JOURNAL OF VIROLOGY, Oct. 1968, p. 1096-1101 Vol. 2, No. 10
Copyright © 1968 American Society for Microbiology
Printed in U.S.A.

Evidence for a New Endonuclease Synthesized by λ Bacteriophage

ROBERT C. SHUSTER[1] AND ARTHUR WEISSBACH[2]

National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014

Received for publication 24 June 1968

Infection of nonlysogenic *Escherichia coli* CR34(S) (Thy^-) with bacteriophage λ C_I857 resulted in the formation of twisted circular double-stranded phage deoxyribonucleic acid (DNA; species I). When such infected bacteria were incubated in the absence of thymine, there was a significant decrease in the amount of species I DNA after 60 min of incubation. A similar loss of species I λ DNA during incubation in a thymine-deficient medium was also observed after infection of the endonuclease I-deficient strain, *E. coli* 1100(S) (Thy^-). This destruction of twisted, circular λ DNA in thymine-deprived cells did not occur in the presence of chloramphenicol nor in lysogenic *E. coli* CR34 carrying a noninducible λ prophage. It is therefore concluded that the endonuclease which attacks this circular configuration of λ DNA is newly synthesized after infection and is directed by the phage chromosome.

When λ bacteriophage infects a bacterial cell, a large proportion of the entering linear duplex deoxyribonucleic acid (DNA) molecules are converted to a nonended circular form (1, 16, 17). This form of phage DNA corresponds to species I as described by Bode and Kaiser (1) and can be distinguished by its sedimentation properties in sucrose gradients. At neutral *p*H, species I sediments 1.9 times faster than linear λ DNA (species III), whereas at alkaline *p*H it sediments almost four times as rapidly as species III. In neutral sucrose, a third form of λ DNA can be separated; this form has a sedimentation rate of 1.14 times that of linear λ DNA. It is designated species II and is believed to consist of double-stranded circular DNA containing one or more single-strand breaks. Centrifugation of species II in alkaline sucrose causes denaturation of the DNA which then sediments at nearly the same rate as species III. Thus, centrifugation in alkaline sucrose clearly separates species I from species II and III but usually does not permit the distinction between species II and III.

In a continuing study of the role of circular phage DNA in λ development, we have examined the formation of twisted circular λ DNA (species I) after infection of thymine auxotrophs in the presence and absence of thymine. As expected, the infection of thymine-requiring cells with phage λ led to a conversion of between 20 and 40% of the incoming linear λ DNA to the twisted circular configuration, whether or not thymine was present. This twisted circular λ DNA was found to be quite stable and persisted up to the time of lysis when thymine was present, but was almost completely destroyed within 1 hr in the absence of thymine. Furthermore, once the twisted, circular λ DNA was degraded, it was not reconstituted when thymine was added back to the cells, even though subsequent phage formation was apparently normal.

In this paper, we present evidence which suggests that species I is not initially involved in the replication of λ DNA. It is also shown that, during incubation in the absence of thymine, a new endonuclease, which causes the disappearance of parental species I λ DNA, is produced by the bacteriophage.

MATERIALS AND METHODS

Bacterial strains. *Escherichia coli* CR34(S) (nonlysogenic) *leu*$^-$, *thr*$^-$, *thy*$^-$, *thi*$^-$ was obtained from M. Meselson. *E. coli* CR34 *leu*$^-$, *thr*$^-$, *thy*$^-$, *thi*$^-$, λ*ind*$^-$ was isolated in this laboratory. It was immune to infection with wild-type λ but sensitive to infection with λ_{vir}. The prophage could not be induced by ultraviolet light. *E. coli* W3110 (λ C_I857) is a heat-inducible lysogen obtained from M. Gottesman (15). *E. coli* 1100 is an endonuclease I^- mutant isolated by H. Hoffman-Berling and obtained from M. Gellert. Thymine-requiring mutants of *E. coli* 1100 were isolated by the method of Stacey and Simson (14).

Reagents. Thymine-*methyl*-^3H (specific activity, 7 c/mmole) and thymidine-*methyl*-^{14}C (specific activity, 5 c/1.4 moles) were obtained from New England

[1] Present address: Department of Biochemistry, Emory University, Atlanta, Ga., 30322.

[2] Visiting Scientist, Roche Institute of Molecular Biology.

Nuclear Corp., Boston, Mass. Salt-free lysozyme was obtained from Worthington Biochemical Corp., Freehold, N.J.

Media. Cells were grown in a synthetic medium the composition of which has been previously described (12). Cultures of *E. coli* CR34 contained 0.1% Casamino Acids (Difco) and 60 μM thymidine. Cultures of *E. coli* 1100 (Thy$^-$) were routinely supplemented with 120 μM thymidine.

Preparation of ^{3}H-labeled bacteriophage. A culture of *E. coli* W3110 (λ C_I857) was grown in 1,500 ml of synthetic medium at 34 C with vigorous aeration to a density of 4×10^8 cells/ml. The cells were heated at 41 C for 15 min at the same cell density, followed by incubation at 37 C. Radioactive thymidine-*methyl-^{3}H* was added at 15-min intervals in 0.4-mc portions at the beginning of the 41-C heating period. Lysis occurred 60 to 70 min after transfer to 37 C. The phage were then isolated and banded in cesium chloride as previously described (12). The total phage yield was 1.6×10^{13} plaque-forming units (PFU) containing 4×10^7 counts/min.

Infection of bacteria. Bacteria were grown to a concentration of 2×10^8 cells/ml in synthetic medium containing thymidine or thymine. They were centrifuged, washed once in the same medium lacking thymine, and resuspended in a thymine-deficient medium at the same cell concentration. After incubation at 37 C for 5 min, the cells were infected with radioactive phage at a multiplicity of 5 phage/cell, unless otherwise noted. After incubation for 15 min at 37 or 39 C, where indicated, the infected cells were transferred to a Waring Blendor in a cold room (2 C) and were sheared at top speed for 4 min in order to remove uninjected phage (1). No loss in bacterial viability occurred under these conditions. The cells were then centrifuged and resuspended in the same volume of thymine-free medium (zero-time). Incubation of the infected bacterial cells was continued at 37 or 39 C; 100-ml samples were taken at intervals and were immediately chilled in ice. The infected cells were collected by centrifugation and were suspended in 15 ml of a solution containing 0.1 M ethylenediaminetetraacetic acid (EDTA) and 0.6 M tris(hydroxymethyl)-aminomethane (Tris), *p*H 8.0. Cell lysis and extraction of the DNA with phenol was as previously described (13). In some experiments, the infected cells were suspended in 0.015 M citrate and 0.15 M saline rather than in KCN-EDTA-Tris buffer. After lysis, 1 mg of Pronase per ml, previously heated at 80 C for 10 min, was added and the samples were incubated overnight at 37 C. These lysates were not dialyzed or treated with phenol but were sedimented directly as described below. The method had the advantage of eliminating the 40 to 60% losses of radioactivity that normally occur after phenol treatment. No differences in sedimentation properties of the phage DNA components were noted between the two methods of deproteinization.

Zone centrifugation. The DNA preparations were concentrated to 1.5 ml by pervaporation, under reduced pressure, through a collodion filter at 2 C, and 0.1 ml of 5 N NaOH was added. The entire sample was then layered onto 26 ml of a sucrose gradient and was centrifuged in the SW25 head of a Spinco model L-4 for 16 hr at 23,000 × *g*. A gradient of 10 to 30% sucrose in 0.5 N NaOH, 10^{-3} M EDTA was used. The bottom layer contained 6 ml of the 30% sucrose solution. On top of this, we serially layered 5 ml each of 25, 20, 15, and 10% sucrose solutions. Fractions of 0.7 ml were collected and analyzed for radioactivity as previously described (13).

RESULTS

Instability of twisted circular λ C_I857 after infection and incubation in a thymine-deficient medium. To determine whether thymine starvation had any effect on λ DNA, a culture of *E. coli* CR34(S) was infected with radioactive λ C_I857 at a multiplicity of 3 phage/cell. After incubation at 39 C for 15 min in thymine-deficient medium, unabsorbed phage were removed by shearing and centrifugation as described in Materials and Methods. Incubation was continued at 39 C in the absence of thymine, and samples were taken at 0, 15, 30, 45, and 60 min. The distribution of radioactivity in phage DNA was determined after alkaline sucrose gradient centrifugation (Fig. 1). The faster sedimenting peak of radioactivity (around fraction 5) is the closed, twisted circular form of λ DNA (1). The per cent of radioactivity in this form of phage DNA was 20% at zero-time. During incubation in the absence of the thymine, the percentage decreased to 8, 2, 1, and <1% after 15, 30, 45, and 60 min, respectively. The slower sedimenting material (around fraction 20) would include both single-stranded linear DNA and circular DNA containing single or double strand breaks.

In another experiment, when the multiplicity of infection was 1 PFU/bacterial cell, the number of infective centers was assayed at each time period and found to be unchanged during incubation in thymine-deficient media (Table 1). Table 1 also indicates that thymine-starved cells absorb phage as do normal cells. In this experiment, 21% of the λ DNA was in the form of species I at zero-time. After 60 min of incubation in the absence of thymine, species I λ DNA was undetectable.

Failure of species I λ DNA to reform after the addition of thymine. An experiment was conducted to determine whether the twisted circular form of parental phage DNA could be reconstituted once it had disappeared. At 60 min, in another experiment, when the per cent of twisted circular λ DNA had decreased from 31 to 4.5%, unlabeled thymine was added at a concentration of 60 μM. Samples were taken at 0, 5, 10, 20, and 30 min after the addition of thymine, and the DNA was analyzed as before. There was no change in the per cent of twisted circular λ DNA (less than 5%) or of total radioactive phage DNA

extracted at any time after the addition of unlabeled thymine. Cell lysis occurred between 40 and 45 min after thymine had been added with a phage yield of approximately 60 PFU/infected bacteria.

De novo synthesis of species I λ DNA after the addition of thymine. Although species I λ DNA had almost completely disappeared after 60 min of incubation in the absence of thymine, phage maturation could apparently proceed normally after thymine had been added, as evidenced by cell lysis in the previous experiment. In addition, the results in Table 1 showed that the number of infective centers did not change even after species I had disappeared completely. To determine whether species I was synthesized de novo both before and after parental species I had broken down, cells were grown and infected with ^{3}H-labeled bacteriophage as before. One group was immediately supplemented with 60 μM of thymine containing 10 μc of ^{14}C. The second group was incubated for 60 min in the absence of thymine at which time the same concentration of ^{14}C-thymine was added. In the first group, parental species I DNA was still present (49% of total phage DNA), whereas in the second group the amount of parental species I had decreased to 10% of total phage DNA at the time ^{14}C was added. Incubation of both groups was continued for 30 min after the addition of ^{14}C-thymine, and the DNA was extracted and analyzed as before. It was found that de novo twisted, circular DNA containing ^{14}C was synthesized to the same extent in both groups. Approximately 2% of the radioactivity in the newly synthesized DNA was present as twisted, covalent circles. However, the de novo synthesis of species I was independent of the amount of parental twisted, circular DNA present at the time DNA synthesis began.

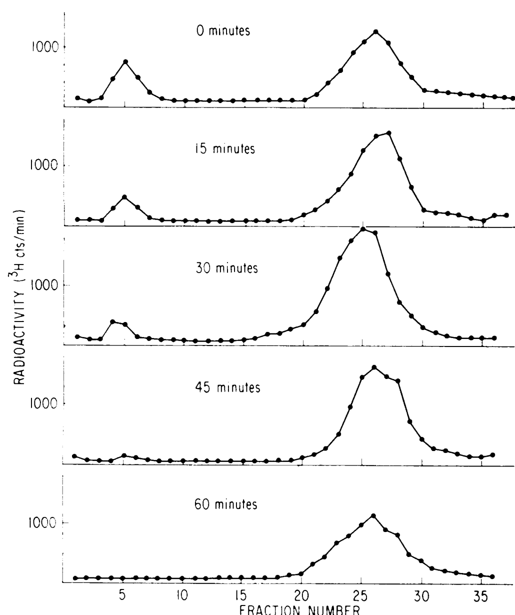

FIG. 1. *Disappearance of twisted circular λ DNA in the absence of thymine after infection and incubation of E. coli CR34(S) (Thy⁻) with labeled ^{3}H-thymine bacteriophage λ $C_{I}857$. A 500-ml culture of E. coli CR34(S) was grown and washed in a medium lacking thymine. After incubation for 5 min at 39 C in thymine-deficient medium, ^{3}H-thymine labeled λ $C_{I}857$ bacteriophage were added at a multiplicity of 3 phage/cell. Incubation was continued for 15 min at 39 C. Unabsorbed phage were removed by shearing and centrifugation. The cells were resuspended in 500 ml of the medium lacking thymine. Incubation was continued at 39 C, and 100-ml samples were removed at 0, 15, 30, 45, and 60 min. Infected cells were lysed, deproteinized by incubation with Pronase, and centrifuged in alkaline sucrose. Fractions of 0.7 ml were collected and assayed for radioactivity in a Nuclear-Chicago Mark I liquid scintillation counter. Sedimentation was from right to left.*

TABLE 1. *Determination of infective centers after thymine starvation*[a]

Time of incubation at 39 C	No. of infective centers/ml	No. of bacterial colonies/ml
min		
0	117×10^{6}	74×10^{6}
15	135×10^{6}	
30	133×10^{6}	
45	129×10^{6}	
60	132×10^{6}	39×10^{6}

[a] *E. coli* CR34(S), at a concentration of 2×10^{8} cells/ml, was infected in the absence of thymine with λ $C_{I}857$ at a multiplicity of infection of 1 phage/cell. After shearing and removal of unattached phage by centrifugation, the infected complexes were incubated at 39 C for the times indicated in the absence of thymine. Samples were plated on a lawn of *E. coli* C600 at 39 C to assay infective centers. The number of colony-forming bacteria was determined by plating samples on tryptone agar at 39 C.

A similar result, demonstrating the de novo synthesis of species I λ DNA after lysogenic induction of *E. coli* CR34(λ), has been previously reported (10).

Stability of species I λ DNA during the infection of nonlysogenic E. coli CR34(S) with λ $C_{I}857$ in the presence of thymine. Since parental twisted, circular phage DNA was not regenerated after the addition of thymine, it is possible that the breakdown of parental species I circular λ DNA normally occurs during the course of phage DNA synthesis. To determine whether parental circular λ DNA is stable during normal vegetative condi-

tions, the bacteriophage were infected and incubated in the presence of thymine. It was found that the twisted circular DNA was completely stable for at least 30 min during incubation in the presence of thymine (Fig. 2). In another experiment, there was no loss of twisted circular DNA in infected cells after incubation with thymine for 40 min. Cell lysis began at approximately 45 min. Thus, species I is stable during the normal development of the phage and breaks down only during thymine deprivation. This finding is in agreement with results obtained after the induction of lysogenic *E. coli* CR34(λ); in this study, newly synthesized species I λ DNA was also stable during the course of phage maturation (10).

Breakdown of twisted circular phage DNA in an endonuclease I^-, thy^- mutant. The previous experiments demonstrating the destruction of species I of λ DNA suggested that an endonuclease was synthesized or activated when cells were incubated in the absence of thymine. To determine whether endonuclease I was involved in the disappearance of species I, labeled bacteriophage were permitted to infect an endonuclease I^-, thymine-requiring mutant. This strain, grown on glycerol, absorbed the phage poorly, and it was necessary to substitute maltose as an energy source (0.2% in the synthetic medium). The experiments showed that there was extensive breakdown of the twisted circular form of λ DNA in this strain 60 min after incubation in a thymine-deficient medium. The results were the same as those found with the endonuclease-containing strain, i.e., *E. coli* CR34.

Effect of chloramphenicol on the disappearance of twisted circular λ DNA. Since the twisted circular form of the incoming phage DNA was broken down in an endonuclease I-deficient host strain, it is possible that breakdown of species I was initiated by a process requiring the synthesis of a new protein. To investigate this possibility, chloramphenicol (40 μg/ml) was added to the thymine-deficient medium and analysis of the phage DNA after incubation was carried out as before. At 60 min, the culture incubated in the presence of chloramphenicol showed no decrease in the percentage of species I λ DNA, whereas the sample lacking chloramphenicol showed the usual extensive loss of twisted circular phage DNA (Fig. 3).

Stability of species I λ DNA upon infection of E. coli CR34(λ ind^-) in the absence of thymine. From the previous experiments, it could not be determined whether the disappearance of species

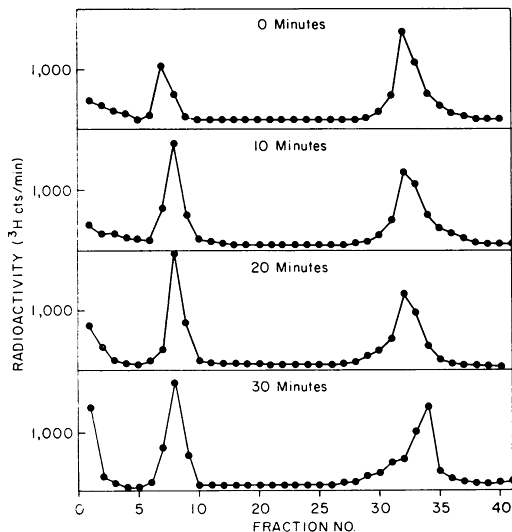

FIG. 2. *Stability of twisted circular λ DNA in the presence of thymine after infection of E. coli CR34(S) (Thy^-) with labeled bacteriophage λ C_I857. Cells were grown and infected at a multiplicity of 5 phage/cell as described in Fig. 1, except that thymine was present throughout each step. Deproteinization of cell lysates was by the phenol procedure. Fractions were collected after centrifugation and were assayed for radioactivity described in Fig. 1.*

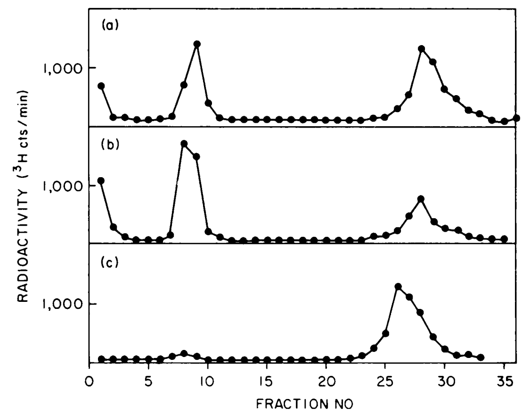

FIG. 3. *Effect of chloramphenicol on the disappearance of twisted circular λ DNA during incubation in the absence of thymine. E. coli CR34(S) was grown, washed, and infected with labeled λ C_I857 at a multiplicity of 5 phage/cell as described in Fig. 1. Incubation was continued for 60 min in the absence of thymine. The phage DNA was extracted, deproteinized with phenol, centrifuged in alkaline sucrose, and assayed for radioactivity as described in Fig. 1. (a) Incubation for 0 min after phage infection. (b) Incubation for 60 min in the absence of thymine but in the presence of chloramphenicol (40 μg/ml). Chloramphenicol was also present at the same concentration during infection and absorption of the phage. (c) Incubation for 60 min in the absence of thymine without chloramphenicol. Chloramphenicol was also absent during phage infection and absorption.*

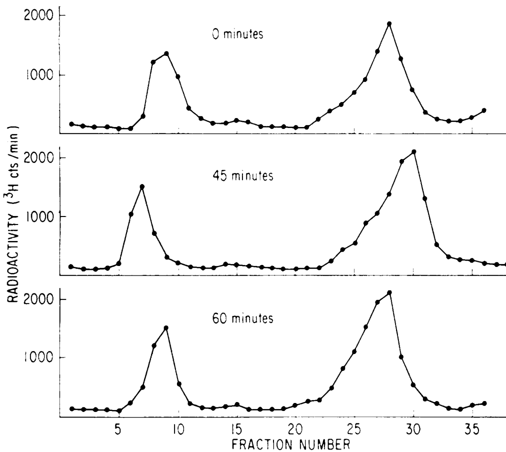

FIG. 4. *Stability of twisted, covalent, circular λ DNA in E. coli CR34 λ ind⁻ after infection and incubation in the absence of thymine. Bacteria were grown and infected with radioactive λ C_I857 at a multiplicity of 5 phage/cell as described in Fig. 1. Incubation was continued at 39 C in the absence of thymine for the times indicated. Infected cells were lysed and deproteinized by incubation with Pronase. They were then centrifuged, and fractions were assayed for radioactivity as described in Fig. 1.*

I during incubation in the absence of thymine was under the control of the host bacteria or the infecting phage. To answer this question, *E. coli* CR34 lysogenic for a noninducible λ prophage was used as the host bacteria. The repressor synthesized by this prophage is not inactivated either by thymine starvation or by heating at 39 C, so that the genes of the superinfecting λ C_I857 are not expressed under the conditions of the experiment (8, 15). Therefore, if the enzyme was phage-directed, the twisted, circular λ DNA should be stable in the absence of thymine, but if the enzyme was host-controlled, then the circles should disappear.

E. coli CR34 (λ*ind*⁻) was therefore grown and infected with λ C_I857 as previously described. The infected cells were incubated in the absence of thymine at 39 C, and samples were taken at 0, 45, and 60 min. The cells were lysed and sedimented in alkaline sucrose; then fractions were assayed for radioactivity (Fig. 4). The twisted, circular form of phage DNA in the Ind⁻ lysogen was stable during incubation in the absence of thymine. Since the superinfecting phage was repressed in this host, the results strongly suggested that the enzyme responsible for the disappearance of the twisted, covalent circular phage DNA is mediated by the bacteriophage.

DISCUSSION

The results of these experiments indicate that the disappearance of the twisted circular form of phage DNA during incubation in the absence of thymine is due to an enzyme synthesized by the bacteriophage. The fact that its activity is manifested in the absence of thymine, and therefore presumably in the absence of DNA synthesis, would classify the enzyme as an early function in phage maturation (9). In addition, the disappearance of species I probably begins soon after infection since, after 15 min of incubation in the absence of thymine, the percentage of radioactive phage DNA sedimenting as species I decreases 50% (Fig. 1).

The stability of the twisted circular form of λ DNA during infection and incubation in the presence of thymine is still unexplained. It is possible that some of the incoming λ DNA does not form a covalent circle because there are gaps of one or more nucleotides in either DNA chain. However, these gaps may be repaired in the presence of thymine so that, while some of the twisted, covalent circular phage DNA is being broken by the enzyme, more is continuously being formed; therefore, the total amount of parental species present at any time does not vary markedly. In addition, nonradioactive species I and other λ DNA intermediates are being formed de novo (13, 18) and may compete with radioactive species I for the enzyme.

Endonucleases (synthesized early by the bacteriophage) which are able to break the twisted, covalent circular phage DNA may have a function in the establishment of lysogeny, the initiation of phage DNA replication, or in other phage processes. It has been suggested that the insertion of the phage DNA into the host chromosome involves the prior formation of the covalent, circular form of phage DNA (3). Before the phage DNA can be integrated into the host chromosome, the covalent circular configuration would have to be broken at a specific locus. The integration negative mutants of λ (5, 6) could conceivably be lacking in this enzymatic activity.

A phage-directed endonuclease synthesized early in development could also be important for the initiation of DNA synthesis. It is known that the supressor-sensitive mutants of λ, *susO* and *susP*, are unable to synthesize DNA after infection of a nonpermissive, *su*⁻ host (2, 4, 7). These phage inject their DNA but it does not replicate intracellularly. It is therefore possible that the initiation of DNA synthesis requires a breaking or nicking of the double helix at the point where DNA synthesis begins. Recently, it has been re-

ported that replication of the phage DNA begins in the adenine-thymine-rich half of the right side of the phage genome (11). This site would differ from the site of breakage for integration, so that two or more separate enzymes may be responsible for the breaking of species I observed in these experiments. We are currently examining various phage mutants to determine whether any are deficient in the ability to break species I during incubation in the absence of thymine.

Although the twisted circular form of phage DNA may be an essential component at a discrete stage of the replicative process, the presence of this species I is not required to initiate phage DNA synthesis. Once species I λ DNA disappears after incubation in a thymine-deficient medium, it is not regenerated from parental DNA when thymine is readded. However, subsequent to the readdition of thymine, cell lysis occurs and there is no decrease in the number of infective centers. Thus, phage development can apparently proceed normally even though species I λ DNA is not initially present. A similar situation may occur during lysogenic induction of *E. coli* CR34 (λ) containing DNA labeled with radioactive thymine. When these cells were induced by thymine starvation, no formation of labeled species I λ DNA was observed (10). However, when thymine was readded after induction, there was de novo synthesis of species I phage DNA. These results indicate that the initial synthesis of λ DNA does not require the presence of a twisted circular DNA template and may be initially copied from a linear or open circular structure of phage DNA. In these experiments, we could not determine whether the twisted-covalent circular λ DNA underwent a single or double strand scission. The answer to this question will be determined in future experiments.

LITERATURE CITED

1. Bode, V. C., and A. D. Kaiser. 1965. Changes in the structure and activity of lambda DNA in a superinfected immune bacteria. J. Mol. Biol. **14**:399–417.
2. Brooks, K. 1965. Studies in the physiological genetics of some suppressor-sensitive mutants of bacteriophage lambda. Virology **26**:489–499.
3. Campbell, A. 1962. Episomes. Advan. Genet. **11**:101–145.
4. Eisen, H. A., C. R. Fuerst, L. Siminovitch, R. Thomas, L. Lambert, P. DeSilva, and F. Jacob. 1966. Genetics and physiology of defective lysogeny in K12(λ): studies of early mutants. Virology **30**:224–241.
5. Gingery, R., and H. Echols. 1967. Mutants of bacteriophage lambda unable to integrate into the host chromosome. Proc. Natl. Acad. Sci. U.S. **58**:1507–1512.
6. Gottesman, M. E., and M. B. Yarmolinsky. 1968. Integration-negative mutants of bacteriophage lambda. J. Mol. Biol. **31**:487–506.
7. Joyner, A., L. N. Isaacs, H. Echols, and W. S. Sly. 1966. DNA Replication and messenger RNA production after induction of wild-type λ bacteriophage and λ mutants. J. Mol. Biol. **19**:174–186.
8. Korn, D. 1966. Study of the development of resistance to λ ind$^-$ repression during thymineless induction of *E. coli* K12(λ) thy$^-$. Virology **24**:570–577.
9. Korn, D., and A. Weissbach. 1964. The effect of lysogenic induction on the deoxyribonucleases of *Escherichia coli* K12(λ). Virology **22**:91–102.
10. Lipton, A., and A. Weissbach. 1966. The appearance of circular DNA after lysogenic induction in *E. coli* CR34(λ). J. Mol. Biol. **21**:517–525.
11. Makover, S. 1968. A preferred origin for the replication of lambda DNA. Proc. Natl. Acad. Sci. U.S. **59**:1345–1348.
12. Salzman, L. A., and A. Weissbach. 1966. Studies on the formation of circular lambda DNA. Virology **31**:70–77.
13. Salzman, L. A., and A. Weissbach. 1966. Formation of intermediates in the replication of phage lambda DNA. J. Mol. Biol. **28**:53–70.
14. Stacey, K. A., and E. Simson. 1965. Improved method for the isolation of thymine-requiring mutants of *Escherichia coli*. J. Bacteriol. **90**:554–555.
15. Sussman, R., and F. Jacob. 1962. Sur un systeme de repression thermosensible chez le bacteriophage lambda d' *Escherichia coli*. Compt. Rend. **254**:1517–1519.
16. Weissbach, A., A. Lipton, and A. Lisio. 1966. Intracellular forms of λ deoxyribonucleic acid in *Escherichia coli* infected with clear or virulent mutants of bacteriophage λ. J. Bacteriol. **91**:1489–1493.
17. Young, E. T., and R. L. Sinsheimer. 1964. Novel intracellular forms of lambda DNA. J. Mol. Biol. **10**:562–564.
18. Young, E. T., and R. L. Sinsheimer. 1967. Vegetative bacteriophage λ DNA. II. Physical characterization and replication. J. Mol. Biol. **30**:165–201.