*ENZYMATIC SYNTHESIS OF DNA, XXIII. SYNTHESIS OF CIRCULAR REPLICATIVE FORM OF PHAGE φX174 DNA**

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Replication of a helical DNA template by *Escherichia coli* DNA polymerase yields a macromolecule with the chemical characteristics expected of a complementary copy of the template.[1] However, the product as seen in the electron microscope is a branched rather than a simple linear fiber,[2] renatures very readily after a denaturing treatment,[2] and shows no biologic activity unequivocally distinguishable from that in the associated template.[3] The physical anomalies, which can be ascribed to a failure to replicate both strands of a helical template simultaneously from one end, were absent when single-stranded circular DNA from phage M13 or φX174 was the template.[4] But a test of biologic activity of the product strand was still not practicable inasmuch as the product strand was linear and only circular molecules are known to be infective.[5]

With the discovery of enzymes that join properly aligned ends of a DNA molecule,[6–10] the way was open to see whether DNA polymerase in conjunction with a DNA-joining enzyme can synthesize circular φX174 DNA and whether such synthetic molecules are infective. This report describes the synthesis, isolation, and characterization of a fully covalent duplex circular product from φX174 DNA templates. A succeeding report[11] will describe (1) the isolation of the synthesized complementary circles from these duplexes, (2) the ability of these single-stranded circles to serve as templates for the production of completely synthetic duplex circles, and (3) the capacity of enzymatically synthesized single and duplex circular forms to produce phage particles when mixed with spheroplasts of *E. coli*.

Experimental Procedure.—Materials: Unlabeled deoxynucleoside triphosphates were purchased from Pabst and purified by chromatography on DEAE-Sephadex (A-25) using a triethylammonium bicarbonate gradient. α-P^{32}-dCTP and α-P^{32}-dATP were obtained from Volk, H^3-methylthymidine from New England Nuclear, DPN from Sigma, and CsCl (optical grade) from Harshaw. dBUTP was prepared by bromination and deamination of dCTP.[12] Ethidium bromide was a gift from Boots Pure Drug Co., Nottingham, England. *E. coli* DNA polymerase was purified by the method of Jovin *et al.*[13] and *E. coli* polynucleotide-joining enzyme by the method of Olivera and Lehman.[8] Pancreatic DNase was the Worthington 1× recrystallized product.

Boiled extract of *E. coli* was the supernatant fluid obtained by centrifuging a sonic extract of *E. coli* strain 1100 (deficient in endonuclease I, obtained from Prof. H. Hoffman-Berling) which had been heated at 100° for 5 min as described by Olivera and Lehman.[8]

H^3-φX174 DNA RF-form I (750 cpm/mμmole) was a gift from Prof. R. L. Sinsheimer; RF-form II was generously provided by Dr. P. Pouwels.

Methods: Sucrose gradient sedimentations were carried out in 4-ml linear 5–20% sucrose gradients, in buffers indicated in figure legends and using an IEC B-60 preparative ultracentrifuge with an SB-405 rotor. Fractions were collected and precipitated on filter paper disks with 10% trichloroacetic acid, washed with 0.01 *N* HCl and then with ethanol, dried, and counted in a toluene-base scintillator solution. Standards were processed in the same way.

H^3-φX174 DNA was prepared from the lysis-defective amber mutant *am* 3 using a procedure based on that of Sinsheimer.[14] The thymine-requiring, nonpermissive host, *E. coli* 15 THU^-, was grown in 2 l of TPG 3A medium containing supplements of L-histidine (20 μg/ml), uracil (10 μg/ml), and thymidine (4 μg/ml). At the time of infection (4×10^8 bacteria/ml, multiplicity

of 3), H^3-thymidine (4 mc, 0.14 μg) was added. After the lysis procedure,[14] the phage were purified first by CsCl equilibrium density gradient centrifugation and then by velocity sedimentation in a sucrose gradient. The DNA, extracted from the purified phage with phenol, had a specific activity of 13,000 cpm/mμmole based on absorbancy at 260 mμ in 0.01 *M* Tris-HCl, pH 8.0, and 1 mM EDTA and a molar absorbancy of 9,500.[15] Molarity of DNA refers to concentration of nucleotide residues. Electron microscopy was carried out with the protein film technique of Kleinschmidt *et al.*[16] as modified by Inman *et al.*[17] The hypophase was water or 0.1 *M* ammonium acetate, as indicated. Sterile solutions and vessels were used wherever possible, to minimize nuclease contamination. Operations involving BU-containing DNA were carried out in subdued light.

Results.—Replication of phage (+) *circles and isolation of duplex circular product:* When H^3-ϕX174 DNA was the template for replication by *E. coli* DNA polymerase in the presence of P^{32}-dCTP and three unlabeled deoxynucleoside triphosphates, *E. coli* polynucleotide-joining enzyme, DPN, and a boiled extract of *E. coli*, covalently closed duplex circles were formed. The reaction was carried out at 25°. The amount of nucleotide incorporation proceeded to approximately one replication of the input DNA. The products were fractionated by equilibrium sedimentation in CsCl in the presence of ethidium bromide.[18] More ethidium bromide is bound to incomplete (or nicked) circles and linear molecules than to covalent duplex circles; for this reason the latter have a higher buoyant density.[18] As shown in Figure 1, about half of the phage DNA (51%) was found in the heavier band (partially synthetic duplex circles),[19] in which there was an equimolar ratio of product to template across the peak; in other experiments 38 to 57 per cent of the phage DNA was in the heavy band. The excess of product over template in the lighter band may be due to priming by polynucleotides in the boiled extract or to synthesis beyond one replication[4] in circular molecules in which joining of the replica failed to take place.

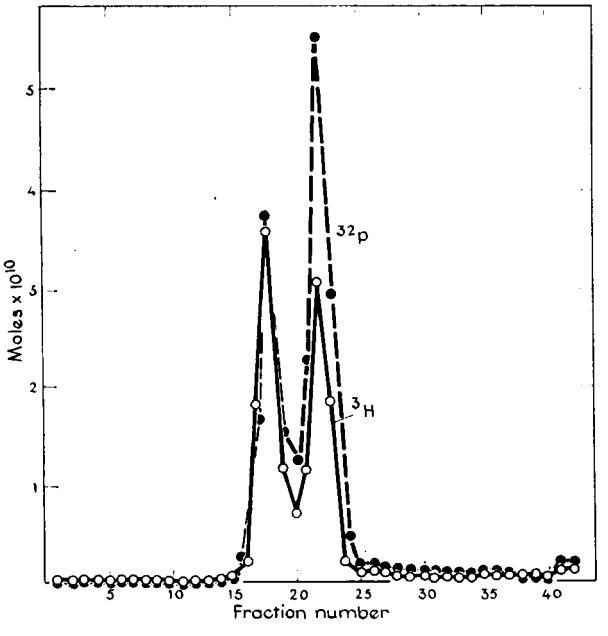

FIG. 1.—Fractionation of duplex circles by CsCl–ethidium bromide equilibrium sedimentation. The incubation mixture (0.50 ml) contained 0.18 mM H^3-ϕX174 *am* 3 DNA, 0.45 mM each of dCTP, dTTP, dGTP, and α-P^{32}-dATP (60,000 cpm/mμmole), *E. coli* DNA polymerase (1,600 units/ml), *E. coli* joining enzyme (2 units/ml), 8 μM DPN, *E. coli* boiled extract (40 μl/ml), 5 mM $MgCl_2$, 20 mM potassium phosphate buffer, pH 7.0, 1 mM β-mercaptoethanol, and albumin (40 μg/ml). After incubation for 180 min at 25°, the mixture was adjusted to 3 ml with 10 mM Tris-citrate buffer, pH 7.6, containing 10 mM EDTA, 300 μg of ethidium bromide, and 2.25 gm of CsCl. The resulting mixture (ρ = 1.56) was centrifuged in the IEC B-60 preparative ultracentrifuge using the SB 405 rotor at 45,000 rpm for 40 hr at 20°. An aliquot of 1 μl from each fraction was precipitated on 3 MM paper disks for counting. In this and succeeding figures, ordinate values represent total moles of nucleotide per fraction.

Replication was also carried out with dTTP replaced by the bromouracil analogue, dBŪTP. The CsCl–ethidium bromide equilibrium pattern of the products (Fig. 2) was similar to that in Figure 1. The BŪ-labeled duplex circles in the heavy band were separated and used as starting material for isolation of (−) circles as described in a later report.[11] These duplex circles were also used for electron microscopic examination (see below).

Velocity sedimentation of partially synthetic duplex circles: An alkaline sucrose gradient analysis of the molecules in the heavier band of the CsCl–ethidium bromide equilibrium sedimentation provided further evidence that these molecules were duplex covalent circles (Fig. 3). They sedimented in an alkaline sucrose gradient coincident with the natural form of ϕX174 duplex covalent twisted circles (RF-form I)[20] at a rate approximately three times that of phage DNA or of RF-form II (which is nicked and separates into single strands at alkaline pH).

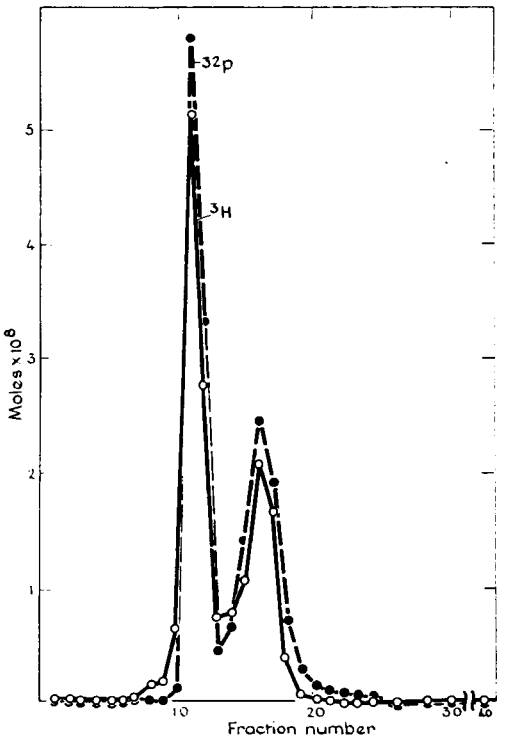

FIG. 2.—Fractionation of duplex circles, containing BŪ in the synthetic (−) strand, by CsCl–ethidium bromide equilibrium sedimentation. The conditions for incubation and fractionation by density gradient centrifugation were the same as for Fig. 1 except that the α-P^{32} label was in dCTP (50,000 cpm/mμmole) instead of dATP and dBŪTP replaced dTTP.

Sedimentation at neutral pH indicates the extent of supercoiling (twisting) in a duplex molecule. Supercoiled duplex molecules are more compact and sediment more rapidly than duplex circles which are relaxed.[21] If the partially synthetic duplex circles were supercoiled, their sedimentation would resemble that of RF-form I; with little or no twist they would sediment more like RF-form II which is nicked and relieved of its twist. At neutral pH and low ionic strength the partially synthetic duplex circles sedimented less rapidly than RF-form I and had the same sedimentation coefficient as the small amount of form II contaminating the preparation of RF-form I (Fig. 4). The partially synthetic duplex circles sedimented at a rate of 0.76 times that of the natural RF-form I and this may be compared to the value of 0.71 determined for natural form II.[22] At neutral pH and high ionic strength (1 *M* NaCl), the partially synthetic duplex circles also sedimented more slowly than natural RF-form I; the rate relative to form 1 was 0.85, whereas the value determined for form II was 0.79,[21] and again the position coincided with contaminating form II.

Electron microscopy of the partially synthetic duplex circles: The enzymatically synthesized molecules were examined in the electron microscope using water rather than salt solution as the hypophase. All molecular forms were photographed

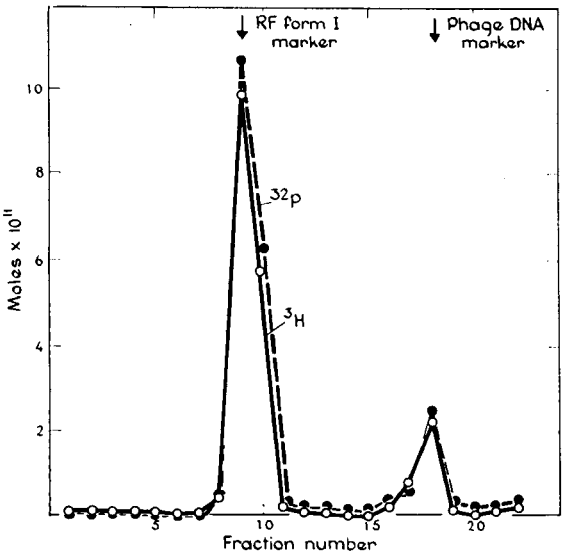

FIG. 3.—Alkaline sucrose gradient sedimentation of purified partially synthetic duplex circular molecules. Fractions 17 and 18 from the gradient shown in Fig. 1 were combined and dialyzed, first against 1 *M* NaCl, 10 mM Tris-HCl, pH 8, 1 mM EDTA, and then against the same buffer without NaCl. A 3 μl-aliquot of the dialyzed fraction was centrifuged in a sucrose gradient in 0.2 *M* NaOH, 0.8 *M* NaCl, 1 mM EDTA at 60,000 rpm for 75 min at 10°. Additional tubes in the same centrifugation contained samples of H^3-ϕX174 DNA and H^3-natural RF-form I, both alone and mixed with the dialyzed CsCl fraction, to serve as markers for their relative positions in the gradient.

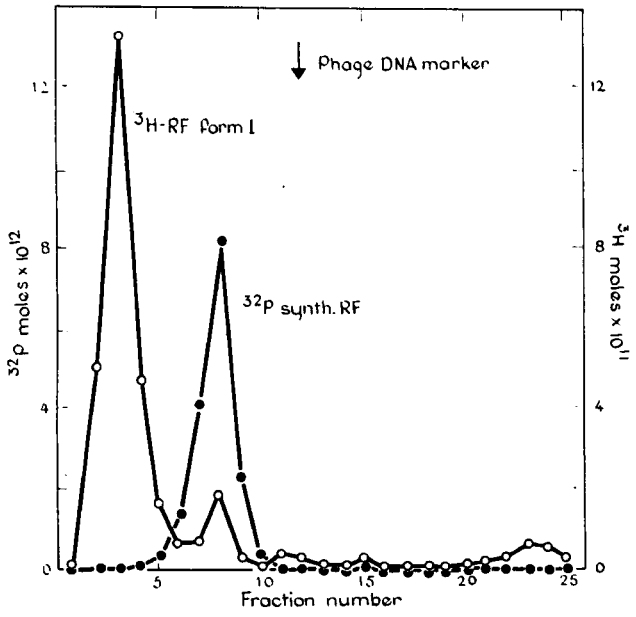

FIG. 4.—Neutral, low-salt, sucrose gradient sedimentation of purified partially synthetic duplex circular molecules. The dialyzed and purified preparation of duplex circles was the same as in Fig. 3. Sucrose gradient sedimentation was carried out in 9 mM NaCl, 1 mM Tris-HCl, pH 8, 0.1 mM EDTA, at 60,000 rpm for 210 min at 10°. The same reference markers were used as in Fig. 3, and were placed in the tube containing the sample and also run separately. For clarity only the P^{32} of the synthetic duplex circles and H^3 of the natural RF are shown; the H^3 label of the synthetic duplex circles coincided with the P^{32} as in Fig. 3. The data given here are from two separate gradients; the results were identical when synthetic and natural RF were run together in the same gradient.

serially without selection, and of these, 75 per cent were simple circles (Table 1, Fig. 5). Highly twisted forms like those of RF-form I[23, 24] were not present. The average contour length was 1.97 μ (Table 1) with a distribution skewed toward the shorter molecules. A preparation of natural RF-form II was examined for contour length and showed approximately the same fraction of simple circles with an average length of 2.07 μ. When the same natural RF preparation was spread on 0.1 *M* ammonium acetate as hypophase, the average length (1.85 μ) was in better agreement with published values of 1.64 $\pm$ 0.11[22] and 1.77 $\pm$ 0.08[23] in which the salt hypophase was used. This capacity of salt in the hypophase to shorten the contour

TABLE 1

DISTRIBUTION AND CONTOUR LENGTHS OF CIRCULAR FORMS IN RF PREPARATIONS

RF preparation	Hypophase	Total forms photographed	Linear forms	Circles*	Circles with branches	Contour Length—Circles measured	Contour Length—Average and S.D. (μ)
Partially synthetic[19]	Water	301	11	225	12	203	1.97 ± 0.12
Natural	Water	73	9	51	3	46†	2.07 ± 0.07
Natural	Salt‡	56	6	41	2	41	1.85 ± 0.14

* Includes only those circles whose circumference could be traced clearly; aggregated, superimposed, or partially twisted forms constituted the remainder of the structures not classified in this table.
† Does not include three large circles (see text).
‡ 0.1 *M* ammonium acetate.

length has been clearly demonstrated by Inman.[25] A branchlike structure which varied in length from 0.05 to 0.36 μ was found on a few of the molecules, synthetic as well as natural. Another anomaly appeared among the natural RF circles. Of 97 circles examined, 3 had lengths of 4.20, 4.25, and 4.42 μ, values just twice the average size; no such form was seen among 237 partially synthetic duplex circles.

Influence of various factors on replication and the synthesis of duplex circles: Optimal yields of the covalently closed duplex circles required all the components of the complete system (Table 2). There was an absolute requirement for polymerase, triphosphates, and phage DNA, whereas DPN, the cofactor for the joining enzyme,[26, 27] had the least influence due to its presence in the boiled extract. Joining enzyme was active at a level expected of its activity in completing λ circles.[28] The rather high conversion in the absence of added joining enzyme suggests that the purified polymerase may be contaminated with polynucleotide-joining enzyme; however, adequate assays have not yet been performed to test this possibility.[29] The function of the boiled extract will be discussed below.

Discussion.—Replication of the circular phage DNA by DNA polymerase alone had been judged to be complete chiefly on the basis of observations in the electron microscope of duplex circles with contour lengths of 2 μ.[4] However, even the appearance of a full helical circle would not have disclosed a discontinuity in the molecule of as much as 0.5 per cent, representing a gap of 30 nucleotides. Since the polynucleotide-joining enzyme requires that the 3′-hydroxyl and 5′-phosphate termini be closely aligned for joining, the success of the conjoint effort of this enzyme with polymerase in completing a fully covalent duplex circle (RF) makes it more likely that replication of the phage circle by polymerase does go to completion. Also remarkable was the efficiency with which the replicas were joined; the values for the amount of RF synthesized indicate that near 50 per cent of the phage DNA was converted.

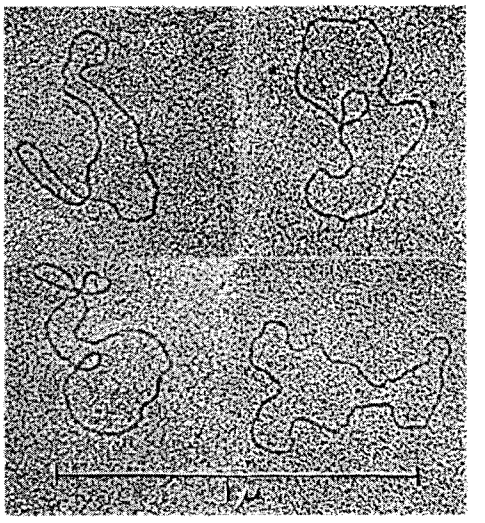

FIG. 5.—Electron micrographs of partially synthetic duplex circles. The DNA sample was obtained from the peak tubes of the heavy band in Fig. 2 and dialyzed (see legend to Fig. 3). For details of electron microscopy, see the text.

TABLE 2

	Conversion of phage DNA to RF (%)
Complete system	38
Minus polymerase	<0.2
" dTTP	<0.2
" phage DNA	<0.2
" DPN	26
" joining enzyme	4.5
" boiled extract	1.9

The complete system, in 14 μl, was the same as described for Fig. 1. After incubation, each mixture was made 60 mM in EDTA and centrifuged in a sucrose gradient in 0.2 *M* NaOH, 0.8 *M* NaCl, 1 mM EDTA at 60,000 rpm for 60 min at 10°. The gradient was then divided into 10 fractions and the H^3 in the rapidly sedimenting fractions (see Fig. 3) was pooled and is expressed as % of total H^3 in the gradient.

Although the experiments described in this report have been limited to the replication of ϕX174 by *E. coli* polymerase, studies with another phage DNA and with another DNA polymerase have yielded comparable results. Phage M13 DNA, which resembles ϕX174 DNA in structure and in size,[4, 30] was also converted by the *E. coli* polymerase–polynucleotide-joining enzyme system to a duplex circular molecule. The product, synthesized under conditions described for ϕX174 DNA (Fig. 1), had the same characteristics as partially synthetic ϕX174 RF in equilibrium sedimentation in the presence of ethidium bromide, and in velocity sedimentation at alkaline and neutral pH.

A duplex circular molecule was also produced by the combined action of *E. coli* and phage T4 DNA polymerases. A 3′-hydroxyl initiator site, required by the phage polymerase,[31] was provided by annealing small pieces of complementary (−) strands to M13 DNA circles. Such fragments were prepared by partial replication of M13 circles with *E. coli* polymerase and subsequent fractionation of the products on alkaline sucrose gradients. The M13 circle with fragments annealed to it replicated by the phage polymerase and the complementary circle was closed to form a duplex by joining-enzyme activity (ligase)[7, 10] that contaminated the purified phage T4 polymerase preparation.

The electron microscopic appearance of the partially synthetic RF is that of a circle of the same size as RF isolated from infected *E. coli* but with little or no supercoiling. Branchlike structures, which occurred in only 5 per cent of the circular molecules, were no more common than in the natural RF population and may represent a tightly twisted portion of the circle. Although large molecules, twice the average size, were seen in 3 of 97 natural RF molecules examined, no such giant forms were found in 237 synthetic molecules nor were there any midgets, either. The significance of the giant forms of RF, possibly a result of an intracellular recombinational event between two RF molecules, deserves further investigation.

Gellert[6] and Gefter *et al.*[9] have reported that λ DNA circles closed *in vitro* sediment more slowly in neutral sucrose gradients than natural duplex circles, and under some conditions resemble nicked duplex circles. The partially synthetic duplex circles described here have a similar relationship to natural RF-forms I and II or ϕX174 DNA in sedimentation at neutral pH. We assume that the structures synthesized in these studies, as in the case of enzymatically closed λ circles, have, under the conditions employed, little or no twist. The extended appearance of the synthesized circles in the electron microscope supports this assumption. The mechanism by which twist is introduced at the time of closure is not yet clear, but the effects of salt and temperature are probably very important. The influence of salt concentration on DNA structure in general,[32] and particularly as it applies to duplex circles,[6] is well documented. The recent work of Wang[33] makes it clear that the temperature at the time of closure of the duplex circle has a profound effect. This effect may account to a major extent for the lower amount of twist in the duplex

circles formed enzymatically at 25° in our studies as compared to those produced in *E. coli* at 37°.

An aspect of the RF synthesis which is not properly clarified is the stimulation of the over-all reaction by some component in a boiled extract of *E. coli*. Exploratory studies concerning the function of boiled extract in this system have shown that (1) the presence of joining enzyme reduced polymerase replication of ϕX174 DNA to a value of 12 per cent in its absence; addition of boiled extract restored it to a level of 120 per cent; (2) the extract stimulated replication of circular DNA 1.8-fold in the absence of joining enzyme; (3) the latter stimulation was even greater when the extract was first treated with pancreatic DNase; and (4) the DNase-treated extract was inhibitory in the replication-joining system at concentrations optimal for the replication reaction alone but retained activity when used at lower levels. The effect of boiled extract therefore appears to be on the replication rather than the joining part of the over-all synthesis. Some of the uncertainties regarding the start of replication of a circular template[4] suggest that the effect may be in the initiation phase of the reaction. Perhaps a very small piece of DNA in the extract adheres to the phage circle and provides a priming point for replication. The effect may also be related to the inhibitory action of the joining enzyme on replication, which is also unexplained. More details on how replication by *E. coli* polymerase starts and proceeds on a circular template might contribute to the explanation of the stimulation by boiled extract.

The capacity of DNA polymerase and joining enzyme to produce a circular complementary copy of phage DNA has now made it possible to isolate these copies, demonstrate their infectivity, and proceed with the total enzymatic synthesis of infective RF.[11] The implication for studies of mutagenesis becomes clear at once, inasmuch as a variety of base analogues or ribonucleotides can now be incorporated into an infectious molecule. In the same vein, defective polymerases from phages with mutations in the polymerase structural gene can be examined and exploited for errors in their replicative activity. Finally, it should be possible and worthwhile to carry out the replication of the circular DNA's from other viruses, such as polyoma, and from cellular organelles, such as mitochondria and chloroplasts. With these duplex circular DNA's as with RF of ϕX174,[11] controlled nuclease cleavage and denaturation should release (+) and (−) circles as templates for replication and eventually total synthesis of the duplex structures.

Summary.—Phage ϕX174 DNA [(+) circles] were converted by the combined action of *E. coli* DNA polymerase and polynucleotide-joining enzyme to a duplex circular form (RF). The partially synthetic RF has (1) the sedimentation and dye-binding properties expected of a fully covalent duplex circle, (2) a circular appearance in the electron microscope, and (3) the contour length of natural RF. Partially synthetic RF was also prepared with bromouracil in place of thymine in the (−) circle to facilitate isolation of the (−) circle, to enable the synthesis of a fully synthetic RF, and to demonstrate infectivity of synthetic molecules in studies to be reported.

We gratefully acknowledge the contribution of Miss Maria Schnös in preparing and examining the specimens for electron microscopy. We also appreciate the helpful advice of Prof. R. L. Sinsheimer and Mr. John Newbold in the preparation of H^3-ϕX174 *am* 3 DNA.

Abbreviations: RF, replicative form; DEAE, *O*-(diethylaminoethyl)cellulose; dATP, dBUTP, dCTP, and dTTP, deoxynucleoside 5′-triphosphates of adenine, bromouracil, cytosine, and thymine; DPN, diphosphopyridine nucleotide; EDTA, ethylenediaminetetraacetate.

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[1] Kornberg, A., *Enzymatic Synthesis of DNA*, Ciba Lectures in Microbial Biochemistry (New York: John Wiley & Sons, Inc., 1961).

[2] Schildkraut, C. L., C. C. Richardson, and A. Kornberg, *J. Mol. Biol.*, **9**, 24 (1964).

[3] Richardson, C. C., C. L. Schildkraut, H. V. Aposhian, A. Kornberg, W. Bodmer, and J. Lederberg, in *Informational Macromolecules* (New York: Academic Press, 1963), p. 13.

[4] Mitra, S., P. Reichard, R. B. Inman, and A. Kornberg, *J. Mol. Biol.*, **24**, 429 (1967).

[5] Fiers, W., and R. L. Sinsheimer, *J. Mol. Biol.*, **5**, 424 (1962).

[6] Gellert, M., these PROCEEDINGS, **57**, 148 (1967).

[7] Weiss, B., and C. C. Richardson, these PROCEEDINGS, **57**, 1021 (1967).

[8] Olivera, B. M., and I. R. Lehman, these PROCEEDINGS, **57**, 1426 (1967).

[9] Gefter, M. L., A. Becker, and J. Hurwitz, these PROCEEDINGS, **58**, 240 (1967).

[10] Cozzarelli, N. R., N. M. Melechen, T. M. Jovin, and A. Kornberg, *Biochem. Biophys. Res. Commun.*, **28**, 578 (1967).

[11] Goulian, M., R. L. Sinsheimer, L. L. Bertsch, and A. Kornberg, to be submitted.

[12] Bessman, M. J., I. R. Lehman, J. Adler, S. B. Zimmerman, E. S. Simms, and A. Kornberg, these PROCEEDINGS, **44**, 633 (1958).

[13] Jovin, T. M., P. T. Englund, and L. L. Bertsch, in preparation.

[14] Sinsheimer, R. L., in *Procedures in Nucleic Acid Research*, ed. G. L. Cantoni and D. R. Davies (New York: Harper and Row, 1966), p. 569.

[15] Sinsheimer, R. L., *J. Mol. Biol.*, **1**, 37 (1959).

[16] Kleinschmidt, A. K., D. Lang, D. Jacherts, and R. K. Zahn, *Biochim. Biophys. Acta*, **61**, 857 (1962).

[17] Inman, R. B., A. Kornberg, and C. L. Schildkraut, *J. Mol. Biol.*, **11**, 285 (1965).

[18] Radloff, R., W. Bauer, and J. Vinograd, these PROCEEDINGS, **57**, 1514 (1967).

[19] The term "partially synthetic duplex circle," as used here, refers to a structure in which the (+) circle is phage DNA and the (−) circle is enzymatically synthesized.

[20] Burton, A., and R. L. Sinsheimer, *J. Mol. Biol.*, **14**, 327 (1965).

[21] Vinograd, J., and J. Lebowitz, *J. Gen. Physiol.*, **49**, No. 6, Pt. 2, 103 (1966).

[22] Kiger, J. A., Jr., E. T. Young, II, and R. L. Sinsheimer, personal communication.

[23] Kleinschmidt, A. K., A. Burton, and R. L. Sinsheimer, *Science*, **142**, 961 (1963).

[24] Roth, T. F., and M. Hayashi, *Science*, **154**, 658 (1966).

[25] Inman, R. B., *J. Mol. Biol.*, **25**, 209 (1967).

[26] Olivera, B. M., and I. R. Lehman, these PROCEEDINGS, **57**, 1700 (1967).

[27] Zimmerman, S. B., J. W. Little, C. K. Oshinsky, and M. Gellert, these PROCEEDINGS, **57**, 1841 (1967).

[28] Assuming one covalent bond closure by the joining enzyme for each complementary circle, a value of 0.04 $\mu\mu$mole min^{-1} $unit^{-1}$ was obtained compared to 0.06 for closure of hydrogen-bonded λ circles[8] and the value of 1 under optimal assay conditions.[8]

[29] The boiled extract which by direct assay[8] contained, per ml, less than 0.01 unit of joining enzyme activity, is ruled out as a source.

[30] Ray, D. S., H.-P. Bscheider, and P. H. Hofschneider, *J. Mol. Biol.*, **21**, 473 (1966); Ray, D. S., A. Preuss, and P. H. Hofschneider, *J. Mol. Biol.*, **21**, 485 (1966).

[31] Goulian, M., Z. J. Lucas, and A. Kornberg, submitted for publication.

[32] Studier, F. W., *J. Mol. Biol.*, **11**, 373 (1965).

[33] Wang, J. C., personal communication.