ENZYMATIC SYNTHESIS OF DNA, XXIV.
SYNTHESIS OF INFECTIOUS PHAGE φX174 DNA*

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Past attempts at *in vitro* replication of transforming factor present in DNA have given negative or inconclusive results.[1-3] Rigid proof was lacking that template material had been excluded from the synthetic product. Even if a rigorous demonstration of net synthesis of transforming factor for a given genetic marker were forthcoming, it would still prove only that some relatively short sequence of nucleotides, sufficient for replacement of the mutant locus, had been synthesized. If enzymatic synthesis of infectious bacteriophage DNA were achieved, it would be made clear at once that relatively few, if any, mistakes had been made in replicating a DNA sequence of several thousand nucleotides.

Escherichia coli DNA polymerase can replicate single-stranded circular DNA from phage M13 or φX174[4] and in conjunction with a polynucleotide-joining enzyme produces a fully covalent duplex circle.[5] Analyses of this product by equilibrium and velocity sedimentation and by electron microscopy have shown it to be indistinguishable, except for supercoiling, from replicative forms (RF)[6] of the viral DNA.[5] By substitution of bromouracil for thymine in the complementary strand ((−) circle), it should be possible on the basis of density difference to isolate this strand from the duplex circle and determine whether it has the infectivity known to reside in (−) circles.[7,8]

This report will describe: (1) the isolation of infective, synthetic (−) circles from the partially synthetic replicative form, (2) the ability of the isolated (−) circles to serve as templates for the production of infective, completely synthetic duplex circles, and (3) the isolation of infective, synthetic (+) circles from the latter.

Thus, DNA polymerase carries out the relatively error-free synthesis of the φX174 genome from the four deoxyribonucleoside triphosphates on direction from phage DNA templates.

Results.[9]—Isolation of synthetic (−) circle and test of infectivity: A duplex circle was synthesized by replicating H^3-φX174 DNA with DNA polymerase in the presence of a polynucleotide-joining enzyme. Details for the production and isolation of this partially synthetic RF, containing $\overline{BU}$ and P^{32} in the (−) circle, were described in an earlier report.[5] Separation of the synthetic (−) circle from the duplex form followed the plan outlined in Figure 1. The duplex circles were exposed to pancreatic DNase to an extent sufficient to produce a single scission in one of the strands in about half of the molecules. The resulting mixture of intact and nicked molecules was denatured by heating. The mixture, which now contained circular and linear H^3-T (+) strands, and P^{32}-$\overline{BU}$ (−) strands, in addition to intact RF, was fractionated by equilibrium density-gradient sedimentation in CsCl (Fig. 2).[10] Three peaks of radioactivity were evident, corresponding, in order of decreasing density, to single-stranded DNA containing $\overline{BU}$, a duplex hybrid
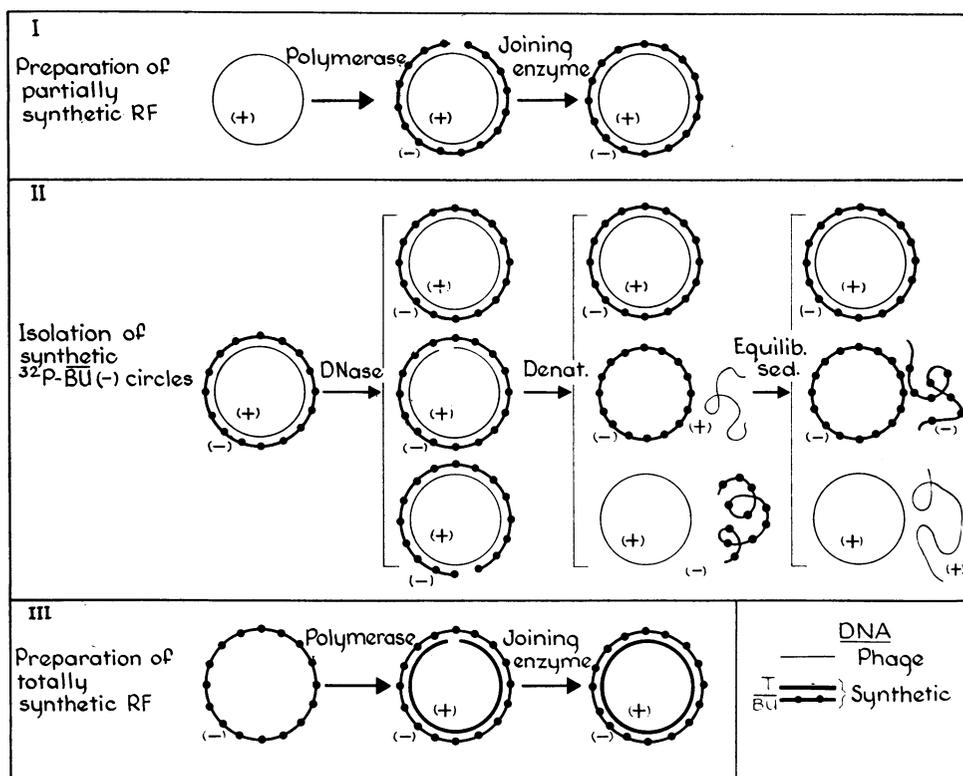
FIG. 1.—Schematic representation of the preparation of synthetic (−) circles and RF.   For details see text and Figs. 2 and 6.

of $\overline{\text{BU}}$ and T, and single strands containing T, with mean densities of 1.809, 1.747, and 1.722 gm/ml, respectively.   These values may be compared to the values of 1.732 and 1.725 previously determined[13] for the native hybrid and T (+) single strands prepared *in vivo* or to the calculated values[14] of 1.815 and 1.753 for the $\overline{\text{BU}}$ (−) strands, and the hybrid, respectively.   In addition to the three peaks, there was an area on the heavy side of the hybrid zone, which in other experimental trials appeared as a more distinct shoulder and is attributable to some duplex circles that had failed to renature after the heat treatment (Fig. 2).

Inasmuch as the (−) circle is infectious in the spheroplast assay,[7, 8] it was possible to test the enzymatically synthesized material directly for biologic activity.   Four peaks of infectivity were found (Fig. 2).   One corresponded to the position of heavy, P^{32}-$\overline{\text{BU}}$, synthetic (−) single strands and another to that of light, H^3-T (+) single strands.   Specific infectivity values for the single-stranded regions could not be determined from these data because there was an unknown quantity of linear strands.   The P^{32}-$\overline{\text{BU}}$ and H^3-T peaks were therefore each subjected to velocity sedimentation in a neutral, low-salt sucrose gradient to give a partial separation of the circles from linear forms.   As seen in Figure 3 for the P^{32}-$\overline{\text{BU}}$ (−) strands, and in Figure 4 for the H^3-T (+) strands, the infective material was found, in each case, in the leading shoulder of the peak which contains the circles

FIG. 2.—Equilibrium density-gradient sedimentation analysis of partially synthetic RF after limited DNase action and denaturation. Partially synthetic RF, with P[32] and $\overline{BU}$ in the synthetic (−) strand,[5] was incubated for 20 min at 20° at a concentration of 0.1 mM, in 0.2 ml of 10% glycerol–10 mM Tris HCl (pH 7.6)–2 mM MgCl$_2$–0.25 mμg/ml pancreatic DNase. (The DNase (Worthington 1× recrystallized), 5 mg/ml in 0.01 N HCl, was stored at 0°[11] and diluted immediately before use in 10 mM Tris acetate (pH 5.5)–5 mM MgCl$_2$–0.2 M KCl–50% glycerol.) The reaction was stopped by addition of EDTA to 8 mM. The mixture was heated at 90° for 2 min and adjusted to a volume of 9.8 ml with 0.01 M Tris HCl (pH 7.6); EDTA was added to 1 mM, as well as 1 mg of bovine plasma albumin and 9.961 gm of CsCl. Centrifugation of this mixture (ρ = 1.750) was carried out in the Spinco no. 50 angle rotor at 45,000 rpm at 25° for 50 hr. Aliquots from each fraction were assayed for radioactivity on filter paper disks,[5] and for infectivity by the spheroplast assay of Guthrie and Sinsheimer.[12] Inhibition by CsCl in the spheroplast assay was avoided by dilution.
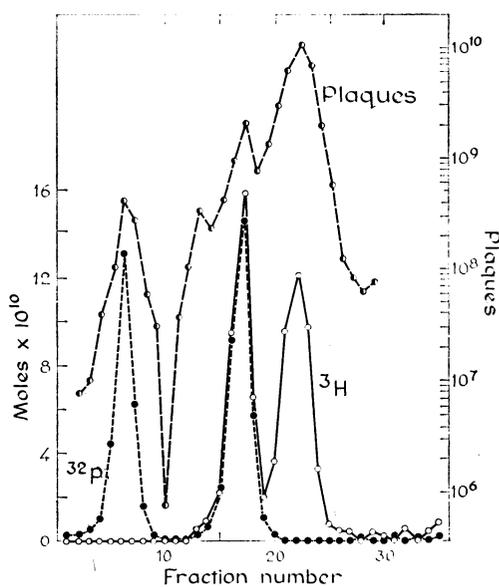
because of their more rapid sedimentation. Because of their content of $\overline{BU}$, the (−) circles had a distinctly higher sedimentation rate than their T (+) complements (compare sedimentation values relative to the DNA marker in Figs. 3 and 4). The specific infectivities estimated for the synthetic (−) circles and template (+) circles were 0.074 and 0.80, respectively (Table 1).

The other two peaks of infectivity in Figure 2 corresponded to the position of denatured and native forms of duplex hybrid molecules. Their respective specific infectivities were 0.066 and 0.012 (Table 1).

Proof that the infectivity of the P[32]-$\overline{BU}$ peak resides in the enzymatically synthesized DNA: (1) A peak of infectivity coincides with the P[32]-$\overline{BU}$ peak[17] in the density gradient (Fig. 2) and is separated from neighboring peaks. (2) Phage (+) circles are absent from the single-stranded, P[32]-$\overline{BU}$ peak as judged by the absence of detectable H[3]-labeled material. In view of the sensitivity of the radioactivity measurements, the *upper* limit for the amount of template material in the synthetic peak is 8 $\mu\mu$moles/ml; this concentration is one-tenth of that necessary to account for the infectivity of the peak. (3) In velocity sedimentation in sucrose gradients, the peak of infectivity corresponds to the position of intact P[32]-$\overline{BU}$ (−) circles, and sediments more rapidly because of the presence of $\overline{BU}$ than the analogous peak of intact H[3]-T (+) circles. (4) The photoinactivation of P[32]-$\overline{BU}$ (−) DNA as compared with H[3]-T (+) DNA (Fig. 5) demonstrates the more rapid inactivation of most of the infectious particles in the CsCl gradient peak corresponding to P[32]-$\overline{BU}$ (−) strands and is consistent with the known greater photosensitivity of $\overline{BU}$-containing DNA.[18] The presence of approximately 5 per cent of the infectious material displaying an inactivation rate similar to that of T DNA[19] (Fig. 5) indicates the extent of contamination by T (+) circles. Inasmuch as the (+) circles of Figure 2 have about ten times the specific infectivity of these (−) circles, the residual content of phage DNA in the $\overline{BU}$ fraction is estimated to be closer to 0.5 per cent than 5 per cent.
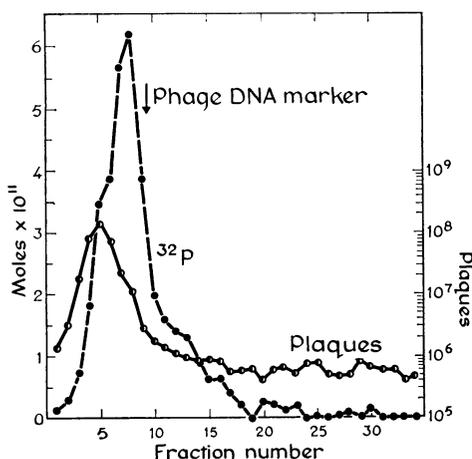
FIG. 3.—Velocity sedimentation of P^{32}-$\overline{BU}$, (−) synthetic DNA derived from partially synthetic RF. The P^{32}-$\overline{BU}$ peak fractions were pooled, dialyzed against 2 mM Tris HCl (pH 7.6)–0.2 mM EDTA and then concentrated five fold to a volume of 0.1 ml by rotary evaporation under reduced pressure. An aliquot of 20 μl was centrifuged in a 5–20% sucrose gradient in 5 mM NaCl–5 mM Tris HCl (pH 7.6)–1 mM EDTA, at 60,000 rpm and 10° for 360 min. H^3 was not detectable (<0.3 μμmole/fraction). The position of φX174 DNA was obtained from a separate tube containing this DNA as marker.

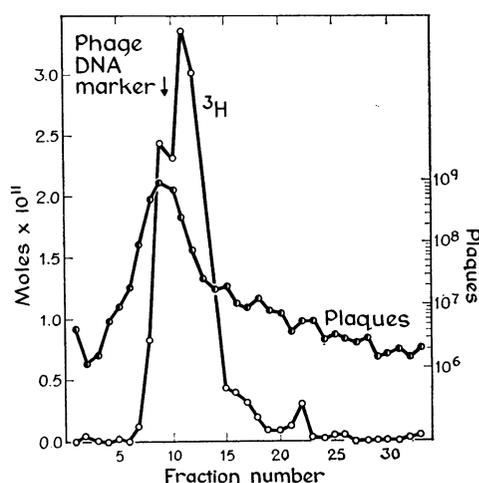FIG. 4.—Velocity sedimentation of H^3-T, (+) phage DNA derived from partially synthetic RF. The H^3-T peak (Fig. 2) was treated as described in Fig. 3 for P^{32}-$\overline{BU}$ except that half as much H^3-T was placed on the sucrose gradient.

Replication of synthetic (−) *circle, isolation of fully synthetic replicative forms, and a test of infectivity:* The synthetic, P^{32}-$\overline{BU}$ (−) circles, separated from phage (+) circles, could now be used as templates for the production of fully synthetic RF (Fig. 1) which proved to be infective. Incubation conditions for synthesis of the RF were as previously employed,[5] except that H^3-dCTP was the labeled sub-

TABLE 1

INFECTIVITIES OF NATURAL AND SYNTHETIC φX174 DNA

	Plaques (ml^{-1} × 10^{-8})	DNA (μμmole ml^{-1})	Specific infectivity (plaques/ particle)	Relative infectivity	Ref.
(+) Circle, natural	37	64	0.80*	1.0	Fig. 4
(−) Circle, natural				~0.2	8
"　　" , synthetic	6.2	150	0.074*	0.09	Fig. 3
RF (native), natural				0.05	15
"　　" , part. synthetic	6,000	200,000	0.058	0.07	†
"　　" , part. synthetic	61	9,100	0.012	0.01	Fig. 2
RF (denat.), natural				1.0	16
"　　" , part. synthetic	9.5	260	0.066	0.06	Fig. 2
"　　" , fully synthetic	24	120	0.36	0.3	Fig. 6

Specific infectivity was calculated on the basis of 1.1 × 10^8 particles/μμmole of nucleotide residues for single-stranded molecules and half that value for the duplexes. *Relative infectivity* of the natural (+) circle was arbitrarily taken as 1.0 and the other figures adjusted, with inclusion of a correction, for variations between different assays, from phage DNA standards that were run in each assay. Technical difficulties resulting from the low concentrations of DNA have thus far prevented reliable estimates of the specific infectivities of native, fully synthetic RF and synthetic (+) circles.

* Includes a correction for estimated contamination with linear forms.
† Sample assayed prior to exposure to DNase as in Fig. 2.

strate, dTTP replaced dBUTP, and
the pooled P^{32}-$\overline{BU}$ ($-$) peak from the
CsCl gradient (Fig. 2) was the tem-
plate. Evidence that a duplex circle
was synthesized was obtained by ve-
locity sedimentation analysis in an
alkaline sucrose gradient (Fig. 6). The
hybrid peak contained H^3 and P^{32} in
approximately equimolar amounts and
had the S value expected of a covalent
duplex circle in alkali. The infectivity
coincided exactly with the radioac-
tivity, and the specific infectivity
values were within the range expected
for the denatured form of natural RF
(Table 1). Additional evidence for
the covalent duplex structure was ob-
tained by density-gradient centrifuga-
tion in the presence of ethidium bro-
mide (Fig. 7). This analysis was pre-
ceded by an initial density-gradient
centrifugation with ethidium bromide

FIG. 5.—Photoinactivation of synthetic P^{32}-$\overline{BU}$ DNA and H^3-T, phage DNA. The P^{32}-$\overline{BU}$, ($-$) and H^3-T, ($+$) peaks (Fig. 2), dialyzed and concentrated as described in Fig. 3, were each diluted into 10 mM Tris HCl (pH 7.6)–1 mM EDTA. The diluted DNA's were exposed in identical fashion to a 15-watt daylight fluorescent tube at a distance of 3 cm; 0.05-ml aliquots were placed in the dark at the indicated times and subsequently assayed for infectivity. The ratio of the initial slope for $\overline{BU}$-DNA to that for T-DNA is 12.6.

in which a peak of higher buoyant density was identified as corresponding to
the duplex covalent zone by alkaline sucrose gradient analysis of each fraction
(legend to Fig. 7).

Isolation of a synthetic ($+$) *circle from the fully synthetic replicative form:* A
procedure similar to the one employed to separate synthetic ($-$) circles from
partially synthetic RF forms was used (Fig. 1). A limited digestion by pancreatic
DNase, followed by alkaline denaturation, achieved the release of H^3-T ($+$) circles
from the fully synthetic RF containing H^3-T ($+$) and P^{32}-$\overline{BU}$ ($-$) circles. The
mixture was fractionated directly in an alkaline sucrose gradient (Fig. 8). The
synthetic H^3-T ($+$) circles, complementary to the synthetic P^{32}-$\overline{BU}$ ($-$) circles
and now corresponding in structure to the original ($+$) phage DNA template,
were evident as a H^3-labeled shoulder, with corresponding infectivity, partially
separated from the slower sedimenting linear forms. Trailing from this infective
($+$) strand peak obscured the position of the more rapidly sedimenting and less
infective $\overline{BU}$ ($-$) template circles (Fig. 8; also note legend for method of collecting
the fractions); all hybrid molecules which had remained were pelleted under the
conditions used.

Discussion.—Physical studies on the partially synthetic RF prepared by enzy-
matic replication of phage DNA showed its structure to be like that of the RF form I
isolated from infected cells.[5] The only distinction was the relative absence of
supercoiling in the partially synthetic molecule and this can be attributed, at least
in part, to the difference between the *in vitro* and *in vivo* conditions of salt and
temperature[20] at the time of strand closure to form the circular duplex. The
test of infectivity is a more rigorous and meaningful measure of the accuracy of
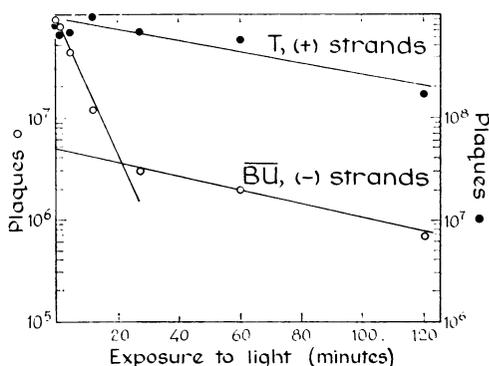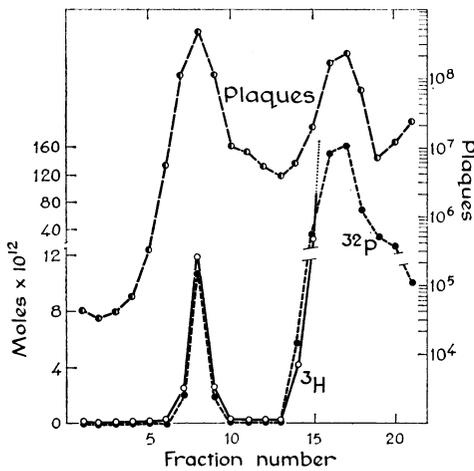replication and ring closure of phage DNA by polymerase and joining enzyme.

FIG. 6.—Alkaline sucrose-gradient sedimentation of fully synthetic RF. P^{32}-$\overline{BU}$, ($-$) strands (40 μl of peak sample from Fig. 2, dialyzed and concentrated as described in Fig. 3) were replicated in a volume of 0.1 ml as described previously;[5] the labeled nucleotide was H^3-dCTP (Schwarz BioResearch, 1000 cpm/$\mu\mu$mole), and dTTP rather than d$\overline{BU}$TP was used. After 180 min, the mixture was made 20 mM in EDTA, 0.1 M in NaOH, and centrifuged in a sucrose gradient in 0.2 M NaOH–0.8 M NaCl–1 mM EDTA, at 60,000 rpm and 1° for 100 min. The fractions were neutralized with 1 M Tris citrate (pH 5) before being assayed for radioactivity and infectivity.
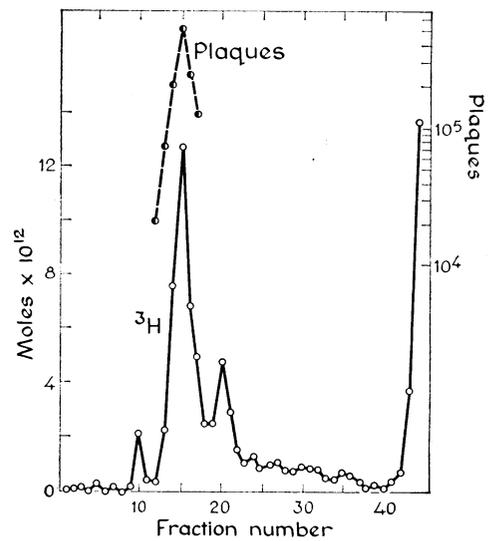
FIG. 7.—Density-gradient sedimentation of synthetic RF in the presence of ethidium bromide. The synthetic RF, prepared as described in Fig. 6, was purified by a preliminary density-gradient centrifugation in CsCl-ethidium bromide, as described previously.[5] The covalent duplex zone, identified by alkaline sucrose-gradient sedimentation of aliquots from the fractions, was collected and refractionated in the same type of CsCl-ethidium bromide gradient with results shown above. Fractions were diluted 200-fold for the spheroplast assay but were not otherwise treated to remove CsCl or ethidium bromide. P^{32} in the $\overline{BU}$, ($-$) template was not measurable due to radioactive decay and low recoveries.

Numerous ϕX174 mutants are known[21] in which the change of a single nucleotide results in loss of infectivity under the assay conditions employed. The fact that isolated synthetic circles and fully synthetic RF forms made with these circles as templates had specific infectivity values in the range measured for natural forms of viral DNA (Table 1) attests to the precision of the enzymatic operation.

It should now be possible to apply the techniques used in this work to the synthesis of the duplex circular genomes of other viruses, such as phage λ and animal viruses, and DNA molecules of comparable structure from cellular organelles. Such synthetic efforts will permit the insertion of base and nucleoside analogues in a manner and variety not attainable with *in vivo* systems. In addition, base changes generated by replication of the DNA with defective polymerases can now easily be studied in combination with standard genetic tools. It is of interest that DNA of approximately normal specific infectivity has been synthesized here without the use of any methylated nucleotide. This result may be related to the lack of host modification or restriction in the *E. coli* C-K12 pair and might not be applicable to other viral DNA's.

Since the conversion of phage DNA to RF-form I is accomplished *in vivo* by
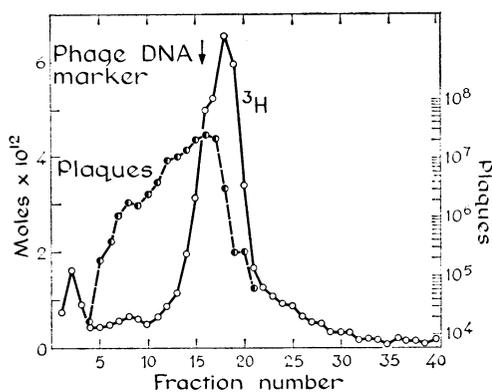
FIG. 8.—Identification of synthetic, (+) circles by alkaline sucrose-gradient sedimentation of synthetic RF exposed to limited DNase action. The rapidly sedimenting fractions of synthetic RF in the alkaline sucrose gradient (Fig. 6) were pooled, dialyzed against 10 mM Tris HCl (pH 7.6)–1 mM EDTA and incubated for 20 min at 20° (final volume of 2 ml) at a concentration of 50 $\mu\mu$moles/ml in 10 mM Tris HCl (pH 7.6)–5 mM $MgCl_2$–0.1 mg/ml bovine plasma albumin–1.2 mμg/ml pancreatic DNase. The mixture was then made 15 mM in EDTA, reduced in volume to 0.15 ml by rotary evaporation under reduced pressure, brought to pH 12 with NaOH, and centrifuged in a sucrose gradient in 0.2 M NaOH–0.8 M NaCl–1 mM EDTA, at 60,000 rpm and 10° for 240 min. The bottom of the tube was punctured with a hollow needle and the contents were displaced by saturated CsCl solution (containing Blue Dextran from Pharmacia) using a peristaltic pump. The sucrose-gradient fractions were collected from the top of the tube via a fine polyethylene tube in a stopper at the top. The fractions were neutralized (as in Fig. 6) prior to assays. The fractions were numbered in the reverse order of their collection, in order that the direction of sedimentation conform to the illustration of velocity sedimentations in the other figures. P^{32} in the $\overline{BU}$, template strand was not measurable due to radioactive decay and the small amounts of DNA employed.

host enzymes, and since the DNA polymerase and polynucleotide-joining enzyme are so effective in converting phage DNA to RF-form I *in vitro*, it appears likely that these enzymes are used by infected *E. coli* cells to carry out this conversion *in vivo*. Although the predominant pathway of phage replication appears to involve the open RF-form II,[22] the two forms are in fact interconvertible *in vivo*. Questions of the roles of these enzymes and the replicative forms in the production of (+) circles for progeny phage require further study.

The fact that *E. coli* DNA polymerase can synthesize biologically active DNA does not establish its function in the replication of the bacterial chromosome. However, the effectiveness of the combined action of the polymerase and the polynucleotide-joining enzyme in forming infective DNA may have considerable significance for chromosomal replication. In an earlier paper,[4] a mechanism was suggested whereby polymerase, with a then hypothetical polynucleotide-joining enzyme, might function in the simultaneous replication of both strands of helical DNA. The subsequent discovery of this joining enzyme, the requirement for it in phage T4 DNA synthesis,[23] its persistence in the most purified *E. coli* and phage T4 DNA polymerase preparations,[5] as well as the current demonstration of its conjoint action with polymerase, all strengthen the suggestion of this replication mechanism.[4]

Summary.—A partially synthetic, closed replicative form (RF) of ϕX174 DNA, consisting of phage DNA as the (+) circle and a bromouracil-containing complement synthesized by DNA polymerase as the (−) circle, was used as the source of synthetic (−) circles. The latter were separated from template strands by limited DNase action on the RF followed by denaturation and density-gradient equilibrium sedimentation. The isolated (−) circles were infectious and had the buoyant density, sedimentation velocity, and radiation sensitivity expected for DNA containing bromouracil. These (−) circles served as templates for a second round of replication which produced a fully synthetic RF with the specific infectivity

of natural RF. Infective synthetic (+) circles, corresponding to the original phage DNA, were isolated from the synthetic RF after DNase treatment, as in the previous isolation of synthetic (−) circles. These results imply a relatively error-free synthesis of the φX174 genome by DNA polymerase.

Note added in proof: A study by Okazaki, R., T. Okazaki, K. Sakabe, and K. Sugimoto (*Jap. J. Med. Sci. Biol.*, **20**, 255 (1967)) of DNA replication in *E. coli* supports a mechanism of discontinuous 5′ → 3′ chain growth on the 5′ template strand (see *Discussion*).

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[6] Abbreviations used are: RF for replicative form; T for thymine; $\overline{BU}$ for bromouracil; dCTP, d$\overline{BU}$TP, and dTTP for the deoxyribonucleoside triphosphates of cytosine, $\overline{BU}$, and T, respectively; (+) circle for phage DNA; (−) circle for complementary copy of (+) circle.

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[8] Siegel, J. E. D., and M. Hayashi, *J. Mol. Biol.*, **27**, 443 (1967).

[9] Experimental procedures were as described previously[5] or as detailed in the figure legends.

[10] In this figure, and in all succeeding ones, the ordinate values represent the total moles of nucleotide or plaques per fraction. The fractions, except where indicated otherwise, are numbered in the order of their collection from the bottom of the tube.

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[12] Guthrie, G. D., and R. L. Sinsheimer, *Biochim. Biophys. Acta*, **72**, 290 (1963).

[13] Denhardt, D. T., and R. L. Sinsheimer, *J. Mol. Biol.*, **12**, 647 (1965).

[14] The ρ values for $\overline{BU}$-containing DNA were calculated from the base composition (Sinsheimer, R. L., *J. Mol. Biol.*, **1**, 43 (1959)), and the figure of 0.2 gm/ml determined by Baldwin and Shooter (*J. Mol. Biol.*, **7**, 511 (1963)) for the difference in ρ between dAT and dA$\overline{BU}$.

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

[17] The possibility of a facilitative effect of the synthetic DNA upon the infectivity of a small contaminant of natural DNA was tested by mixing synthetic DNA molecules (P³²-$\overline{BU}$; Fig. 2; amber mutant) and natural DNA (γh, temperature-sensitive mutant).[13] The plaque count for the amber mutant was 398 for the $\overline{BU}$ DNA alone and 459 for the mixture, whereas the corresponding figures at similar dilutions for the temperature-sensitive mutant, alone and mixed, were 88 and 126, thus indicating the lack of interaction.

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[19] This relatively large amount is unexplained and surprising in view of the low level to which infectivity dips between the P³²-$\overline{BU}$ peak and the denatured hybrid duplex region of the CsCl gradient (Fig. 2).

[20] Wang, J. C., D. Baumgarten, and B. M. Olivera, in preparation.

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[23] Fareed, G. C., and C. C. Richardson, these PROCEEDINGS, **58**, 665 (1967).