Double-Length, Circular, Single-Stranded DNA from Filamentous Phage

FRANCES C. WHEELER, ROLF H. BENZINGER, AND HERMANN BUJARD

Department of Biology, University of Virginia, Charlottesville, Virginia 22901, and Institut fuer Genetik, Universitaet Heidelberg, Germany

Received for publication 25 March 1974

Wild-type and gene 3 mutant filamentous phage stocks, containing different relative amounts of multiple-length particles, were treated exhaustively with DNase and then were highly purified. The phage DNA was extracted and examined by electron microscopy. In all cases, about 0.03% of the molecules were circular dimers. ^3H -labeled phage DNA was separated as to size by sedimentation in a preformed CsCl density gradient. Individual fractions were then examined in the electron microscope, and the percentage of linear and circular monomer and dimer DNAs was determined. A peak of double-length, circular molecules (with the expected sedimentation constant of 38S) was found ahead of the 24S monomer peak. The double-length molecules had been purified 65-fold. As previously found for single-stranded DNA, the contour length of these molecules was strongly dependent upon ionic strength. Possible artifacts were ruled out, and it was shown that the double-length molecules arose from phage particles.

Multiple-length, circular, double-stranded DNA has been found in many organisms, both eukaryotic and prokaryotic (5, 9, 11, 18, 19). The origin of these molecules is often attributed to errors in DNA replication (7). In the case of ϕX174 double-length replicative form DNA, there is evidence for this hypothesis (2, 8) and for the hypothesis that some of the dimers arise through recombination (6, 8).

After infection of *Escherichia coli* by icosahedral (19) or filamentous (10) phage containing single-stranded circular DNA, multiple-length double-stranded circular DNAs are found. Thus, one could ask if these multiple-length replicative forms are sometimes converted to multiple-length single-stranded DNAs. If synthesized, such DNA would not be expected to be packaged in icosahedral phage particles since only a "headful" of DNA can be incorporated (23). The discovery of multiple-length filamentous phage particles (16, 22) which could accommodate multiple-length single-stranded circular DNA prompted a search for such molecules. However, none were found (21). Using refined electron microscopic techniques (4, 13, 14), we now report the discovery of such molecules in filamentous phage and their partial purification on CsCl velocity gradients.

MATERIALS AND METHODS

All filamentous phage were grown on *E. coli* K37 F^+su^+ (17), whereas ϕX174 phage were propagated on

E. coli C. ^3H -labeled filamentous phage was isolated from infected culture containing 250 μg of deoxyadenosine per ml (3) and 1 μCi of [^3H]thymidine per ml. The phage purification procedure was modified from Yamamoto et al. (24) by treating all crude lysates with 1 μg of pancreatic DNase per ml for 6 h at 4 C; this treatment was repeated at 25 C in 0.02 M Mg acetate buffer (pH 6.8) before the CsCl buoyant density centrifugation step (if the DNase treatments were omitted, contaminating host DNA could be found in the phage DNA electron micrographs). Twenty to sixty percent of the optical density at 260 nm could be accounted for by infective filamentous phage; 30% of the optical density at 260 nm was accounted for by infective ϕX174 phage.

Phage DNA was isolated after incubation with 1% sodium dodecyl sulfate or 2.5% Sarkosyl 90 (Geigy), followed by at least three extractions with redistilled phenol. Phenol was removed by dialysis, and the DNA was stored in 0.01 M sodium phosphate buffer, or 0.1 M Tris buffer, pH 7.6, containing 1 mM EDTA. The DNA preparations were stored under sterile conditions at 4 C.

Velocity sedimentation of fd $^+$ DNA in a CsCl gradient: a 0.1-ml sample of [^3H]fd $^+$ DNA was layered on to an 11-ml preformed linear gradient of CsCl (from density 1.0 to 1.2) in 0.1 M Tris-1 mM EDTA, pH 8.5, and was centrifuged in an SW41 rotor at 40,000 rpm for 1 h at 20 C. Fractions (0.27 ml each) were collected from a hole punctured in the bottom of the tube. A 20- μl iter sample from each fraction was dried on a GFC-2 (Whatman) filter and was counted to determine [^3H]thymidine radioactivity. A background of 25 counts/min was subtracted for each sample. The recovery of radioactive counts from the gradient was 73%.

Sedimentation constants in the CsCl gradient were calculated by using the equations of Martin and Ames (15). These $s_{20, \text{Cs}}$ values were converted to $s_{20, \text{w}}$ values by comparison with the known 24S sedimentation constant for single-stranded, circular monomer fd DNA (21).

Filamentous bacteriophages were prepared for electron microscopy by the diffusion method (4, 14), using 0.2 M ammonium acetate as the bulk solution. After unfolding in the presence of formaldehyde and dimethylsulfoxide, phage-extracted DNA was also prepared for electron microscopy by the diffusion method. CsCl gradient fractions, containing very low concentrations of DNA (less than 0.01 $\mu\text{g/ml}$), were prepared for electron microscopy by using a modification of the drop diffusion method of Lang (13).

After shadowing with platinum, the preparations were examined in a Siemens Elmiskop IA or an Hitachi HU-11E. The phage particles or DNA molecules were counted on the fluorescent screen by scanning nonoverlapping fields. Quantitative evaluation of the electron micrographs and length measurements were performed as described previously (4, 14).

In the CsCl gradient fractions, the length distribution of the DNAs was determined from measurements of at least 100 circular monomers and 10 linear monomers; all double-length molecules (open and crossed-strand circles, as well as linear molecules) were photographed and measured.

The percentage of circular dimers per fraction was determined by examining at least 750 molecules in each fraction and counting the number of dimers observed. DNA rings were scored as double-length circles only if in an open configuration (no crossing of the strands) as shown in Fig. 1 and 4. The percentages of double-length circles are thus lower limits. Crossed-strand double-length circular molecules are listed separately (see Table 2).

RESULTS

An examination of wild-type filamentous phage stocks showed that they contained between 0.2 and 0.3% double-length particles (Table 1). When the amber mutant phage stock 3-H5 was examined, 21% of the particles were of multiple lengths (Table 1; Fig. 1) as expected from the results of Pratt et al. (16).

When DNA was extracted from the various phage stocks, 0.02 to 0.05% of the molecules were double-length circles (Table 1). Figure 2 shows an example of such a double-length molecule. Eleven double-length circular molecules were found in the filamentous phage DNA preparations; however, none were found among the 30,000 ϕX174 phage DNA molecules examined (Table 1).

Since a total of only eleven double-length DNA molecules were observed after direct extraction from filamentous phage (Table 1), further purification and characterization of these molecules was attempted in order to

firmly establish their existence in phage particles. ^3H -labeled wild-type fd phage DNA was sedimented on a neutral CsCl velocity gradient; after determining the radioactive peak of circular monomer phage DNA (Fig. 3), individual fractions of the gradient were examined by electron microscopy. Since the absolute concentration of DNA could not be determined by this technique, the percentage of double-length circular molecules was measured for each fraction (Fig. 3). When a peak of such molecules is reached, one expects a constant, high percentage of double-length circular molecules which should not change further for the faster sedimenting fractions. Indeed, Fig. 3 shows that a constant 1.95% of the DNA molecules were double-length circles from fraction 15 to fraction 12. The peak position of the double-length circles at fraction 15 corresponds to an S value of 38, exactly as predicted by Salivar et al. (21). Furthermore, the 1.95% of double-length circles in fractions 15 to 12 represents a 65-fold purification of these molecules over the 0.03% found in phage DNA before CsCl velocity sedimentation (Table 1). Figure 4, showing two adjacent double-length DNA circles from the gradient, underscores this purification.

Table 2 presents a detailed distribution of the relative frequency of six different kinds of DNA molecules found in fractions 25 to 12 in the CsCl velocity gradient of Fig. 3: double-length circles, double-length crossed circles ("figure-eights"), double-length linear molecules, monomer circles, monomer linear molecules, and contaminating double-stranded DNA. The following facts are apparent from this tabulation. (i) A total of 129 double-length circles was observed just in the analysis of this CsCl gradient. (ii) Although changing salt concentrations affected contour lengths by as much as 11%, the length of the dimer circles was almost exactly twice the length of the monomer circles within each fraction. (iii) No double-length circles were observed in the monomer peak or ahead of this peak until fraction 21. (iv) Double-length crossed circles ("figure-eights") were found in a peak closely following the completely unfolded dimers, indicating that many are not artifacts due to the juxtaposition of two monomer circles. Thus, the true percentage of circular dimers in fd⁺ phage DNA may be as high as 0.06%. (v) Only eight double-length linear molecules were observed and most of these were found in a peak with a calculated S value of 31 (fraction 20) between monomer and dimer circles. (vi) The fraction of linear monomer molecules varied from only 2.4 to 5.7% with no discernible peak; the expected peak fraction for linear mono-

TABLE 1. *Length distribution of different phage and phage DNAs*

Determination	Type of phage or phage DNA			
	fd	M13	M13 am3-H5	ϕ X174
No. of phage particles examined	5,000	5,000	1,000	
% of particles of different length in units of normal length phage ^a				
1 $\times$	99.7	99.8	79	
1.5 $\times$	0	0	4	
2 $\times$	0.3 $\pm$ 0.08	0.2 $\pm$ 0.06	13	
3 $\times$	0	0	2	
4 $\times$	0	0	2	
No. of DNA molecules examined	20,000	10,000	8,000	30,000
% of molecules of different length in units of normal length molecules (absolute numbers in parentheses)				
1 $\times$	99.98	99.97	99.05	100
1.5 $\times$	0	0	0	0
2 $\times$	0.02 $\pm$ 0.01 (4)	0.03 $\pm$ 0.02 (3)	0.05 $\pm$ 0.03 (4)	0
2 $\times$	0	0	0	0

^a Phage preparations gave identical results over a fivefold range of concentrations, indicating that the observed multiple-length particles did not arise by accidental end-to-end aggregation of the phage on the cytochrome *c* film. Some of the intermediate (1.5 $\times$) length phage particles may have been produced by fragmentation artifacts, as their fraction increased among aged stocks.

mers (fraction 30) was not examined. (vii) No contaminating double-stranded DNA molecules were found.

Another experiment was conducted to confirm that the circular dimers were single-stranded, and not double-stranded, DNA. The contour lengths of circular monomer and dimer DNA from fraction 19 of the gradient (Fig. 3) were measured at three different ionic strengths. Table 3 shows that the contour lengths of both kinds of molecules changed by 15 and 24% when compared at salt concentrations of 0.1, 0.15, and 0.2 M. This parallels the change in contour length found for single-stranded DNA by Bujard (4). Lang et al. (14) showed that there is no more than a 2% change in length of double-stranded DNA at these salt concentrations.

Finally, a remote possibility remained that the double-length DNA circles were in reality linear DNA molecules generated by a unique single-strand break and converted nearly quantitatively to a circular dimer form by annealing of cohesive ends in the high salt CsCl velocity gradient. To rule out this possibility, DNA directly from fraction 19 of the gradient (Fig. 3) was incubated with 0.1 N NaOH for 10 min at room temperature to disrupt any cohesive ends, then was diluted and prepared for electron microscopy. Table 4 shows that the relative frequencies of circular and linear molecules, as well as monomers and dimers, remained unchanged by this treatment.

DISCUSSION

Since circular dimer phage DNA occurs very rarely in filamentous phage (Table 1), it was necessary to rule out several possible artifacts which might accidentally generate these molecules during isolation and electron microscopy. A possible electron microscopic artifact is the fortuitous end-to-end juxtaposition of two linear single-length molecules to apparently form a double-length circle; this is ruled out by comparing the frequency of double-length linear molecules with the frequency of double-length circular molecules across the CsCl gradient. The former would have two ends touching, the latter four ends touching. Thus, if the circular dimers were an artifact, they would occur much less frequently than linear dimers. In fact, the detailed data of Table 2 show that linear dimers are 16 times less frequent than the circular dimers, eliminating this hypothesis.

Another hypothesis postulates that the circular dimers were actually formed by two circular monomers broken at the same site and then reannealed to form circles by their cohesive ends in the high salt environment of the CsCl gradient. If this were the case, alkaline denaturation of the circular dimers should cause their conversion to linear molecules; however, Table 4 shows that the relative frequency of circular and linear molecules is not changed by this treatment.

Another possibility is the generation of circular dimers in the infected cell and their adhe-

FIG. 1. *M13 am3H5* polyphage particles grown on *E. coli* K37 su^+ ; the purified phage were prepared for electron microscopy by the diffusion method and were shadowed with platinum. Single-length, one and a half-length, and double-length particles are visible. The bar represents the length of a normal phage. Magnification 4.6×10^4 .

FIG. 2. A double-length, circular, single-stranded DNA molecule isolated from *fd*⁺ phage, surrounded by single-length molecules. Magnification 3.1×10^4 .

FIG. 3. The velocity sedimentation profile of ^3H -labeled fd^+ DNA in a linear CsCl gradient. Sedimentation was from right to left. One count is the equivalent of 10^8 fd -DNA molecules. The percentage of circular dimers in each fraction was determined as described in Materials and Methods. A detailed analysis of the CsCl gradient is given in Table 2. Symbols: -----, $[\text{}^3\text{H}]$ thymidine DNA counts per minute per fraction; —, percentage of dimer circular DNA.

sion to phage particles during the phage isolation procedure. This appears most unlikely since the phage were treated exhaustively with pancreatic DNase both in the crude lysate and before the CsCl buoyant density gradient centrifugation step. Furthermore, Table 2 shows that no double-stranded DNA was observed in the fractions examined after CsCl velocity sedimentation of the phage DNA. Since double-stranded host DNA and double-stranded phage DNA are the predominant species in the infected cell, one would expect these as contaminants along with the circular single-stranded dimers from the host cell.

Further evidence supporting the existence of circular single-stranded DNA dimers in filamentous phage comes from the following facts: a total of 160 such molecules in a completely spread configuration have now been observed in electron micrographs (see Tables 1 and 2 and Fig. 2 and 4). After CsCl velocity sedimentation, the dimer molecules were found in a sharp peak with the expected S value of 38 (21) and no dimer molecules were observed in the monomer peak (Fig. 3 and Table 2). The latter result also supports the single-stranded nature of the dimer DNA, since under the conditions employed in our gradient double-stranded relaxed phage DNA monomers would have an expected S value of 21 (fraction 29, Fig. 3), as opposed to an S value of 33 (fraction 19, Fig. 3) for double-stranded relaxed DNA dimers.

Final proof of the single-stranded nature of the dimer DNA comes from the dependence of contour length on ionic strength (Table 3): the contour length is changed by 15 and 24% in changing the salt concentration from 0.1 to 0.15 or 0.2 M. Double-stranded DNA would be

expected to change by no more than 2% under these conditions (14).

The existence of the dimer single-stranded DNA circles in filamentous phage poses two obvious questions. What is their origin and what is their fate? Specifically, what kind of phage particles can incorporate this DNA? Pratt et al. (16) and Scott and Zinder (22) showed that multiple-length particles of filamentous phage exist; they are present in wild-type stocks at a frequency of about 0.3% (Table 1). Thus, at least one in 10 double-length phage particles may contain a double-length DNA molecule. Mutations of filamentous phage which result in a high fraction of polyphage (16, and Table 1) do not cause an increase in the percentage of double-length DNA.

If the double-length DNA circles are contained in the rare double-length phage particles, isolation of these phage free of normal-length phage should provide an opportunity to obtain pure double-length single-stranded circular DNA. Both column chromatography (21) and polyacrylamide gel electrophoresis (1) have been used to obtain pure double-length phage stocks; CsCl velocity sedimentation of DNA extracted from these phage should yield pure double-length circles. Once this purified DNA is available, its fate may be examined by looking at the progeny phage produced after single transfection of spheroplasts. Where such experiments have been conducted with double-stranded SV40 circular DNA (12), double-length molecules generated double-length progeny DNA. Rush and Warner (20) examined the progeny ϕX174 phage released from *E. coli* spheroplasts transfected by double-length double-stranded ϕX174 DNA circles. Only the

FIG. 4. A field from the electron micrographic analysis of the CsCl gradient fractions of Fig. 3. In this peak fraction (No. 15) double-length circles have become so enriched that two of them are visible in the same field. Magnification 3.3×10^4 .

TABLE 2. Detailed analysis of the CsCl velocity sedimentation of Fig. 3^a

Fraction	25	24	23	22	21	20	19	18	17	16	15	14	13	12	Totals
Total no. of molecules examined	3,232	3,123	3,017	3,448	3,442	2,954	3,509	2,080	1,364	990	773	821	777	761	30,393
Double-length circles	0	0	0	0	2 (2.93 μ)	7 (2.87 μ)	14 (2.86 μ)	15 (3.12 μ)	15 (3.07 μ)	15 (2.98 μ)	15 (2.87 μ)	16 (2.82 μ)	15 (2.82 μ)	15 (2.81 μ)	129
Double-length crossed circles	0	0	0	4	10 (2.92 μ)	12 (2.85 μ)	16 (2.89 μ)	17 (3.04 μ)	18 (3.12 μ)	17 (2.99 μ)	16 (2.88 μ)	15 (2.87 μ)	17 (2.85 μ)	15 (2.83 μ)	157
Double-length linear	0	0	0	0	1 (2.91 μ)	3 (2.78 μ)	1 (2.93 μ)	1 (2.98 μ)	1 (3.20 μ)	0	0	1 (2.83 μ)	0	0	8
Monomer circles	3,150 (1.39 μ)	3,027 (1.39 μ)	2,926 (1.40 μ)	3,361 (1.47 μ)	3,293 (1.45 μ)	2,782 (1.46 μ)	3,290 (1.46 μ)	1,928 (1.56 μ)	1,250 (1.53 μ)	907 (1.50 μ)	703 (1.48 μ)	747 (1.47 μ)	705 (1.44 μ)	695 (1.42 μ)	28,864
Monomer linear	82 (1.38 μ)	96 (1.40 μ)	91 (1.39 μ)	83 (1.46 μ)	136 (1.46 μ)	150 (1.46 μ)	188 (1.45 μ)	119 (1.54 μ)	60 (1.54 μ)	51 (1.49 μ)	39 (1.48 μ)	42 (1.48 μ)	40 (1.40 μ)	36 (1.42 μ)	1,213
Double-stranded DNA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

^aNumbers given in parentheses represent the measured contour length of the molecules. All sample standard deviations were between 3 and 10%.

FIG. 5. A double-length linear fd DNA molecule from fraction 20 of the CsCl gradient of Fig. 3. Magnification 3.2×10^4 .

TABLE 3. Dependence of contour length of single-stranded monomer and dimer fd⁺ DNA (from fraction 19 of the CsCl gradient-Fig. 3) on ionic strength

Ionic strength (M)	Contour length (μm)	Observed change (%)	Expected change for ss-DNA (%) ^a	Expected change for ds-DNA (%) ^c
Monomer circles ^a				
0.1	1.73 ± 0.09			
0.15	1.46 ± 0.06	16	15	2
0.2	1.32 ± 0.04	24	24	2
Dimer circles ^a				
0.1	3.38 ± 0.17			
0.15	2.86 ± 0.15	15	15	2
0.2	2.54 ± 0.12	25	24	2

^a At each salt concentration, twenty molecules were measured for monomer circles, and 10 molecules were measured for dimer circles.

^b Bujard (4).

^c Lang et al. (14).

normal small size virus particles were observed; this result was inconclusive because double-length DNA may not be packaged in ϕ X174 capsids and thus would be denied transfer to another host cell. The absence of double-length circular phage DNA from among 30,000 ϕ X174 DNA molecules (Table 1) seems to bear out this hypothesis. However, examination of the DNA inside ϕ X174 phage-infected cells may yield double-length single-stranded ϕ X174 DNA.

ACKNOWLEDGMENTS

We thank Ronald Dowlen and Pat Pollock for diligent technical assistance and Robert Huskey for critical comments and help in calculating sedimentation constants. F.C.W. thanks Carl Schnaitmann and Hermann Bujard for introduction to the techniques of electron microscopy and Pat Scheible for help with the preparation and shadowing of grids.

This work was supported by grant no. GB 4388 from the National Science Foundation and Public Health Service grant no. GM 13224 and no. FR 5646 from the National Institute of General Medical Sciences and the Division of Research Facilities and Resources (H.B.), as well as grant no. AI-08572 from the National Institute for Allergy and Infectious Diseases and a research career development award to R.H.B. from the Institute for General Medical Sciences (6-KO4-GM-50284 GEN). F.C.W. was a fellow under the NDEA Title IV Fellowship Program and was also supported by National Institutes of Health training grant no. 5-T01-GM-01450.

LITERATURE CITED

1. Beaudoin, J., T. J. Henry, and D. Pratt. 1974. Purification of single- and double-length M13 virions by polyacrylamide gel electrophoresis. *J. Virol.* **13**:470-477.
2. Benbow, R. M., M. Eisenberg, and R. L. Sinsheimer. 1972. Multiple-length DNA molecules of bacteriophage ϕ X174. *Nature. N. Biol.* **237**:141-144.

TABLE 4. Effect of alkaline denaturation on the relative frequency of circular and linear, monomer, and dimer fd DNA from fraction 19 of the CsCl gradient

Determination	Before NaOH	After NaOH
Total no. of molecules examined	3,509	3,602
Double-length circles	14 (0.40) ^a	15 (0.42)
Double-length crossed-circles	16 (0.46)	14 (0.39)
Double-length linear molecules	1 (0.03)	2 (0.06)
Monomer circles	3,290 (93.76)	3,356 (93.17)
Monomer linear molecules	188 (5.36)	215 (5.97)

^a Numbers in parentheses represent percentages of the total number of molecules examined.

3. Boyce, P., and R. B. Setlow. 1962. Simple method for increasing the incorporation of thymidine into the DNA of *E. coli*. *Biochim. Biophys. Acta* **61**:618-620.
4. Bujard, H. 1970. Electron microscopy of single-stranded DNA. *J. Mol. Biol.* **49**:125-137.
5. Clayton, D. A., and J. Vinograd. 1967. Circular dimer and catenate forms of mitochondrial DNA in human leukaemic leucocytes. *Nature (London)* **216**:652-657.
6. Doniger, J. C., R. C. Warner, and I. Tessman. 1973. Role of circular dimer DNA in the primary recombination mechanism of bacteriophage S13. *Nature N. Biol.* **242**:9-12.
7. Goebel, W., and D. R. Helinski. 1968. Generation of higher multiple circular DNA forms in bacteria. *Proc. Nat. Acad. Sci. U.S.A.* **61**:1406-1413.
8. Hofs, E. B. H., J. H. van de Pol, and G. A. van Arkel. 1972. Dimeric circular duplex DNA of bacteriophage ϕ X174 and recombination. *Mol. Gen. Genet.* **118**:161-172.
9. Hudson, B., and J. Vinograd. 1967. Catenated circular DNA molecules in HeLa cell mitochondria. *Nature (London)* **216**:647-652.
10. Jaenisch, R., P. H. Hofschneider, and A. Preuss. 1969. Isolation of circular DNA by zonal centrifugation. Separation of normal length, double length and catenated M13 replicative form DNA and of host specific "episomal" DNA. *Biochim. Biophys. Acta* **190**:88-100.
11. Jaenisch, R., and A. Levine. 1971a. DNA replication in SV 40-infected cells. V. Circular and catenated oligomers of SV 40 DNA. *Virology* **44**:480-493.
12. Jaenisch, R., and A. Levine. 1971b. Infection of primary African green monkey cells with SV 40 monomeric and dimeric DNA. *J. Mol. Biol.* **61**:735-738.
13. Lang, D. 1971. Individual macromolecules: preparation and recent results with DNA. *Phil. Trans. Roy. Soc. London* **261**:151-158.
14. Lang, D., H. Bujard, B. Wolff, and D. Russell. 1967. Electron microscopy of size and shape of viral DNA in solutions of different ionic strengths. *J. Mol. Biol.* **23**:163-181.
15. Martin, R. G., and B. N. Ames. 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. *J. Biol. Chem.* **236**:1372-1379.
16. Pratt, D., H. Tzagoloff, and J. Beaudoin. 1969. Conditional lethal mutants of the small filamentous coliphage M13, II. Two genes for coat proteins. *Virology* **39**:42-53.

17. Pratt, D., H. Tzagoloff, and W. S. Erdahl. 1966. Conditional lethal mutants of the small filamentous coliphage M13. I. Isolation, complementation, cell killing, time of cistron action. *Virology* **30**:397-410.
18. Roth, T., and D. R. Helinski. 1967. Evidence for circular DNA forms of a bacterial plasmid. *Proc. Nat. Acad. Sci. U.S.A.* **58**:650-657.
19. Rush, M. G., A. K. Kleinschmidt, E. Hellman, and R. C. Warner. 1967. Multiple length rings in preparations of ϕ X174 replicative form. *Proc. Nat. Acad. Sci. U.S.A.* **58**:1676-1683.
20. Rush, M. G., and R. C. Warner. 1967. Multiple-length rings of ϕ X174 replicative form, II. Infectivity. *Proc. Nat. Acad. Sci. U.S.A.* **58**:2372-2376.
21. Salivar, W. O., T. H. Henry, and D. Pratt. 1967. Purification and properties of diploid particles of coliphage M13. *Virology* **32**:41-51.
22. Scott, J. R., and N. D. Zinder. 1967. Heterozygotes of phage f1, p. 211-218. *In* J. S. Colter and W. Paranchych (ed.), *The molecular biology of viruses*. Academic Press Inc., New York.
23. Streisinger, G., J. Emrich, and M. M. Stahl. 1967. Chromosome structure in phage T4: III. Terminal redundancy and length determination. *Proc. Nat. Acad. Sci. U.S.A.* **57**:292-295.
24. Yamamoto, K., B. Alberts, R. Benzinger, L. Lawhorne, and G. Treiber. 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large scale virus purification. *Virology* **40**:734-744.