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The Rolling Circle for ϕX DNA Replication, III. Synthesis of Supercoiled Duplex Rings*

David Dressler and John Wolfson

THE BIOLOGICAL LABORATORIES, HARVARD UNIVERSITY, CAMBRIDGE, MASSACHUSETTS 02138

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Abstract. During ϕX duplex ring synthesis, the first supercoils to acquire radioactivity after the addition of tritiated thymidine are labeled only in their negative strands. In longer pulses, this asymmetry of labeling progressively disappears. This finding supports the rolling circle model for DNA replication due to the structural asymmetry of its replicating intermediate, but is not predicted by the Cairns or Yoshikawa models.

Introduction. Self-replicating molecules often exist as duplex rings.[1] Some diverse examples are DNAs from the mitochondrion, the tumor-inducing virus polyoma, the lysogenic bacteriophage lambda, and the lytic coliphage ϕX. The precise molecular mechanisms involved in the replication of these duplex rings are unknown. There are at present, however, three models which attempt to explain the process. These are the rolling circle model,[2] and the models of Yoshikawa[3] and of Cairns.[4,5] As is discussed below, the rolling circle model is distinguished from the Cairns and Yoshikawa models by the asymmetries which it requires of the replicating DNA molecule and of the products of replication. In this paper we will consider, and confirm experimentally, one of the main asymmetric predictions of the rolling circle model as applied to the replication of ϕX double-stranded circles.

The first of several asymmetries which characterize DNA replication in the rolling circle mechanism results because the two strands of the parental duplex ring are processed differently at initiation. A nick is introduced into a specific base sequence of the "positive" strand. In this way the strand is converted into a DNA rod with a 3′-OH terminus and a 5′-phosphate terminus. DNA synthesis begins when the 5′-terminus is peeled back and nucleotide precursors are condensed upon the 3′-terminus (Fig. 1*A*). A continuous elongation of the positive strand around the circular negative strand template results in the displacement of a free single-stranded tail of increasing length. The synthesis of negative strand material, which appears in pieces of less than unit length, converts the tail to a region of double-stranded DNA.

As the growing point travels around the template circle for the second time, the linear tail becomes longer than one genome. In this state, the tail contains homologous DNA sequences a genome's length apart; the tail can therefore be used to construct circular daughter chromosomes, by either a generalized or a site-specific recombination process. In the case of a site-specific recombina-

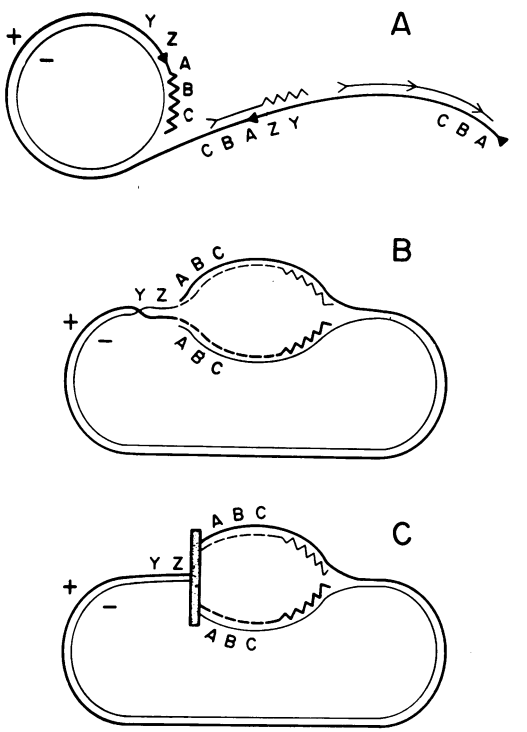

FIG. 1. Three models for the replication of circular DNA.

(*A*) The replicating intermediate of the rolling circle model is redrawn from Gilbert and Dressler.[2] The intermediate is characterized by its structural asymmetry: one strand of the parental duplex ring is nicked and elongated while the other is maintained as a permanent circular template.

(*B*) The intermediate of the Yoshikawa model[3] (see also Alberts[9]) is structurally symmetric. In contrast to the rolling circle, not just one but both parental DNA strands are elongated.

(*C*) The Cairns form is redrawn from Cairns and Davern.[5] The stippled rectangle represents the swivel, to which the two daughter origins and the terminus of the partially replicated chromosome are attached. The Cairns intermediate is structurally symmetric. As in the case of the Yoshikawa intermediate, this symmetry is eventually reflected in the equivalence of the two daughter chromosomes.

Solid lines indicate parental polynucleotide strands. Dashed lines indicate daughter polynucleotide strands (for *B* and *C*). Jagged lines indicate strands labeled during a pulse.

Of the three models for the replication of duplex rings, only the rolling circle model employs an intermediate for DNA replication which is inherently asymmetric and is therefore expected to produce a population of asymmetrically labeled supercoils. Perhaps the Yoshikawa and Cairns models could be modified to generate asymmetric progeny chromosomes. For example, asymmetry in these two models could result from some as yet unknown event which occurs in the "membrane replication site." A molecular mechanism designed to confer asymmetry would, however, require an addition to the Cairns and Yoshikawa models.

tion process, sequence-recognizing endonucleases would introduce pairs of staggered nicks into the tail at genome length intervals. This would allow the excision of a DNA rod with single-stranded, complementary termini (like lambda DNA[6,7]). This type of DNA rod is intrinsically capable of circularization, and could be sealed into a supercoil[8] by the ligase.

A striking prediction of the rolling circle model is that, after the addition of tritiated thymidine to ϕX-infected cells making duplex rings, the first supercoils to acquire radioactivity will be labeled only in their negative strands. This asymmetry of labeling results from the fact that supercoils are derived from the tail of the rolling circle intermediate. As label begins to flow into the rolling circle, the tail is composed of an unlabeled positive strand which is serving as a template for the growing negative strand (Fig. 1*A*). The first radioactive supercoil to be excised from the tail is expected to contain both the unlabeled positive strand template and the pulse-labeled negative strand: the supercoil will therefore be asymmetrically radioactive. As the length of exposure to

tritiated thymidine increases, radioactivity originally incorporated into the long positive strand on the rolling circle itself (Fig. 1*A*) is rotated into the tail, where it is in a position to be matured into a supercoil. Therefore, after a longer period of labeling, the supercoils excised from the tail should contain equivalent amounts of radioactivity in both positive and negative strands.

Asymmetrically labeled supercoils are not the expected products of replication under two alternative models concerned with the replication of circular chromosomes—the models which have arisen from the experiments of Yoshikawa with *Bacillus subtilis*[3] and from the work of Cairns with *Escherichia coli*.[4] In contrast to the rolling circle model, the Yoshikawa and Cairns models are characterized by the symmetry of their replicating intermediates and by the equivalence of the progeny chromosomes. As a consequence of their structural symmetry, these intermediates are expected to produce, as their first radioactive progeny during a pulse, supercoils which are labeled in *both* positive and negative strands.

For example, let us consider the replicating intermediate of the Yoshikawa model. This intermediate is characterized by the elongation of not just one, but both parental DNA strands. As the growing points move the whole way round the circle (Fig. 1*B*) the elongated strands eventually become double length, the newly-synthesized ends of the daughter strands are then in a position to be joined into the origins of the parental strands. In this way a double-size duplex ring may be produced.[9] A site-specific recombination event then cleaves the circular dimer into a pair of equivalent progeny duplex rings.

A short pulse of radioactivity incorporated by the replicating intermediate of the Yoshikawa model would label both positive and negative strand material symmetrically (Fig. 1*B*). And as the first radioactive duplex rings are derived from the intermediate, and sealed into supercoils by the ligase, they should form a population of molecules labeled in *both* DNA strands.

In the case of the Cairns model (Fig. 1*C*), as in the case of the Yoshikawa model, the replicating intermediate is structurally symmetric. It is therefore expected to generate a pair of progeny duplex rings which are equivalent. Thus, during a period of pulse-labeling, as the first radioactive circular chromosomes are completed and sealed by the ligase, they should form supercoils which as a population are labeled in both positive and negative strands.

To distinguish between the three models for the replication of circular genomes on the basis of their different predictions about the labeling of supercoils, the following experiment was performed. Cells producing a normal yield of ϕX were allowed to incorporate tritiated thymidine during the period of active double-stranded circle synthesis. The first supercoils to acquire radioactivity were recovered and found to be asymmetrically labeled in their negative strands, confirming the prediction of the rolling circle model.

Isolation of Pulse-Labeled Supercoils. ϕX DNA replication occurs in two phases: a period of double-stranded circle synthesis, followed by a period of single-stranded circle synthesis. It is expected that, early after infection, a pulse of tritiated thymidine given to ϕX-infected cells will label intermediates involved in the replication of duplex rings and eventually product supercoils.

Accordingly, cells whose endogenous DNA synthesis had been arrested with mitomycin were infected with φX at a low multiplicity and, 6 min after the initiation of synchronous phage growth, exposed to tritiated thymidine (Fig. 2). At 40, 70, 100, and 150 sec after the addition of labeled thymidine, equal aliquots of the culture were harvested and lysed (Fig. 3). The unfractionated cell

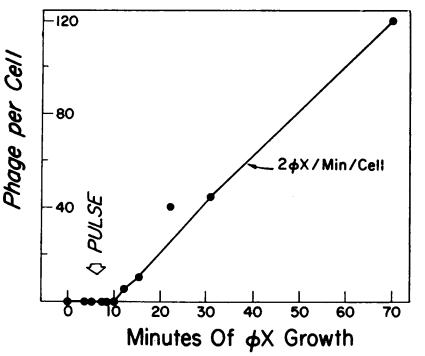

FIG. 2. Infection and pulse-labeling.

Cells with their endogenous DNA synthesis arrested with mitomycin were infected with φX and pulse-labeled with tritiated thymidine during the period of duplex ring synthesis.

The details of the infection* are as follows: *E. coli* strain HF 4704 (hcr^-thy^-) was grown to a titer of 6×10^8/ml at 37°C in 200 ml of mT3XD medium. During the final 20 min of growth, mitomycin C (Calbiochem) was present at 100 μg/ml. The cells were washed, resuspended in 30 ml of holding buffer that contained mitomycin, starved with aeration at 37°C for 20 min, and then infected at a multiplicity of 2 with φX am-3 (defective in lysis). After >99% of the phage had eclipsed (10 min later), 30 ml of double-strength m3XD (containing 1.0 μg/ml of thymine) was added to initiate phage growth.

The purpose of synchronizing the infection and of using a low multiplicity of phage was to insure that the conversion of infecting positive strand rings to double-stranded rings would occur *prior* to the period of pulse-labeling. Control experiments with tritium-labeled phage showed that the formation of parental duplex rings was entirely completed within 4 min after the addition of nutrients. Therefore, at 6 min when pulse-labeling was begun, there should have been few if any infecting positive strand circles in the process of conversion to parental duplex rings. Supercoils asymmetrically radioactive in their negative strands at the end of the pulse ought, therefore, to reflect patterns of duplex ring replication, and not the conversion of the original positive strands to double-stranded rings.

Six min after the initiation of synchronous phage growth, tritiated thymidine was added to the culture. Aliquots of the culture were harvested at 40, 70, 100, and 150 sec after the addition of label, lysed, and the radioactive supercoils recovered (see Fig. 3).

lysates, containing all of the labeled intracellular DNA forms, were denatured and sedimented on a preparative scale through alkaline sucrose gradients in order to recover the fast-sedimenting supercoils.

The radioactivity profiles of the four gradients are shown in Fig. 3. The profile of the material labeled for 40 sec shows that about 10% of the radioactivity is recovered in supercoils (fractions 12–15). The remainder of the 40 sec pulse-label sediments in a broad distribution between fractions 5 and 12, and represents single-strands released by alkaline denaturation of replicating structures. As the length of the labeling period increases, radioactivity builds up in the supercoil position; after 150 sec of labeling, 25% of the counts are recovered as supercoils.

Asymmetrically Labeled Supercoils. The supercoils that had become radioactive by the end of each period of labeling (fractions 12–14, Fig. 3) were recovered from the gradients. That these labeled structures were in fact supercoils was confirmed in two different ways: by sedimentation in secondary alkaline sucrose gradients where the material again formed fast-moving (54 S) peaks, characteristic of denatured φX supercoils; and by centrifugation in secondary neutral sucrose gradients where the material formed sharp peaks at 40 S, characteristic of denatured and rapidly-reneutralized φX supercoils.[10]

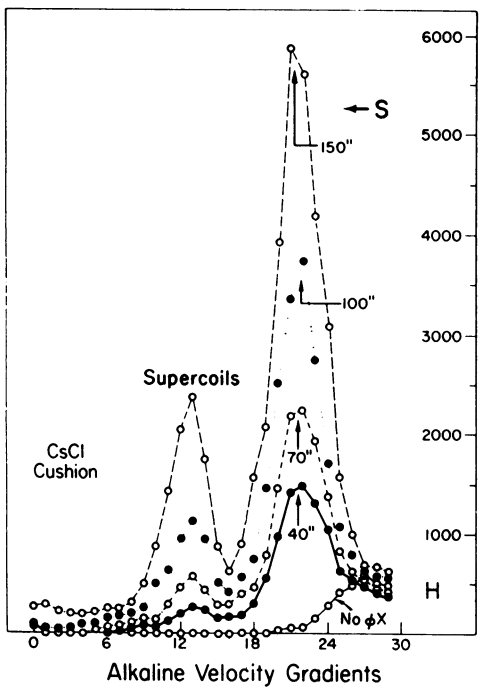

FIG. 3. Isolation of pulse-labeled supercoils.

Radioactive supercoils were recovered from φX-infected cells after 40, 70, 100, and 150 sec of pulse-labeling.

The pulsed cells were harvested, resuspended in buffer (0.01 M Tris, pH 8; 0.1 M NaCl, 0.01 M KCN, 0.01 M iodoacetate, 0.001 M EDTA) and broken open with lysozyme (400 μg/ml, 37°C, 20 min) and detergent (2% sarkosyl, 65°C, 20 min). To guard against nuclease activity during the isolation of intracellular DNA forms, tRNA (40 μg/ml) and denatured DNA from lambda phage (20 μg/ml) were added just before lysis. To recover supercoils, each lysate was denatured by the addition of NaOH to 0.2 M, and then sedimented through a 25-ml alkaline sucrose density gradient (10–30% sucrose, 0.8 M NaCl, 0.2 M NaOH, 0.001 M EDTA; underlaid with a saturated CsCl-sucrose cushion). Centrifugation was at 24,000 rpm for 16 hr at 8°C in a Beckman SW 25.1 rotor.

All gradients contain the contents of about 2×10^{10} cells. 2.5% of each fraction was assayed for radioactivity.

As a control to monitor the suppression of host DNA synthesis by mitomycin, an equivalent aliquot of the original culture was not infected. It was, however, exposed to tritiated thymidine for 90 sec beginning at 6 min, and processed in parallel with the infected cultures. The resulting gradient showed that there was essentially no incorporation of label into macromolecular DNA by the uninfected cells.

The covalently interlocked strands of the pulse-labeled supercoils were separated from each other, after the introduction of a limited number of single-strand nicks, by heating to 100°C for 3 hr (see the legend to Fig. 4). The released strands were then centrifuged to equilibrium in CsCl at pH 12.5. Under these conditions, φX positive and negative strands band at two different densities because of their different thymine contents.[11] To define the positions of both φX positive and negative strand material, two markers were included in the centrifugation: ^{14}C-positive strands (*isolated from mature phage*) and ^{32}P-negative strands (synthesized *in vitro*).

Fig. 4*A* shows that the supercoils contained almost all of their radioactivity in negative strand material after 40 sec. That is, almost all of the ^{3}H came to equilibrium with the ^{32}P-negative strand marker. Thus, the first supercoils to appear after the addition of label are radioactive in their negative strands, an observation which is in accord with the rolling circle model.

Supercoils labeled for longer periods contained an increasing percentage of their radioactivity in positive strand material. After 150 sec or longer exposure to tritiated thymidine, the population of labeled supercoils contained positive and negative strand radioactivity in about equal amounts (Fig. 4*C*).

The data of this and similar experiments indicate that positive strand label

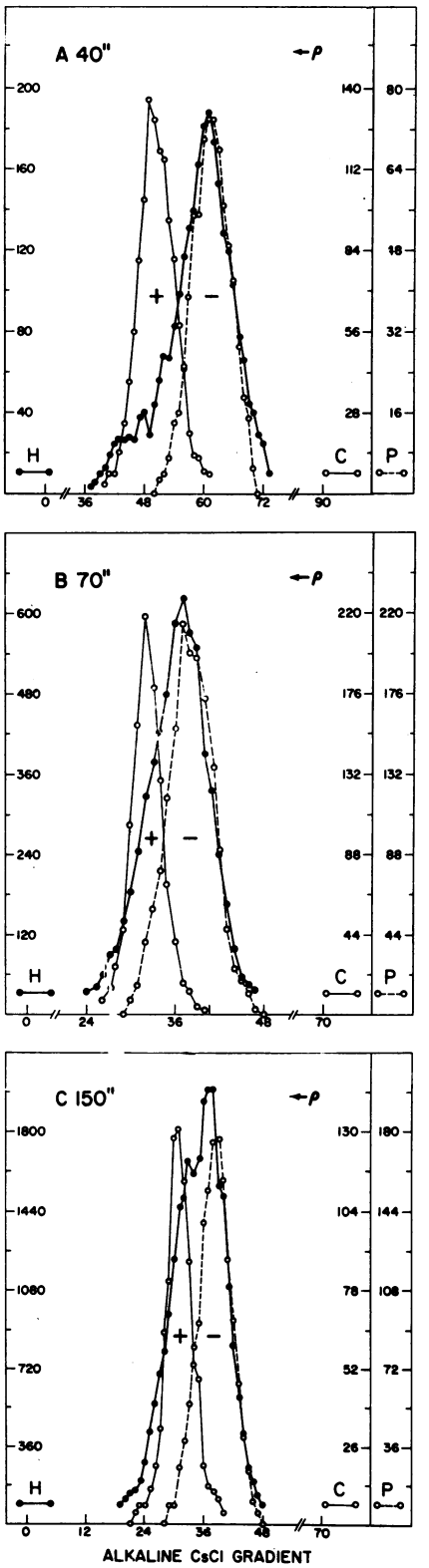

FIG. 4. Asymmetrically labeled supercoils. Supercoils pulse-labeled for 40, 70, 100, or 150 sec were recovered from the gradients shown in Fig. 3 (fractions 12–14), and analyzed to determine the distribution of radioactivity between the positive and negative strands.

After neutralization of the solutions (HCl, 0.05 M Tris to pH 8.6) the supercoils were heated to 100°C for 3 hr. Sedimentation of the supercoils in alkaline sucrose before and after heating showed that the initially pure supercoils had been nicked into fragments which were of approximately half the size of ϕX.

The strands from the supercoils were dialyzed and taken up to 2500 μl with 0.05 M Na_3PO_4 that contained: 0.001 M EDTA, 3 μg of denatured lambda DNA, and two markers: ^{14}C-ϕX positive strands (isolated from phage) and ^{32}P-negative strands (synthesized *in vitro* by Dr. David Denhardt according to the method of Goulian and Kornberg.[12]) 3.325 g of CsCl were added and the pH was adjusted to 12.5 with KOH.

Centrifugation was at 40,000 rpm for 60 hr at 17°C in the angle 60 rotor of the IEC B60 centrifuge. Each gradient was fractionated into 60-100 parts. Recoveries were near 100%.

The supercoils, after 40 sec of labeling, contained most of their radioactivity in negative strand material (Panel A). After 70, 100 (not shown), or 150 sec of labeling, the supercoils contained an increasing percentage of label in positive strand material (Panels *B* and *C*).

The radioactivity profiles of the alkaline CsCl gradients have been normalized to compensate for the fact that, in labeling with [^{3}H]thymidine, 56 radioactive thymines are incorporated into positive strand material for every 44 which are put into negative strand material.[13]

In some experiments, as a control, the ^{14}C-(positive) and ^{32}P-(negative) strand markers were added to the tritium-labeled supercoils before heating at 100°C. This procedure did not affect the subsequent banding positions of the markers.

The asymmetric labeling of supercoils after 40 sec was not due to the incorporation of more cpm into negative strand than positive strand material during the initial moment of pulse-labeling. This was checked when the total 40 sec pulse-label (supercoils plus single strands from Fig. 2) was examined in alkaline CsCl and found to consist of equivalent amounts of radioactivity in both positive and negative strand material.

follows negative strand label into supercoils with a delay of about 20 sec. This delay means that the newly-synthesized positive strand material has spent a longer time in some percursor form. In terms of the rolling circle intermediate, this result is interpreted to mean that positive strand label is first incorporated into the circular part of the intermediate and must be displaced into the tail before it may be matured into a supercoil. The 20 sec delay would then represent the time required for the growing point to move around the circle (5500 bases).

The rolling circle model ascribes the synthesis of asymmetrically labeled supercoils to a replicating intermediate with a growing positive strand which is longer than unit length and a growing negative strand which is shorter than unit length (Fig. 1*A*). Such intermediates are present in ϕX-infected cells making duplex rings (manuscript in preparation).

Discussion. Our model, and our experiments thus far, support the idea that both the early and late stages of ϕX DNA replication (the synthesis of double-stranded circles and the synthesis of single-stranded circles) are united under one replicating intermediate (Fig. 1A). Early in the phage life cycle, a duplex ring would be excised from the tail of the rolling circle; later the tail is prevented from becoming duplex (except for the limited region required for the site-specific recombination) and an essentially single-stranded circle is the product of excision. The experiments which showed the rolling circle structure of the late life cycle replicating intermediate[2,14] have been confirmed by Sinsheimer and his co-workers for ϕX,[15,16] and by Ray[17] and Wirtz and Hofschneider (personal communication) for the filamentous phage M13.

However, our view of the early ϕX life cycle is at this stage at some variance with the view developed by Knippers, Komano, Whalley, and Sinsheimer[15,18]. These researchers have presented experiments which led them to conclude that (1) during the period of double-stranded DNA replication, the parental duplex ring passes through a rolling circle state in which the intermediate is composed of a long *negative* strand associated with a circular *positive* strand template; and (2) during the period of double-stranded circle synthesis, the parental duplex ring replicates repeatedly while the progeny duplex rings remain inert, as supercoils. The data and interpretations of these authors, if taken together, are not compatible with our supercoil experiment. In fact, their conclusions, collectively, require that pulse-labeled supercoils should be radioactive asymmetrically in their *positive* strands. However, we believe that the effects they observed were in some cases rather small and were not sufficiently pronounced to establish all of their conclusions (for instance: in ref. 18, Fig. 3*A*, the crucial experiment shows only a marginal enrichment for long negative strands, and most of the counts do not band in ϕX positions).

To check our experimental result, we have performed the supercoil experiment several times, under varying conditions. For example: Mitomycin was not used to inhibit host DNA synthesis. The supercoils were labeled for 30, 50, 80, 120, and 180 sec, beginning 8 min (instead of 6) after the initiation of synchronized phage growth (multiplicity of infection = 1). The pulse-labeled supercoils were separated from labeled host and viral DNA by the use of two suc-

cessive CsCl-ethidium bromide gradients. They were then nicked with DNase I, and the separated strands were banded in alkaline CsCl along with marker ^{14}C positive strands and ^{32}P negative strands. At the end of 30 sec of labeling, the supercoils contained 92% of their radioactivity in negative strand material. And again, this asymmetry of labeling progressively disappeared and was gone after 3 min of labeling.

The experiments of this paper thus show that throughout the period of ϕX double-stranded circle synthesis the production of supercoils is associated with an asymmetry. We interpret this asymmetry as reflecting the production of duplex rings by a rolling circle intermediate which is composed of a long positive strand and a circular negative strand template (Fig. 1*A*).

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* This paper is the fourth in a series dealing with the mechanism of replication of ϕX DNA. The other papers are Dressler and Denhardt (*Nature*, **219**, 346, 1968), Gilbert and Dressler (*Cold Spring Harbor Symp. Quant. Biol.*, 1968), and Dressler and Gilbert (*Proc. Nat. Acad. Sci., USA*, in press). These papers contain detailed descriptions of materials and methods.

[1] Thomas, C., and L. MacHattie, *Annu. Rev. Biochem.*, **36**, 485 (1967).
[2] Gilbert, W., and D. Dressler, *Cold Spring Harbor Symp. Quant. Biol.* (1968).
[3] Yoshikawa, H., *Proc. Nat. Acad. Sci. USA*, **58**, 312 (1967).
[4] Cairns, J., *Cold Spring Harbor Symp. Quant. Biol.* (1963).
[5] Cairns, J., and C. Davern, *J. Mol. Biol.*, **17**, 418 (1966).
[6] Hershey, A., E. Burgi, and L. Ingraham, *Proc. Nat. Acad. Sci. USA*, **49**, 748 (1963).
[7] Strack, H., and A. Kaiser, *J. Mol. Biol.*, **12**, 36 (1965).
[8] Supercoil: a duplex ring composed of two covalently closed, intertwined single-stranded circles, Dulbecco, R., and M. Vogt, *Proc. Nat. Acad. Sci. USA*, **50**, 236 (1963); Vinograd, J., J. Lebowitz, R. Radloff, R. Watson, and P. Laipis, *Proc. Nat. Acad. Sci. USA*, **53**, 1104 (1965).
[9] Alberts, B., *Fed. Proc.*, **29**, 1154 (1970).
[10] Pouwels, P., C. Knijnenburg, J. von Rotterdam, J. Cohen, and H. Jansz, *J. Mol. Biol.*, **32**, 169 (1968).
[11] Vinograd, J., J. Morris, N. Davidson, and W. Dove, *Proc. Nat. Acad. Sci. USA*, **49**, 12 (1963).
[12] Goulian, M., and A. Kornberg, *Proc. Nat. Acad. Sci. USA*, **58**, 1723 (1967).
[13] Sinshemier, R., *J. Mol. Biol.*, **1**, 37 (1959).
[14] Dressler, D., thesis, Harvard University (1969).
[15] Sinsheimer, R., R. Knippers, and T. Komano, *Cold Spring Harbor Symp. Quant. Biol.* (1968).
[16] Knippers, R., A. Razin, R. Davis, and R. Sinsheimer, *J. Mol. Biol.*, **45**, 237 (1969).
[17] Ray, D., *J. Mol. Biol.*, **43**, 631 (1969).
[18] Knippers, R., J. Whalley, and R. Sinsheimer, *Proc. Nat. Acad. Sci. USA*, **64**, 275 (1969).