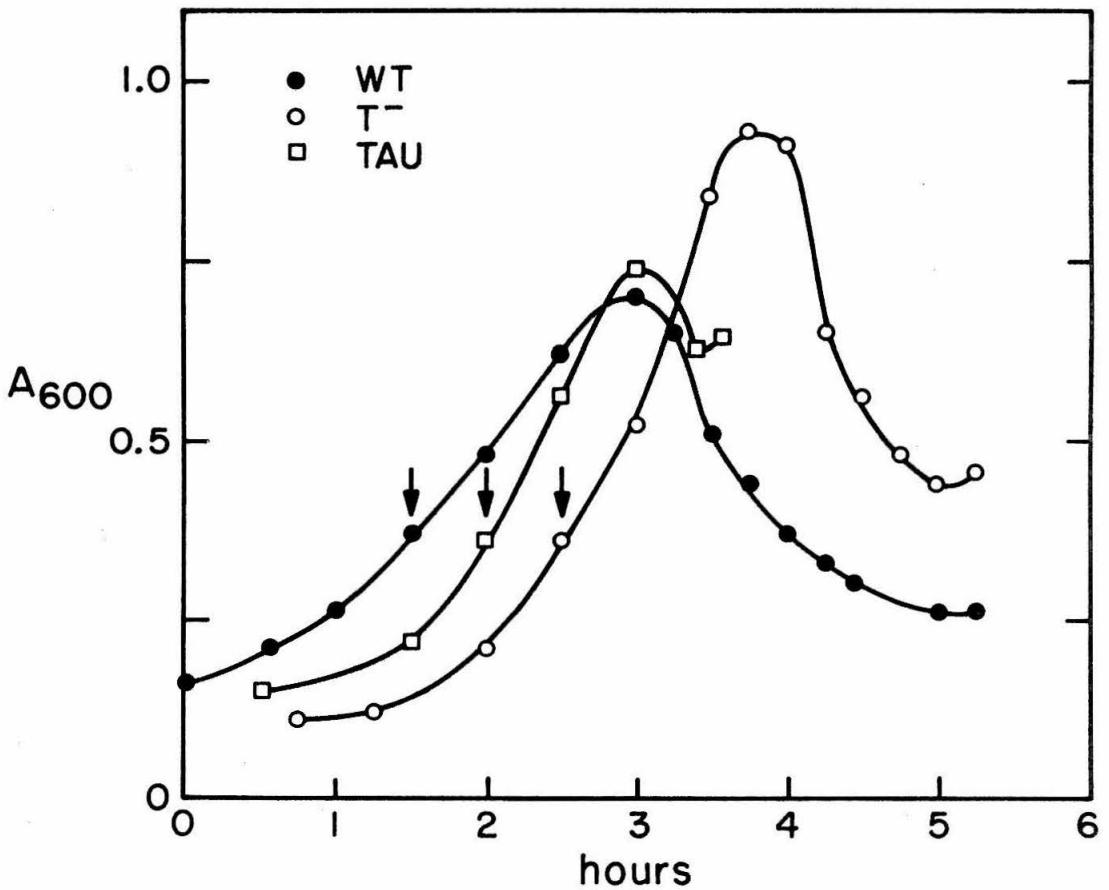
the media described above. At a cell concentration of 2-3 $\times 10^8$ cells/ml, mitomycin C (Nutritional Biochemical Company) was added to give a final concentration of 3 μg/ml. Aeration was continued for another 15 min. The cells were pelleted by centrifugation at 7000 rpm for 10 min in a Sorvall centrifuge, resuspended in 200 ml of fresh medium without mitomycin, and aeration continued. The culture became foamy and the turbidity started to decrease about 1 hr after resuspension. The total turbidity decrease was usually 20-30%. Bacterial growth was stopped when the turbidity again started to increase, by adding $CHCl_3$ and cooling to 0°.

For uv induction, 20 ml of a cell suspension (2-3 $\times 10^8$ cell/ml) in a Petri dish with a solution depth of less than 5 mm were gently swirled under a 15 watt germicidal lamp at a distance of about 40 cm. Typical growth curves are shown in Fig. 1. Exposures of 0.5, 1, and 2 min were tested; 1 min exposure was optimal. Under these conditions a λ lysogen also gives maximal lysis (Parkinson, 1969).

Unlysed bacteria and bacterial debris were removed by centrifugation in a Sorvall. Phage were then pelleted by centrifugation in a Spinco Model L centrifuge at 22,000 rpm for 3 hrs with an SW 25.2 rotor or at 30,000 rpm for 3 hrs with an SW 50.1 rotor, depending on the volume. The phage pellet was resuspended in a small volume of 0.1 M NaCl, 0.01 M Tris, 0.01 M $MgSO_4$ (pH 8). This solution was used for plaque assay and colicin tests. For the study of the DNA, the phage were further purified by adding CsCl to ρ = 1.50 g/ml and

Fig. 1. Typical growth curves for uv induction. The E. coli strains indicated in the figure were grown in 20 ml of medium as described in the text. Turbidity at 600 mμ was measured. Arrows indicate where the cell suspensions were irradiated with ultraviolet light for 1 min.

Fig. 1



buoyant banding. The phage band was collected and dialyzed against 0.1 M NaCl, 0.01 M Tris, 0.01 M $MgSO_4$ (pH 8) for storage. About 2×10^{13} phage particles corresponding to one milligram of DNA were typically obtained from a 200-ml culture.

Colicin tests with the phage suspension were made as described by Mukai (1960).

(d) DNA extraction

Native DNA was extracted from the phage suspension after dilution to an A_{260} of 0.2, either by heating for 10 min at 80° C in 0.1 M NaCl, 0.01 M EDTA (pH 7.3) or by dialysis against 8 M urea, 0.1 M NaCl, 0.01 M EDTA (pH 8.0) at 25° C, followed by dialysis against 0.1 M NaCl, 0.01 M EDTA. (All EDTA buffers were prepared by adjusting the pH of an Na_2EDTA solution with NaOH.) Both procedures gave essentially complete lysis of the phage and good native DNA molecules.

(e) Isolation of closed circular DNA molecules during induction

The cells were harvested 15 min and 30 min after induction with mitomycin C. NaN_3 at a final concentration of 0.01 M was added to the cell suspension which was quickly chilled. The closed circular DNA was then isolated by a gentle lysis, buoyant banding in CsCl-ethidium bromide procedure (Radloff, Bauer, and Vinograd, 1967; Lee, Davidson, and Scaletti, 1968).

(f) DNA renaturation

DNA was denatured and then renatured for electron microscopy by alkaline denaturation followed by formamide renaturation. This

procedure minimizes the number of single-strand breaks introduced during the manipulations. Ten μl of phage suspension, containing 5 μg of DNA, were diluted to 0.4 ml with 0.022 M EDTA (pH 8.0). Lysis and denaturation were achieved by addition of 50 μl of 1.0 M NaOH. After 10 min the solution was neutralized by the addition of 50 μl of 1.8 M Tris-HCl, 0.2 M Tris-OH (pH ~ 7). Renaturation at room temperature was then achieved by the addition of 0.5 ml of formamide (Malinckrodt, 99%) and incubation in the resulting final volume of 1.0 ml for 1-10 hrs. The pH before renaturation was 8.5 and the final pH after standing was 7.5-8.0. Renaturation was stopped by dialysis against 0.1 M NaCl, 0.01 M EDTA (pH 7.3) at 5° C.

The hybridization of a mixture of coliphage 15 (WT) and coliphage 15 (TAU) DNA's was carried out similarly except that the mixture contained 5 μg of each kind of DNA. For one experiment, in which sheared wild type DNA was used with whole TAU DNA, the shearing was accomplished by forcing an alkaline DNA solution from a 0.5 ml disposable syringe through a 27 gauge needle with maximum thumb pressure 30 times. This treatment resulted in fragments of single-strand DNA with an average size of 1/7 that of the whole strand.

(g) Electron microscopy

(1) Phage. Phage particles were negatively stained with 2% phosphotungstic acid (pH 8) or 1% aqueous uranyl acetate.

(2) DNA. DNA was prepared for electron microscopy by the basic protein film technique (Kleinschmidt and Zahn, 1959). Two

variations of this technique were used:

Aqueous technique. This technique is essentially that described by Davis and Davidson (1968) and in more detail in Davis (1969). Thirty μl of a solution containing about 0.5 μg/ml of DNA and 0.1 mg/ml of cytochrome C in 0.5 M ammonium acetate (pH 7) were spread onto a clean glass slide. This solution (hyperphase) was then allowed to flow down the glass slide onto a clean surface of 0.25 M NH_4Ac (hypophase). The protein-DNA mixed film was picked up on a parlodion coated grid and stained with uranyl acetate. In some cases the grids were also rotatory shadowed with platinum-palladium.

Formamide technique. When single-strand DNA is mounted by the aqueous technique it is collapsed upon itself through nonspecific intrastrand base interactions. By using a suitable concentration of formamide, both single- and double-stranded DNA can be displayed in an extended form suitable for contour length measurements and topological observations (Westmoreland, Szybalski, and Ris, 1969). One particular prescription for this procedure is the following: The hyperphase is made by mixing 10 μl of solution containing 0.05 μg of DNA with 10 μl of 1 mg/ml cytochrome C in 1 M ammonium acetate, 0.1 M Tris (pH 7.9); 40 μl of water; and 40 μl of formamide. The hypophase contains 0.01 M Tris (pH 7.9) and 10% formamide. Fifty μl of the hyperphase is spread onto the hypophase as described above. Grids were usually stained with uranyl acetate and rotatory shadowed with platinum-palladium to obtain maximal contrast.

With the formamide technique, both single- and double-stranded DNA appear as well extended threads. The latter is usually slightly thicker and more heavily contrasted. A reasonably long stretch of a DNA molecule can usually be classified with confidence as either single- or double-stranded; however, it is at present difficult to precisely locate a junction between single- and double-stranded DNA.

(3) Contour length measurements. Electron micrographs were taken on 35-mm film with a Philips EM 300 electron microscope using a 50 μ objective aperture and 60 kv accelerating voltage. Tracings were made by projecting the negative with a Nikon shadowgraph and were measured with a map measurer.

In our experience the most effective way of obtaining accurate reproducible molecular length measurements for single- and double-stranded DNA molecules is to mount the DNA molecule of unknown length and a standard DNA molecule, either single- or double-stranded to correspond with the unknown, in the same basic protein film and to photograph them simultaneously. This procedure virtually eliminates magnification errors and minimizes differential environment effects on the length of the DNA. Under favorable conditions, relative length measurements are reproducible to ± 0.2%. Even for a homogeneous DNA there is a distribution of contour lengths on a single grid due, we believe, to inherent fluctuations, at the molecular level, in the length per base pair of the DNA trapped in the film. Further information about this topic is presented in the Results section below. It is discussed in detail by Davis (1969) and by Davis, Simon, and Davidson (1969).

3. Results and Discussion

(a) Properties of coliphage 15 particles

When strains of E. coli 15 wild type (WT), T^-, TAU, and TAU-bar were treated with uv light or mitomycin, the production of phage occurred for all cases except TAU-bar (Fig. 1) in agreement with previous results (Ryan, et al., 1955; Mukai, 1960; Mennigmann, 1965; Endo, et al., 1965, Sandoval, et al., 1965; Cozzarelli, et al., 1968). The essential results of the colicin tests, as summarized in Table 1, are that coliphage 15 (WT) and coliphage 15 (T^-) were colicinogenic to both strains, but not to E. coli 15 TAU and 15 TAU-bar. The particles of coliphage 15 (TAU) gave negative results with all E. coli 15 strains in this test. This defective colicinogenicity is probably due to the morphological defect described below.

Productive viral infections were not obtained with either coliphage 15 (WT) or (T^-) with any of the hosts of E. coli tested, including strains 15 WT, 15 T^-, 15 TAU, 15 TAU-bar, W3110, C600, S/6, and B/5.

The morphology of coliphage 15 (WT) as shown in the electron micrographs in Plate I (a), (b), (c), and (d) is quite consistent with the earlier observations of Mennigmann (1965) and Endo, et al. (1965). The phage has a regular hexagonal head with a diameter of 600 Å connected to a sheath of length 1000 Å and width 200 Å by a narrow neck of length 120 Å. In some cases an end plate is visible [Plate I (c)]. Many phage particles have a sheath which is quite precisely one half the full sheath length, but with a protruding core

TABLE 1

Properties of coliphage 15

Strains of E. coli 15	Inducibility (UV and mitomycin C)	Morphology of phage	Plaque assay on various E. coli strains	Colicin tests				
				WT	T^-	TAU	TAU-bar	C600*
Wild type (WT)	+	intact phage free heads free tails	-	+	+	-	-	-
T^-	+	same as above	-	+	+	-	-	-
TAU	+	free heads no tails	-	-	-	-	-	-
TAU-bar	-							

* In addition to C600, W3100 and B/5 were used as controls.

Plate I. Coliphage 15 (WT) and coliphage 15 (TAU) particles.

(a) An intact coliphage 15 (WT) particle from the crude lysate

(b) Common type of (WT) particle from the crude lysate showing a half sheath and core

(c) Free (WT) tail showing an end plate with spikes

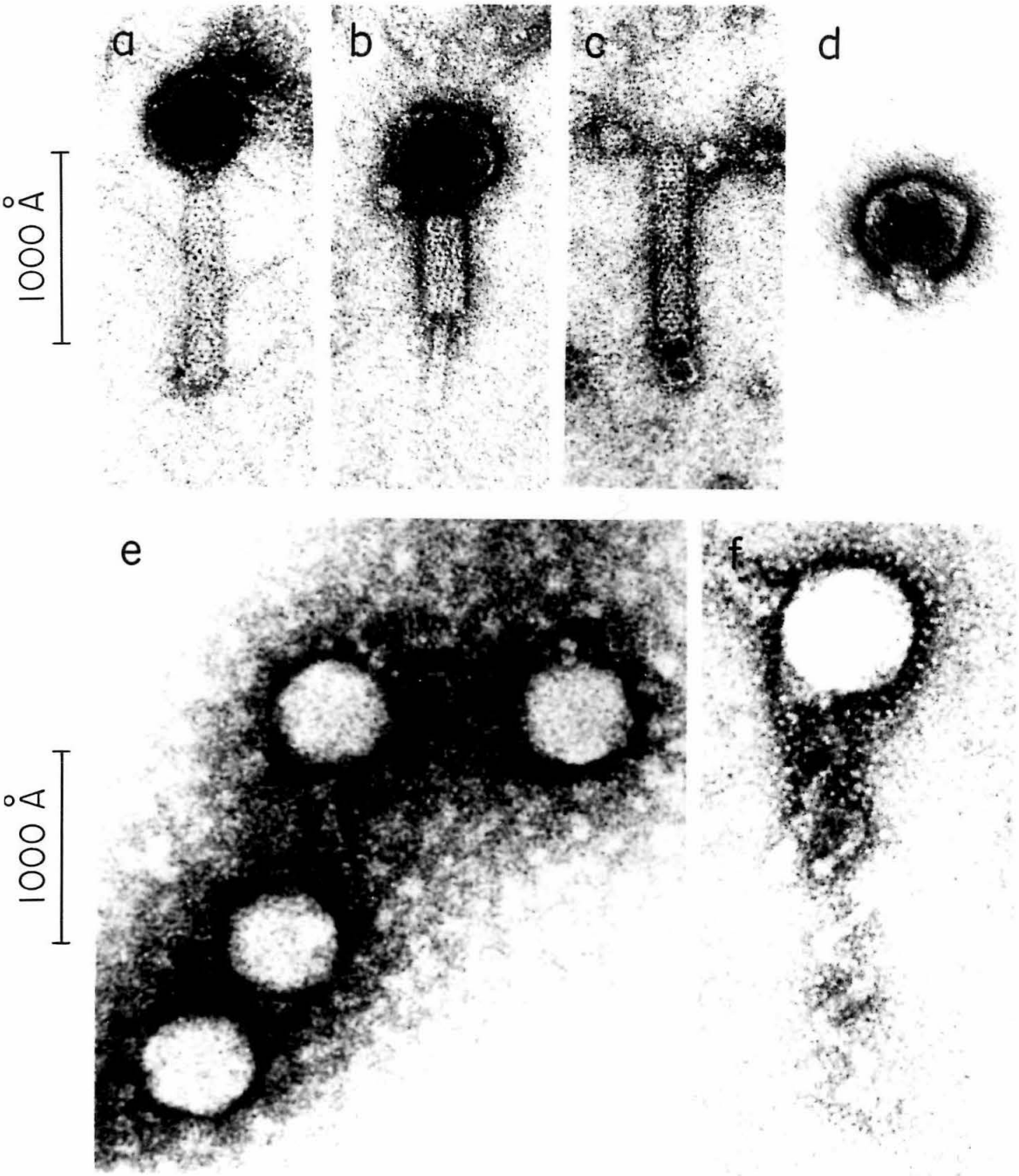
(d) Head of coliphage 15 (WT) after CsCl purification

(e) Head of coliphage 15 (TAU) from the crude lysate

(f) Unusual phage-like particle from 15 (TAU) crude lysate

Plates (a), (b), (c), and (d) were stained with uranyl acetate. Plates (e) and (f) were stained with phosphotungstic acid.

Plate I



[Plate I (b)]. It is difficult to judge whether the sheath has become thicker (by a factor of $2^{1/2}$) as might be expected if it were a contracted full sheath. Alternatively, the full sheath may be composed of two subunits of equal length, one of which is missing in the half sheath. The lysates generally contained many more free tails than free heads as is common in phage growth. Similar observations were made with a preparation of coliphage 15 (T^-).

The coliphage 15 (TAU) preparations consisted entirely of tailless phage [Plate I (e)]. The dimensions and the shape of the head are very similar to those of the free head of coliphage 15 (WT) [Plate I (d)]. In a few cases spiral-like structures are seen attached to the head [Plate I (f)]. The absence of tails in the coliphage 15 (TAU) lysate could be the reason for its noncolicinogenicity, since in an earlier work it was inferred that the phage tail is the colicinogenic component (Endo et al., 1965). In spite of the difference in morphology between the TAU phage and the other strain 15 phages, they are closely related as shown by the DNA homology tests described later.

(b) DNA from mature phage particles

The native DNA molecules extracted by heat shock from the coliphage 15 (WT) and (TAU) particles appear in the electron microscope as double-stranded linear DNA [Plate II (a)] with a number average molecular length of 12.8 μ (Fig. 2).

As already mentioned, accurate ratios of contour lengths for two different DNA molecules can be measured by mounting them both on

Plate II. Mature linear and in vivo circular coliphage 15 DNA.

(a) Mature linear coliphage 15 (TAU) DNA prepared by heat-shocking phage particles

(b) In vivo circular DNA observed after induction of E. coli TAU The large molecule is a covalently closed supercoiled coliphage 15 (TAU) DNA molecule. The small circular molecules are E. coli 15 minicircles.

(c) Same as (b) except that the circular coliphage 15 (TAU) DNA has an open structure.

(Mounted by the aqueous technique, stained with uranyl acetate, and shadowed with Pt-Pd.)

Plate II

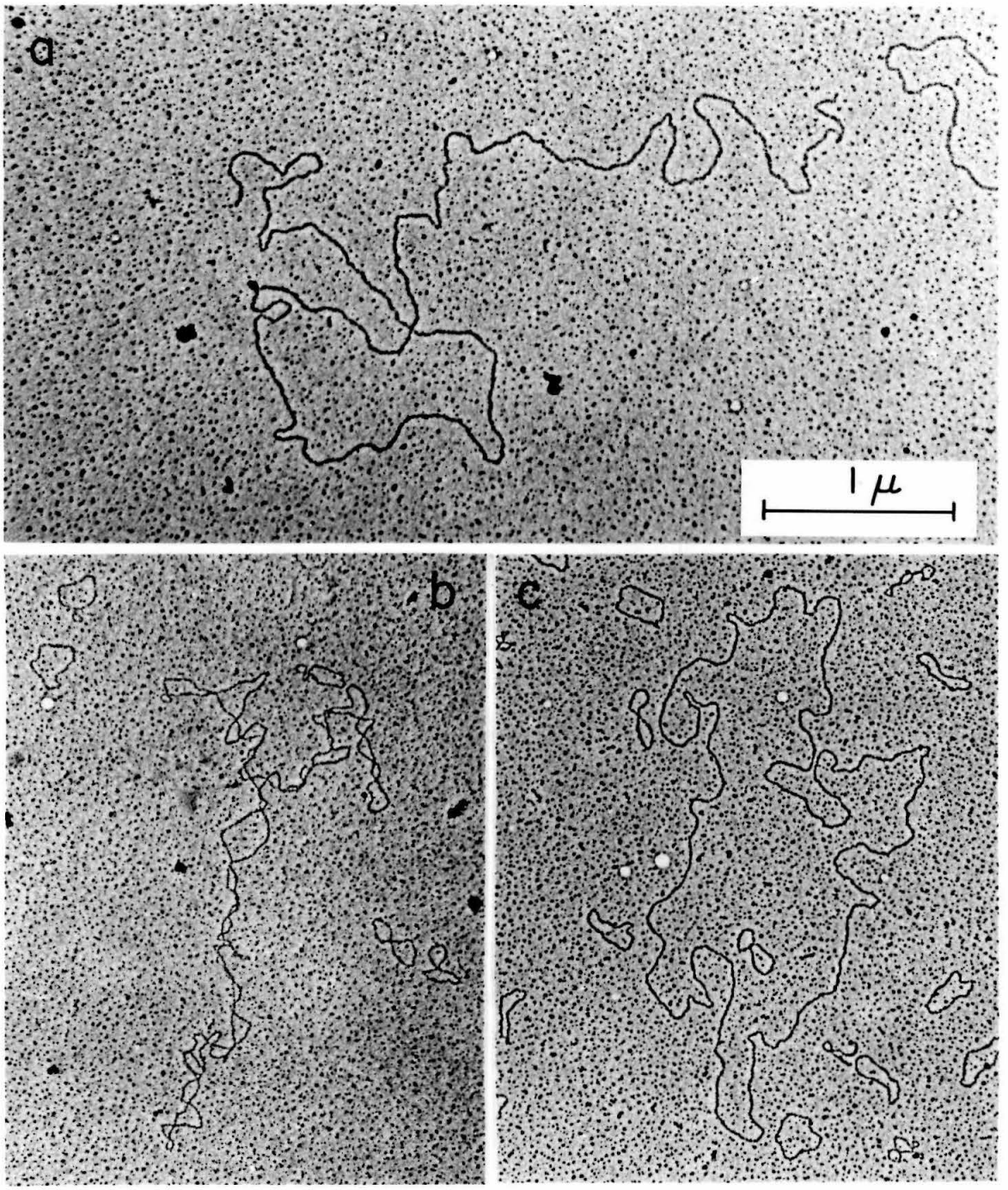
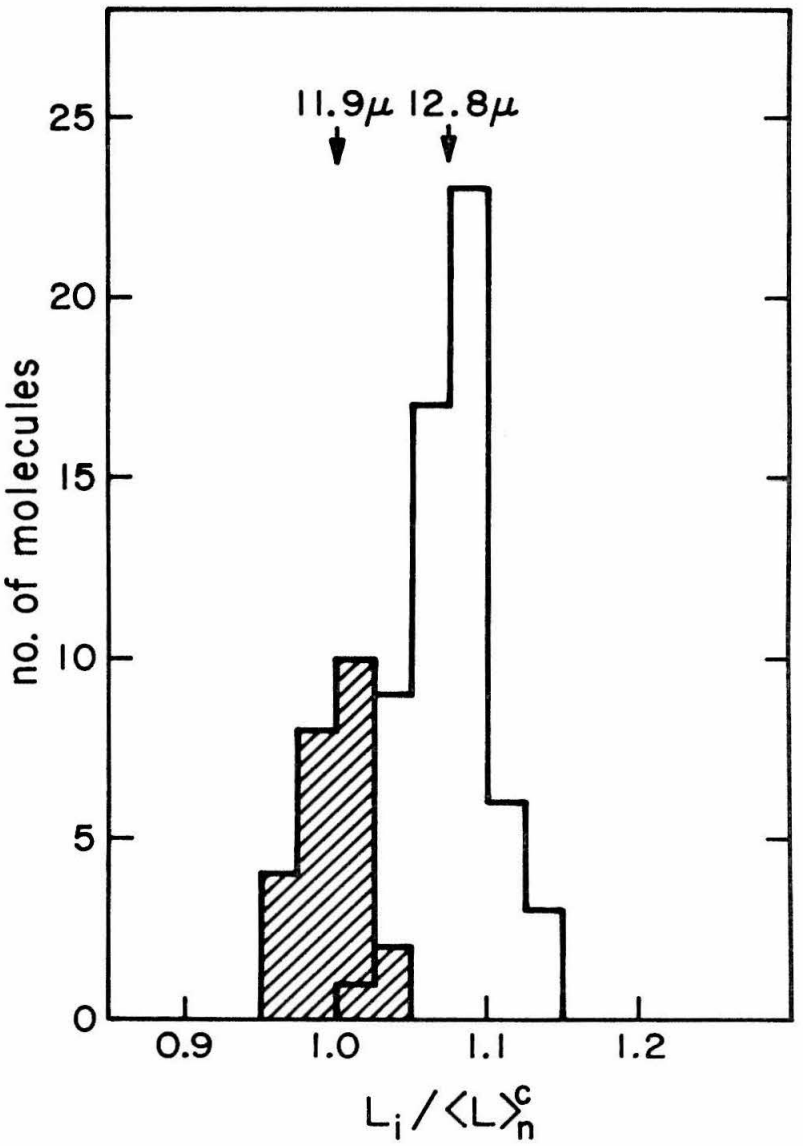


Fig. 2. Histogram of molecular lengths of mature linear phage DNA and in vivo circular DNA. The in vivo circular DNA and the mature linear phage DNA were mixed and mounted on the same grid. Linear and circular molecules were alternately photographed without refocussing. Thus, an accurate, direct comparison of the relative lengths of the two species was possible. The absolute lengths of the two species were obtained using a diffraction grating for calibration and are, thus, only approximate. Since the relative length of the two species is the most significant quantity, each distribution is plotted relative to the mean length of the circular DNA. The unshaded area is for 59 linear molecules. The shaded area is for 24 circular molecules. $\underline{L}_i$ is contour length. The number average molecular length of the linear molecules, $\langle \underline{L} \rangle_{\underline{n}}^{\underline{l}}$, is 12.8 μ, with a r of 2.7%. The number average molecular length of the circular molecules is $\langle \underline{L} \rangle_{\underline{n}}^{\underline{c}} = 11.9\,\mu$, with a r of 2.2%. The difference in mean lengths, 0.90 μ, divided by the mean length of the circular molecules is 0.075; this quantity is measurable with a standard error of 0.005.

Fig. 2


11.9μ
12.8μ
25
20
15
10
5
0
no. of molecules
0.9
1.0
1.1
1.2
$L_i / \langle L \rangle_n^c$

the same grid and photographing them alternately. In this laboratory (Davis, Simon, and Davidson, 1969), we find a ratio of contour lengths of λc_{26} DNA and φX 174 RF II DNA of 9.0 (± 0.1). If the molecular weight of the latter is taken as 3.4×10^6 (Sinsheimer, 1959), the molecular weight of λ DNA is calculated as $30.6(\pm 0.3) \times 10^6$. Values varying from 31 to 33×10^6 have been recommended by different authors (31×10^6, Burgi and Hershey, 1964; 33×10^6, MacHattie and Thomas, 1964; 32×10^6, Richardson and Weiss, 1966; 33×10^6 Studier, 1965).

The ratio of contour lengths of the linear DNA of mature coliphage 15 and φX RF II DNA was measured as 7.8(±0.1); the ratio of coliphage 15 DNA to λc_{26} DNA was measured as 0.875(± 0.01). For purposes of calculation, we take the molecular weights of φX RF II and λc_{26} DNA as 3.4×10^6 and 31×10^6; the molecular weight of coliphage DNA is then $27.0(\pm 0.5) \times 10^6$.

No circular DNA molecules were produced by slowly cooling the linear molecules from 80° C in either 0.1 M NaCl, 0.01 M EDTA (pH 7.4) or in 2 M NaCl, 0.01 M EDTA. These are conditions which produce efficient cyclization in the lambdoid phages (Wang and Davidson, 1966a) and the N phages (Lee and Davidson, 1969). We conclude that coliphage 15 DNA does not have cohesive ends.

The complexity of the DNA (number of base pairs in one non-repeating sequence of the DNA sample) may be determined by measuring the rate of renaturation (Wetmur and Davidson, 1968). An approximate measure of the complexity of coliphage 15 DNA was obtained

from our incidental observations of the rate of renaturation of samples prepared for electron microscopy. Under the conditions described in section (f) of Materials and Methods, λ DNA is about 50% renatured in one hour. The coliphage 15 DNA was observed to be about 64% renatured in one and one-half hours and 91% renatured in ten hours. In both cases, the degree of renaturation was scored by counting the number of two-stranded molecules and single-strand bushes in the electron microscope. Thus, the rate of renaturation is about the same as that of λ DNA. Thus, the complexity of the DNA is about equal to the number of base pairs in one mature phage molecule. The observations recorded above are probably accurate to better than a factor of two; they are certainly sufficient to eliminate the possibility that the phage DNA is a random sample of the bacterial chromosome.

The renaturation result described in a later section shows that the majority of the phage molecules do not contain any single-strand breaks.

(c) Covalently closed circular DNA molecules in vivo after induction

A culture of E. coli 15 TAU was induced with mitomycin C, samples were drawn 15 and 30 min after induction, and closed circular DNA molecules were extracted by gentle lysis and CsCl-ethidium bromide banding. Two kinds of covalently closed circular molecules were found [Plate II (b), (c)]: the E. coli minicircles of Cozzarelli et al. (1968) and a species with a contour length of 11.9 μ.

A quantitative comparison of the lengths of this circular DNA and the linear phage DNA was made by mounting a mixture of the two on the same specimen grid and photographing the two kinds of molecules alternately. For the histograms shown in Fig. 2, the difference between the number average molecular lengths of the linear and circular forms is 7.5% of the mean length of the circular form. In our experience, a difference in contour length of 0.5% can be detected by the relative length technique used here.

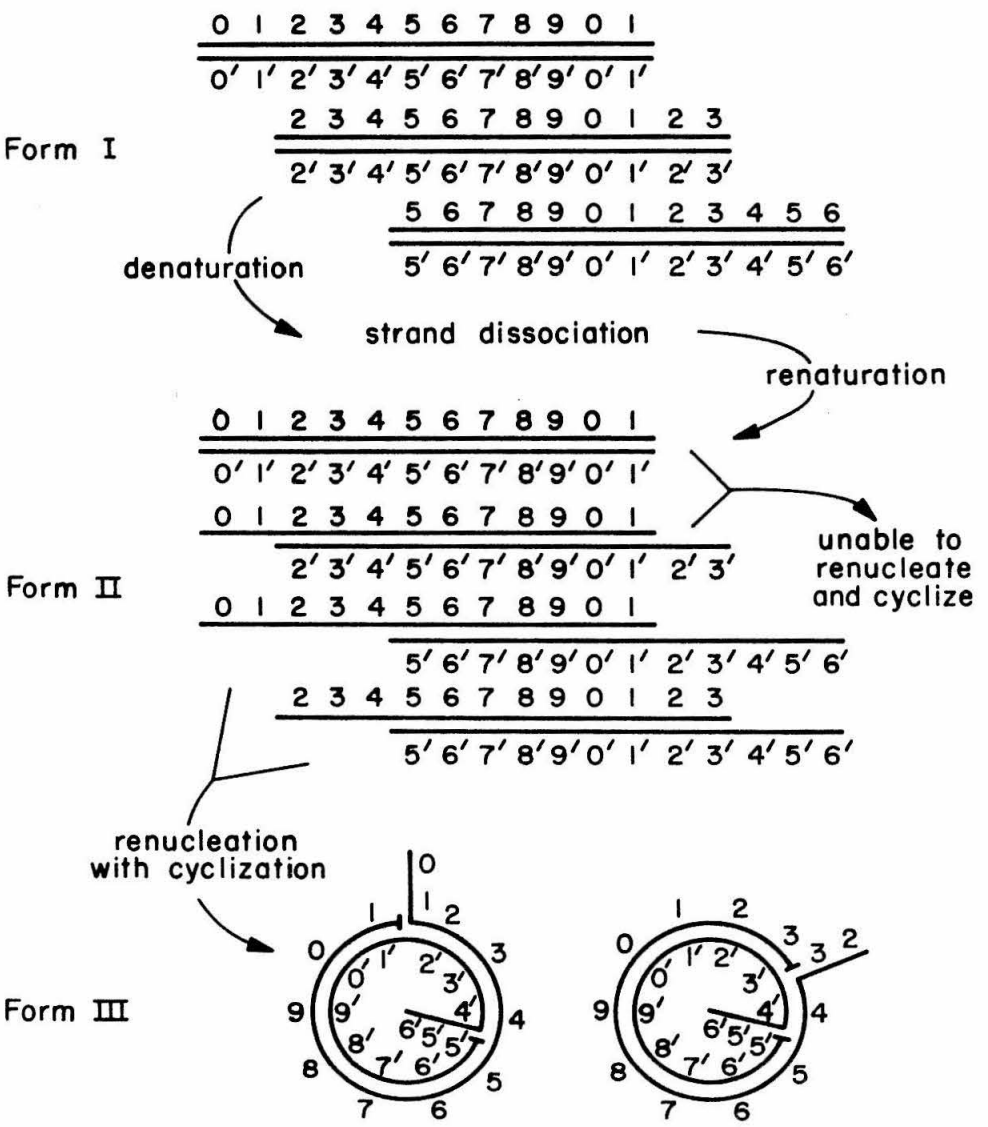
(d) Circular permutation and terminal repetition

If the intracellular closed circular form of DNA is indeed related to the mature linear phage DNA, the fact that the circular molecule is 7.5% shorter could be due to a terminal repetition of this length in the mature linear phage DNA. Several other phage DNA's which have a relatively large terminal repetition of this magnitude are also circularly permuted (T2, MacHattie, Ritchie, Thomas, and Richardson, 1967; P22, Rhoades, MacHattie, and Thomas, 1968). Mature P1 DNA is 12% longer than the closed circular prophage DNA, and is circularly permuted and terminally redundant (Ikeda and Tomizawa, 1968). We therefore searched for circular permutations and terminal repetitions in the DNA by the denaturation-renaturation method of Thomas and MacHattie (1964). The principles of this method are illustrated in Fig. 3.

Denaturation followed by renaturation of an ensemble of circularly permuted molecules labeled Form I in Fig. 3 will initially

Fig. 3. Experimental method for studying circularly permuted and terminally repetitious DNA (slightly modified from Fig. 3 of MacHattie, et al., 1967).

Fig. 3


Form I
denaturation
strand dissociation
renaturation
Form II
unable to renucleate and cyclize
renucleation with cyclization
Form III

lead to the collection of linear molecules labeled Form II in the figure. Renaturation of two strands which begin at different points initially leads to a linear duplex with single-strand ends. In some of the Form II molecules the single-strand ends are mutually complementary and the molecules can cyclize to give Form III molecules. If terminal repetition occurs, the resulting circular molecules have two single-strand branches consisting of repetitious pieces. The length of the branches is the length of the terminal repetition. The separation of the branches is related to the distance between the starting points of the two strands in the renatured duplex. Linear molecules with single-strand ends also occur (Form II); some of these molecules have short single-strand ends of length less than the terminal repetition and are unable to cyclize; others have longer single-strand ends with mutually complementary regions but have failed to cyclize for kinetic reasons.

Single-strand DNA appears as a bush when mounted in the basic protein film from an aqueous medium; it appears spread out as a filament when mounted at a suitable formamide concentration, as discussed in Materials and Methods. When coliphage 15 DNA molecules are denatured by alkali, renatured in formamide, and mounted from an aqueous medium for electron microscopy, many circular molecules with single-strand bushes are seen. Plate III (a and b) gives examples of such molecules. Plates IV and V have several examples of circular molecules mounted from a formamide

Plate III. Renatured coliphage 15 DNA.

(a) Renatured circular coliphage 15 (TAU) DNA molecule showing two single-stranded repetitious branches. This molecule corresponds to Form III of Fig. 3.

(b) Same as (a) except the bushes are closely spaced showing a different permutation

(c) Renatured linear coliphage 15 (TAU) DNA molecule showing two single-stranded ends (end bushes). In the upper right corner is a whole single strand of coliphage 15 (TAU) DNA. Note that the end bushes are of equal size. This molecule corresponds to Form II of Fig. 3.

(d) Renatured sheared coliphage 15 (WT) DNA. Note the short duplex length and the absence of any large end bush. This molecule is designated as a fragment/fragment homoduplex. [Section (g) in Results].

(e) Renatured whole coliphage 15 (TAU) DNA with sheared coliphage 15 DNA. This molecule is designated as a whole/fragment heteroduplex. Note the short duplex length and the difference in size of the end bushes. The right end bush is almost as large as a whole single strand of DNA [upper right corner of (c)].

The DNA was mounted by the aqueous technique and stained with uranyl acetate.

Plate III

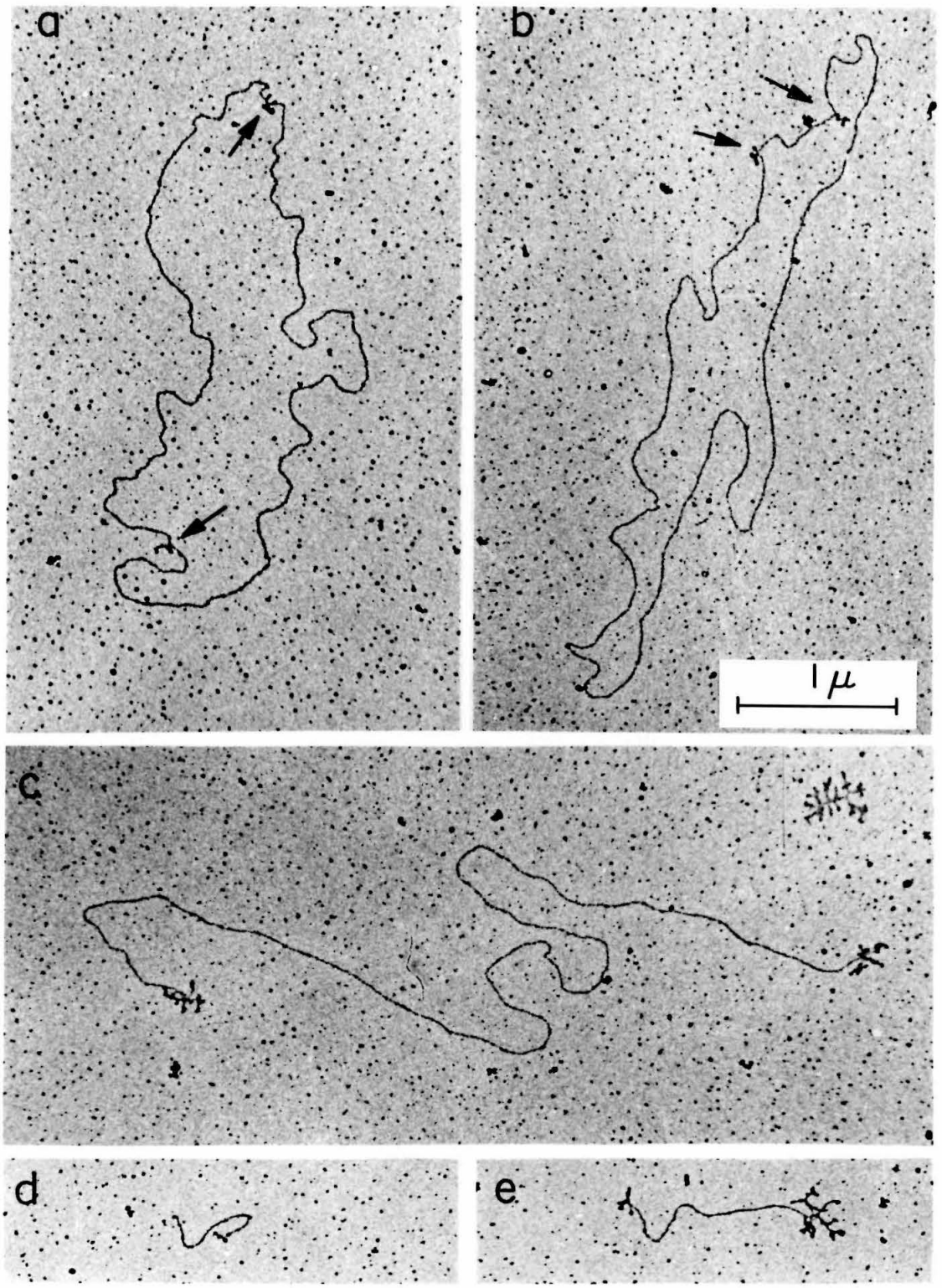


Plate IV. Renatured coliphage 15 DNA with the single-stranded repetitious branches extended by formamide.

Two renatured circular coliphage 15 (WT) DNA molecules. The arrows mark the single-stranded repetitious branches. All whole circular molecules observed showed two such branches. The DNA was mounted by the formamide technique, stained with uranyl acetate, and shadowed with Pt-Pd as described in Materials and Methods.

Plate IV

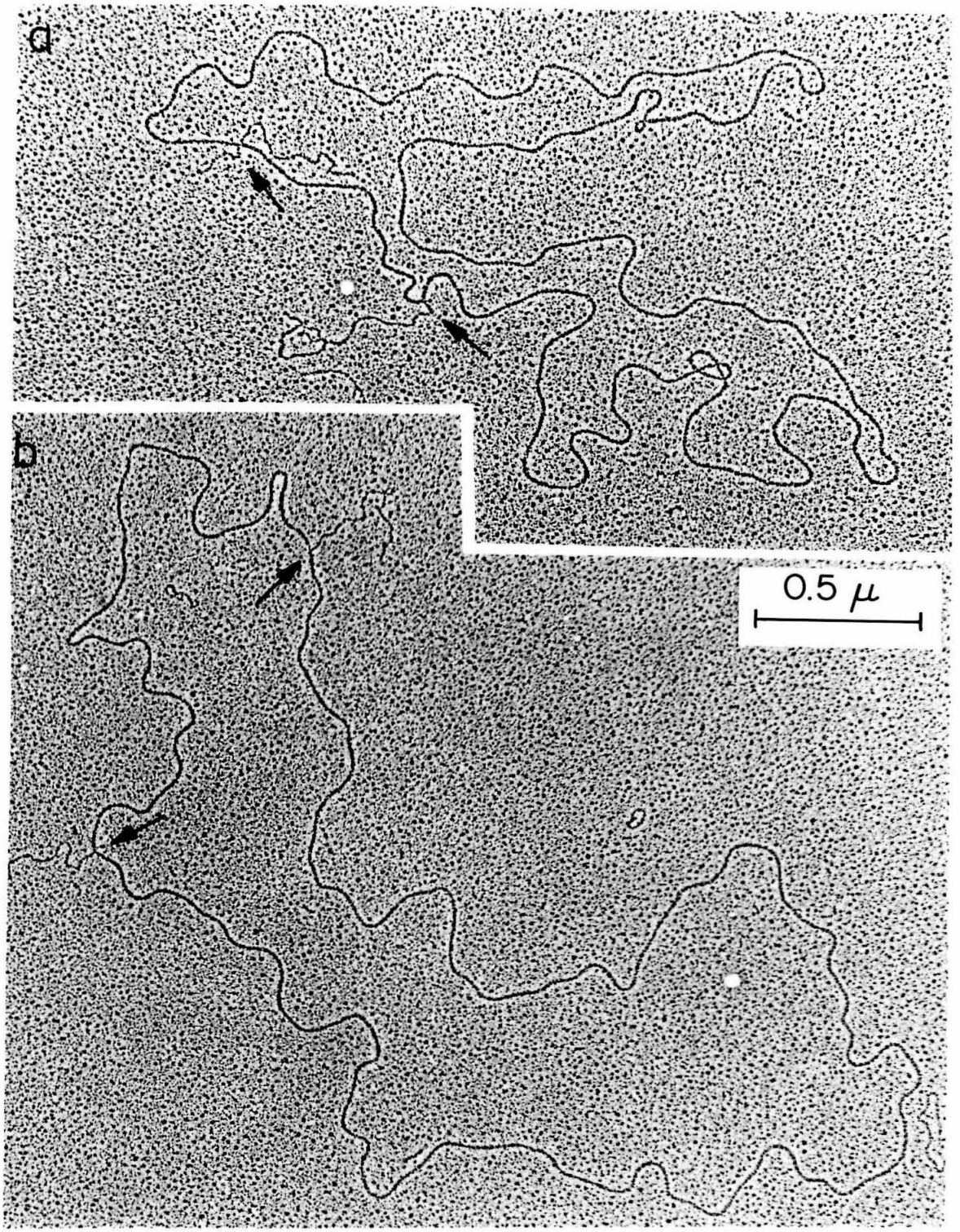


Plate V.

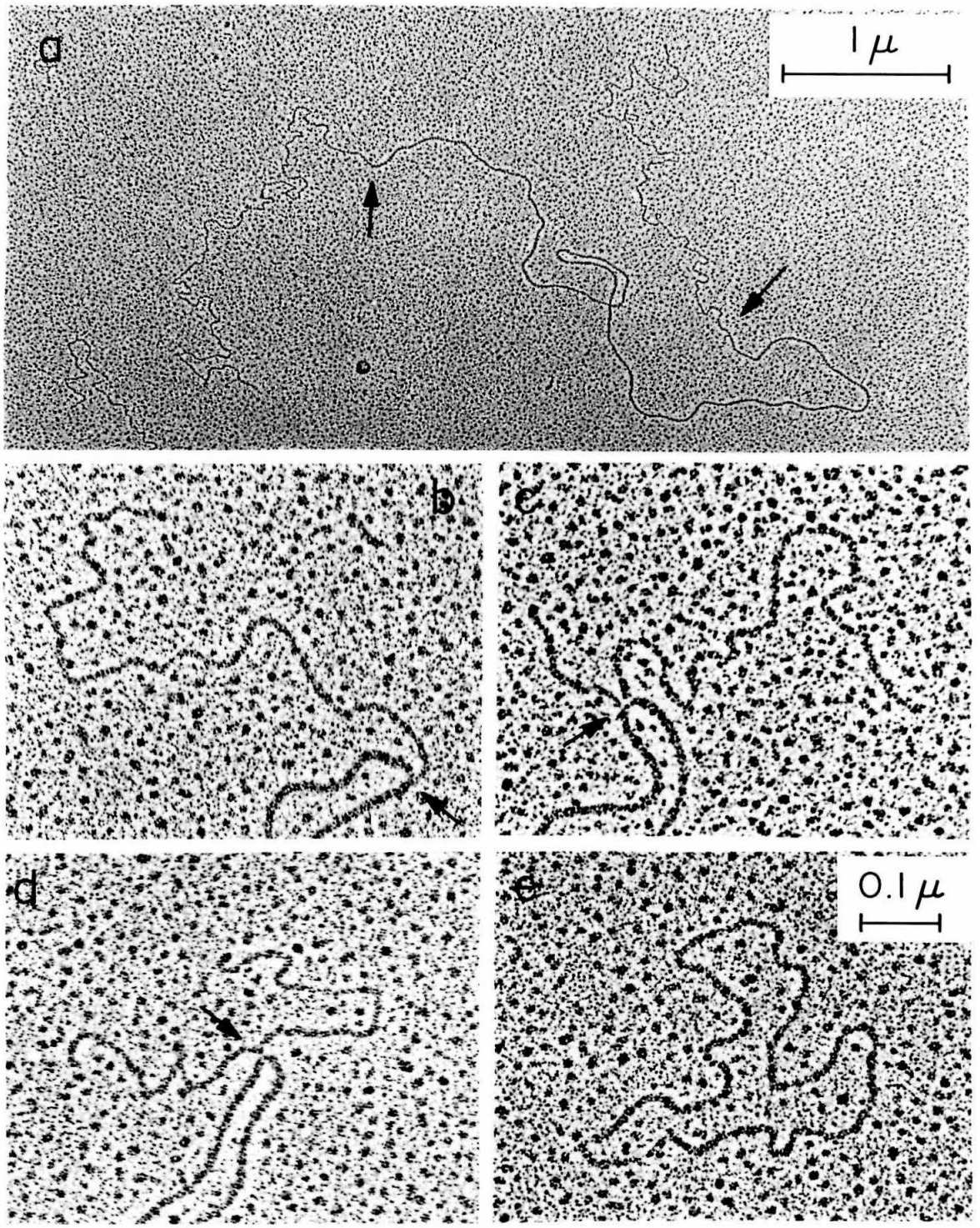
(a) Renatured linear coliphage 15 (WT) DNA molecule with single-stranded ends: The arrows mark the approximate junction points between single-stranded and double-stranded DNA.

(b), (c), and (d) Enlargements of unforked (b) and forked [(c) and (d)] branches.

(e) Single-stranded φX 174 DNA used for the calibration of the length measurements of the repetitious branches shown in (b), (c), and (d).

The φX DNA was mounted on the same grid that was used for Plates IV and V (b), (c), and (d).

Plate V



solution so that the single-strand ends are extended into visible filaments which we call branches. A number of linear molecules with single-strand ends are also seen. [Plate III (c) and Plate V (a)]. Under our experimental conditions over 50% of the DNA renatured; the relative amounts of circular and linear species in the renatured preparations were about 40% and 60%, respectively.

If the mature phage molecules are circularly permuted, the contour lengths (circumferences) around the circles for the renatured molecules should be constant and equal to the contour length of the in vivo circular molecules. Histograms of the circumferences for the renatured molecules gave a mean length equal, within experimental error, to 11.9 μ, and a standard deviation of 0.4 μ.

We conclude that coliphage 15 DNA is indeed circularly permuted and terminally redundant.

(e) Distribution of beginning points for permuted sequences

We wish to investigate the distribution of beginning points in the ensemble of circularly permuted linear molecules from the mature phage. Information pertaining to this distribution can be obtained from measurements of the length of the double-strand regions in Form II molecules and the separation of bushes in the Form III molecules.

Let C be the length of the nonrepetitious sequences of the DNA molecules, and let T be the length of the terminal repetition. The length of the mature linear molecule is then C + T. Let p be the phase

difference between the beginning points of two strands which have reassociated, by which we mean that the physical distance between their beginning points is pC.

For Form II molecules the length, D, of the double-stranded region is then

$$D = C(1 - p) + T \qquad (1)$$

A Form II molecule has complementary ends of length $pC - T$ if $pC > T$. Such molecules are presumably, capable of cyclization.

In Form III molecules the circumference of the circle has a length C. The protruding single-strand branches have a length T. For a given $p < 0.5$ the separation of the branches in the shorter direction around the circle varies between $pC - T$ and $pC + T$ (depending on the phenomenon of branch migration as discussed later). Therefore, the separation of the branches in the longer direction around the circle, B, varies in the interval

$$C(1 - p) + T > B > C(1 - p) - T \qquad (2)$$

We use the longer distance around the circle as a measure of the branch separation because it is more directly comparable to the quantity D of Form II molecules. The distribution of p values may conceivably then be inferred from the observed distribution of D and B values. A histogram of observed D/C and B/C values is shown in Fig. 4. Among Form III (circular) molecules there are a few with B/C values close to 0.5 but the number increases irregularly to about

Fig. 4. Distribution of the relative distance between single-strand branches on renatured molecules. All observations were made on molecules mounted by the aqueous technique so that the single-strand branches appeared as "bushes". A histogram (shaded area) of the length of the double-stranded portion between the end bushes of 78 linear molecules is given with the horizontal coordinate, $\underline{D}/\underline{C}$, being the length of the double-stranded portion between the end bushes of the linear molecules divided by the mean length of the renatured circular molecules. Similarly, a histogram of the quantity $\underline{B}/\underline{C}$ which is the longer distance around the circle between the two bushes of a renatured circular molecule divided by the circumference of that circular molecule is displayed (unshaded area). One hundred fifty circular molecules and 78 linear molecules from the photographs of coliphage 15 (TAU) were chosen at random for this plot. A similar plot was obtained for coliphage 15 (WT) DNA. To obtain a valid distribution for all molecules, the value of the vertical coordinate for the linear molecules in the plot above should be multiplied by 3 since they actually represent about 60% of the total population of renatured molecules, but only 78 out of 228 were scored for the histogram. The distributions are plotted in intervals of approximately 1 σ relative to the observed values of $\underline{C}$.

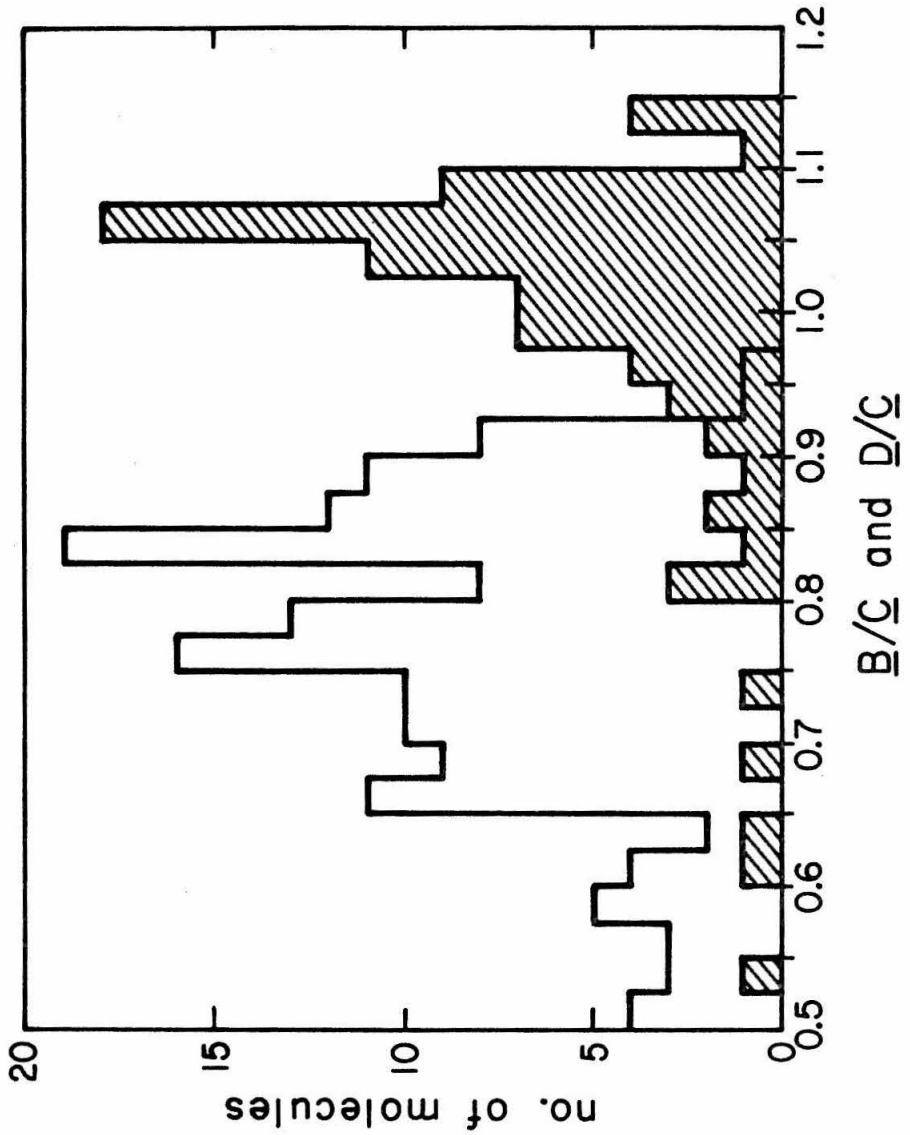


Fig. 4

0.85 and then falls to a very low value for B/C close to 1.0. For linear molecules there are many with D/C values greater than 1.0, as expected if there is terminal repetition, and a decreasing number with decreasing D/C values.

The fact that there are some renatured molecules with B/C or D/C values in all intervals between 0.5 and 1.0 shows that a number of coordinates of beginning points, corresponding to p values between 0.0 and 0.5, occur in the population. A discussion of other features of the shape of the histogram in Fig. 4 will be given later.

(f) Size and heterogeneity of the terminal repetition

In previous physical work, the size of the terminal repetition of a DNA has been estimated mainly by the E. coli exonuclease III digestion method (MacHattie, et al., 1967; Ritchie, Thomas, MacHattie, and Wensink, 1967; Rhoades, et al., 1968). More accurate values can be derived by electron microscopy.

We estimate the size of the terminal repetition as 0.075(±0.005) C from the difference in lengths of the mature linear and the in vivo closed circular DNA's.

However, the most accurate values are obtained by direct measurements of single-strand branch lengths on micrographs of Form III molecules mounted under conditions in which the single-strand DNA is extended. For measurements of this type, it is essential that a single-strand DNA of known length be mounted on the same grid as an internal standard.

The phenomenon of branch migration should be mentioned before the branch length measurements are discussed. Electron micrographs of the branches due to terminal repetitions are shown in Plates IV and V. In some cases each branch consists of one single strand with one free end. In other cases the branch has forked into two sub-branches and there are two free ends. A schematic presentation of the mechanism of forking is shown in Fig. 5. On consideration of the physicochemical forces involved, it is expected that branch migration and forking will indeed occur, as observed.

The total branch length is the sum of the lengths of the two sub-branches. The relative fork position is defined as the length of the longer sub-branch divided by the total branch length. The probability of different fork positions has been studied by plotting the histogram of relative fork positions shown in Fig. 6. It appears that all fractional fork positions are equally probable, but unforked branches occur in about 20% of the cases and thus with greater than random probability.

The distribution of length of the branches and of the φX 174 standards is plotted in Fig. 7. The ratio of the number average molecular length of the branches to the number average molecular length of φX 174 is 0.536. (The corresponding ratio of weight average lengths is 0.554.) Since the length of the mature linear coliphage 15 DNA is 7.81 times that of double-stranded φX 174 RF II DNA, the number average length of the terminal repetition is $0.536/(7.81 - 0.536) = 0.074$ of the length, $\underline{C}$, of the nonrepetitious part of the mature phage

Fig. 5. Schematic representation of the mechanism of branch migration. An enlargement of a portion of the circular Form III molecule in the lower right of Fig. 3 is shown. Since a protruding single-strand branch in the top figure has the same base sequence as an adjacent strand which is in the duplex, the branch can fork and migrate without changing the total number of base pairs.

Fig. 5

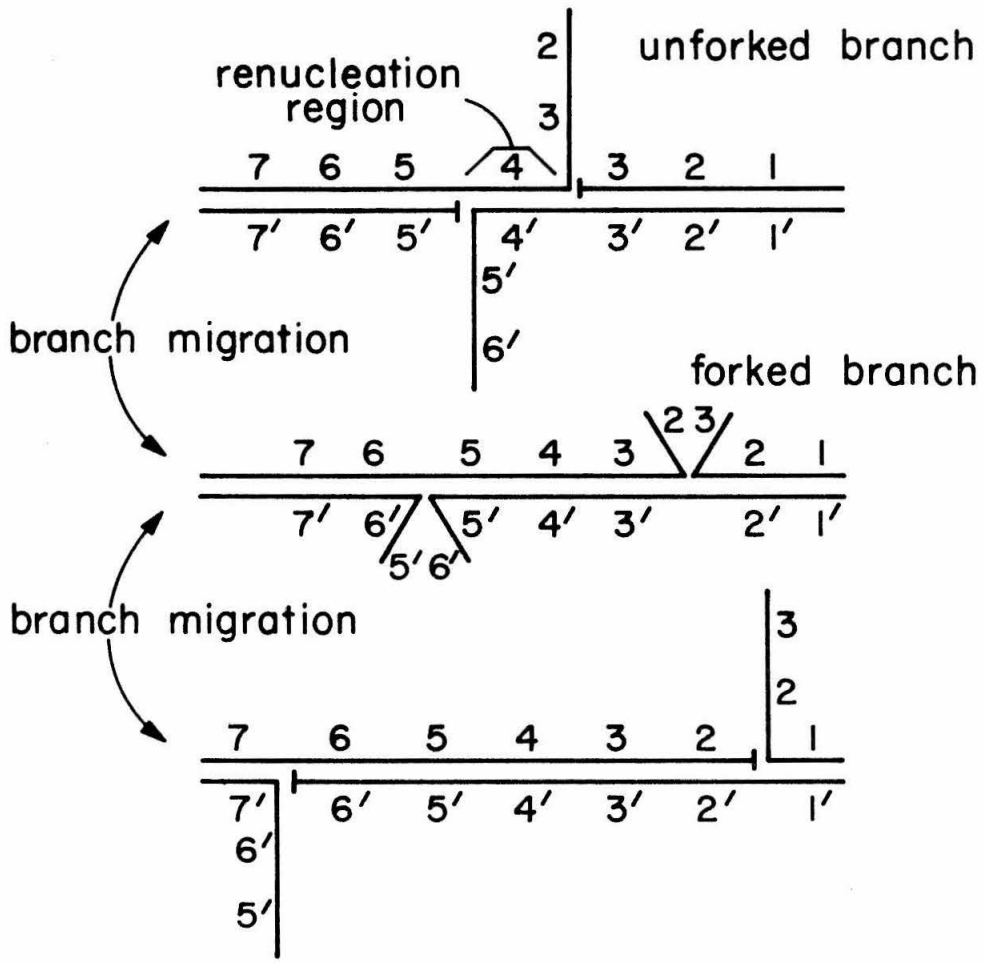

renucleation region
unforked branch
branch migration
forked branch
branch migration

Fig. 6. Relative fork position of repetitious branches. Coliphage 15 (WT) DNA was denatured and renatured for 6 hrs in 50% formamide at 25° C. The DNA was mounted for electron microscopy in the presence of formamide and the length of the repetitious branches measured. The relative fork position is evaluated by dividing the distance from the fork position to the end of the longest branch by the total length of the branch. Therefore, a relative fork position of 1.0 would represent a linear branch, while a relative fork position of 0.5 would represent a forked branch in which the lengths of the two single strands are equal. The distribution of the relative fork position for 91 repetitious branches is plotted here.

Fig. 6

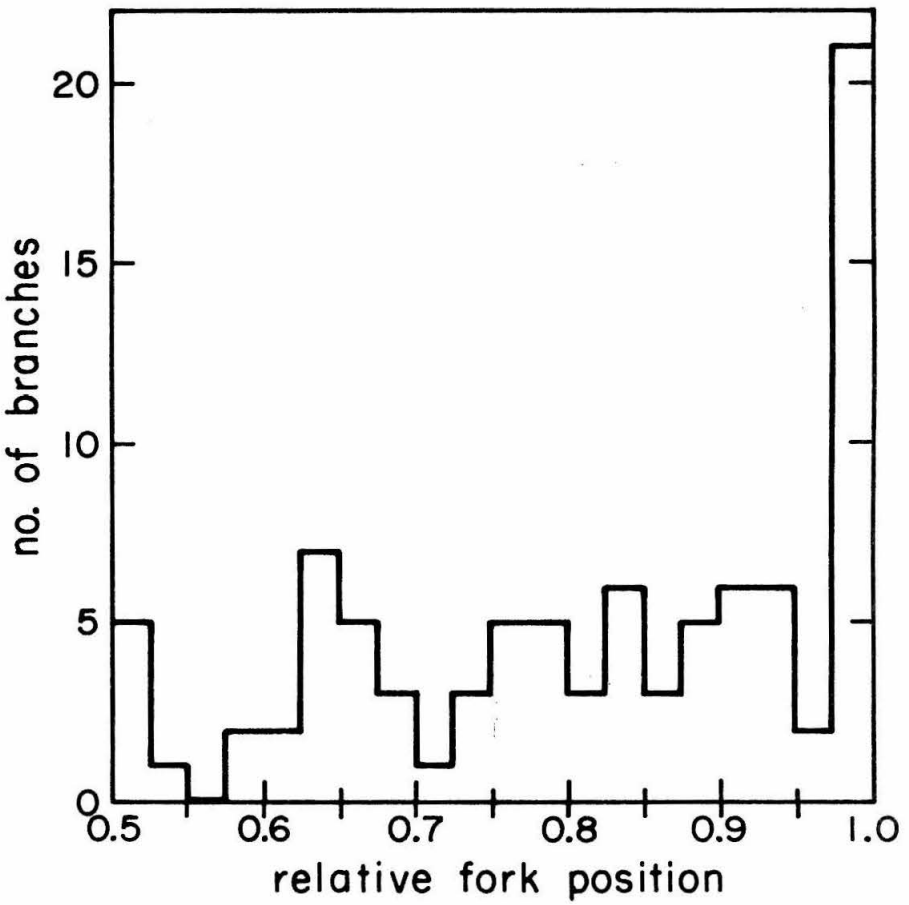
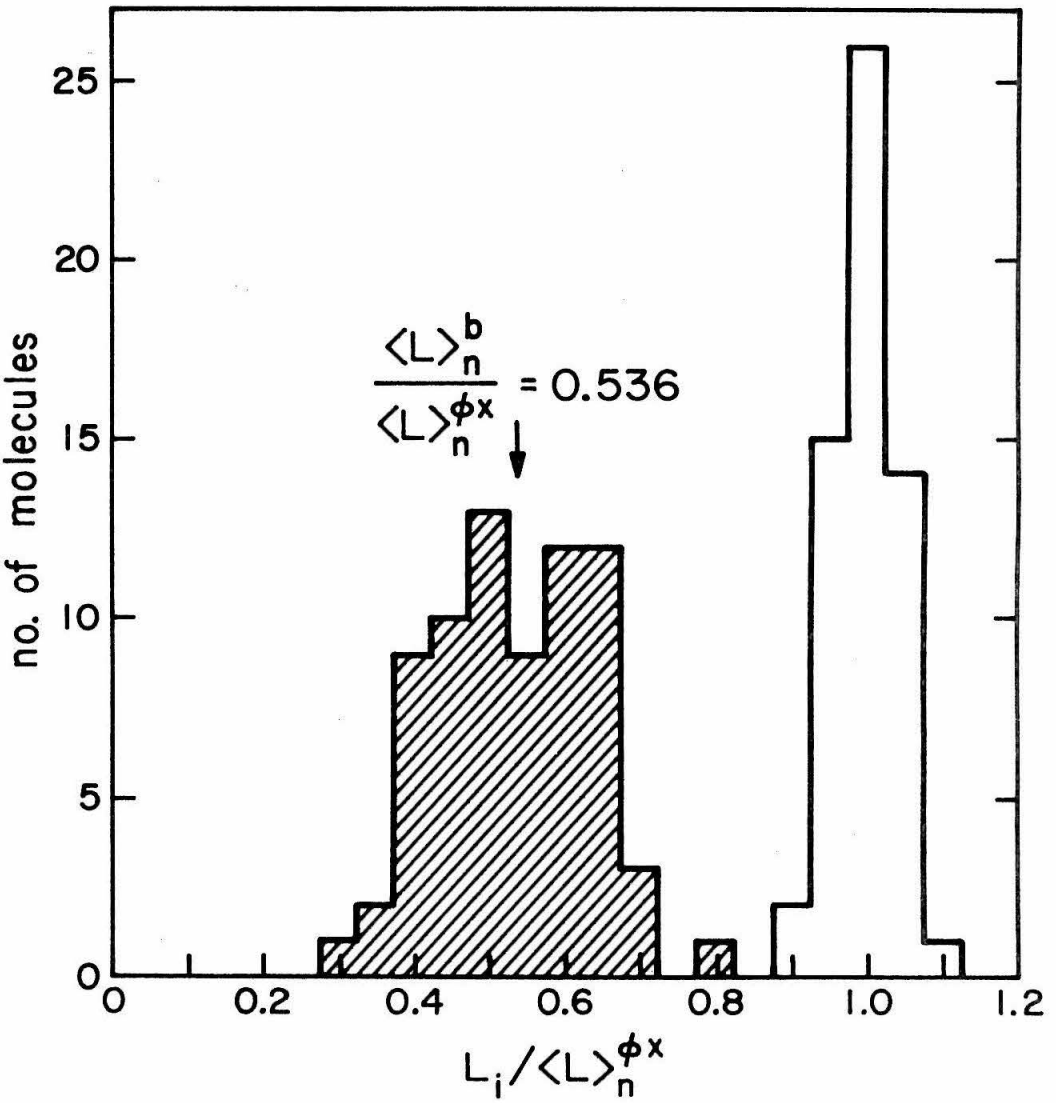

20
15
10
5
0
no. of branches
0.5
0.6
0.7
0.8
0.9
1.0
relative fork position

Fig. 7. Length distribution of the repetitious single-stranded DNA branches relative to φX 174 DNA. Renatured coliphage 15 (WT) was mounted along with single-stranded φX 174 DNA in the presence of formamide. Micrographs of both the repetitious single-stranded DNA branch (shaded area) and φX 174 DNA (unshaded area) were alternately taken. Only those renatured circular DNA molecules which showed two unambiguous repetitious single-stranded DNA branches are included in this distribution. The quantities $\underline{L}_i$, $\langle \underline{L} \rangle_n^{\varphi X}$, and $\langle L \rangle_{\underline{n}}^{\underline{b}}$ are contour length of a branch or molecule, number average length of φX, and number average length of the branches, respectively.

Fig. 7


25
20
15
10
5
0
no. of molecules
$\frac{\langle L\rangle_n^b}{\langle L\rangle_n^{\phi x}}$ = 0.536
0
0.2
0.4
0.6
0.8
1.0
1.2
$L_i/\langle L\rangle_n^{\phi x}$

DNA. This value agrees well with the less exact value deduced previously. It corresponds to 2800 base pairs.

It is obvious from Fig. 7 that the fractional standard deviation of the branch lengths is greater than the fractional standard deviation of φX 174 lengths. The fractional standard deviations are 0.19 for the branches and 0.043 for φX DNA. We attribute this large fractional standard deviation for the branch lengths to a true heterogeneity in length of the terminal repetition.

In order to discuss the heterogeneity quantitatively, we must first discuss the distribution in lengths for a homogeneous DNA population in electron micrographs. It is observed (Davis, et al., 1969) that for a homogeneous DNA population, the square of the standard deviation of a distribution of contour lengths on a micrograph is proportional to the mean length of the DNA. In fact, it is found that

$$(\sigma_d/\underline{L}_d) = 0.037(\pm 0.007)\underline{L}_d^{1/2} \qquad (3)$$

and

$$(\sigma_s/\underline{L}_s) = 0.049(\pm 0.12)/\underline{L}_s^{1/2} \qquad (4)$$

where $\underline{L}$ represents contour length and subscripts $\underline{d}$ and $\underline{s}$ refer to double- and single-stranded DNA, respectively. The numerical constants in the equation above apply when double- and single-strand lengths are measured in units of the length of φX RF II double-stranded DNA and the length of φX single-stranded DNA, respectively. These relations were deduced from studies of DNA's with a large

range of lengths and apply under a wide range of mounting conditions. We believe that the primary sources of fluctuation in length occur in the structure of the DNA-protein film, and not in magnification or measuring errors.

The predicted fractional standard deviation for φX 174 lengths from eq. 4 is 0.05(±0.01), in satisfactory agreement with the value observed for this sample of 0.043.

The predicted fractional standard deviation for a homogeneous population with the mean length of the terminal repetition is 0.07 (±0.01). Therefore we believe that the fractional standard deviation in length of the terminal repetition due to its heterogeneity is $(0.19^2 - 0.07^2)^{1/2} = 0.18$, or 500 base pairs. A variation in length of the terminal repetition of this magnitude corresponds to a fractional variation of 0.013 in length of the mature phage DNA, which is too small to be reliably observed.

(g) DNA-DNA homology studies by electron microscopy

The phage from wild type and TAU strains of E. coli 15 are morphologically similar, except that the latter have no tails. The DNA's have the same contour length and the same circular permutation-terminal repetition characteristics. We now ask the question as to the degree of sequence relationship between the DNA's.

A mixture of coliphage 15 (WT) and (TAU) DNA's was denatured, renatured, and mounted for electron microscopy in the presence of formamide. The resulting mixture will contain homoduplexes of the two types and, if there is any significant sequence homology, hetero-

duplexes with one strand each from the two kinds of DNA. In such heteroduplexes, regions of nonhomology are visible as single-strand loops of various kinds (Davis and Davidson, 1968; Westmoreland, et al., 1969; Davis, et al., 1969). In our experience, deletions, additions, or substitutions of nonhomologous regions as small as 50 to 100 base pairs are observable by this method. Point mutations cannot be detected.

No nonhomology regions were found by electron microscopic examination of the renatured mixture. Therefore, either there is no significant sequence relationship between the two DNA's so that no heteroduplexes form or the relationship is very close, within the limits stated above.

The hypothesis of no sequence relationship at all was eliminated by hybridizing a mixture of sheared wild type phage DNA and unsheared TAU DNA under conditions that only a relatively small amount of renaturation occurs. This method has been described previously in connection with a similar problem concerning several M. lysodeikticus phages (Lee and Davidson, 1969). If there is any sequence homology between the sheared and the unsheared DNA, heteroduplexes, each of which consists of a sheared piece of DNA and an unsheared strand, will form. These heteroduplexes can be recognized as molecules with a short inner double-strand region and two single-strand ends, one or both of which is large. The DNA is trapped in the cytochrome film from aqueous solution, so that single-strand regions appear as bushes.

The length of a single-strand piece can be approximately estimated from the perimeter of the bush (Davis and Davidson, 1968). Plate III (d) and (e) show relevant micrographs; the scoring of the observed grids is presented in Table 2. The molecules of interest are the whole/fragment heteroduplexes [Plate III (e)]. These are readily distinguished from whole/whole homoduplexes, which are either circular, or linear with short equal size end bushes and most frequently with a long double-strand region. Fragment/fragment homoduplexes have only short end bushes [Plate III (d)].

According to the steric hindrance effect in renaturation, which is explained in more detail in a later section, a fragment which is homologous to a region close to the topological center of a whole strand has a lower probability of penetrating the random coil and forming a heteroduplex than does a fragment which is homologous to a region close to the topological outside of the whole strand. The observations are in agreement with this theory and the whole/fragment heteroduplexes most frequently have one large and one small end bush. The occurrence of whole/fragment homoduplexes due to the presence of single-strand breaks in the unsheared TAU preparation can only be evaluated by the control sample of TAU alone.

It is clear from the scoring in Table 2 that whole/fragment heteroduplexes do occur in the large amount expected in, and only in, the renatured mixture of sheared wild type and unsheared TAU DNA. We therefore conclude that the two kinds of DNA are closely related and there are no sequence dissimilarities of length greater than 50 to 100 base pairs.

TABLE 2

Numbers of different kinds of molecules in heteroduplex preparation

Classes of molecules	No of molecules	
	Whole TAU	Whole TAU + sheared WT
Long linear or cyclic double-stranded region with small equal sized end bushes (A)	80	14
Short double-stranded regions with large equal sized end bushes (B)	2	3
Long double-stranded region with small unequal sized end bushes (C)	10	3
Short double-stranded region with large unequal sized end bushes (D)	9	66
Short double-stranded region with small end bushes (E)	11	192
Whole single-stranded molecules (F)	99	14
TOTAL	211	292
Probable identification of scored molecules		
Whole/whole homoduplexes = A + B	82	17
Whole/fragment homoduplexes = C	10	3
Whole/fragment heteroduplexes = D	9	66
Fragment/fragment homoduplex = E	11	192
Percent heteroduplexes*	5%	64%

* The percentage of whole DNA strands hybridized to a small fragment = $100\,D/[2(A + B) + C + D]$

4. Recapitulation and Further Discussion

(a) Phage properties

Auxotrophic mutants of wild type E. coli 15 have in general been derived by uv mutagenesis and the standard penicillin selection procedure. E. coli 15 T^- was so derived (cited by Barner and Cohen, 1954); it was the mother strain for further uv mutagenesis and selection to produce E. coli 15 TAU (Barner and Cohen, 1958) and TAU-bar.* Of these four strains, only TAU-bar does not produce phage particles after uv or mitomycin C induction. [Our observations on this point agree with those of Cozzarelli, et al. (1968), and disagree with those of Bolton, et al. (1964).] Furthermore, the in vivo closed circular DNA species was found after induction in 15 WT and 15 TAU, but not in TAU-bar. It is possible then that either TAU-bar has been cured of the prophage or the prophage has mutated to a noninducible form by the uv mutagenesis.

We noted that in all strains studied there was a considerable decrease in the number of the 0.67 μ plasmid DNA circles after mitomycin C treatment.

Whereas the lysates of induced E. coli 15 WT and T^- contained phage with attached tails and excess free tails, the TAU lysate con-

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* We are informed by Professor P. C. Hanawalt that the TAU-bar was derived by R. Wax from an auxotrophic mutant E. coli 555-7 that R. Weatherwax originally isolated from 15 T^-. It was named TAU-bar by P. C. Hanawalt. It has also been named WWU by S. Person.

tained only tailless phage and no free tails. The studies reported here show that the DNA's are very similar in base sequence, and possibly differ only by some point mutations. Presumably, one or several such mutations have made the TAU DNA defective for the production of tails.

The coliphage 15 (WT) and (T^-) particles are colicinogenic to 15 WT and 15 T^- bacteria; coliphage 15 (TAU) particles are not. These observations provide further evidence that tail particles are the colicinogenic component (Endo, et al., 1965).

Both TAU and TAU-bar bacteria are resistant to the killing action of the (WT) and (T^-) phage. These strains were isolated after uv irradiation of T^- which induces the prophage; the selection procedure would therefore favor selection of a resistant strain.

In agreement with previous workers (Sandoval, et al., 1965) Mennigmann, 1965; Endo, et al., 1965) we have not succeeded in finding a host on which the phage particle produces a productive infection; the authors cited have suggested that the phage is defective. Our studies, however, show that the phage particles have a unique circularly permuted, terminally repetitious sequence with a complexity approximately equal to the molecular weight. Thus, this phage is not like the defective B. subtilis phage (Okamoto, Mudd, and Marmur, 1968) which contains a random sample of the bacterial chromosome as its DNA. Furthermore, Cowie and Szafranski (1965) have reported that

coliphage 15 (TAU) DNA has 16% homology with host DNA.

(b) Properties of the circular permutation and terminal repetition

The DNA's of coliphage 15 (WT) and (TAU) are identical in size and base sequence by the criteria available.

Like the virulent phages T2 and T4 and the temperate phages P22 and P1 (Thomas and Rubinstein, 1964; Thomas and MacHattie, 1964; MacHattie, et al., 1967; Rhoades, et al., 1968; Thomas, et al., 1968; Ikeda and Tomizawa, 1968), the DNA of the E. coli 15 phages is terminally repetitious and circularly permuted.

The mean size of the terminal repetition is 0.074 of the whole genome, or 2800 base pairs. For T2 DNA, the size is reported to be 2000 to 5000 base pairs or 0.01 to 0.03 of the genome size; for P22 it is roughly 1000 base pairs or 0.025 of the genome size (Thomas, Kelly, and Rhoades, 1968); for P1, it is reported to be 0.12 of the genome or 10,000 base pairs (Ikeda and Tomizawa, 1968).

The accurate electron microscope methods used here have made it possible to observe that the size of the terminal repetition is somewhat variable, with a fractional standard deviation of 0.18 of the mean size of the terminal repetition, or 500 base pairs, or 0.013 of the genome size. This variability shows that the mechanism for cutting the DNA for packaging in the phage head is not precise. In particular, the variability is plausible for the "headful" mechanism of packaging DNA (Streisinger, Emrich, and Stahl, 1967). The cutting mechanism is, however, sufficiently precise so that every phage

has a full complement of genes.

After induction and before phage maturation, there is an intracellular closed circular DNA and the mature phage DNA is 0.075 longer than this intracellular form. The amount of this DNA increases from 15 min to 30 min after induction. It is highly probable that this closed circular molecule contains one complete genome of the coliphage 15 DNA with no terminal repetition.

(c) Excluded volume effects and the renaturation of circularly permuted DNA's

In order to explain the observed dependence of the rate of renaturation on strand length, Wetmur and Davidson (1968) suggested, as one of several possibilities, that there is an excluded volume effect so that points near the physical center of a random coil are less available for the initial joining event in renaturation than are points close to the outside. Random walk theory shows that points near the topological center of a random coil have a smaller radius of gyration with respect to the center of mass of the coil than do points near the topological outside of the coil. Therefore, a point near the topological end of a strand is, on the average, closer to the physical outside of the coil and is more available for the initiation of a renaturation reaction than is a point close to the topological center of the strand.

This hypothesis has been confirmed by Wulff, Jamieson, and Davidson (1969) who showed that the rate of renaturation of sheared fragments of λDNA with whole strands was greater for sheared strands

homologous to points near the topological outside of the whole strand. Essentially the same observations by the same method are reported here in Table 2 in our study of the renaturation of sheared fragments of coliphage 15 (WT) DNA with the whole strands of coliphage 15 (TAU) DNA.

For two complementary whole strands with widely spaced beginning points (renaturing to duplexes with a relative p value close to 0.5 in eq. 1 and a relative branch separation close to 0.5 in Fig. 4) in a population of circularly permuted molecules, the outside of one molecule is the interior of the other, and vice versa; whereas, for two strands with beginning points close to each other (relative p values close to zero and a branch separation close to 1.0) the topological outsides are homologous. According to the excluded volume hypothesis, two strands of the latter type will renature more rapidly. We believe that the excluded volume phenomenon will have a significant effect on the relative rates of formation of molecules with different p values, but we do not have available an independently derived theoretical or experimental quantitative expression for the magnitude of the effect. In the discussion that follows, we shall assume that the probability of renaturation of two strands decreases linearly as a function of their relative p value, from a value of 1.0 for $p = 0$ to a value of 0 for $p = 0.5\,[1 + (T/C)]$ (this being the maximum possible value of p).

(d) The physical chemistry of branch migration

Consider a molecule with a forked branch. The exact position of the fork does not affect the total number of base pairs in the duplex or

the steric interaction at the point of the fork. Thus, it is reasonable that all fork positions are equally probable. There are 2800 ways to have a forked branch and two ways to have an unforked branch. Experimentally, the ratio of unforked to forked branches is 1:4. If this is an equilibrium ratio at 300° K, and in view of the statistical factor of 1:1400, there is an intrinsic difference in free energy between an unforked and a forked branch of 3500 cal/mole in favor of the unforked branch. The steric interactions at the junction of the two sub-branches are different than the steric interactions with only a single branch, and a free energy difference of the magnitude calculated is not unreasonable.

Let τ be the jump time for the migration of a branch by one nucleotide. The mean time to equilibrate the fork position over 2800 nucleotides is then of the order of $(2800)^2 \tau$. The sample scored in the histogram of fork positions (Fig. 6) was allowed to renature for about 10^4 sec; thus an upper limit on τ is 10^{-3} sec, if equilibrium has indeed been achieved. There are no strictly comparable measurements in the literature for comparison, but this relaxation time is of the same order as that observed by Massie and Zimm (1969) for the relaxation time per nucleotide in ordinary renaturation under comparable conditions. Better measurements of the equilibrium and kinetics in branch migration would appear to be both useful and practicable.

(e) The distribution of beginning points

The question at issue is whether the circular permutation around the genome is complete, or whether there are certain points or regions

along the genome which have a higher than random probability of being beginning points.

The histogram of Fig. 4 does not show a uniform occurrence of molecules with all possible branch positions and hence, at first thought, suggests a nonuniform distribution of beginning points in the population of DNA molecules. We shall argue below that there are several physical factors which contribute to the shape of the histogram and that there is no evidence in Fig. 4 against the hypothesis of a uniform distribution of beginning points. With reference to Fig. 4, for renatured linear molecules with a double-strand length of $\underline{D}$, the separation $\underline{p}$ of the beginning points of the two strands in units of the genome size, $\underline{C}$, is $\underline{p} = 1 - (\underline{D}/\underline{C}) + \underline{t}$, where $\underline{t} = \underline{T}/\underline{C}$ is the fractional length of the terminal repetition. In Fig. 4 there are renatured linear molecules with values of $\underline{D}/\underline{C}$ in the interval 1.0 to $1.0 + \underline{t}$. These are molecules with $\underline{p}$ values in the interval $0 < \underline{p} < \underline{t}$. They do not have mutually complementary regions at their ends and are incapable of cyclization. The important point is that these are a class of molecules with beginning points of the two strands less than one terminal repetition apart.

There are some renatured linear molecules with values of $\underline{p} > \underline{t}$. These molecules presumably do have complementary regions on their ends and are capable of cyclizing but have not done so for kinetic reasons. The rate of cyclization is presumably proportional to the length of the complementary ends and therefore slower the shorter the ends. However, it should be noted that for all but an insignificant number of such molecules, the complementary ends are greater than 200 base pairs long and, according to arguments given previously (Wang and Davidson, 1966a), the equilibrium under renaturing conditions strongly favors cyclization.

For renatured circular molecules, we define the fractional branch separation by the shorter distance around the circle as $\underline{b} = 1 - (\underline{B}/\underline{C})$, where $\underline{B}$ was defined previously as the separation of the branches by the longer way around the circle. In principle, molecules with values of $\underline{p} > \underline{t}$ are capable of cyclization. Due to branch migration, the branch separation, $\underline{b}$, for a given value of $\underline{p}$ can be anywhere between $\underline{p} - \underline{t}$ and $\underline{p} + \underline{t}$. As we shall see, this affects the distribution function for $\underline{b}$ values.

The histogram of Fig. 4 shows an increase in the number of renatured circular molecules with decreasing $\underline{b}$ values between 0.5 and 0.15 but a sharp fall in the number of such molecules as $\underline{b}$ goes from 0.15 to 0. Thus, the results show that a number of positions along the chromosome occur as beginning points.

We now attempt to interpret the shape of the distribution curve of Fig. 4. The increase in the number of molecules with decreasing $\underline{b}$ values between 0.5 and 0.15 can be attributed, partly or solely, to the selective renaturation phenomenon proposed in a preceding section. Furthermore, the sharp decrease in the number of molecules as $\underline{b}$ goes from 0.15 to 0 can be attributed to branch migration, as discussed below. Thus, there is no evidence in the histogram against the hypothesis of a random distribution of beginning points.

The argument about the effect of branch migration goes as follows: Let $\underline{f}(\underline{p})d\underline{p}$ be the fractional number of circular molecules and molecules which are in principle capable of cyclization with $\underline{p}$ values

in the interval $\underline{p}$ to $\underline{p} + d\underline{p}$. The range for $\underline{p}$ is $0 < \underline{p} < 0.5(1 + \underline{t})$. (A renatured molecule with a $\underline{p}$ value, $\underline{p}_1$, greater than $0.5(1 + \underline{t})$ gives the same branch separation as though its $\underline{p}$ value were $(1 + \underline{t} - \underline{p}_1)$ which is less than $0.5(1 + \underline{t})$. In fact, the experimental method used here cannot distinguish between circular permutations over half the chromosome and over all of the chromosome.) For a given $\underline{p}$, the branch separation $\underline{b}$ lies between $\underline{p} - \underline{t}$ and $\underline{p} + \underline{t}$ due to branch migration. We assume that complete equilibration of all branch positions has occurred and that for a given $\underline{p}$ there is a uniform probability of any value of $\underline{b}$ between $\underline{p} - \underline{t}$ and $\underline{p} + \underline{t}$. (The experimental fact is that there is a slightly higher probability of the values $\underline{p} - \underline{t}$ and/or $\underline{p} + \underline{t}$ than any of the intermediate values, but we neglect this.)

Let $\underline{N}(\underline{b})^{d\underline{b}}$ be the number of renatured circular molecules with a branch separation in the interval $\underline{b}$ to $\underline{b} + d\underline{b}$. We wish to evaluate $\underline{N}(\underline{b})$, given $\underline{f}(\underline{p})$ and the phenomenon of branch migration.

A molecule with a branch separation $\underline{b}$ such that $2\underline{t} < \underline{b} < 0.5\text{-}1.5\ \underline{t}$ receives contributions from all $\underline{p}$ values in the range $\underline{b} - \underline{t}$ to $\underline{b} + \underline{t}$. However, molecules with small $\underline{b}$ values $(0 < \underline{b} < 2\underline{t})$ receive contributions only from a smaller interval of $\underline{p}$ values between $\underline{t}$ and $\underline{b} + \underline{t}$. This is the important effect which decreases the number of molecules with small $\underline{b}$ values. The case for $\underline{b}$ values greater than $0.5\text{-}1.5\ \underline{t}$ is a little more complicated and not important here; the answer is correctly stated in the range of conditions given for the integrations below.

Assuming complete equilibration of all fork positions, the function $\underline{N}(\underline{b})$ is given by

$$\underline{N}(\underline{b}) = \int_{\underline{t}}^{\underline{b}+\underline{t}} \underline{f}(\underline{p})d\underline{p} \quad \text{for} \quad 0 < \underline{b} < 2\underline{t} \tag{5}$$

$$\underline{N}(\underline{b}) = \int_{\underline{b}-\underline{t}}^{\underline{b}+\underline{t}} \underline{f}(\underline{p})d\underline{p} \quad \text{for} \quad 2\underline{t} < \underline{b} < 0.5-1.5\underline{t} \tag{6}$$

$$\underline{N}(\underline{b}) = \int_{\underline{b}-\underline{t}}^{\underline{b}+\underline{t}} \underline{f}(\underline{p})d\underline{p} + \int_{1-\underline{b}-\underline{t}}^{0.5+0.5\underline{t}} \underline{f}(\underline{p})d\underline{p}$$

$$\text{for} \quad 0.5 - 1.5\underline{t} < \underline{b} < 0.5 - 0.5\underline{t} \tag{7}$$

$$\underline{N}(\underline{b}) = \int_{\underline{b}-\underline{t}}^{0.5+0.5\underline{t}} \underline{f}(\underline{p})d\underline{p} + \int_{1-\underline{b}-\underline{t}}^{0.5+0.5\underline{t}} \underline{f}(\underline{p})d\underline{p}$$

$$\text{for} \quad 0.5 - 0.5\underline{t} < \underline{b} < 0.5 \tag{8}$$

To calculate $\underline{N}(\underline{b})$ we need to know $\underline{f}(\underline{p})$. The function $\underline{f}(\underline{p})$ is affected by the actual distribution of beginning points and by the selective renaturation phenomenon. The latter causes $\underline{f}(\underline{p})$ to decrease with increasing $\underline{p}$. To calculate a theoretical curve, we assume that $\underline{f}(\underline{p})$ decreases linearly from $\underline{f}(\underline{p}) = 1$ at $\underline{p} = 0$ to $\underline{f}(\underline{p}) = 0$ for $\underline{p} = 0.5(1 + \underline{t})$ due to the selective renaturation phenomenon; that is,

$$\underline{f}(\underline{p}) = 1 - \underline{p}/0.5(1 + \underline{t}) \tag{9}$$

The curve so calculated from equations **5**, 6, 7, and 8 is presented as a theoretical curve in Fig. 8. To compare it with the observed

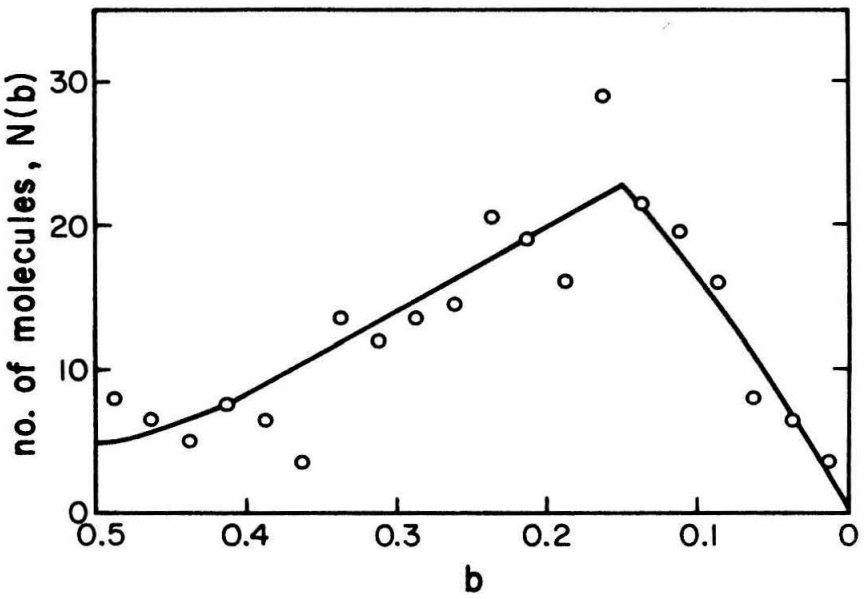
histogram of Fig. 4, we must add a correction to the latter for the linear molecules with values of $\underline{p} > \underline{t}$ which have failed to cyclize. This correction is made by assuming that such a linear molecule with a given value of $\underline{p}$ contributes uniformly to all values of $\underline{b}$ between $\underline{p} - \underline{t}$ and $\underline{p} + \underline{t}$. The histogram of $\underline{b}$ values from Fig. 4, so corrected, is shown as a set of points in Fig. 8. There is good agreement between the "theoretical" curve and the experimental points. The most important features of the curve, the maximum around $\underline{b} = 2\underline{t}$, and the decrease of $\underline{N}(\underline{b})$ to zero as $\underline{b}$ goes to 0, are not strongly dependent on the exact shape of the $\underline{f}(\underline{p})$ curve, provided it is relatively uniform for $0 < \underline{p} < 4\underline{t}$.

In view of all of the experimental errors and the theoretical assumptions, we can only conclude that the present data are consistent with the idea of a uniform distribution of beginning points, with the observed distribution of branch separations modified by the selective renaturation phenomenon and branch migration.

Finally, we note that Ikeda and Tomizawa (1968) have reported a histogram which is qualitatively similar to that of Fig. 4 for the distribution of branch separations in renatured P1 DNA molecules. They observe an increase in the number of renatured molecules with decreasing $\underline{p}$ values for $\underline{p} > 0.1$ (approx.). Their tentative explanation that linear polymerization is more probable than cyclization for renatured Form II molecules with widely spaced beginning points and therefore with large complementary ends is in our opinion not acceptable, since the relative probability of cyclization and linear

Fig. 8. Comparison of "theoretical" (see text) and experimental distribution curve of branch separations, $\underline{b}$. The theoretical curve has been normalized to have about the same area as the experimental curve.

Fig. 8



polymerization depends on the concentration of DNA and not on the length of the complementary ends which are equally available for both reactions (Wang and Davidson, 1966b).

The histogram presented by Ikeda and Tomizawa also shows a paucity of renatured cyclic molecules with branch separation less than the size of the terminal redundancy. Each of the several factors discovered above for coliphage 15 DNA may also be operative for P1 DNA.

Finally, we note that the phenomenon of branch migration and the experimental variability would make it difficult, by the methods used here, to recognize a distribution curve for beginning points with several maxima spaced one terminal repetition apart. Such a distribution is expected for models which involve replication of a moderately long concatenate starting at a specific origin of replication with the concatenate being cut in accordance with the headful mechanism at approximately constant lengths of one genome plus one terminal repetition.

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Chapter 5

Some Physicochemical Studies on the Minicircular DNA in *E.coli* 15.

Summary

Some studies on the minute circular plasmid DNA in E. coli 15 have been undertaken. Its weight average length is 0.670 μ corresponding to a molecular weight of 1.47 X 10^6 daltons. Dimers and higher multimeric forms are found in a DNA preparation from E. coli 15 wild type. These species amount to about 5% of the total plasmid molecules. Renaturation kinetics measurements show that all the plasmids in a bacterium have the same unique base sequence. It seems that the replication of this minicircular DNA is random, whether or not the the host DNA synthesis is synchronized. Two presumed replicating species (σ form and θ form) of the minicircular DNA are observed in the electron microscope.

1. Introduction

Numerous cytoplasmic elements have been found in bacteria which are able to replicate independently of the host chromosome. The most well known among them are sex factors, colicinogenic factors and drug resistance factors (Scaife, 1967). It has recently been discovered that there exist minute plasmid DNA molecules in E. coli 15 with a molecular weight of 1.5×10^6 (Cozzarelli, Kelly and Kornberg, 1968) and in M. lysodeikticus with a molecular weight of 0.9×10^6 (Lee and Davidson, 1968; Chapter 3 in this thesis). The latter size of circular DNA molecules also exists in the kinetoplasts of Trypanosoma cruzi (Riou and Paoletti, 1967; Riou and Delain, 1969). A class of small circular DNA molecules which are heterogeneous in size have been found in HeLa cells (Radloff, Bauer and Vinograd, 1967). The biological functions of these small circular DNA's, all having molecular weights of less than 2×10^6, are not known at present.

Normally, one extrachromosomal determinant is present per chromosome, whereas E. coli 15 harbors about 15 copies of plasmids per chromosome (Cozzarelli, et al, 1968). It has been shown that M. lysodeikticus contains one minicircular DNA per bacterium (Lee and Davidson, 1968; Chapter 3 in this thesis). In this respect the minicircle (mc) in M. lyso is basically different from that in E. coli 15. In an attempt to elucidate the biological function of the mc in E. coli 15, the following fundamental questions could be asked:

(a) Are all the copies of the mc in a cell the same with respect to base sequence?

(b) A plasmid DNA replicates autonomously by definition. However, the number of copies of mc per chromosome is constant throughout exponential growth (Cozzarelli, et al, 1968), suggesting that the number of mc replicated is somehow regulated by the host. A general question would be how the host regulates the replication of the mc. More specifically, do all the copies of mc replicate simultaneously and, if they do, at what stage of the host DNA replication do these mc replicate?
(c) Perhaps the presence of multiple copies of mc indicates an amplification of some genes in the host chromosome. The amplification of ribosomal RNA genes in oocytes has been observed (Brown and Dawid, 1968). Thus a question arises whether there is any degree of base sequence homology between the mc and the host chromosome.
(d) In order for the mc to be biologically functional, it should be transcribed and a messenger RNA(s) produced. Of course, the occurrence of transcription does not necessarily mean that there also occurs translation to functional proteins. However, the question may still be asked whether there is any in vivo production of m-RNA, the base sequence of which is homologous to that of the mc. Verification of this should be compromised with (c) above.

The first two questions have been investigated and the results are reported in this thesis. It will be shown that the complexity of the mc expressed as daltons as determined from renaturation kinetics is the same as the molecular weight as determined from the contour length measurements, suggesting that all the copies of the mc in E. coli 15 have the same unique non-repeating base sequence. There occur multimeric forms of mc in E. coli 15 wild type, amounting to 5% of the total mc. An electron microscopic study shows that there seem to be two kinds of replicating forms; one

with two forking points (θ form) and another with a protruded linear branch (σ form).

A covalently closed circular DNA having a molecular weight of 15×10^6 daltons has been found in *E. coli* S/6.

2. Materials and Methods

(a) Bacterial Strains

E. coli 15 wild type, 15 TAU, 15 TAU-bar and S/6 were used in this study. The sources of these strains have been described in the previous chapter.

(b) Growth Media

The media described in the previous chapter were used for the growths of *E. coli* 15 WT, 15 TAU and 15 TAU-bar in normal preparations of mc.

For synchronous growth of *E. coli* 15 TAU-bar (Cerdá-Olmedo, 1967), a tris-salt buffer was supplemented with 0.2% glucose and, when necessary, required amino acids (152 γ/ml arginine, 120 γ/ml methionine, 56 γ/ml tryptophan and 60 γ/ml proline) and bases (30 γ/ml each of uracil and cytosine, and 2 γ/ml thymine). The tris-salt buffer consists of 9% solution A, 90% H_2O, and 1% solution B: Solution A was prepared by mixing 250 ml of tris buffer (pH was adjusted to 7.4 by titrating with concentrated HCl) containing 60 g of tris-OH, and 200 ml of salt mix containing 10 g KCl, 10g NH_4Cl, 3.3 g Na_2HPO_4 and 1.7 g Na_2SO_4. Solution B is 0.25 M $MgCl_2$.

(c) Preparation of Minicircles

E. coli 15 TAU-bar was grown in 5ℓ of the medium described above at 37°C with vigorous aeration. When $A_{600} \sim 2$, cells were pelleted by centrifuging in a Sorvall centrifuge at 8,000 rpm for 10 min. The pelleted cells were resuspended in 150 ml of 0.01 M tris-OH, 0.01 M Na_2EDTA (pH 7) (TE buffer) and 30 mg of lysozyme (Worthington) was

added to the suspension followed by an incubation at 37°C for 15 min. To this spheroplast suspension, 1.5 ml of pronase stock solution (10 mg/ml, preincubated at 37°C for 1 hr) was added. After another 5 min incubation, 15 ml of 5% SDS (sodium dodecyl sulfate) was added and the lysate incubated at 37° for 1 hr with occasional gentle stirring.

Solid CsCl was added to this viscous cell lysate to give a concentration of 1 M. After standing in the cold for 2 hrs or longer, the lysate was centrifuged in a Spinco model L2-50 centrifuge with an SW 25.2 rotor at 22,000 rpm for 2 hrs. This procedure removes the SDS precipitate, the bacterial debris and most of the large pieces of host DNA. The supernatant was carefully drawn out and $CHCl_3$- extraction was performed as described in Chapter 1. After removal of $CHCl_3$ by ether extraction, the remaining ether was blown out by bubbling N_2 gas through the solution. In order to reduce the volume, the DNA was pelleted by centrifuging at 45,000 rpm for 24 hrs with a Ti 50 rotor. The DNA pellet was resuspended in 20-30 ml of TE buffer. This DNA solution was treated with RNAse and extracted with freshly distilled phenol.

The DNA was then banded in a CsCl-ethidium bromide medium by the method of Radloff, Bauer and Vinograd (1967). The concentration of ethidium bromide was 0.2 mg/ml and the density of the medium was around 1.55 g/ml. The centrifugation was done with an SW 41 Ti rotor at 31,000 rpm or an SW 50.1 rotor at 40,000 rpm for 48 hrs. The mc bands were collected and subjected to a second banding for a further purification. For optical studies such as renaturation kinetics, a third banding was necessary to obtain an optically clean preparation.

After the second banding, the contamination of host DNA was less than 5% by weight as observed in the electron microscope. A typical second banding profile is shown in Fig. 1. After removal of ethidium bromide either by i-amyl alcohol extraction or by Dowex-50 column chromatography (Radloff, et al, 1967), the DNA was dialyzed vs a desired buffer and stored at -20°C.

(d) Sonication of DNA

Argon gas was bubbled through one to two ml of DNA solution for 15 min before sonication.

The sonicator tip was cleaned by sonicating redistilled water and then the same buffer as the DNA solution until no more uv absorbing material was released. A Bronson sonicator was used with a power level 2. DNA samples were sonicated for 3 min at 0°C under an argon atmosphere (repeats of 15 sec sonication and 15 sec rest). The sonicated DNA samples were characterized by electron microscopy and analytical band sedimentation in alkali.

(e) Electron microscopy

This method has been described in detail in the previous chapter.

(f) Analytical ultracentrifugation

The band velocity centrifugation was done essentially as described in Part II except that a photoelectric scanner was used.

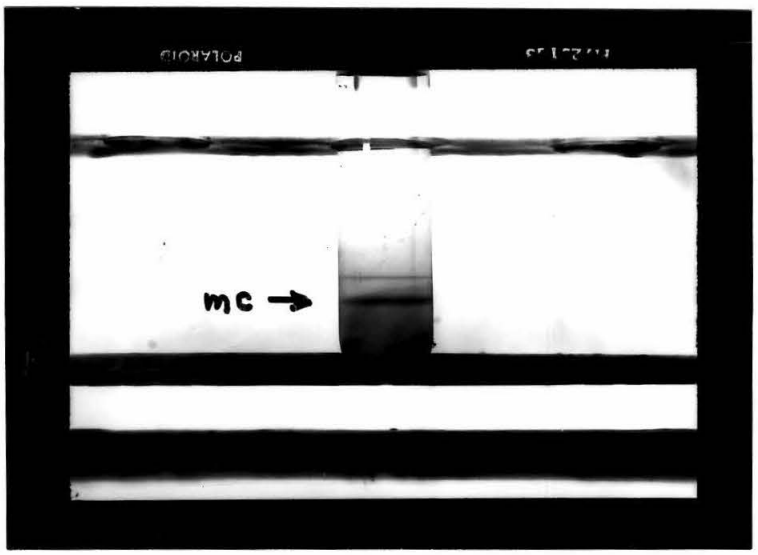
(g) Measurements of Renaturation Rates

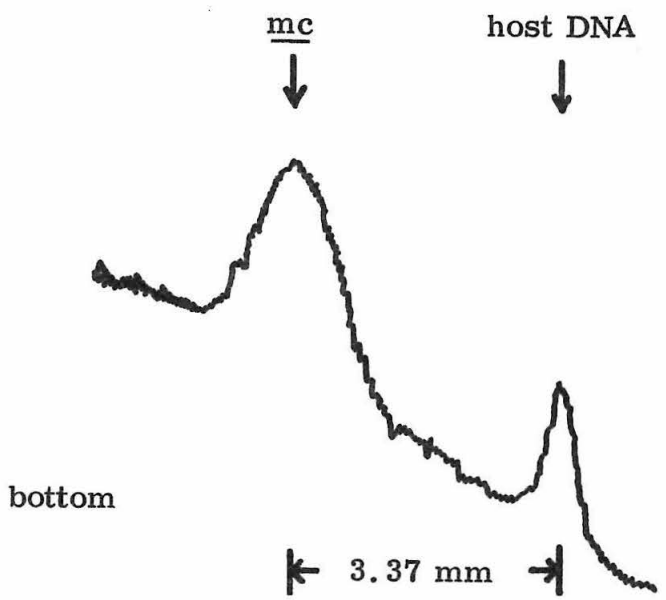
The method employed for renaturation rate measurements of sonicated, denatured DNA is in principle the same as described in Wetmur and

Fig. 1. Banding profile of minicircles in a CsCl-ethidium bromide medium.

Photographs of fluorescent bands were taken with a Polaroid camera using a type 46-L film. The photograph was traced with a microdensitometer (magnification X10). The separation of the two bands is 3.37mm in a ½" X 2" centrifuge tube.

Fig. 1





Davidson (1968).

The apparatus consisted of a Cary model 14, two water baths appropriately connected to a copper cell-holder in the Cary, and a tele-thermometer. One water bath was maintained at around 10°C above Tm and the other at (Tm-25) ± 2°C. Two three-way stop-cocks were attached in such a way that any one of the two water baths or cool water from an outside source could circulate through the cell-holder. Circulation of cool water from an outside source was necessary to lower the temperature quickly from Tm + 10°C to Tm-25°C. In this system the temperature of water in the quartz microcell within the cell-holder drops from about 95°C to about 55°C within 5 min. This time lapse was small compared to the half-renaturation reaction time (usually about 2 hours). A half ml of DNA solution ($A_{260} \sim 0.5$, phosphate buffer, pH 7.0) in a quartz cell was degassed by suction for 10 min prior to rate experiments. A reference DNA solution was treated simultaneously under identical conditions, since ionic strength variation significantly affects the rate of renaturation. DNA was denatured by circulating hot water through the cell-holder. After maintaining Tm +10°C for 10 min, the temperature of the DNA solution was quickly brought to Tm-25°C. The decrease in uv absorption at 260 mμ due to renaturation was followed with time. The temperature of water in a neighboring reference cell was monitored by a tele-thermometer. The thermometer's response time was less than 10 sec for the temperature change from 80°C to 25°C or vice versa. At the end of the reaction, the DNA was again denatured and its spectrum was taken to verify that the

hyperchromicity was solely due to denaturation. These rate measurements were repeated with the same samples giving identical results.

(h) Synchronization of DNA Replication

The synchronization of E. coli DNA replication was accomplished essentially by the methods of Maaløe and Hanawalt (1961), Hanawalt and Wax (1964), and Cerdá-Olmedo, Hanawalt and Guerola (1968).

When an exponentially growing culture of E. coli 15 TAU-bar reached a concentration of 2 - 3 X 10^8 cells/ml, the cells were pelleted in a Sorvall centrifuge (10,000 rpm, 7 min), resupended in tris-salt buffer and pelleted again. The cell pellet was resuspended in a fresh medium with all the required nutrients except for amino acids. After 90 min of incubation, the cells were again washed as above and resuspended in a medium with all the required nutrients except thymine. After 45 min of incubation, thymine was added and synchronous growth began.

Forty-five ml portions were taken out at 0, 5, 10, 20, 40, 60, 80, 100, 120 and 150 min after the addition of thymine. They were quickly chilled and NaN_3 was added to give a concentration of 0.01 M. The cells were pelleted and treated as described earlier to determine the amount of mc in each sample. A general outline of E. coli 15 TAU-bar synchronous growth is shown in Fig. 2.

(i) Determination of DNA concentrations

In the analyses of the amounts of mc during synchronous growth, concentrations were too low to be determined optically. Thus, the following electron microscopic method was used. An mc preparation

Fig. 2. Synchronous growth of E. coli 15 TAU-bar.

A 500 ml culture was grown at 37°C with vigorous aeration. The cell concentration was measured by turbidity at 600 mμ. From a relation between the turbidity and the number of cells, the concentration in terms of cells/ml was estimated.

a; pelleting and washing of cells.

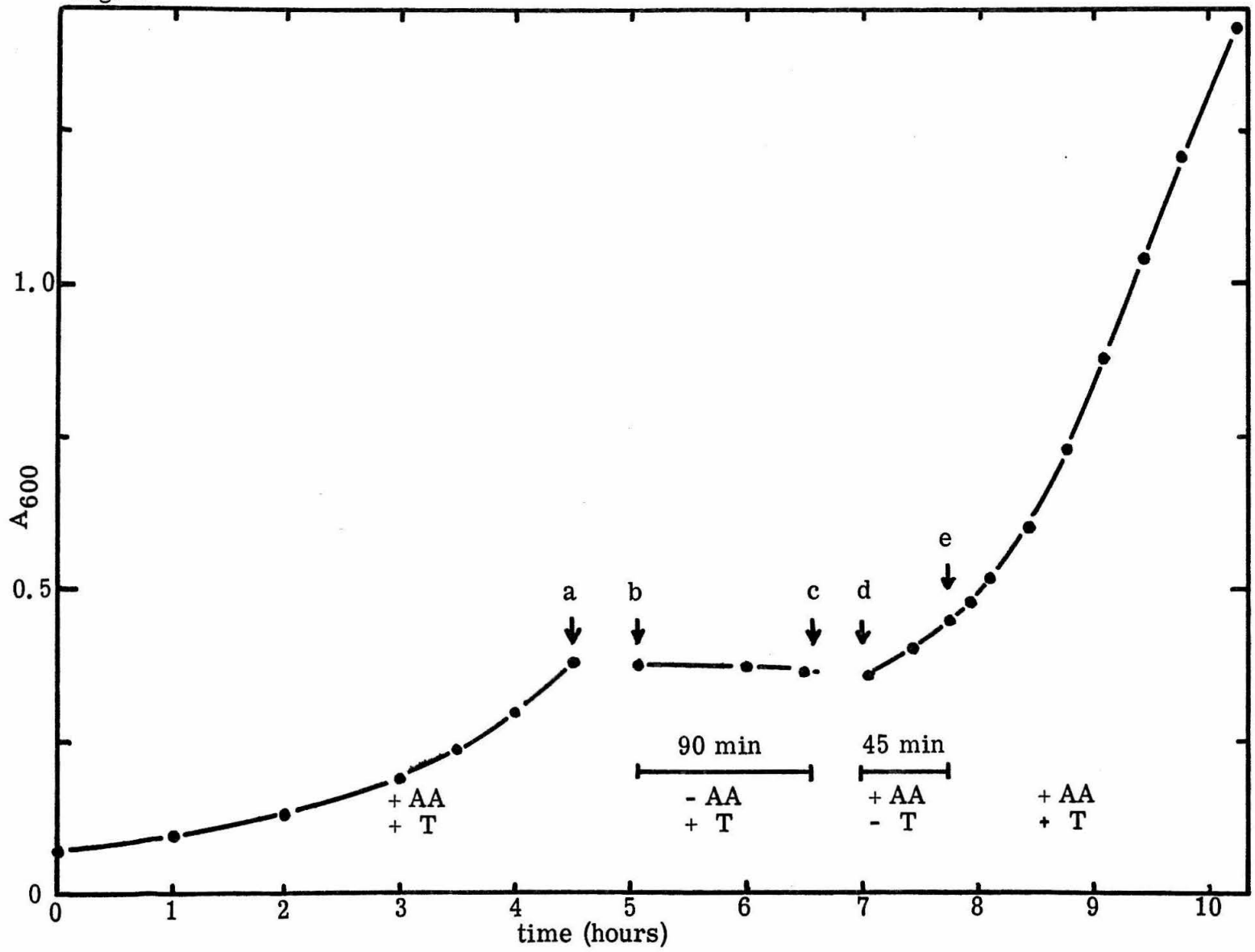
b; resuspension in a new medium with thymine but without amino acids.

c; pelleting and washing.

d; resuspension in a medium with amino acids but without thymine.

e; addition of thymine.

Fig. 2


1.0
0.5
0
A_{600}
a
b
c
d
e
90 min
45 min
+ AA
+ T
- AA
+ T
+ AA
- T
+ AA
+ T
0
1
2
3
4
5
6
7
8
9
10
time (hours)

was mixed with a known amount of ΦXRFI DNA (a generous gift from Mr. J. Sedat). The mixture was mounted on an electron microscope grid and the relative numbers were scored directly on the fluorescent screen or from the photographs taken. Because of the size difference between mc and ΦXRF DNA (ΦXRF DNA is 2.3 times longer than mc), they were easily distinguishable. This method gave very reproducible results.

The concentrations of host DNA were determined by the diphenylamine colorimetric method of Burton (1956), which is a slight modification of Dische (1955). The pellets of bacterial debris and large pieces of host DNA were resuspended in TE buffer. The milky suspension was dialyzed vs TE buffer exhaustively to remove SDS. This DNA solution was combined with the main DNA fraction from the CsCl-ethidium bromide banding and the diphenylamine method was performed. To verify the specificity of the reaction the assay was used on a portion of DNA solution which had been subjected to pancreatic DNAse treatment and an extensive dialysis to remove resulting nucleotides. Less than 5% of the original concentration was detected. E. coli DNA of a known concentration was assayed simultaneously and used as a reference.

3. Results

(a) Minicircles and Other Circular DNA Species in E. coli 15

A minute circular DNA species (called "minicircles" or mc in this thesis) has been found as a plasmid in E. coli 15 strains irrespective of their colicin 15 productions (Cozzarelli, et al, 1968).

The mc prepared from the lower band in a CsCl-ethidium bromide centrifugation show both twisted and open structures under an electron microscope. Its contour length is 0.670 μ. Based on the ratio (2.32) of contour lengths of ΦXRF DNA and mc mounted on the same electron microscope grid and the molecular weight of 3.4×10^6 daltons (Sinsheimer, 1959), the molecular weight of mc is calculated to be 1.47×10^6 which is in excellent agreement with the value previously reported (Cozzarelli, et al, 1968). The histogram of contour lengths of mc and ΦXRF DNA is shown in Fig. 3(a). The sedimentation coefficient ($S^o_{20,w}$) of mc at pH 7.4 is 17.2 S. The ratio of uncorrected S values at pH 13 and at pH 7.4 is 2.56 which agrees very well with the ratio obtained for polyoma DNA (Vinograd, et al, 1965). The uncorrected S value of 27.2 at pH 13 is as expected from this size DNA (Clayton and Vinograd, 1967).

To investigate whether any multimeric forms of mc exist in E. coli 15, mc preparations from a wild type(WT), TAU and TAU-bar were examined under an electron microscope. The results of this study are shown in Fig. 3(b) and 4. In an mc preparation from 15 WT, there are about 5% multimeric forms of mc by number. Fig. 3(b) shows length distributions of multimeric forms. As also seen in this histogram, the frequency of the occurrence of different multimeric forms decreases sharply as the order of multimers increases. Preparations from 15 TAU and

Fig. 3(a). Length comparison of Minicircle and ΦX 174 RF DNA.

Minicircle and ΦXRF DNA were mounted on the same electron microscopic grid for a direct and accurate comparison. The number of molecules are plotted vs lengths relative to the weight average length of mc ($\langle L \rangle_w^{mc}$).

The ratio, $\langle L \rangle_w^{\Phi X} / \langle L \rangle_w^{mc}$, is 2.316. The weight average lengths of mc and ΦXRF DNA are 0.670 (±0.030) μ and 1.550 (±0.040) μ, respectively. The molecular weight of mc is 1.47×10^6 daltons, based on the ΦXRF DNA molecular weight of 3.4×10^6.

Fig. 3(b). Length Histogram of A Minicircle Preparation from E. coli 15 WT.

The plotting is the same as above. The population of mc is, in reality, much larger than shown here. The number of multimers was 27 molecules out of a total of 527 circles.

Fig. 3

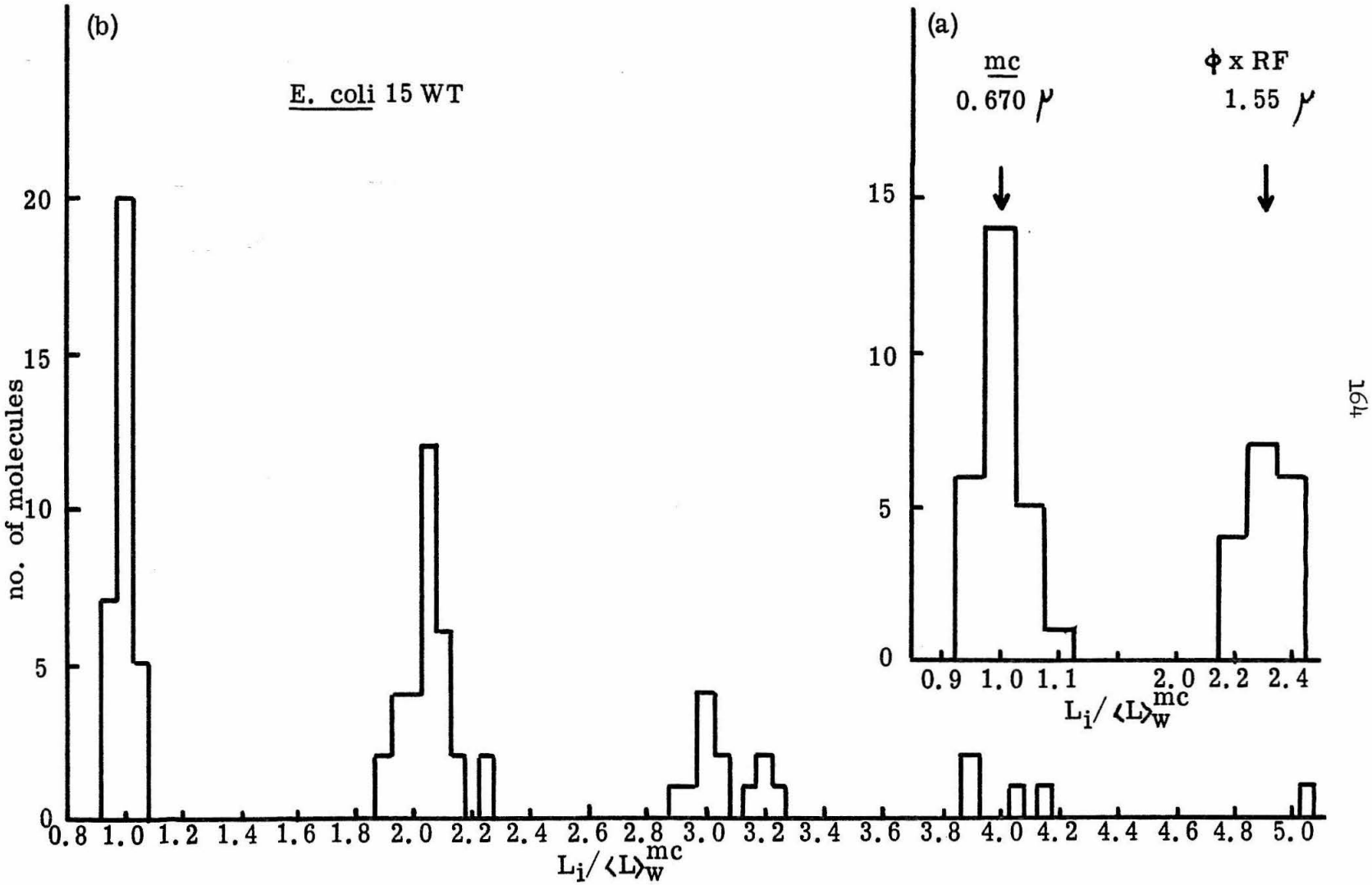
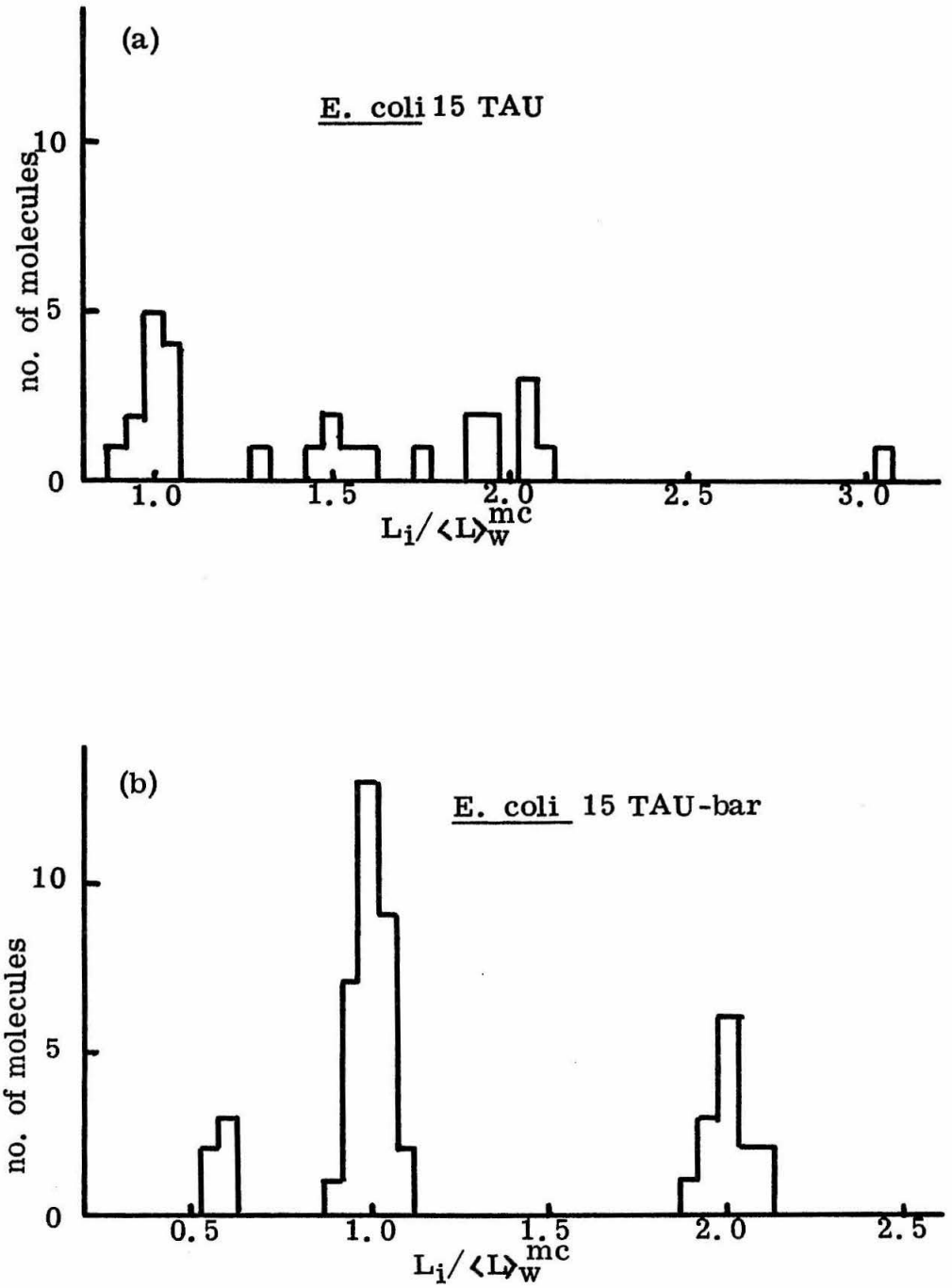

(b)
E. coli 15 WT
no. of molecules
0
5
10
15
20
0.8 1.0 1.2 1.4 1.6 1.8 2.0 2.2 2.4 2.6 2.8 3.0 3.2 3.4 3.6 3.8 4.0 4.2 4.4 4.6 4.8 5.0
$L_i / \langle L \rangle_w^{mc}$
(a)
mc
0.670 μ
φ x RF
1.55 μ
0
5
10
15
0.9 1.0 1.1 2.0 2.2 2.4
$L_i / \langle L \rangle_w^{mc}$

Fig. 4. Length Histograms of Minicircle Preparations from E. coli 15 TAU (a) and 15 TAU-bar (b).

The plotting is the same as Fig. 3. The population of multimers was around 0.1-0.5% of the total circular DNA molecules.

Fig. 4


(a)
E. coli 15 TAU
no. of molecules
10
5
0
1.0
1.5
2.0
2.5
3.0
$L_i/\langle L\rangle_w^{mc}$
(b)
E. coli 15 TAU-bar
no. of molecules
10
5
0
0.5
1.0
1.5
2.0
2.5
$L_i/\langle L\rangle_w^{mc}$

15 TAU-bar show, however, much less frequent multimeric forms of the order of 0.1-0.5% of the total circular DNA species. Furthermore, as shown in Fig. 4, several molecules 1.5 times the size of a monomer were observed in the 15 TAU preparation, and some molecules with half the size of a monomer were observed in the 15 TAU-bar preparation.

Some examples of mc and other circular molecules from E. coli 15 TAU-bar are shown in Plate I. Species A is a monomeric form, Species B has the size of a dimer and Species C is half of the monomer size. Species D is probably catenated multimers. Such a circular DNA species has been observed previously in other systems (Hudson, Clayton and Vinograd, 1968; Rhoades and Thomas, 1968). In Plate I, also shown are some examples of "chained" molecules. They probably are catenated molecules and/or replicating molecules (cf. Plate III). It seems quite unlikely that they are accidentally overlapped molecules, in view of the fact that such overlapping does not normally occur in our electron microscopic technique. Such complex molecular species have also been observed in kinetoplasts (Riou and Delain, 1969).

One incidental observation made in the course of this study is of interest. One large batch of mc preparation yielded a population of circular DNA molecules of various sizes (Plate II). The length distribution of these heterogeneous circular DNA molecules (HCD) is shown in Fig. 5. The length variation is not random. But it shows a collection of molecular populations having certain discrete sizes. Each population may have its own unique base sequence, since there seem to be no multimeric relations. In fact, as will be shown later, the kinetic

Plate I. Electron Micrographs of a Minicircle Preparation from E. coli 15 TAU-bar.

DNA was mounted by a basic protein film technique. After staining with a uranyl salt the grid was rotary-shadowed (see the previous chapter). Species D at the low right hand corner was shadowed from one direction after rotary-shadowing. See text for explanation of letters.

Plate I

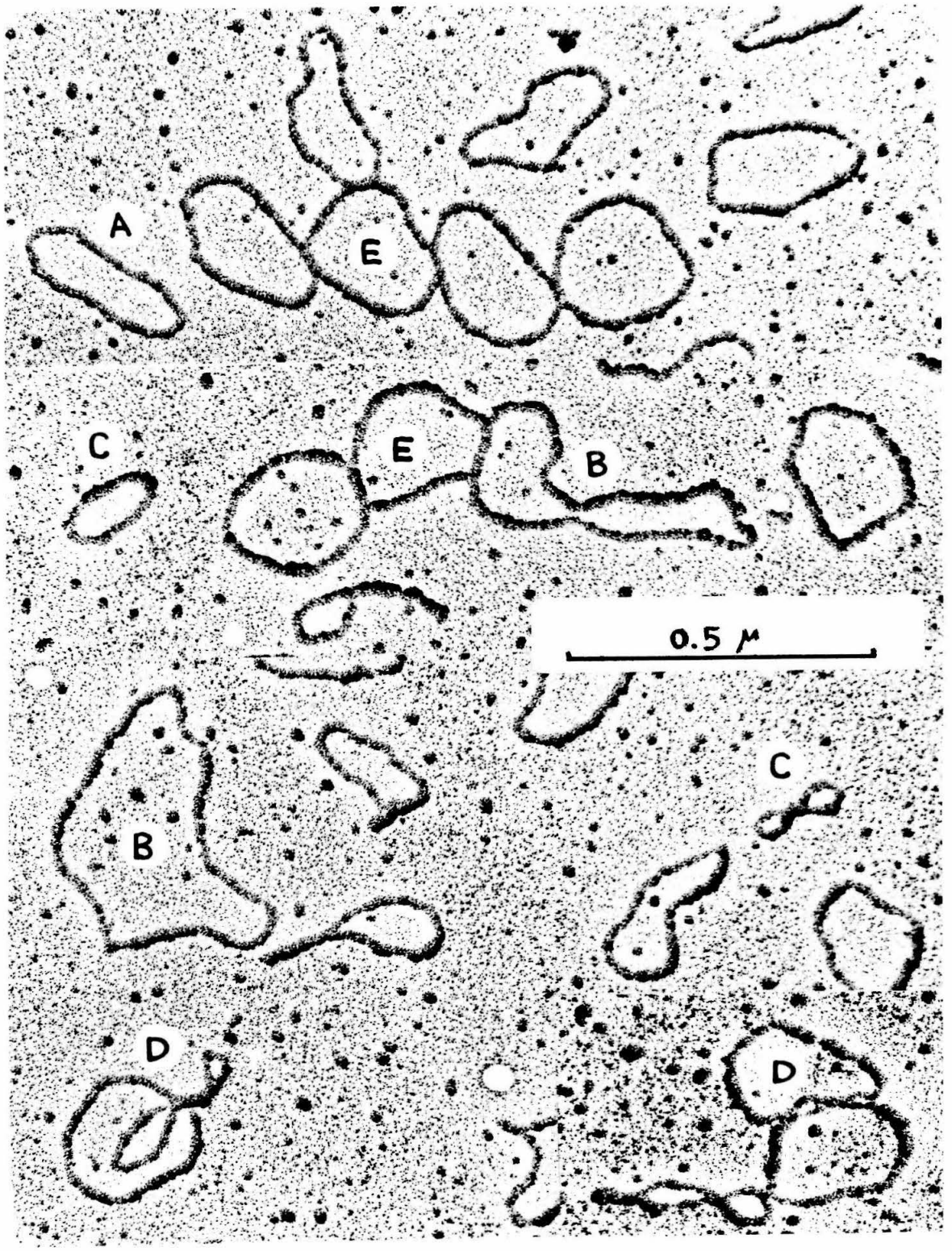


Plate II. Electron Micrographs of Heterogeneous Size Circular DNA Molecules in One Preparation from *E. coli* 15 TAU-bar. The smallest circular DNA species seen are *mc*.

Plate II

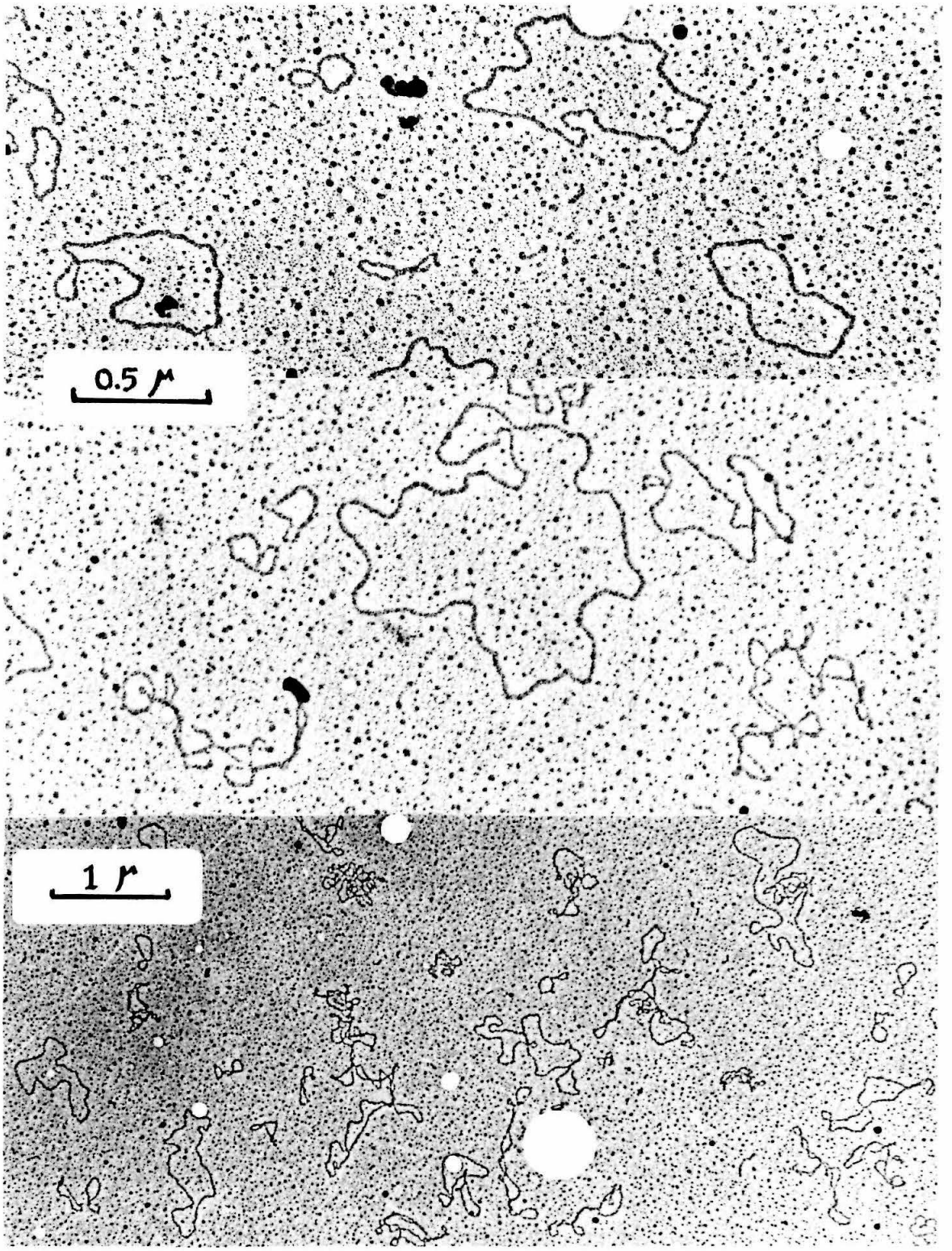


Fig. 5. Length Histogram of Heterogeneous Circular DNA Molecules in One Preparation from E. coli 15 TAU-bar. The lengths shown are relative to mc length.

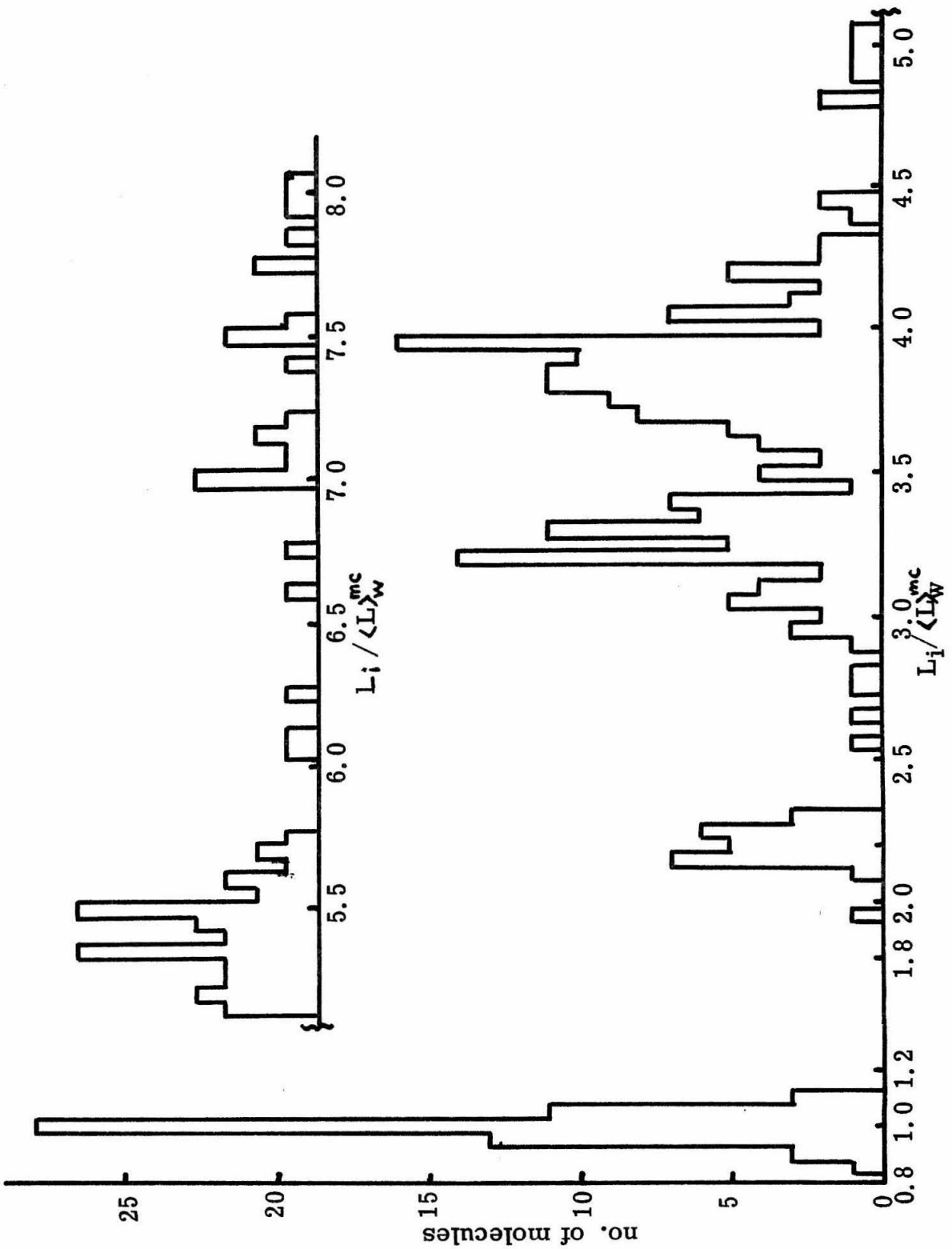

no. of molecules
25
20
15
10
5
0
0.8 1.0 1.2 1.8 2.0 2.5 3.0 3.5 4.0 4.5 5.0
$L_i / \langle L \rangle_w^{mc}$
5.5 6.0 6.5 7.0 7.5 8.0
$L_i / \langle L \rangle_w^{mc}$

Fig. 5

complexity of HCD is even larger than that of T7 DNA. Unfortunately, the production of HCD could not be repeated in later experiments because of reasons unknown.

(b) Molecular Weights of Sonicated DNA

To determine the complexity of the mc, the mc and other DNA's of interest were sonicated as described in Materials and Methods. Two methods, electron microscopy and band velocity sedimentation analysis, were used to determine the molecular weights of sonicated DNA samples.

The distribution of contour length of sonicated DNA is shown in Fig. 6. As expected, the distribution is rather heterogeneous with a standard deviation of 40%. The expected standard deviation of the length distribution of homogeneous double-stranded DNA molecules of this size is 10% (see the previous chapter). However, the important point for our purpose is that the shape of the distributions is the same for four sonicated DNA somples. The molecular weights given in Table 1 are half the values for double-stranded DNA fragments evaluated from contour lengths in Fig. 6.

Sedimentation coefficients of sonnicated DNA's were determined by alkaline band velocity centrifugations. The sedimentation coefficients $\left(s^{o\,pH\ 13}_{20,w}\right)$ and the molecular weights evaluated by the Studier relation (1965) are summarized in Table 1. The agreement between the molecular weights determined from contour lengths ($\langle L \rangle_w$) of double-stranded DNA and from the sedimentation coefficients are satisfactory. The small difference observed could easily be due to the inappropriateness of the Studier relation in this range of molecular weight. Another

Fig. 6. Length Histograms of Sonicated DNA

The lengths shown are in cm on tracing paper. They are converted to μ and the weight average molecular weights calculated with ΦX 174 RF DNA as a standard are as follows: 0.272×10^6 for T7 DNA, 0.292×10^6 for HCD, 0.281×10^6 for ΦXRF DNA, and 0.269×10^6 for mc.

Fig. 6

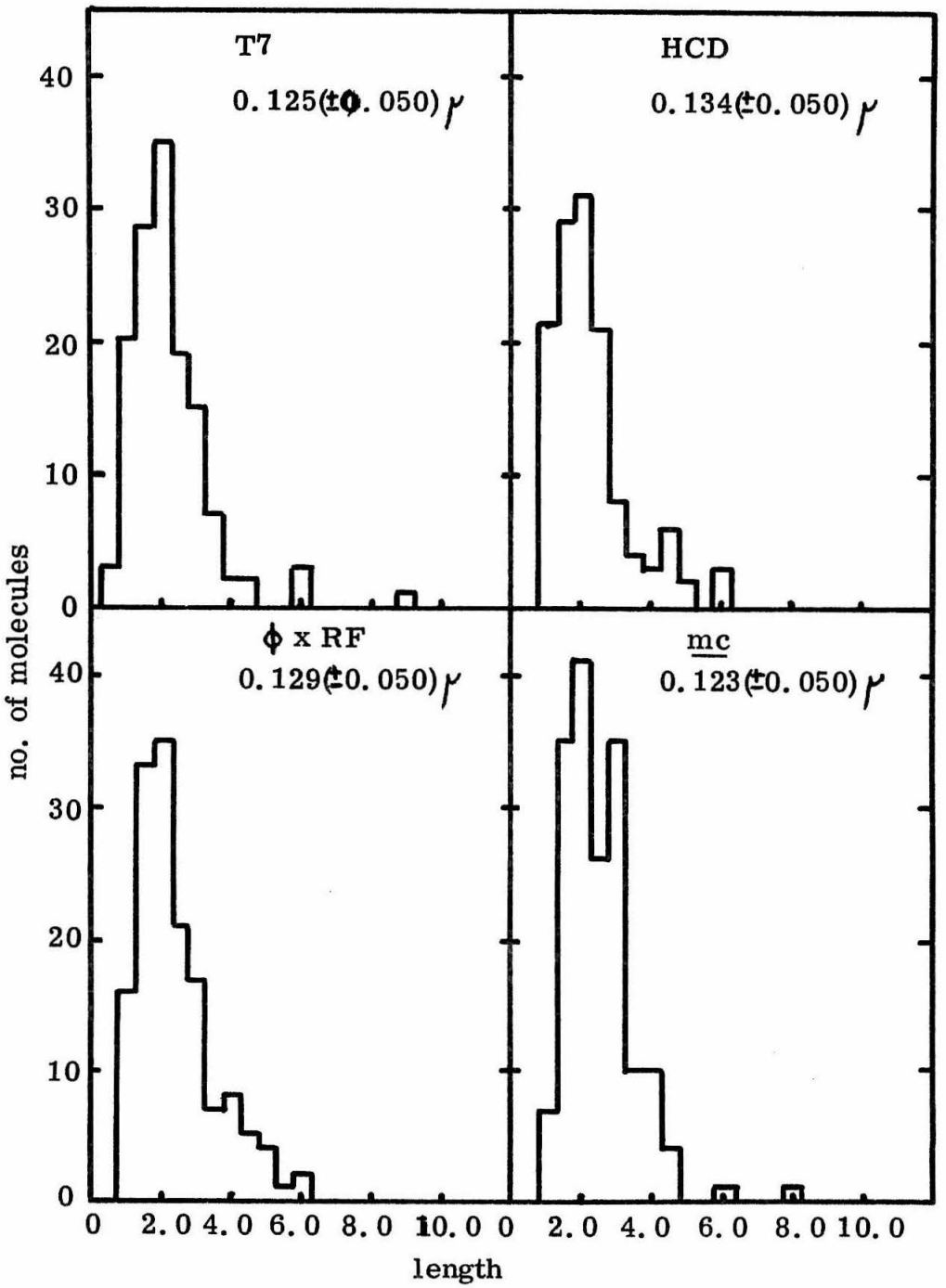

T7
0.125(±0.050) μ
HCD
0.134(±0.050) μ
φ x RF
0.129(±0.050) μ
mc
0.123(±0.050) μ
no. of molecules
length
0
10
20
30
40
2.0
4.0
6.0
8.0
10.0

TABLE 1

Molecular Weights of Sonicated Denatured DNA's

DNA's	EM		Sedimentation Coefficients	
	$\langle L \rangle_w$ (μ)	mol. wt. of single-stranded DNA (X 10^6) (1)	$s^o_{20,w}$ pH 13	mol. wt. X 10^6 (2)
T7	0.125	0.136	5.30	0.10
HCD	0.134	0.146	6.24	0.15
ΦXRF	0.129	0.140	5.39	0.11
mc	0.123	0.135	5.26	0.10

(1) Molecular weights of double-stranded DNA's based on $\langle L \rangle_w$ of ΦXRF were divided by two.

(2) Based on the following relation (Studier, 1965);

$$s^{o\,pH\ 13}_{20,w} = 0.0528\ M^{0.40}$$

possibility is that there are a small number of single-strand breaks on the double-stranded DNA fragments. These would not be revealed in the electron microscope. Single-strand breaks could have occurred during sonication. If the latter possibility is assumed correct, the number of breaks per single-stranded DNA can be calculated by the following relation (Tanford, 1961);

$$\frac{Mw}{Mw_o} = \frac{2(e^{-s} + s - 1)}{s^2}$$

where $Mw_o \equiv$ weight average molecular weight of original polymer

$Mw \equiv$ weight average molecular weight of degraded polymer

$s \equiv$ number of breaks in the original polymer.

In our case, $Mw_o = 0.14 \times 10^6$ from the contour length measurements and $Mw = 0.10 \times 10^6$ from alkaline sedimentation coefficients. The above relation yields 1.1 single-strand breaks per strand.

(c) Complexity of Minicircle

E. coli 15 strains harbor the *mc* as a plasmid and the number of *mc* per chromosome is 12-15 (Cozzarelli, *et al*, 1968). Normally, one copy of a plasmid or an episome exists per host chromosome, the number being somehow regulated by the host (Jacob, Brenner and Cuzin, 1963). The question is raised, do all 15 copies of the *mc* have the same base sequences or do some of them contain different sequences? A kinetic study of the renaturation of denatured DNA solves this problem. The rate of renaturation is determined by the probability that a given single-strand segment will find a correct mating strand in the solution.

The empirical second-order rate constant for renaturation of denatured DNA is known to be inversely proportional to the complexity of the DNA (Bolton, et al., 1965; Wetmur and Davidson, 1968). Further, the complexity is directly related to the molecular weight of a genome with a non-repeating base sequence. Thus, for instance, if there were 15 different copies of mc present in the bacterium, the rate of renaturation would be 15-fold slower than if there were only one.

Renaturation of sonicated denatured mc, ΦX 174 RF DNA, T7 DNA and HCD has been performed as described in Materials and Methods. The following equation was used to determine the second-order rate constants (Wetmur and Davidson, 1968).

$$\frac{A_o - A_\infty}{A - A_\infty} = 7.35 \times 10^{-5} A_\infty k_2 t + 1$$

where A_o ≡ absorbance at 260 mμ of fully denatured DNA

A_∞ ≡ absorbance of native DNA

A ≡ absorbance during renaturation

k_2 ≡ seconder-order rate constant (ℓ/mole-sec)

t ≡ time (sec)

The rate constant, k_2, was determined from the slope of the change in absorbance vs time plot. Such plots are shown in Fig. 7. The observed rate constants are 3.23 and 1.55 ℓ/mole-sec respectively for mc and ΦXRF DNA in 0.03 M $[Na^+]$. They are 2.72 and 2.02 ℓ/mole-sec for T7 DNA and HCD in 0.06 M $[Na^+]$. The rate constants were corrected for molecular weights (Table 1) by the following relations (Wetmur and Davidson, 1968);

Fig. 7. The Second-Order Rate Plots.

The straight lines are least square plots of experimental points. Minicircle and ΦXRF DNA are in 0.01 M NaH_2PO_4, 0.01 M Na_2HPO_4 (pH 7.0). T7 DNA and HCD are in 0.02 M NaH_2PO_4, 0.02 M Na_2HPO_4 (pH 7.0).

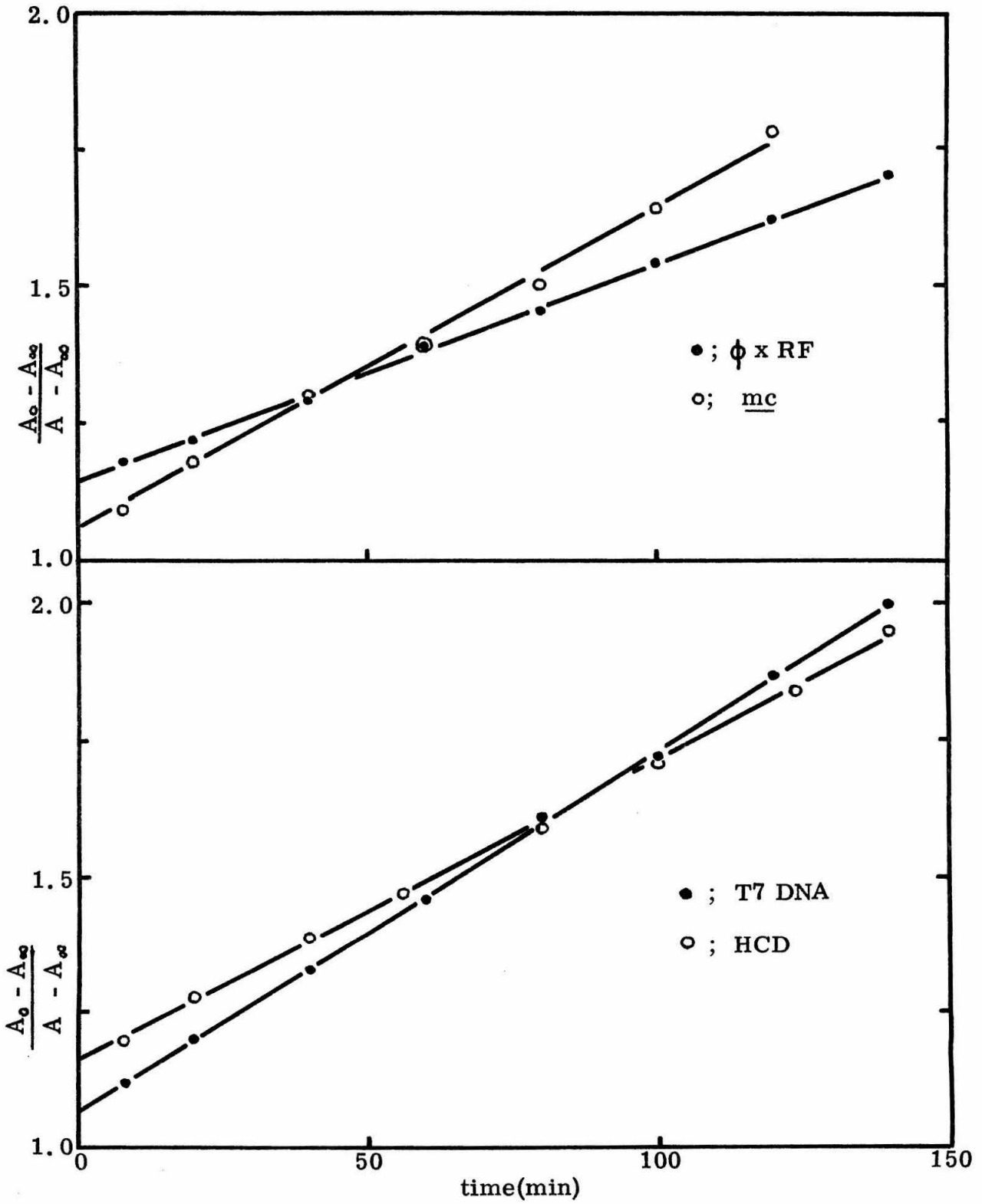
mc; A_∞= 0.410, slope = 5.90 X 10^{-3} min^{-1}, intercept = 1.05

ΦXRF DNA; A_∞= 0.578, slope = 3.94 X 10^{-3} min^{-1}, intercept = 1.14

T7 DNA; A_∞= 0.555, slope = 6.67 X 10^{-3} min^{-1}, intercept = 1.06

HCD; A_∞= 0.628, slope = 5.59 X 10^{-3} min^{-1}, intercept = 1.16

Fig. 7


2.0
1.5
1.0
$\frac{A_0 - A_\infty}{A - A_\infty}$
● ; φ x RF
○ ; mc
2.0
1.5
1.0
$\frac{A_0 - A_\infty}{A - A_\infty}$
● ; T7 DNA
○ ; HCD
0
50
100
150
time(min)

$$k_{2_{correct}} = k_{2_{obs}} \left(\frac{L}{L_{rf}}\right)^{0.5} \text{ and } k_{2_{correct}} = k_{2_{obs}} \left(\frac{s_{o\,20,w}^{pH\ 13}}{s_{o\,20,w\ rf}^{pH\ 13}}\right)^{1.25}$$

The reference DNA's were ΦXRF DNA for mc and T7 DNA for HCD. The corrected k_2's and the complexities are summarized in Table 2. The complexities in terms of daltons are 1.67×10^6 and 30×10^6 for mc and HCD, respectively. It is evident that the kinetic complexity of mc is in close agreement with the analytical complexity (1.47×10^6). Thus, it is concluded that all of the mc present in a bacterium have the same base sequence. The complexity of HCD suggests that the various sizes of circular molecules are neither multimeric forms of smaller molecules nor random fractions of bacterial genome. They represent several populations each with a unique base sequence for a total complexity of 30×10^6.

(d) Replication of Minicircles During Synchronous Replication of Host Chromosome

For a steady state growth the number of mc per chromosome is constant at 12-15 copies (Cozzarelli, et al, 1968). The constancy of this number suggests the mc replication is somehow regulated by the host chromosome replication and/or by the cell division. We may then ask whether all 15 copies of mc double simultaneously at a certain stage of host DNA replication or they replicate randomly throughout a generation irrespective of the synchrony of the host.

Synchronized initiation of host DNA replication in E. coli 15 TAU-bar has been performed by a method of amino acid starvation and successive

TABLE 2

The Second-Rate Constants and the Complexities of Several DNA Samples

DNA	MW(X 10^6)	$k_{2_{obs}}$ 0.06M $[Na^+]$	$k_{2_{obs}}$ 0.03M $[Na^+]$	$k_{2_{correct}}$ (2) $\langle L \rangle_w$	$k_{2_{correct}}$ (2) $s^o_{20,w}$ pH 13	Complexity (X 10^6) $\langle L \rangle_w$	Complexity (X 10^6) $s^o_{20,w}$ pH 13
T7 (1)	25	2.72		2.72	2.72	25	25
HCD		2.02		2.09	2.62	33	26
ΦXRF (1)	3.4		1.55	1.55	1.55	3.4	3.4
mc	1.47		3.23	3.17	3.13	1.66	1.68

(1) T7 DNA and ΦXRF were used as references and thus complexities given are their molecular weights.

(2) Corrections were based on molecular weights determined by electron microscopy and band sedimentation in alkali.

thymine starvation (see Materials and Methods). This method has been used by others (Hanawalt and Wax, 1964; Cerdá-Olmedo, et al, 1968). A 90 min period of amino acid starvation in the presence of glucose and all other nutrional requirements permits completion of a DNA synthesis cycle but not initiation of a new round of replication (Maaløe and Hanawalt, 1961; Lark, Repko and Hoffman, 1963). During a subsequent 45 min period of thymine starvation in the presence of all required amino acids, all the proteins necessary for initiation of DNA replication are synthesized. The addition of thymine at this stage allows synchronous initiation of host DNA replication.

Samples were taken out at intervals after the introduction of thymine and the amounts of host DNA and mc in each sample were determined. Results are shown in Fig. 8. Fig. 8(a) shows there is some delay in the synthesis of host DNA at the beginning contrary to the expected linear increase. This slower rate has also been observed by others who have used this method (Cerda-Olmedo, et al, 1968). These workers presented two possible explanations for this behavior; it may be due (1) to a lack of synchronous initiation in some cells or (2) to a lower initial rate of replication in all cells starting together. The former was favored from their experimental results.

The number of chromosomes per cell can be estimated from Fig. 8(a) knowing the molecular weight of the E. coli chromosome (3×10^9 daltons). The number of cells in each sample was deduced from the turbidity measurements (A_{600}) with a previously obtained relationship between A_{600} and the number of cells per ml. This relationship had been obtained

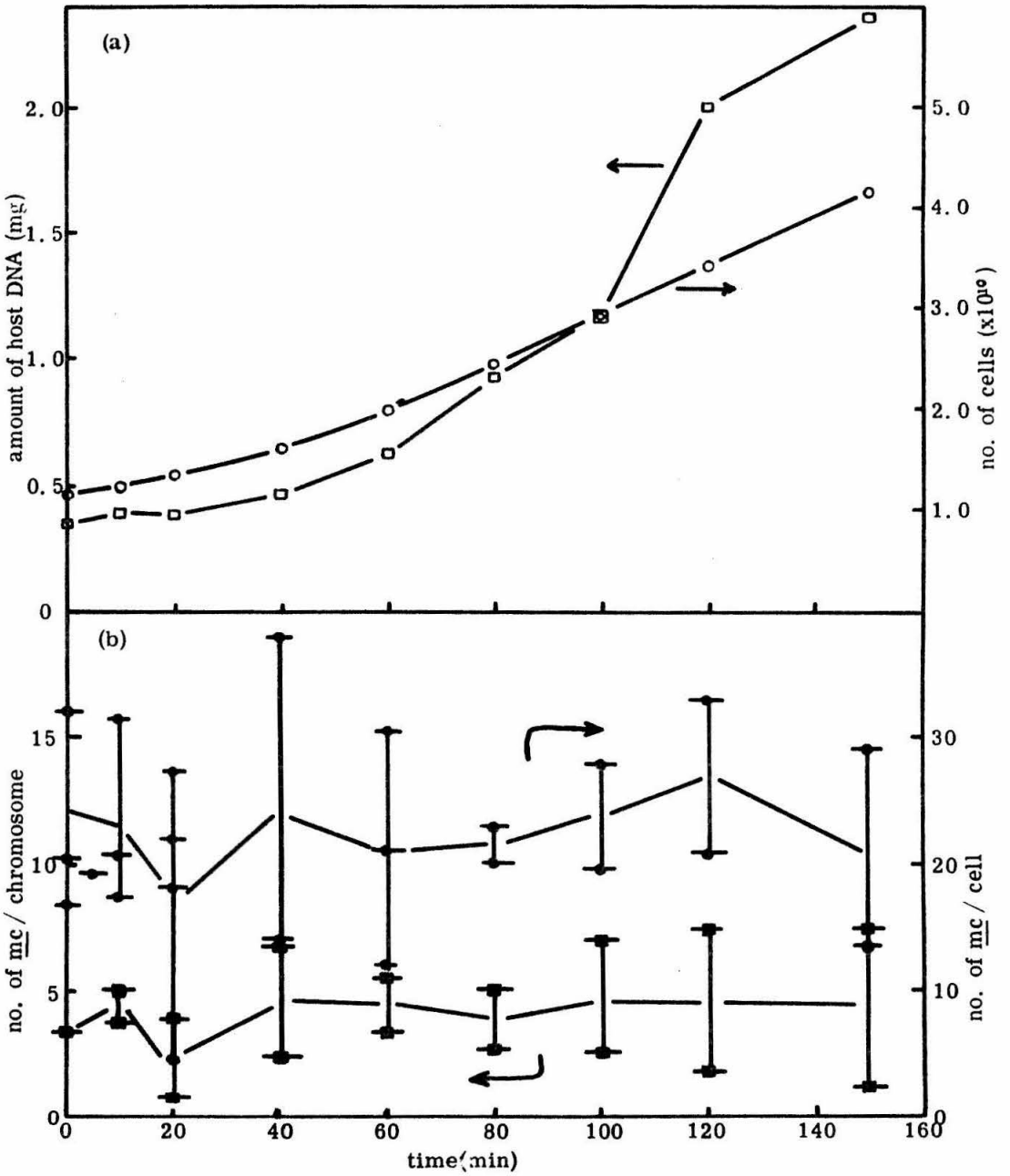
Fig. 8(a) E. coli DNA Synthesis in Synchronized Cell Growth.

The total number of cells (open circles) and the total amount of host DNA present (open squares) in each sample are plotted vs the time after addition of thymine. The number of cells per sample was estimated from turbidity measurements (A_{600}) shown in Fig. 2 and a known linear relationship between A_{600} and cells/ml obtained from a normal exponential growth. Details of cell growth and determination of DNA concentrations are described in Materials and Methods.

Fig. 8(b) Behavior of the mc Replication During Synchronous Host DNA Replication.

The number of mc per cell (filled circles) and per host chromosome (filled squares) is plotted vs time. The vertical bars indicate variations of two or more independent experiments.

Fig. 8


(a)
amount of host DNA (mg)
no. of cells (x10⁸)
2.0
1.5
1.0
0.5
0
5.0
4.0
3.0
2.0
1.0
(b)
no. of mc / chromosome
no. of mc / cell
15
10
5
0
30
20
10
0
0
20
40
60
80
100
120
140
160
time (min)

from a normal exponential growth. Therefore, it is not entirely certain that this relation be used for a synchronous cell growth. However, for the present purpose of determining the number of host chromosomes per cell, this uncertainty would not affect much. The data indicate the average number of chromosomes per cell is about six. The number reported is 2-3 chromosomes per cell in a glucose medium (Lark, 1966). Several control experiments were performed in an attempt to elucidate this discrepancy. In the first, we wished to verify that the diphenylamine reaction was indeed specific for DNA. Thus, the DNA solution was subjected to pancreatic DNAse. After removal of resulting nucleotides by dialysis, the diphenylamine method was applied. The result showed that more than 95% of color positive material had been removed by the treatment above. A further diphenylamine assay was performed on $CHCl_3$-extracted DNA. The $CHCl_3$-extraction procedure has been commonly used for the isolation of DNA from bacteria. Only 20-30% of the material positive in the color reaction was extractable by $CHCl_3$ and the rest remained at the interphase of aqueous and $CHCl_3$ layers. Similar results were obtained from normally grown cells.

Changes in the number of mc per cell and per chromosome during synchronous growth are shown in Fig. 8(b). The quantity, the number of mc per cell, is not quite relevant here, since the number of cells was deduced from turbidity measurements as described before. However, this quantity is adopted simply to compare with the experimental data obtained by other workers. Experimental variations are rather large to draw firm conclusions. These large variations are perhaps caused

by some irregularities in the synchronization from one experiment to the other and by some irregular trapping of replicating mc on the membrane. The amount of mc present in the host DNA band was less than 10% of the total mc. Despite such experimental variations it seems that the numbers of mc remain fairly constant at about 4 per chromosome and 24 per cell. The latter value is in agreement with Cozzarelli, et al. (1968), as the former if it is assumed that the number of E. coli chromosome per cell is two rather than six. It has been shown in this study and in Cozzarelli, et al. (1968) that the number of mc per cell is constant throughout a normal exponential growth. Thus, it seems likely that individual copies of mc are selected at random for replication regardless of the timing of host DNA replication. However, another possibility can not be excluded here that the periodical fluctuations seen in Fig. 8(b) are real and each maximum corresponds to the end of a generation. The minima could be explained if the replicating mc were attached to cell membrane and lost during the mc preparation.

(e) Replicating Forms of Minicircles

Replicating forms of DNA's from several microorganisms have been observed directly in the electron microscope (Okawa, Tomizawa and Fuke , 1968; Weissbach, Bartl and Salzman, 1968; Huberman, 1968; Kirschner, Wolstenholme and Gross, 1968; Thomas, Kelly and Rhoades, 1968; Knippers, Razin, Davis and Sinsheimer, 1969). The following three species of replicating λ DNA have been visualized; a long concatenate, a circular DNA molecule with a long protruded linear double-stranded DNA (σ form) (Weissbach, et al, 1968), and a circular

molecule with two forks, one presumably corresponding to the origin of replication and the other corresponding to the replicating point (θ form) (Okawa, et al, 1968). Because mc is a small DNA and multiple copies are present in a cell, it was of interest to study its replicating forms. When the main DNA band containing host linear DNA, nicked mc and presumably replicating forms of mc was examined with an electron microscope, it was indeed observed that there occur two kinds (σ form and θ form) that we tentatively identify as of replicating mc. Of course, long concatenates, if any, could not be distinguished from host DNA fragments in this examination. Some examples of these two species are shown in Plate III.

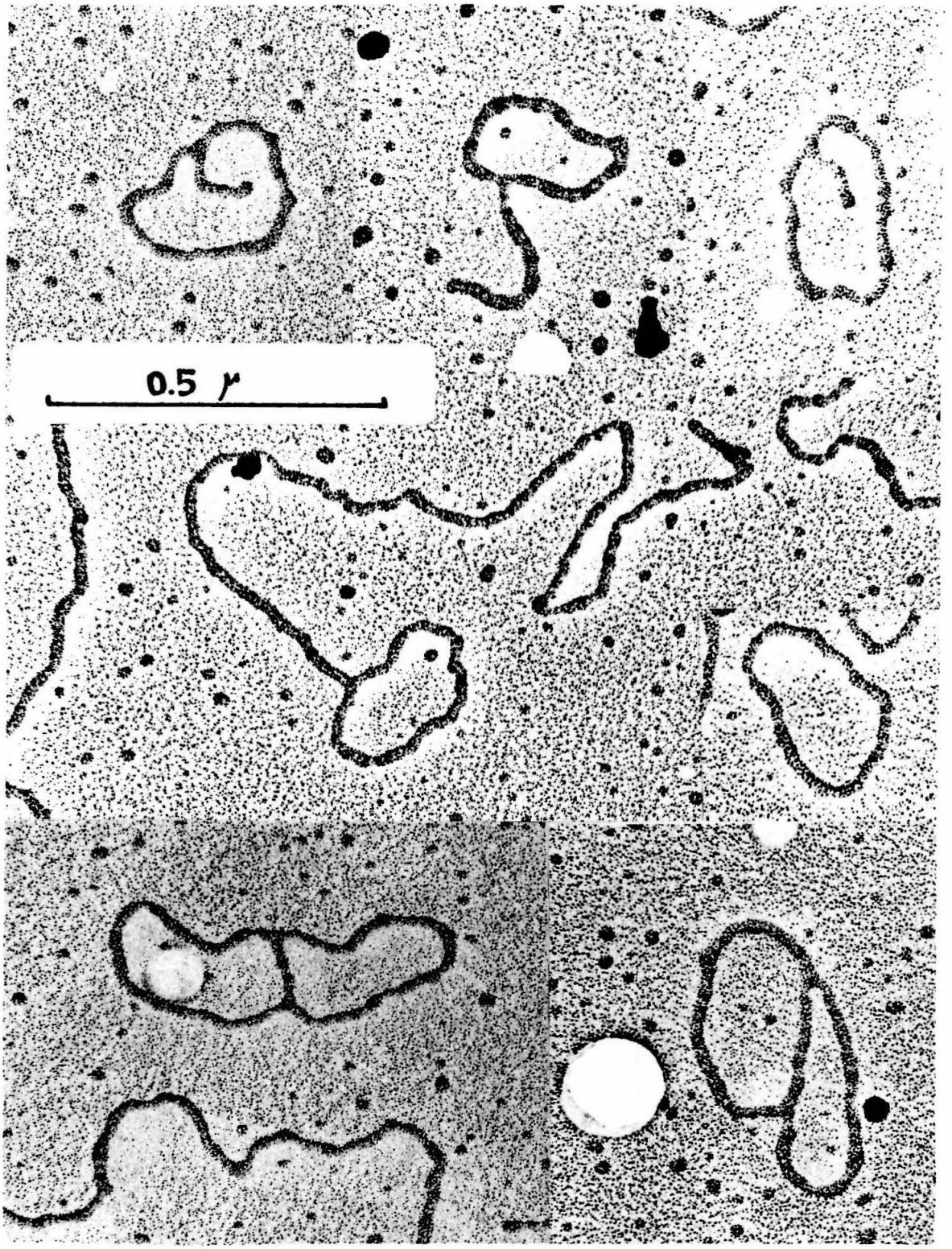
The average length of the circular portions of the replicating forms agrees with the average mc length previously measured. The standard deviation was 0.04 μ comparable to 0.03 μ given already. Out of 28 molecules examined, 5 molecules were the θ form and 23 molecules the σ form. The lengths of branches in the σ form varied from 0.1 to 10 μ. In fact, 7 out of 23 σ forms had a branch length longer than the size of mc.

Replicating mc in the main DNA band represents about 5% of nicked mc. Since the amount of nicked mc was less than 10% of the total mc, the replicating forms amount to 0.5% or less of the total. This amount is about as expected if the rate of replication is the same in both the mc and the host, and if an mc replicates once on the average per one round of host replication.

Plate III Replicating Forms of Minicircle.

After banding in a CsCl-ethidium bromide medium, the upper band was collected, and the CsCl and ethidium bromide removed as described in Materials and Methods. This preparation was examined under an electron microscope. Four σ forms and two θ forms are shown here.

Plate III



It might be argued that the σ forms could be accidentally formed from any linear molecules. Therefore, E. coli S/6 which does not carry mc was treated and examined under identical conditions. No such species in which its length of circular part is the size of mc was detectable. However, the possibility still remains that σ forms would be formed by an accidental overlap of an mc and a linear molecule. The small probability of this kind of overlapping has been already described in the discussion of catenated molecules.

(f) Covalently Closed Circular DNA in E. coli S/6

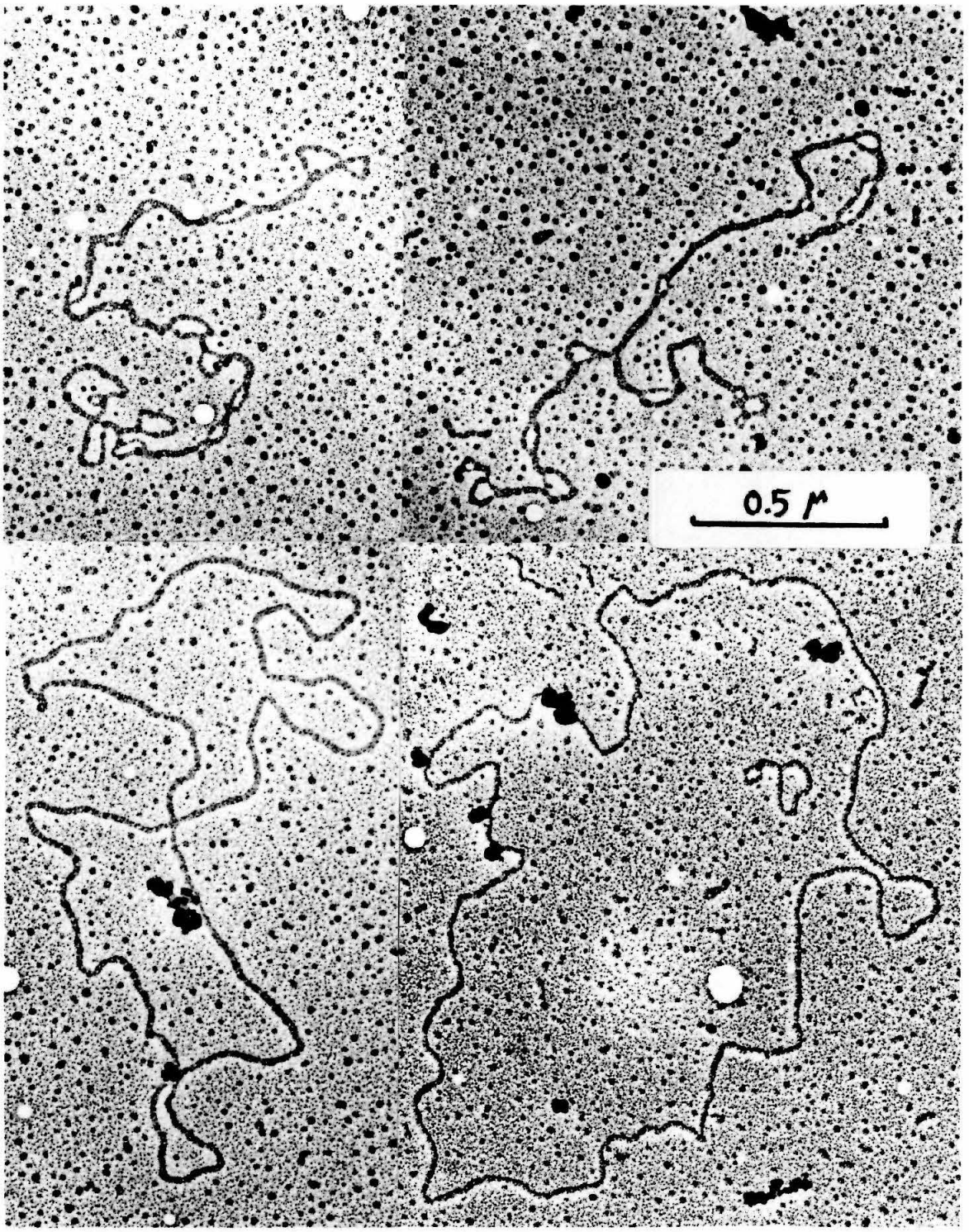
As described in the section above, E. coli S/6 was used as a control for the study of mc replicating forms. A covalently closed circular DNA was found in the E. coli S/6 lysate as shown in Plate IV.

The length of this circular DNA is 6.82 (± 0.15) μ, corresponding to a molecular weight of 14.9×10^6 daltons.

The possibility that this DNA may be a defective P2 prophage will be discussed later.

Plate IV Covalently Closed Circular DNA in E. coli S/6. E. coli S/6 was grown and the cells were treated in the same way as the mc preparation. The closed circular DNA band was examined with an electron microscope. Twisted and open structures are shown here. For a size comparison, a small amount of mc was added to one preparation. An mc is shown inside a larger circle.

Plate IV



4. Discussion

All strains of *E. coli* 15 possess the *mc* as a cytoplasmic element regardless of coliphage 15 production (Cozzarelli, *et al*, 1968). The number of copies per chromosome is unusually high (12-15 copies) making this plasmid DNA different from others. Multiple copies of a drug resistance factor have been found only when this factor was transferred from a normal host *E. coli* to *Proteus mirabilis* (Rownd, Nakaya and Nakamura, 1966). However, the latter strain may not be considered a normal host, since higher multimeric forms of several colicinogenic factors have been observed only when transferred to this strain from *E. coli* (Goebel and Helinski, 1968).

One may ask then whether all the copies of the *mc* are genetically identical despite their uniform size. The complexity of the *mc* determined by renaturation kinetics shows that there is only one DNA base sequence in the *mc* population. Thus, it was speculated that the presence of multiple copies of a unique base sequence might be due to some sort of gene amplification, as was observed in ribosomal RNA genes in oocytes (Brown and Dawid, 1968). However, the hybridization of *E. coli* r-RNA to the *mc* was not successful. Nevertheless, a possibility still remains that the presence of multiple copies might be due to an amplification of some other gene.

Results from the study of *mc* replication under conditions of synchronous host DNA synthesis are not entirely conclusive because of several anomalies. As pointed out in Results, the main contributing factor could be some irregularities in the initiation of host DNA

replication. This irregular synchronization can result in the observed slower rate at the beginning of DNA synthesis. This slower rate has been observed by Cerda-Olmedo, et al. (1968). In their study of gene mapping by nitrosoguanidine mutagenesis, peaks due to reversion of some genes appear broad. Such a broadening has been explained by variance in the synchronous initiation. A similar interpretation has been drawn by Lark and Renger (1969) in a different type of experiments. In all cases mentioned above, the uncertainty of synchronization is of the order of 10-20 min. This uncertainty is probably much longer than the replication time of mc DNA and therefore could give the results obtained by us. At this moment we can only say that the replication of the mc seems to be random regardless of the synchronous host DNA synthesis. A similar mode of replication has been observed with the R-factor in P. mirabilis that individual copies of the R-factor are selected at random for replication during exponential growth without discrimination as to whether any particular copy has been duplicated one or more times during that generation (Rownd, 1969).

Two kinds of presumed replicating forms of mc were observed in this study. The σ form is expected as a consequence of a "rolling circle" model (Gilbert and Dressler, 1968). This form has been observed in the replication of λ DNA and ΦX 174 RF DNA (Weissbach, et al, 1968; Whalley, 1969). The θ form is consistent with the Cairns' model (1963) and has been observed in λ DNA and in mitochondrial DNA (Okawa, et al, 1968; Kirschner, et al, 1968). The simultaneous presence of these two forms could be a further reason that the replication of mc is random with respect to host DNA synthesis.

The presence of a circular DNA species in E. coli S/6 is worth further consideration. This bacterial strain was derived from a strain B. It has been shown genetically that strain B carries a defective prophage P2 and that this prophage contains an immunity region (Cohen, 1959). It is quite possible that the circular DNA observed in S/6 is this defective prophage P2. The molecular weight of mature P2 DNA is 22 X 10^6 daltons (Mandel, 1967) which is about 1.5 times longer than the circular DNA observed in our work. This size difference may be the reason that the prophage is defective and extrachromosomal in nature. An analogous example is found in the λ system (Matsubara and Kaiser, 1968). A DNA-DNA hybridization experiment can decide the issue.

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Part II

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Flow Dichroism of Deoxyribonucleic Acid Solutions

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Synopsis

The flow dichroism of dilute DNA solutions ($A_{260} \approx 0.1$) has been studied in a Couette-type apparatus with the outer cylinder rotating and with the light path parallel to the cylinder axis. Shear gradients in the range of 5–160 sec.$^{-1}$ were studied. The DNA samples were whole, "half," and "quarter" molecules of T4 bacteriophage DNA, and linear and circular $\lambda b_2 b_{\frac{1}{2}} c$ DNA. For the linear molecules, the fractional flow dichroism is a linear function of molecular weight. The dichroism for linear λ DNA is about 1.8 that of the circular molecule. For a given DNA, the dichroism is an approximately linear function of shear gradient, but with a slight upward curvature at low values of G, and some trend toward saturation at larger values of G. The fractional dichroism increases as the supporting electrolyte concentration decreases.

It is well known that native DNA shows strong negative dichroism and flow birefringence in accordance with the expectation for the B form of the Watson-Crick structure in which the planes of the bases are perpendicular to the helix axis. The B form is the common form for DNA fibers at high humidity and presumably the same molecular structure occurs for native DNA molecules in solution.[1] Several experimental studies of the flow dichroism of DNA have been made previously. In one apparatus the observations are made with the light beam propagating perpendicular to a very thin rectangular channel, with the fluid velocity vector along the channel, and with the velocity gradient therefore parallel to the light beam.[2–5] This apparatus requires a rather high DNA concentration. A second popular apparatus uses two concentric cylinders with the inner transparent cylinder rotating, and with the light beam perpendicular to the stream lines and to the cylinder axis, that is, again parallel to the velocity gradient.[6,7] This apparatus also requires rather high DNA concentrations because of the short optical paths (1.4 mm.). Furthermore, there is a distinct possibility that the desired, simple laminar flow pattern does not occur in this apparatus at high shear rates because of Taylor instability (see below).

We therefore felt that it was desirable to construct a flow dichroism apparatus of the conventional Couette type, with the outer cylinder rotating for flow stability, and with the light beam along the cylinder axis so that long light paths and low macromolecule concentrations can be used. With this apparatus the basic flow dichroism parameters, especially the

dependence of the flow dichroism signal on shear gradient and on molecular weight of DNA, have been investigated.

Flow dichroism and flow birefringence are closely related measurements. To some extent they provide the same information; to some extent they complement each other. The dichroism measurement has the advantage that it enables one to observe and compare the orientation of several different chromophores which are attached to the macromolecule and which absorb at different wavelengths.[4,5]

EXPERIMENTAL

Flow Dichroism Apparatus

A schematic diagram is shown in Figure 1. The basic principle is that a velocity gradient is established in an annular ring of liquid which is contained between a rotating outer cylinder C and an inner stator D. A monochromatic light beam, shown as a dotted line, propagates vertically upwards through the liquid, K. The plane of polarization of the light is controlled by the rotatable polarizer, B. If there is flow dichroism, the

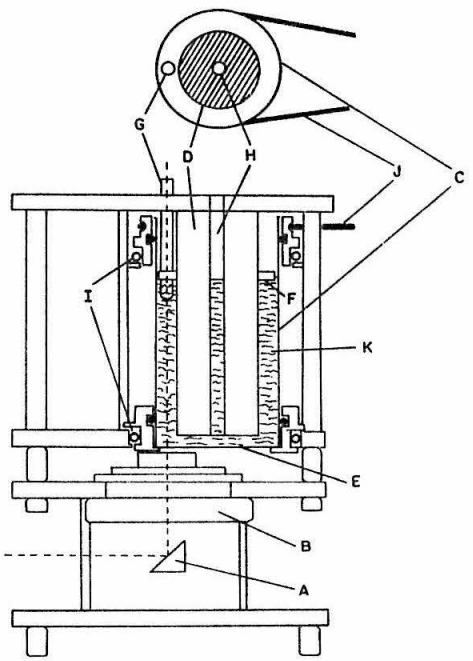

Fig. 1. Flow dichroism apparatus: (A) 45° prism with aluminized face; (B) rotatable Glan-Thompson polarizer; (C) rotor (Pyrex glass cylinder); (D) titanium stator; (E) quartz window; (F) titanium ring; (G) polished quartz rod; (H) hole in the center of the titanium cylinder for filling; (I) ball bearings; (J) O-ring, connected to a motor; (K) solution. The broken line shows the light path. The capacity is 60 ml. (For detailed explanation, see text.)

absorbance of the solution will depend on the angle between the plane of polarization and the stream lines.

The inner stator, D, is made of titanium for corrosion resistance and has a diameter of 2.794 cm. The outer rotor, C, a piece of precision bore Pyrex glass with an inner diameter of 3.810 cm., is clamped by O-rings at the two ends. At these positions, the outer cross-section of the precision inner-bore tubing was ground to be truly round also. The annular gap is 0.508 cm. A quartz window, E, was glued with epoxy resin to the bottom of the outer cylinder and baked at 50°C. overnight. This resulted in a water-resistant seal with no observable birefringence in the window. The purpose of the titanium ring, F, attached to the stator is to prevent the centrifugal rise of the liquid on the inner wall of the rotor. The annular gap between this ring and the outer cylinder is 0.018 cm. The quartz rod, G, is attached to this ring and is immersed below the level of the liquid, which is just below F. Thus, the optics are not affected by the curvature of the liquid meniscus. The surface of the rod is ground and polished to focus the light on the phototube just above the outer end of the rod. The hole, H, is for filling and cleaning. The rotatable Glan-Thompson prism, B, is transparent down to about 220 mμ. The angle of rotation can be adjusted with an accuracy of ±1°. The apparatus is constructed so that it can be attached to the monochromator of a Beckman DU spectrophotometer. A deuterium lamp is used to increase the intensity of the light in the ultraviolet. The light enters horizontally and is reflected at the metallized prism, A. The absorbance changes are measured with a Gilford absorbance indicator and a recorder.

The outer cylinder is driven by a 0–1725 rpm variable-speed d.c. motor with an arrangement of several pulleys for further speed control. The rotation speed is measured with a tachometer.

The temperature was controlled rather crudely at 25(±1)°C. by clamping several of the Beckman thermospacers to the metal plates and circulating water from a constant temperature bath through the spacers.

For Couette flow with the outer cylinder rotating, the shear gradient G at any point r in the annular gap is given by[8]

$$G(r) = \frac{R_i^2\Omega}{R_o^2 - R_i^2} + \frac{\Omega}{[(1/R_i)^2 - (1/R_o)^2]r^2} \tag{1}$$

$$G(r) \approx \frac{R_o + R_i}{2(R_o - R_i)}\Omega$$

$$\text{for } (R_o - R_i) \ll (R_o + R_i) \tag{2}$$

where R_o and R_i are the outer and inner radii and Ω is the angular velocity.

The average gradient is given by

$$\langle G \rangle = \frac{\int Gr\, dr}{\int r\, dr} = \frac{\Omega R_i^2}{R_o^2 - R_i^2} + \frac{2\Omega R_o^2 R_i^2}{(R_o^2 - R_i^2)^2} \ln (R_o/R_i) \tag{3}$$

In the present instance, $R_o = 1.905$ cm., $R_i = 1.397$ cm., $R_o - R_i = 0.508$ cm. Then $\langle G \rangle = 3.17\Omega = 23.3N$, where N is the rotor speed in revolutions per second.

Taylor has discussed the limits of stable laminar flow in a Couette apparatus.[9] With the outer cylinder rotating, this condition depends essentially on a Reynolds' number based on the annular gap, $U(R_o - R_i)\rho/\eta$, where $U = \Omega R_o$, ρ is density, and η is viscosity. The condition for stable flow is

$$U(R_o - R_i)\rho/\eta \leq 2000 \tag{4}$$

For the present apparatus, this limit corresponds to $G = 1900$ sec.$^{-1}$, for $\eta/\rho = 0.01$ cgs units.

As a matter of general interest, we note that, with the inner cylinder rotating, a centrifugal instability known as Taylor instability sets in at much lower values of the Reynolds' number. The helical motions that relieve this instability are clearly visualized in the experiments of Coles.[10] For a narrow gap, the criterion for stability given by Taylor[9] reduces to

$$\frac{U(R_o - R_i)\rho}{\eta} \leq 41[R_i/(R_o - R_i)]^{1/2} \tag{5}$$

For the Shimadzu apparatus,[6] with $(R_o - R_i) = 7 \times 10^{-2}$ cm., $R_o = 1.5$ cm., and $\eta/\rho = 0.01$ cm.^{2}/sec., this limit corresponds to $G \leq 400$ sec.$^{-1}$. Nevertheless, Wada and Kozawa[6] and Wada [7] have reported what appear to be consistent and smooth curves of dichroism versus G up to G values of 3000 sec.$^{-1}$. Possibly the flow is stabilized because the viscosity of the rather concentrated DNA solutions used is greater than 0.01 poise and/or is non-Newtonian; possibly the helical motions that relieve the Taylor instability do occur but do not significantly affect the optical observations.

DNA Preparation

Most of the T4 DNA samples were prepared from T4 bacteriophage preparations kindly donated by Professor W. Dreyer. The bacteriophage had been acid-precipitated at pH 3.8 in the course of isolation, which probably caused a small number of depurinations and single-strand scissions in the DNA. Several small batches of the bacteriophage were grown and purified under milder conditions without acid precipitation by Dr. J. G. Wetmur. No difference in the results was noted. The bacteriophage was purified by several cycles of differential centrifugation. In each cycle, debris was pelleted by two centrifugations at 7000 rpm for $^1/_2$ hr. and the phage then pelleted by spinning at 16,000 rpm for 1 hr. in an SW 25.1 Spinco rotor. The purified phage were suspended at a concentration of about $A_{260} = 10$ in 0.1M NH_4HCO_3, 0.006M $MgSO_4$, pH 8.

The mutant bacteriophage, $\lambda b_2 b_5 c$, was grown by the method of Young and Sinsheimer.[11] The bacterial strain *E. coli* K12W3110 and the phage stock were gifts from Dr. J. Weigle. Debris was pelleted from lysates by centrifugation at 9000 rpm for $^1/_2$ hr. and the phage centrifuged down at 17,000 rpm for $5^1/_2$ hr. by use of the L19 fixed-angle rotor. The phage

was suspended in 0.01*M* Tris, 0.01*M* $MgSO_4$, pH 8, and the differential centrifugation repeated in an SW 25.1 rotor. The phage was then banded in CsCl (ρ = 1.50 g./ml.), 0.01*M* $MgSO_4$, 0.01*M* Tris (pH 8), in an SW 50L rotor at 20°C. at 40,000 rpm for 18 hr., at an initial phage concentration corresponding to $A_{260} \approx 6$. The visible phage band was collected by puncturing the side of the cellulose nitrate tube and was dialyzed against a buffer of 0.01*M* Tris, 0.01*M* $MgSO_4$, pH 8.0.

DNA was prepared from bacteriophage by phenol extraction. Reagent grade phenol was distilled under purified nitrogen, and saturated with buffer prior to use. Bacteriophage solutions at an $A_{260} \approx 5$ to 10 were extracted three times with phenol; the aqueous phase was extracted with ether and exhaustively dialyzed versus a buffer solution containing 1.0*M* NaCl, 0.01*M* Tris, 0.001*M* EDTA, pH 8.0. The DNA solutions were then stored at 5°C. over several drops of $CHCl_3$.

T4 DNA "half" and "quarter" molecules were prepared by subjecting whole molecules to hydrodynamic shear by repeated passage through a capillary in our automatic apparatus.[12,13] The DNA solutions were at a concentration of 30 μg./ml. in 1.0*M* NaCl, 0.01*M* Tris, 0.001*M* EDTA, pH 8.0. Half molecules were produced by 100 passes through the capillary at 25 psi at 5°C., corresponding to a maximum gradient of 5.4×10^4 sec.$^{-1}$. An additional 120 passes at 50 psi ($G_{max} \approx 1 \times 10^5$ sec.$^{-1}$) were used for breakage to quarter molecules.

Circular molecules of $\lambda b_2 b_5 c$ DNA were prepared from the linear molecules as described by Wang and Davidson.[14] Solutions of linear molecules at a concentration of 5 μg./ml. in 1.0*M* NaCl, 0.01*M* Tris, 0.001*M* EDTA (pH 8.0) were heated to 75°C. for 3 min., maintained at 52°C. for 3 hr., and then stored at 5°C. Before making measurements on linear molecules, solutions were heated to 75°C. for 3 min. to dissociate any circles or linear polymers.

DNA preparations were characterized for molecular weight and/or for circularity by band sedimentation and/or by electron microscopy.

Sedimentation Analysis

Analytical band sedimentation runs were done with 30-mm. centerpieces in a Spinco Model E ultracentrifuge at 20°C. The bottom windows were either flat or side-wedge windows and −1° or −2° windows were used on top. Rotor speeds were 21,740 rpm for T4 DNA whole, half, and quarter molecules and 35,600 rpm for λ DNA linear and circular molecules. The DNA concentration of the solution in the pocket was 5 μg./ml. and the bulk medium was 3.0*M* CsCl (ρ = 1.374 g./ml.), 0.001*M* EDTA, pH 8.0.

Photographs were traced with a Joyce-Loebl microdensitometer. Sedimentation coefficients were evaluated and corrected to the standard state.[15,16] The corrected sedimentation coefficients are as follows: 63 ± 2 S. for T4 whole molecules, 44 ± 1 S. for T4 half molecules, 33 ± 1 S. for T4 quarter molecules, 32 S. for $\lambda b_2 b_5 c$ linear molecules, and 36 S. for $\lambda b_2 b_5 c$ circular molecules.

Molecular weights were calculated from sedimentation coefficients by using the equation of Crothers and Zimm:[17]

$$0.445 \log M = 1.819 + \log (s^0_{20,w} - 2.7) \quad (6)$$

The molecular weights so calculated for the T4 whole, half, and quarter molecules are 126×10^6, 53×10^6, and 26×10^6, respectively.

From band sedimentation runs on good circular λ DNA preparations the amount of linear material was estimated as less than 5%.

The photographic band sedimentation method is not very sensitive for the detection of minor components with a heterogeneous distribution of sedimentation coefficients. However, observable amounts of such impurities were not present in the sheared T4 DNA samples. The electron micrographs discussed next give a better picture of the size distribution in these samples.

Electron Microscopy

The specimen mounting of DNA preparations for electron microscopy was done by a modified basic protein film method, essentially as described by Wetmur et al.[18] A solution which contains 100 μg./ml. of cytochrome C, 1 μg./ml. of DNA, and 1*M* NH_4Ac was spread on a 0.3*M* NH_4Ac solution. Films were picked up by touching a Parlodion grid to the surface. The films were stained by dipping in uranyl acetate stain solution for 30 sec. following by rinsing in isopentane for 10 sec. The uranyl acetate stain solution was made by adding four or five drops of a stock solution of 0.005*M* uranyl acetate, 0.05*M* HCl, to 10 ml. of 95% ethanol. Sometimes, in addition to uranyl staining, the grids were shadowed, while rotating, with Pt–Pd at an angle of 8° for further contrast.

The fields were scanned visually in the electron microscope and any molecule seen, which was not too tangled to be measurable, was photographed, regardless of the size of the molecule. Countour lengths were then measured on enlargements with a map measuring device.

In our hands, the weight-average contour length of $\lambda b_2 b_5 c$ DNA molecules is 10.8 μ. The recommended molecular weight for this substance is 25×10^6 dalton.[19,20] The conversion factor for electron microscope determinations of molecular weight is then 2.32×10^6 dalton/μ. This value is significantly higher than that obtained by other workers (for instance, MacHattie and Thomas report 1.92×10^6). However, the value is very reproducible in our laboratory. Inman[21] and Lang et al.[22] have recently pointed out that this difference is apparently due to the effect of salt concentration in the hypophase in the basic protein film technique.

The contour length of a single T4 whole molecule was measured as 55 μ, corresponding to a molecular weight of 128×10^6. Measurements on a sample of 17 molecules in a half-molecule preparation gave length measurements of 1, 2, 2, 7, 20, 21, 21, 22, 22, 23, 25, 25, 27, 27, 30, and 32 μ corresponding to a weight-average length of 23.9 μ and molecular weight of 55.5×10^6. The distribution function of total length of molecules in a given size class versus length has a standard deviation of 5.6 μ.

The lengths observed for a sample of quarter molecules were 1, 1, 2, 3, 4, 5, 8, 8, 8, 9, 11, 12, 13, 14, 14, 14, 14, 15, 15, 15, 15, and 18 μ, corresponding to a weight-average length of 12.6 μ and a molecular weight of 29.4 $\times$ 10^6. The standard deviation of the distribution is 3.4 μ.

The sedimentation coefficients and measurements of contour length are in substantial agreement, and we accept as weight-average molecular weights for the T4 whole, half, and quarter molecule preparations the values 127, 54, and 28 $\times$ 10^6. Probably, the least reliable of our measurements by either method is for the whole molecules, but the value of 130 $\times$ 10^6 is in the range generally accepted for this DNA.[23,24]

RESULTS

General Optical Considerations

We take a local system of coordinates in the flow field with the z axis along the axis of the cylinder, the x axis along the streamlines and the y axis along the radius of the cylinder (which is the direction of the velocity gradient). Let the principal axes of the absorption (or polarizability) tensor be z, ξ, η; the ξ axis makes an angle χ with the x axis and $90° - \chi$ with the y axis, where χ is the extinction angle. As is well known, χ approaches 45° as the gradient approaches zero, and approaches 0° as the gradient approaches infinity.

In the present apparatus in which the light propagates along the z axis, it is possible to measure χ by locating the angles of the polarizer which give the maximum and minimum absorbances. One can then measure the extinction coefficients for light with its electric vector polarized along the ξ and η axes, ϵ_ξ and ϵ_η (whereas in a birefringence experiment, it is only practical to measure the difference in refractive indices $n_\xi - n_\eta$).

For an apparatus in which the light beam propagates along the y direction, it is possible to measure ϵ_x and ϵ_z. The x direction is not a principal axis except at sufficiently high gradients where $\chi \approx 0°$.

For a rodlike molecule with axial symmetry, there are two molecular extinction coefficients ϵ_a and ϵ_p for the electric vector polarized along and perpendicular to the rod axis, respectively. The extinction coefficient, $\bar{\epsilon}$, for isotropic orientation is given by

$$3\bar{\epsilon} = \epsilon_a + 2\epsilon_p \tag{7}$$

The molecular dichroism is expressed by the quantity $\epsilon_{ap} = \epsilon_a - \epsilon_p$.

Let ϵ_α represent the measured extinction coefficient for light polarized along an axis, α. Let $\langle \cos^2 \theta_\alpha \rangle$ be the average square of the cosine of the angle which the rod axes make with the α axis. Then

$$\epsilon_\alpha = \epsilon_a \langle \cos^2 \theta_\alpha \rangle + \epsilon_p \langle \sin^2 \theta_\alpha \rangle \tag{8}$$

and

$$\epsilon_\alpha - \bar{\epsilon} = \epsilon_{ap} (\langle \cos^2 \theta_\alpha \rangle - 1/3) \tag{9}$$

and

$$\epsilon_\alpha - \epsilon_\beta = \epsilon_{ap}(\langle\cos^2\theta_\alpha\rangle - \langle\cos^2\theta_\beta\rangle) \tag{10}$$

where β is another axis in space.

In practice, under the conditions of our measurements, the extinction angle χ is close to 0°, and the quantities of interest to us are

$$(\epsilon_x - \bar{\epsilon})/\bar{\epsilon} = (\epsilon_{ap}/\bar{\epsilon})(\langle\cos^2\theta_x\rangle - 1/3) \tag{11}$$

$$(\epsilon_y - \bar{\epsilon})/\bar{\epsilon} = (\epsilon_{ap}/\bar{\epsilon})(\langle\cos^2\theta_y\rangle - 1/3) \tag{12}$$

$$(\epsilon_y - \epsilon_x)/\bar{\epsilon} = (\epsilon_{ap}/\bar{\epsilon})(\langle\cos^2\theta_y\rangle - \langle\cos^2\theta_x\rangle) \tag{13}$$

Extinction Angle

The orientation of the principal axes of the absorbance ellipsoid with respect to the streamlines was measured for several gradients for some dilute T4 DNA solutions. Unless otherwise specified, measurements are at $\lambda = 260$ mμ. The results in Figure 2 show that the extinction angle χ is 10° at a value of the shear gradient G of 5.4 sec.$^{-1}$ and 3° at $G = 64$ sec.$^{-1}$ in a solution of T4 DNA in 0.001M Na_3EDTA. The values of χ close to 0° have been confirmed for a range of gradients, for DNA's of several molecular weights, and at higher supporting electrolyte concentrations. Thus

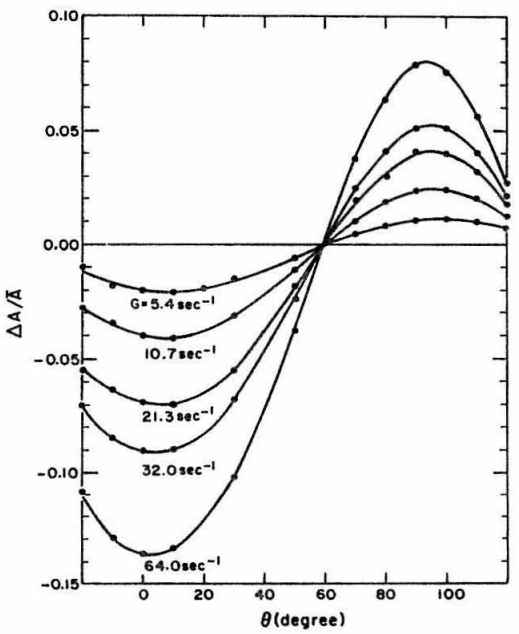

Fig. 2. Fractional dichroism $\Delta A/\bar{A}$ (where ΔA is the change in absorbance from the value $\bar{A}$ at 260 mμ with the rotor stationary) as a function of polarizer angle. The axis for 0° is along the streamlines. The solution contains T4 DNA at a concentration of 5 μg./ml. in 10^{-3}M Na_3EDTA, pH 7.2.

in practice, we routinely set the polarizer only at 0° and 90° with respect to the streamlines. This probably introduces a small error for the lower molecular weight DNA's at small values of G.

Comparison of $\epsilon_x - \bar{\epsilon}$ and $\epsilon_y - \bar{\epsilon}$

Figure 3 is a recorder tracing of a typical experiment, showing the absorbance levels with the rotor stationary and in motion, for two different orientations of the polarizer.

Figure 4 presents typical measurements of $\epsilon_x - \bar{\epsilon}$ and $\epsilon_y - \epsilon$ at 260 mμ. As expected for DNA, the former is negative and the latter is positive. The measured ratio $(\bar{\epsilon} - \epsilon_x)/(\epsilon_y - \bar{\epsilon})$ is 1.83($\pm$0.09) and is independent of G from $G = 32$ to $G = 160$ sec.$^{-1}$. In general, $(\epsilon_x - \bar{\epsilon}) + (\epsilon_y - \bar{\epsilon}) + (\epsilon_z - \bar{\epsilon}) = 0$. Therefore, if $(\epsilon_x - \bar{\epsilon}) = -1.8(\epsilon_y - \bar{\epsilon})$, it follows that $(\epsilon_z - \bar{\epsilon}) = 0.8(\epsilon_y - \bar{\epsilon})$; that is, the distribution function for orientation of the DNA threads around the x axis is almost axially symmetric, but there is slightly more orientation away from the y axis than away from the z axis.

In the measurements reported below, we usually present data for $(\epsilon_y - \epsilon_x)/\bar{\epsilon}$ which we call the fractional dichroism, $\Delta\epsilon/\bar{\epsilon}$.

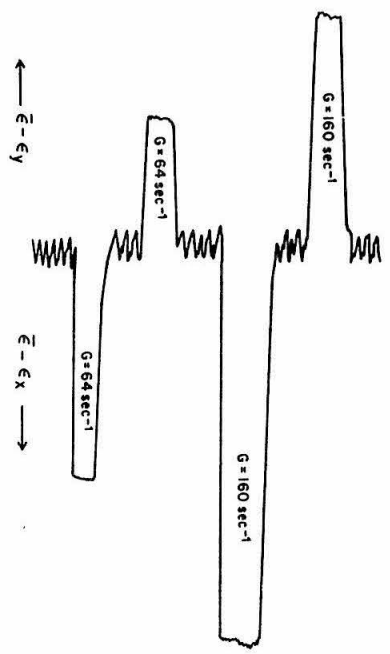

Fig. 3. Recorder tracings of the flow dichroism signals along (x) and perpendicular (y) to the streamlines; the monochromator was set at λ = 260 mμ, with a slit width of 1.8 mm. (band width = 3.2 mμ). The solution contained T4 DNA at an A_{260} of 0.1 (5 μg./ml.) in 1.0M NaCl.

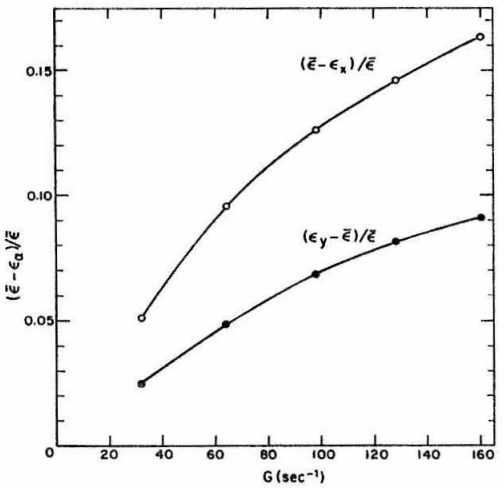

Fig. 4. Typical plot of the absorbance change vs. shear gradient, for light polarized along and perpendicular to the streamlines; T4 DNA at $A_{260} = 0.10$ in 1.0M NaCl, 0.01M Tris, $10^{-4}M$ EDTA.

Wavelength Dependence of $\Delta\epsilon/\bar{\epsilon}$

This topic has not yet been thoroughly investigated. However, it has been observed that the fractional dichroism is independent of wavelength between 250 and 270 mμ, but decreases by about 22% at 220 and 230 mμ. These observations are in qualitative agreement with results already reported by Wada.[7]

Shear Gradient Dependence

Data on the shear gradient dependence of the fractional dichroism, $\Delta\epsilon/\bar{\epsilon}$, are presented in Figures 5, 7, and 9. The curves are approximately linear at intermediate values of G, especially for low molecular weight DNA's. They show some downward curvature for T4 whole and half molecules at the larger gradients. At low gradients, corresponding to values of $\Delta\epsilon/\bar{\epsilon}$ below about 0.02, there is a slight upward curvature, suggesting a G^2 dependence.

Molecular Weight Dependence

The results in Figure 5 at $G = 50$ and $G = 160$ sec.$^{-1}$ are replotted as $\Delta\epsilon/\bar{\epsilon}$ versus molecular weight in Figure 6. At $G = 160$ sec.$^{-1}$, the dichroism signal at constant G is a linear function of molecular weight. There is a very slight upward curvature at $G = 50$ sec.$^{-1}$.

Effect of Concentration

Most of the experiments reported here were done at a DNA concentration of 5 μg./ml. ($A_{260} = 0.10$). Identical results for $\Delta\epsilon/\bar{\epsilon}$ were obtained at a DNA concentration of 2.5 μg./ml. Thus, the dichroism results appear to be independent of concentration over the rather small concentration range studied.

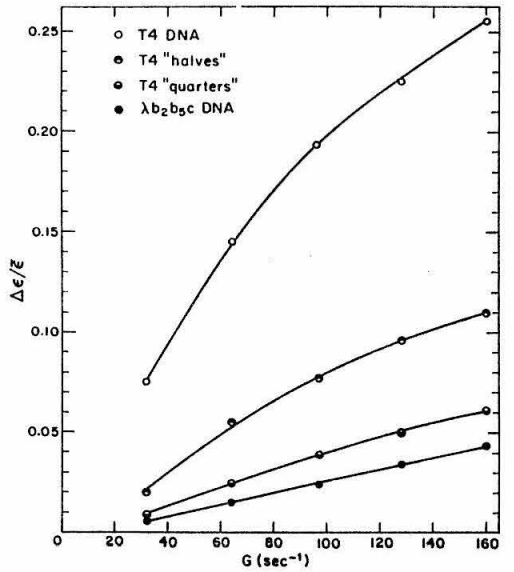

Fig. 5. Fractional dichroism of several DNA's as a function of shear gradient. The measurements were made at a DNA concentration corresponding to $A_{260} = 0.10$ in 1.0M NaCl, 0.01M Tris, 0.001M EDTA (pH = 8.0) at 25°C.

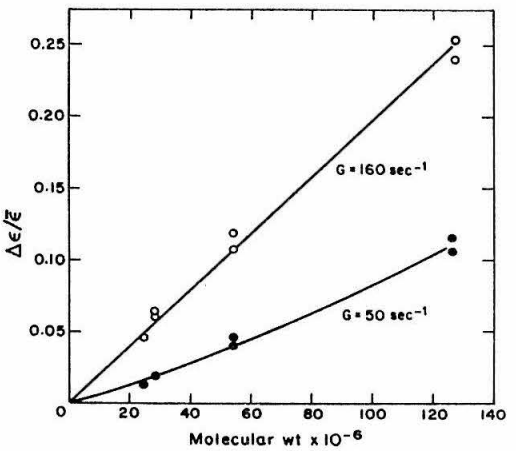

Fig. 6. Fractional dichroism as a function of weight-average molecular weight at $G = 50$ sec.$^{-1}$ and $G = 160$ sec^{-1}. A pair of circles at the same value of M indicates two independent experiments.

Effect of Circularity

Linear λ DNA has short complementary single-strand ends which enable it to form a hydrogen-bonded circular molecule. The cohesive ends dissociate on heating, and the linear molecule reforms.[14,25,26] As is shown in Figure 7, the fractional dichroism of the linear molecule is 1.90(±0.10),

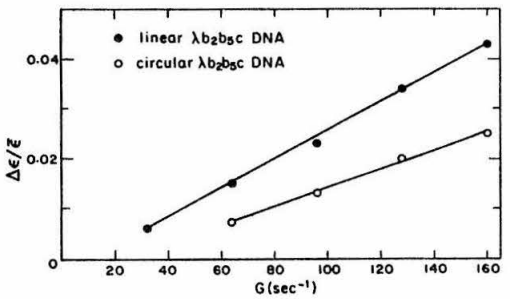

Fig. 7. Fractional dichroism of $\lambda b_2 b_5 c$ DNA linear and circular molecules as a function of shear gradient. The concentration of DNA and the ionic strength are the same as in Fig. 5.

1.75(±0.10), and 1.69(±0.10) that of the circular molecule at G = 80, 120, and 160 sec.$^{-1}$, respectively.

Effect of Ionic Strength

The effect of ionic strength on $\Delta\epsilon/\bar{\epsilon}$ for T4 DNA at G = 32 sec.$^{-1}$ is shown in Figure 8. As the supporting electrolyte concentration decreases, the dichroism increases. The change is small between 1.0M amd 0.1M [Na^+] but there is an increase by a factor of 1.8 between 0.01 and 0.001M [Na^+].

As shown in Figure 9, there is a pronounced downward curvature in the graph of $\Delta\epsilon/\bar{\epsilon}$ versus G for T4 whole molecules at a total sodium ion concentration of 0.0006M. It was observed that the effect of ionic strength is less pronounced at G = 160 sec.$^{-1}$ than at G = 32 sec.$^{-1}$.

DISCUSSION

General

The main results of the present experimental investigation are that the flow dichroism signal is a linear function of molecular weight; that it is an approximately linear function of shear gradient, G, except that there is some downward curvature for large values of G for the higher molecular weight DNA samples and a slight upward curvature for low values of G, corresponding to $\Delta\epsilon/\bar{\epsilon}$ values less than about 0.02, for all the DNA's studied; and the orientability of the DNA increases as the supporting electrolyte concentration decreases. Circular DNA's show a smaller flow dichroism than do linear molecules of the same molecular weight.

Since the absorbance of a solution is proportional to the mass of DNA present and the quantity $\Delta\epsilon/\bar{\epsilon}$ is proportional to molecular weight for homogeneous samples, flow dichroism can be used to measure weight-average molecular weight of a native DNA preparation.

As we shall see in later sections, it is not possible at present to make a meaningful comparison between the experimental results reported here and theory.

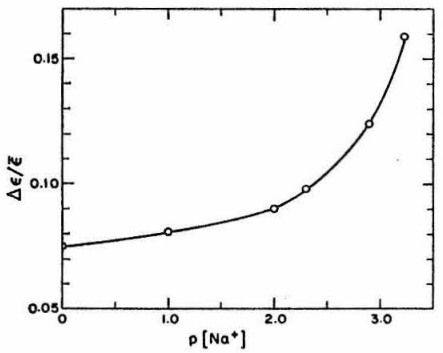

Fig. 8. Fractional dichroism at $G = 32$ sec.$^{-1}$ as a function of ionic strength; $p[Na^+] = -\log [Na^+]$.

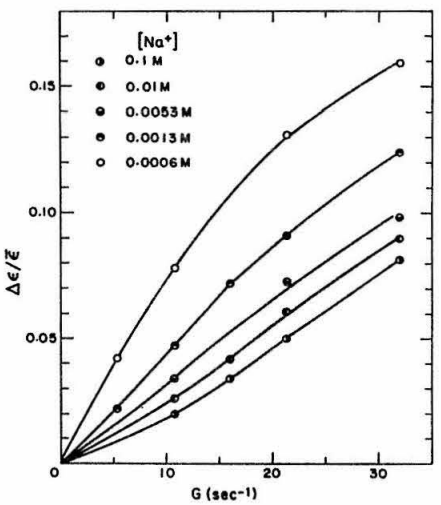

Fig. 9. Behavior of T4 DNA at low shear gradients as a function of ionic strength.

Dichroism and Chain Extension

The main point that we wish to make in this section is that the magnitudes of the observed dichroism signals—for example, values of $\Delta\epsilon/\epsilon$ of 0.25—are quite large and indicate that in the shear gradients used the DNA molecules are very greatly extended as compared to their equilibrium configuration in the absence of the flow field. This important conclusion has already been made by Wada.[7] Existing theories of the effect of flow on a flexible chain are only applicable for small shear gradients and small deformations and hence are not really comparable with our experiments.

Kuhn and Grün[27] have analyzed a simple model for relating the observed optical anisotropy of a flexible polymer to the chain extension. They con-

sider a chain of segments of length d, connected by universal joints. The contour length of the chain is L, it contains L/d segments, and the root-mean-square end-to-end distance is $h_0 = (Ld)^{1/2}$.

The most probable distribution function for the orientation of the segments is calculated imposing the constraint that the end-to-end distance is fixed at h. Thus, it is assumed that the distribution function is axially symmetrical around the end-to-end vector and that the distortion of a segment is independent of its position along the chain.

The result is that the distribution function for orientation of segments in terms of the angle θ_h that a segment makes with the end-to-end vector is

$$dN/N = [\beta/(e^{\beta} - e^{-\beta})] \exp \{\beta \cos \theta_h\} \sin \theta_h \, d\theta_h \tag{14}$$

where β is a Lagrange multiplier which characterizes the distribution.

The ratio of end-to-end distance to contour length is given by

$$\begin{aligned} h/L &= \langle \cos \theta_h \rangle \\ &= [(e^{\beta} + e^{-\beta})/(e^{\beta} - e^{-\beta})] - (1/\beta) \\ &= \mathfrak{L}(\beta) \qquad (15) \\ &\approx \beta/3 \text{ (when } \beta \ll 1) \qquad (16) \end{aligned}$$

where $\mathfrak{L}(\beta)$ is the well-known Langevin function.

Dichroism and birefringence are related to $\langle \cos^2 \theta_h \rangle$, which is given by

$$\langle \cos^2 \theta_h \rangle = 1 - (2/\beta) \, \mathfrak{L}(\beta) \tag{17}$$

$$\approx (1/3) + (2/45) \, \beta^2 \tag{18}$$

If we assume that this distribution function applies to our experiments with the end-to-end vector along the streamlines, then from eq. (13)

$$\begin{aligned} (\epsilon_y - \epsilon_x)/\bar{\epsilon} &= \Delta\epsilon/\bar{\epsilon} \\ &= (3/2)(\epsilon_{ap}/\bar{\epsilon})[\langle \cos^2 \theta_h \rangle - 1/3] \qquad (19) \end{aligned}$$

We now assume that for a perfectly oriented DNA, the dichroic ratio is infinity, that is, in eq. (7), $\epsilon_a = 0$, $\epsilon_{ap} = -\epsilon_p = -(3/2)\bar{\epsilon}$. Then,

$$\Delta\epsilon/\bar{\epsilon} = (9/4)[\langle \cos^2 \theta_h \rangle - 1/3] \tag{20}$$

From an observed $\Delta\epsilon/\bar{\epsilon}$, eq. (20) can be solved for $\langle \cos^2 \theta_h \rangle$; β can then be determined from eq. (17), and h/L from eq. (15). A graph of the solution is given by Wada.[7] (The approximate relation, which is not quite valid for $\Delta\epsilon/\bar{\epsilon} = 0.25$, is $(h/L)^2 = (10/9)(\Delta\epsilon/\bar{\epsilon})$.) For example, at $G = 160$ sec.$^{-1}$ in $1M$ NaCl, we find for T4 DNA that $\Delta\epsilon/\bar{\epsilon} = 0.25$. This corresponds to $h/L = 0.50$.

For this DNA, $L = 55 \times 10^{-4}$ cm. the recommended value of d is 7×10^{-6} cm.,[28] so that $(h_0/L) = 3.6 \times 10^{-2}$. Thus, h/L in the flow is much greater than h_0/L. The simple model used for estimating the chain

extension is not quantitatively reliable, but the conclusion that the chain is greatly stretched beyond its equilibrium configuration to one in which the end-to-end distance is a significant fraction of the total contour length is inescapable.

Comparison with Other Measurements

Even though sufficient data are not available for really good quantitative comparisons, we shall endeavor to compare our measurements with those of Wada[7] and with the flow birefringence measurements of Frisman et al.[29,30]

The observation reported in a preceding section that $(\bar{\epsilon} - \epsilon_x) = 1.8\ (\epsilon_y - \bar{\epsilon})$, implies that $(\epsilon_y - \epsilon_x)/(\epsilon_z - \epsilon_x) = 1.08$, and this is the expected ratio of signals between the Couette apparatus and that used by Wada. Wada's measurements were obtained at higher G values than studied by us; however, extrapolation of his curve for T2 DNA in SSC at 33 μg./ml. down to $G = 100$ sec.$^{-1}$ (Fig. 4 of ref. 7) gives $(\epsilon_z - \epsilon_x)/\bar{\epsilon} \approx 0.09$, whereas we find for T4 DNA in 0.1M NaCl at 5 μg. ml.$^{-1}$ that $(\epsilon_y - \epsilon_x)/\bar{\epsilon} = 0.20$ at $G = 100$ sec.$^{-1}$. T2 and T4 DNA's have about the same molecular weights and should show practically identical flow dichroism behavior. The measurements disagree by a factor of two. However, in view of the large extrapolation involved and the difference in experimental conditions, this disagreement should not be taken too seriously.

Frisman et al.[30] have studied the effect of molecular weight on the flow birefringence of DNA solutions under conditions which should be reasonably comparable with those used here.

A principal obstacle to a quantitative comparison with their results is our ignorance as to the value of the conversion factor between $(\epsilon_y - \epsilon_x)/\bar{\epsilon}$ as measured in flow dichroism and $n_y - n_x$ as measured in flow birefringence. Measurements of both quantities on the same solution at the same shear gradient are needed.

The most suitable data that we know of are from unpublished experiments in our own laboratory a few years ago on the dichroism and birefringence of DNA solutions oriented by an electric field. The dichroism experiments were done by Dr. Walter Huber, and the birefringence measurements were made by Dr. Heiko Ohlenbusch.[31] Measurements were made with pulsed square wave fields of the order of 2000–4000 v./cm. on dilute ($A_{260} \approx 1.0$) DNA solutions at low salt concentrations (10^{-3} to $10^{-4}M$).

Unfortunately, we have been unable to resurrect records of experiments done on the same solution at the same field strengths. The results were not very sensitive to the molecular weight of the samples, but were quite sensitive to the ionic strength. However, typical results for a solution in 1×10^{-4} to 2×10^{-4} Na cacodylate buffer, at a DNA concentration of 50 μg./ml. ($A_{260} = 1.0$) at fields of 3000–4000 v./cm. were $(\epsilon_x - \epsilon_y)/\bar{\epsilon} = 0.6(\pm 0.1)$, and $n_x - n_y = 2 \times 10^{-6}$. We take $\Delta\bar{n}/\Delta c$ for DNA solutions as 0.20 ml./g.; thus, the refractive index increment due to the DNA

is $\Delta\bar{n} = 10^{-5}$, and $(n_x - n_y)/\Delta\bar{n} = 0.20$. Thus, the conversion factor for relating birefringence and dichroism in an oriented DNA solution is

$$(\epsilon_x - \epsilon_y)/\bar{\epsilon} = 3.0(n_x - n_y)/\Delta\bar{n} \tag{21}$$

Unfortunately, this figure cannot be regarded as being reliable to better than a factor of two.

Our results for the smaller DNA's studied (T4 quarters and $\lambda b_2 b_5 c$) may be summarized by the equation:

$$\Delta\epsilon/\bar{\epsilon} = 1.25(\pm 0.2) \times 10^{-11} MG \tag{22}$$

in dilute aqueous salt solutions ($[Na^+] > 0.10M$) at 25°C. Therefore, from eq. (21) and $\Delta\bar{n}/c = 0.20$, we predict

$$(n_x - n_y)/c = 8.3 \times 10^{-13} MG \tag{23}$$

Frisman et al.[29,30] have made flow birefringence measurements more or less in the same range of gradients and DNA concentrations studied by us with sheared and unsheared samples of calf thymus DNA. Molecular weights were estimated from intrinsic viscosities by the equation,

$$[\eta] = 1.45 \times 10^{-6} M^{1.12} \tag{24}$$

as recommended some time ago.[32] In a more recent study, Eigner and Doty[33] and Crothers and Zimm,[17] respectively, concluded that the equations:

$$[\eta] = 6.9 \times 10^{-4} M^{0.70} \tag{25}$$

and

$$([\eta] + 5) = 1.3 \times 10^{-3} M^{0.665} \tag{26}$$

provide improved correlations of viscosities and molecular weights, at least for molecular weights greater than 5×10^6. Equations (25) and (26) are in substantial numerical agreement for the range of molecular weights of interest. The Russian authors find that the birefringence is a linear functions of gradient and that the quantity

$$[(n_x - n_y)/Gc\eta_0]_{G\to 0, c\to 0} \tag{27}$$

has the values 2.80×10^{-3} and 1.05×10^{-3} for two samples of DNA with intrinsic viscosities of 90 and 41.5 dl./g. The calculated molecular weights according to eq. (24) are 9.04×10^6 and 4.55×10^6, respectively. Taking $\eta_0 = 0.01$, we then get that $(n_x - n_y)/c = 3.1 \times 10^{-12} MG$ and $2.3 \times 10^{-12} MG$, respectively. Equations (25) and (26) predict molecular weights of $19.7(\pm 0.7) \times 10^6$ and $6.7(\pm 0.1) \times 10^6$ for the two samples, so that $(n_x - n_y)/c = 1.4 \times 10^{-12}$ and 1.6×10^{-12}, respectively. These values are still somewhat higher than that computed from our flow dichroism measurement [eq. (23)].

The comparisons at present are unsatisfactory. It is both desirable and possible that concordant results should be obtained in several laboratories and it is hoped that the present rather inconclusive discussion will contribute towards this happy *dénouement* (see *Note*, opposite page).

Comparison with Theory

An elegant and attractive theory for the effect of flow on a flexible polymer molecule is that based on a model of the molecule as a chain of beads connected by ideal springs (necklace model). The theory has been developed primarily by Rouse,[34] Zimm,[35] and Reinhold and Peterlin.[36] An alternative model, the Kratky-Porod wormlike chain, has been studied by Harris and Hearst,[37] but this theory is not yet sufficiently well developed for comparison with the experiments reported here.

Reinhold and Peterlin have summarized the present situation for the necklace model. The theory may apply at low shear stress, but, for a number of reasons, it does not apply when the shear stress is sufficiently large so that the molecule is significantly deformed. We have already presented an argument that the observed magnitude of the dichroism indicates that, under our conditions, the molecules are significantly deformed.

An independent, theoretical argument to the same effect is the following. The deformation of the molecules may be characterized by a flow parameter b (β in Peterlin[38] and Reinhold and Peterlin[36]),

$$b = M[\eta]\eta_0 G/NkT \tag{28}$$

where N is Avogadro's number. When $b > 1$, the degree of distortion of the molecule is large compared to its undistorted dimensions. It is not clear to us whether in evaluating b one should use the true intrinsic viscosity at zero shear gradient or the corresponding quantity $[\eta]_G$ measured at the gradient of interest. However, only the former quantity is usually available.

For T4 DNA, with $[\eta] = 3.2 \times 10^4$ cc./g., at $T = 298$°K. and at $G = 100$ sec.$^{-1}$, with $\eta_0 = 0.01$ poise, $b = 160$. For $\lambda b_2 b_5 c$ DNA with $M = 25 \times 10^6$, $[\eta] = 1.1 \times 10^4$ cc./g. at $G = 30$ sec.$^{-1}$, $b = 3.3$. Thus, only in the latter case are our experiments reasonably close to conditions to which the theory may apply.

Note added in proof: Professor R. Harrington [*Biopolymers*, **6**, 105 (1968)] has made a careful study of the flow birefringence of T2 DNA. His results agree with ours on T4 DNA indicating that the extinction angle is close to 0° for gradients above about 50 sec.$^{-1}$ but he finds larger extinction angles at very low gradients (Harrington: $\chi = 25°$ at $G = 5$ sec.$^{-1}$ and $\chi = 7.5°$ at $G = 35$ sec.$^{-1}$, for $c = 3.0$ and 5.3 μg./ml., respectively, in 0.1M NaCl; Lee and Davidson: $\chi = 10°$ at $G = 5.4$ sec.$^{-1}$ and $\chi = 3°$ at $G = 64$ sec.$^{-1}$ for $c = 5.0$ μg./ml. in 0.001M Na_3EDTA). His measurements on T2 DNA show that $n_x - n_y$ is a linear function of G and can be summarized as

$$\frac{n_x - n_y}{cG} = 6.6 \times 10^{-5} \text{ cm.}^3\text{/g.}$$

When compared with our dichroism data, this leads to the result

$$\frac{\epsilon_x - \epsilon_y}{\bar{\epsilon}} = 6.3\frac{n_x - n_y}{\Delta\bar{n}}$$

instead of eq. (21).

According to Peterlin,[38] the birefringence (and similarly for the dichroism) is given by

$$(n_x - n_y)/c = (A/M)b[1 + (0.204b)^2]^{1/2} \tag{29}$$

(where A is a constant depending on the refractive index of the medium and the intensive optical properties of the polymer) for small b. According to this equation, and accepting the approximation that the intrinsic viscosity is proportional to $M^{0.67}$, the birefringence is porportional to G and to $M^{0.67}$ at low G, and to G^2 and $M^{2.3}$ for larger G. As already noted, there is a suggestion of this G^2 dependence at low values of G and $\Delta\epsilon/\epsilon$ in our data (Figs. 5, 7, and 9); clearly, however, most of our results are for values of G corresponding to a degree of molecular extension which is outside the range of applicability of the theory.

Reinhold and Peterlin[36] have modified the necklace theory to correct the assumption of perfect elasticity of the springs connecting the beads, but they assert that this by itself is not sufficient to give a realistic model. However, their Figure 6 indicates that this model can produce a dependence of Δn on G in agreement with our experiments.

Bloomfield and Zimm[39] have discussed the hydrodynamic properties of circular and linear DNA molecules in terms of the necklace model with hydrodynamic interaction. Their results, in conjunction with Zimm's earlier discussion of birefringence, can be used to obtain the equation,

$$\frac{(n_x - n_y)_{\text{linear}}}{(n_x - n_y)_{\text{circle}}} = \left(\frac{[\eta]_{\text{linear}}}{[\eta]_{\text{circle}}}\right)^2 \frac{C_{B\,(\text{circle})}}{C_{B\,(\text{linear})}} \tag{30}$$

where the quantity C_B is defined by Bloomfield and Zimm. Their theory, in conjunction with experimental data on the sedimentation coefficients of the molecules, predicts $([\eta])_{\text{linear}}/[\eta]_{\text{circle}}) = 1.6$, and $(C_{B,\text{circle}}/C_{B,\text{linear}}) = 2.3$. The predicted birefringence ratio is 5.8; the experimental one is 1.8_5. The discrepancy is probably due to the fact that the theory is applicable only for small deformations.

Effect of Concentration

Crothers and Zimm[17] find that in the equation

$$\eta_{sp}/c = [\eta](1 + k'[\eta]c + \dots) \tag{31}$$

for correlating the effect of concentration c (in grams per deciliter) on η_{sp}/c, the value of k' for T4 DNA at very low shear stress is ≈ 0.5. At the concentration of 5×10^{-4} g./dl. used by us, the term $k'[\eta]c$ is 0.08; that is, there is an 8% change in η_{sp}/c between infinite dilution and this concentration. The effect of concentration on η_{sp}/c is still less at the higher shear stresses used by us. We conclude that the observed absence of any concentration depencence in our experiments is in accord with the results of Crothers and Zimm.

We are greatly indebted to Dr. Patrik Callis who has been our collaborator in the later stages of this work and with whom we have discussed all aspects of the problems. The mechanical design and construction of the instrument were done by Mr. R. Ehrich and Mr. E. Mott and we wish to express our appreciation and admiration for their skillful work. This research has been supported by Grant GM 10991 from the Division of General Medical Sciences of the National Institutes of Health. This paper is Contribution No. 3532 from the Gates, Crellin, and Church Laboratories.

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PROPOSITION I

Origin of Replication of Phage λ DNA

A physicochemical method for the precise mapping of the origin for λ DNA replication is proposed. Isolated replicating molecules with two forking points are treated with a proper concentration of methylmercuric hydroxide to produce partial denaturation at specific sites on λ DNA. The location of one of the two forks which is presumably the origin of replication is determined precisely with regard to the position of a particular loop on a partially denatured DNA.

When coliphage λ infects a sensitive bacterium, its DNA replicates autonomously and progeny phage particles are produced. Several replicating forms of λ DNA have been observed (Okawa, Tomizawa and Fuke, 1968; Weissbach, Bartl and Salzman, 1968). Okawa, et al. (1968) found that the replicating form that they isolated has no ends. Instead, it has two fork (Y) points; one is presumably the origin of replication and the other is the growing point. Other evidence by Weissbach, et al. (1968) showed that a long concatenate and a circular structure with a linear DNA branch attached occur as replicating forms. The correlation between the above three forms is not clear at present. However, it seems likely that upon infection, linear DNA is cyclized and replicates to produce more circular DNA molecules. These circular molecules become templates for concatenate synthesis possibly via a "rolling circle"

mechanism (Gilbert and Dressler, 1968).

Replication probably starts at a unique site on λ DNA. It has previously been shown that replication indeed starts somewhere in the right arm ((AT) rich half) of λ DNA and proceeds toward the left (Makover, 1968; LePecq and Baldwin, 1968; Tomizawa and Okawa, 1968). Genetic studies suggest that the origin of replication is probably between gene CII and O (Dove, 1968). This position corresponds to a distance from the right end of about 0.2 of the λ genome (Hogness, Doerfler, Egan and Black, 1966; Parkinson and Davis, 1969). However, the precise location of the origin has not yet been established.

A physicochemical method is proposed here for this purpose. Methylmercuric hydroxide (MMH) denatures DNA, with some selectivity for (AT) rich regions or molecules (Gruenwedel and Davidson, 1966). Under appropriate concentrations of MMH, (AT) rich regions in a DNA can be partially denatured. It is also known that λ DNA contains long intramolecular segments of differing nucleotide composition (Skalka, Burgi and Hershey, 1968). The (AT) rich regions in λ DNA can be partially denatured either by formaldehyde plus heat or by MMH. The denatured portions are visualized as loops in an electronmicrograph (Inman, 1967; Mohr and Davidson, 1969). Mohr and Davidson (1969) showed that at a MMH concentration of 8×10^{-4} M there occur several denatured loops on λ DNA. Most of them are clustered around the b_2 region at 0.42 the length of λ DNA from the right end. However, there occurs a distinct and highly reproducible loop with a length of about 0.1 μ at 0.04 the λ length from the right end. (This loop will be called the "0.04 loop"

for convenience). The distribution of the 0.04 loop positions among many observed molecules is very sharp and the percent standard deviation relative to the whole length of λ DNA is about 1%. This means that the error involved in measuring a position relative to the 0.04 loop is about 1% plus the error involved in measuring any DNA length. This 0.04 loop is used as an internal marker for positioning the origin of λ DNA replication.

The replicating form of Okawa, et al. (1968) is used here, since it has two distinct forks (Y's), one of which is probably the origin. The material will be isolated according to their procedures. For an accurate determination of the lengths, two internal standards are necessary; one for double-stranded regions and the other for single-stranded loops. The natural occurrence of normal circular forms among replicating molecules will serve as the former standard. ΦX 174 DNA will be suitable for the latter.

It can be parenthetically added that any protein molecules which have specific binding sites on λ DNA could be used instead of MMH denaturation. The λ repressor would be one of them. However, the electron microscopic technique required to visualize protein molecules on DNA is not well developed yet and therefore this method is not applicable at present.

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PROPOSITION II

Replication of Coliphage T4 DNA in Minicells

Studies on T4 phage DNA replication at the early stage of infection are proposed here to verify one or more of several replicating models described by other workers. Minicells are ideal hosts for electron microscopic analysis, since they contain normal amounts of RNA and protein but no DNA.

The T4 phage genome is known to be circularly permuted and terminally redundant from genetic studies (Streisinger, Edgar and Denhardt, 1964; Sechaud, et al., 1965) and from physicochemical evidence (Thomas and Rubenstein, 1964; Thomas andMacHattie, 1964;MacHattie, Ritchie, Thomas and Richardson, 1967). The "headful" hypothesis for packaging DNA into the phage head has been proposed on the basis of genetic observations (Streisinger, Emrich and Stahl, 1967). Any models for phage DNA replication must be in accordance with the experimental findings above.

Several models for T4 DNA replication have been proposed recently by Frankel (1968) and by Werner (1968). Earlier, Frankel (1966) found that a replicating form occurs with a sedimentation coefficient of 200 S. This corresponds to about 20 units of mature DNA assuming that the replicating form is linear. This replicating form has some sort of interruptions along its double helical structure which make it susceptible to shear or to enzymes specific for single-stranded DNA. To account for the formation of long, phage-specific DNA strands in T4-infected cells,

he proposed the following two models (1968). In the first, the parental linear DNA is converted to a circular form by a recombinatorial event between the terminally redundant regions and a long concatenate is synthesized from this template according to a "rolling circle" model (Gilbert and Dressler, 1968). In the second, a concatenate is formed by end-to-end recombination of linear molecules. Werner (1968) proposed another model based on his experimental finding that the replicating form has 7 to 8 growing points initiated successively on each parental DNA molecule with distances of 0.15 to 0.19 the mature DNA length between them. His model also is a basically circular structure with several growing points on it. In vivo circle formation is known to occur after P22 infection (Rhoades and Thomas, 1968) and after induction of coliphage 15 (Chap. 4 in this thesis), both DNA's of which have the properties of circular permutation and terminal repetition.

Direct tests of these models have not yet been made. The main difficulty has been that the crucial sedimentation analysis in the early infection process is obscured by the presence of undegraded host DNA (Frankel, 1966). Direct electron microscopic visualization of Frankel's "200 S" form has been made but no obvious conclusion could be drawn from the gigantic mess observed (Huberman, 1968).

It is desirable to study T4 phage DNA replication at an early stage of infection (first 2-10 min) without the interference of the host DNA. It is proposed that such a study is possible by using bacterial minicells as the host. Minicells are produced from a minicell-producing strain of E. coli K12 which was obtained by treatment of a log-phase culture

with triethylenemelamine (Adler, Fisher, Cohen and Hardigree, 1967). Minicells contain normal amounts of RNA and protein but no DNA; they do not divide. Moreover, they apparently have all the machinery required for *in vivo* synthesis of DNA (Cohen, Fisher , Curtiss and Adler, 1968) and they are lysed by phage T6 but not by phage T3 (Adler, *et al*, 1967).

This system is particularly attractive for electron microscopy, because it should provide a clean preparation of replicating DNA molecules. Also, there will be no ambiguity due to the presence of the host DNA in sedimentation analysis. For electron microscopic visualization of single-stranded regions and/or some bizarre structures, if any, the basic protein film technique using formamide will be useful. (Westmoreland, Szybalski and Ris, 1969; Chap. 4 in this thesis)

Additional information with regard to possible membrane attachment of replicating parental DNA molecules and the molecular steps of various amber mutations, in functions, related to the phage DNA replication can be obtained unambiguously from studies in this system. Both sedimentation and electron microscopic analyses will be used for these purposes.

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PROPOSITION III

Transcription on Cross-Linked DNA

The hypothesis that RNA polymerase unwinds and opens up a small portion of the DNA double helix during the transcription process can be directly tested by using a cross-linked DNA. The cross-linked DNA is prepared by treatments with HNO_2 or a bifunctional alkylating agent.

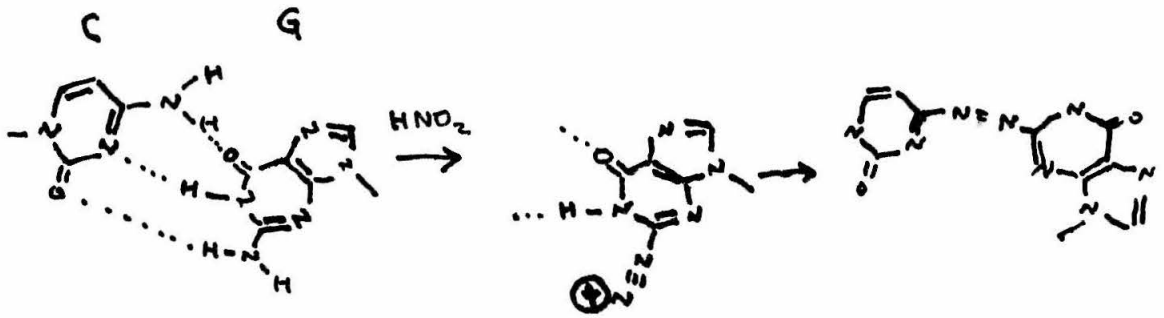
It is commonly believed that a DNA-dependent RNA polymerase unwinds and opens up a small portion of the DNA double helix during RNA synthesis. This hypothesis has not been directly proved. Indirect support was drawn from experiments by Walter, Zillig, Palm and Fuchs (1967) who observed a lag phase in early RNA synthesis at low temperature or at high salt concentration. They suggested that the lag could be the consequence of a limited strand dissociation reaction which is caused by the enzyme on the DNA at the site where RNA synthesis is initiated.

It is proposed here that a study of transcription on a cross-linked DNA will elucidate this hypothetical mechanism and will provide further insights into the transcription process.

Among known cross-linking agents, a chemical mutagen, HNO_2, and a bifunctional alkylating agent, nitrogen or sulfur mustard, are the best studied. Cross-links occur between bases of opposite strands by a covalent linkage. Thus, the resulting cross-linked DNA renatures much faster than noncross-linked DNA since the denatured strands are

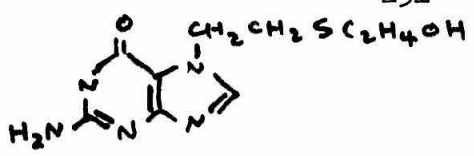
held in close proximity by covalent bonds.

Treatment with HNO_2 (actually a mixture of $NaNO_2$, NaAc and HAc, pH 4.2) produces cross-linked, reversibly denaturable DNA by an unknown mechanism (Geiduschek, 1961; Becker, Zimmerman and Geiduschek, 1964). One possible mechanism would be a diazo bridge formation between the amino groups at C-2 of guanine and at C-6 of cytosine, resulting in a slight distortion of the Watson-Crick base pairing.

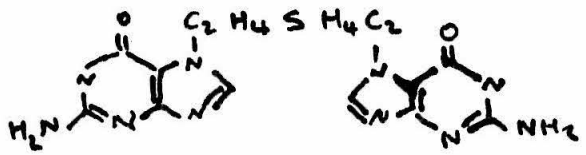


The major reaction is deamination of guanine, adenine and cytosine, producing xanthine, hypoxanthine, and uracil, respectively. However, the cross-linking occurs with a frequency of approximately one per four deaminations at pH 4.2 (Becker, et al., 1964). The cross-links are quite thermo-stable in neutral aqueous solution (Zimmerman and Geiduschek, 1963).

Another class of cross-linking agents employed here are the bifunctional alkylating agents, mustard gases. When DNA is reacted with a mustard gas and hydrolyzed by acid, the following alkylated products are found (Brooks and Lawley, 1961):



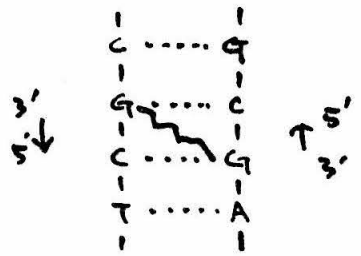
7-β-hydroxyethylthioethyl guanine (I)



di-(β-guanine-7ylethyl) sulfide (II)

The latter product arises from the cross-linking between guanines in opposite strands. The reaction with nitrogen or sulfur mustard is relatively fast and the alkylated products are quite stable in neutral solution at 37° ($t_{\frac{1}{2}} \sim 50$ hours). The ratio of yields of these two products after 15 min of reaction time is (I)/(II)=4.

Examination of a space-filling model indicates that a nitrogen mustard (HN2) cross-link can, with slight distortion of the helix, reach between the N-7 positions of guanine on opposite strands. The cross-links then lie in the major groove and make a connection between G's of adjacent base pairs. The base sequence must be G (3'→ 5')C, not C (3'→ 5') G (Brooks and Lawley, 1961; Kohn, Spears and Doty, 1966). Thus, a partial



opening of the small groove at the cross-linked portion is possible, whereas it is not possible in the HNO_2 cross-linked DNA.

The complementary use of the two different cross-links above will provide some answers as to whether the opening-up of the double-helix is a necessary requirement for RNA synthesis and, if it is, how far the base pairs should be apart. The cross-links probably will not affect RNA polymerase binding. Thus, the attention should be focussed on the propagation step. If RNA polymerase stops at the site of a cross-link, the size of the RNA products will be smaller than that from a normal DNA. When T7 DNA is used, more than 10 cross-links per molecule will be necessary to observe any effects, since under optimal conditions the size of the RNA molecules synthesized <u>in vitro</u> is usually 1.5×10^6 daltons (Hyman, 1969). It is assumed that deamination does not affect the transcription, since the Watson-Crick type base pairing is possible between A and U, between xanthine and C or A, and between hypoxanthine and C or A. This can be easily checked by using a HNO_2 or HN2 treated DNA without cross-links. Cross-links can be removed by aging the DNA solution for a considerable period.

The number of cross-links per DNA molecule can be determined as follows. The cross-linked DNA is sheared to some appropriate size depending on the extent of cross-linking. The sheared fragments are denatured and quenched, and then are banded in a CsCl density gradient. Fragments with cross-links will band in a position typical of native DNA. The number of cross-links can be estimated by comparing the amount in this band with the amount of DNA appearing in a band position typical of denatured DNA. A mathematical analysis using a Poisson distribution is as follows: The probability of not having a cross-link in a T7 DNA

fragment, for example, is given by

$$P(o) = \frac{e^{-n} n^{o}}{0!}$$

where n ≡ average number of cross-links per DNA piece. Assume there are 20 cross-links distributed at random in a T7 DNA. If the fragment size is 1/20 of a whole DNA, n=1. If the fragment is 1/40 whole T7, n=0.5 etc. Therefore, comparison of the relative amounts of denatured and native DNA to the theoretical prediction will provide the number of cross-links per whole T7 DNA. Possible occurrence of cross-links during shear is negligible in this study, since the frequency is only less than 5 cross-links for every 100 new ends formed by shear if it ever happens (Alberts, 1968).

An electron microscope technique can be substituted for this analysis. At a high concentration of formamide, a native DNA is completely dissociated. However, cross-linked regions will not dissociate and will be visible.

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PROPOSITION IV

Molecular Studies on Adaptation in Thermophiles

A certain strain of B. megaterium is an obligate thermophile, whereas this bacterial species normally exists as a mesophile. It is proposed here that experiments in this system will reveal a molecular mechanism of the phenomenon of adaptation to high temperature. The properties of proteins and membranes from thermophilic and mesophilic strains will be compared directly.

Various thermophilic microorganisms occur in nature. Many of the organisms which are found at high temperatures ($> 60^{\circ}C$) are not struggling to survive, but are optimally adapted to the extreme environment (obligate thermophiles) (Brock, 1967). Questions naturally arise; (1) how do thermophiles survive and grow at a high temperature, (2) why is it that they are obligate thermophiles, and cannot grow at all or grow very slowly at a more moderate temperature, and (3) what changes have occurred during evolutionary processes in biological macromolecules in order for these organisms to be adapted to this high temperature extreme?

It is known that thermophiles have enzymes which, in general, are much more resistant to normally denaturing conditions (Brock, 1967). For instance, aldolase from Bacillus stearothermophilus shows an optimal activity at $60^{\circ}C$, the organism's optimal growth temperature, and a much lower activity at $30^{\circ}C$. In contrast, the aldolase from rabbit muscle or yeast is inactivated in 5 min at this temperature, but is normally

active at 30°C (Thompson, Militzer and Georgi, 1958; Thompson and Thompson, 1962). This heat stability may be due to the presence of many disulfide bonds, since treatment with sulfhydryl reagents converts the enzyme to a form which slowly loses its activity at 60°C, and simultaneously increases its activity at 30°C. Further, it has been shown that aldolase from a thermophilic bacterium, YT-1, is fully active at 90°C, but becomes inactive when treated with the chelating agent, EDTA (Brock, 1969). The inference is that this enzyme requires divalent metal ions. However, it is not known that divalent metal ions are specifically responsible for the high temperature stability. Another heat stable enzyme, α-amylase, from B. stearothermophilus is interesting, because it exists in the native state as a well hydrated, semi-random or random coil (Manning, Campbell and Foster, 1961).

Protoplasts produced from B. stearothermophilus or YT-1 by lysozyme action are stable to osmotic shock or to boiling for up to one hour, suggesting that membranes play an essential role in thermophiles.

These observations may support the hypothesis that the thermophily is partly due to the heat stability of enzymes and membranes. However, it is not possible to conclusively state, for example, that the presence of disulfide bonds is the adaptive change responsible for thermophily, unless proteins from two closely related organisms, one thermophilic and one mesophilic, are compared. Until recently a meaningful study of the adaptation of thermophiles on a molecular level was impossible because no thermophilic organism had a mesophilic counterpart to which it could be directly compared.

An obligate thermophilic strain of Bacillus megaterium has been recently isolated from a hot-gas well (Golovacheva, Egorova and Loginova, 1965). The bacterium grows optimally at 60°-70°C with a generation time of about 15 min, whereas mesophilic strains of B. megaterium grow at 40°C with a generation time of 22 min (Egorova, 1965).

It is proposed here that investigations concerning the molecular processes involved in high temperature adaptation be performed with this system. Comparative studies should be undertaken on the structures and the properties of enzymes and membranes from both thermophilic and mesophilic strains of B. megaterium. All known techniques in protein and membrane chemistry are directly applicable. For instance, to begin with an enzyme which has a distinct temperature optimum is isolated. Its amino acid sequence and structure are compared with the enzyme with a different temperature optimum. The membrane stability can be compared as prescribed by previous workers. The differences in composition and in structure of the membranes can be explored by chemical analysis and possibly by ORD and CD. Since the use of a mesophile can serve as a control, any differences observed can be assigned to the characteristics of the thermophile which are necessary for its adaptation.

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PROPOSITION V

Mechanism of Cyclic AMP Stimulation of Protein Kinase Activity

Protein kinase isolated from rabbit skeletal muscle phosphorylates seryl residues of various proteins. Its activity is greatly enhanced by cyclic AMP *in vitro*. This enzyme is especially interesting because it converts inactive phosphorylase kinase to an active form in the presence of cyclic AMP. The mechanism of cyclic AMP stimulation of protein kinase activity is not known. A possibility that cyclic AMP acts as a modifier of the protein kinase either by non-covalent binding (*e.g.* allosteric activator) or by covalent binding is proposed. Experimentals for testing these hypotheses are also proposed.

Protein kinase has been isolated from rabbit skeletal muscle (Walsh, Perkins and Krebs, 1968), liver (Langan, 1968) and *E. coli* (Kuo and Greengard, 1969). It transfers the γ-phosphate of ATP to specific seryl residues of various proteins. This phosphorylation reaction has a complete dependence on adenine 3', 5'-mononucleotide (cyclic AMP or c-AMP). No stimulation is observed with any other nucleotides (Walsh, *et al.*, 1968).

The enzyme is particularly interesting, because it converts inactive phosphorylase kinase to an active form by phosphorylation in the presence of c-AMP. Thus, this enzyme probably plays a significant role in the

regulation of glycogenolysis. Glycogen is degraded stepwise to yield glucose-1-phosphate by a phosphorylase. The phosphorylase has two forms; phosphorylase a (active) and phosphorylase b (inactive) (Krebs and Fischer, 1962). Phosphorylase b becomes phosphorylase a by phosphorylation and dimerization by an enzyme, phosphorylase kinase (Krebs, et al., 1964). The phosphorylase kinase as purified is inactive, but becomes slowly active by incubation in the presence of ATP and c-AMP (DeLange, et al., 1968). However, since the discovery of protein kinase it is believed that this activation is due to the slight contamination of protein kinase in the phosphorylase kinase preparation, since it has been found later that only 0.5% by weight of protein kinase is sufficient for the activation of phosphorylase kinase (Walsh, et al., 1968). Thus, it seems that the protein kinase is the enzyme which phosphorylates and thus activates the phosphorylase kinase.

The enhancement of glycogenolysis by hormone stimulation has been observed for long. One of the direct results of epinephrine stimulation is an enhancemant of adenyl cyclase activity and hence c-AMP production (Robinson, Butcher and Sutherland, 1968). Therefore, it may be speculated that the protein kinase serves as a link between the epinephrine stimulation of adenyl cyclase and the activation of phosphoryl kinase.

The mechanism by which c-AMP stimulates protein kinase is at present unknown. One possible aspect of this mechanism is that the c-AMP binds to protein kinase, covalently or non-covalently, in order to convert the inactive form to the active one. Several binding modes and experimentals for its verification are proposed here.

(a) Non-covalent binding

It is possible that the protein kinase is an allosteric protein and c-AMP is an allosteric activator. The enzyme is activated by the binding of c-AMP to the allosteric site on the protein kinase. The c-AMP is known to be an allosteric activator for phosphofructokinase (Mahler and Cordes, 1966).

(b) Covalent binding

In order to achieve an active form, a covalent binding between c-AMP and the protein kinase may occur which results in an enzyme-adenylate complex. The thermodynamic feasibility of c-AMP acting as an adenylating agent has been pointed out (Greengard, Hayaishi and Colowick, 1969). Enzyme-adenylate complexes have been observed in glutamine synthetase (Wulff, 1968) and polynucleotide ligase (Little, et al., 1967; Weiss and Richardson, 1967; Olivera, et al., 1968). It is also conceivable that only the adenine moiety or the phosphate group may bind to the enzyme, and the phosphate group or the adenine group, respectively, are released.

Experimental methods for this binding study will be described below. The protein kinase is purified as described in Walsh, et al. (1968). Binding media can be the same as those for the protein kinase assay except that substrates (ATP and protein to be phosphorylated) should be absent. The media consist of tris buffer (pH 7), appropriate concentrations of $MgSO_4$ and theophylline to suppress c-AMP phosphodiesterase (DeLange, et al., 1968).

First, in order to verify whether the binding occurs at all, the mixture of H^3-and P^{32}-labeled c-AMP and the protein kinase are incubated

briefly and the mixture is subject to dialysis or column chromatography. Any remaining radioactivity in the protein after removal of small molecules indicates stable binding. If both H^3 and P^{32} are present in the protein and they are removable by common protein denaturing agents or by proteolytic enzymes, and if intact c-AMP is recovered, then the binding can be considered non-covalent binding. However, if these treatments can not remove the radioactivity from the protein, the binding can be suspected as covalent binding. An exhaustive digestion with proteolytic enzymes will reveal any amino acid(s) - adenylate complex(es). The binding of only the adenine moiety or phosphate group can be determined by the presence of either H^3 only or P^{32} only in the protein.

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