*STUDIES OF DNA REPLICATION IN VIVO, I. ISOLATION OF THE FIRST INTERMEDIATE OF DNA REPLICATION IN BACTERIA AS SINGLE-STRANDED DNA**

By M. Oishi

DEPARTMENT OF GENETICS, THE PUBLIC HEALTH RESEARCH INSTITUTE OF THE CITY OF NEW YORK, INC.

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The *in vivo* molecular events at the DNA replicating point have been poorly understood. Few papers have appeared which discuss the structure of DNA at the replicating point. Hanawalt and Ray reported the isolation of the replicating point of DNA from *Escherichia coli*.[1] Kidson separated the newly synthesized DNA from bulk DNA by countercurrent distribution. He suggests that such newly synthesized DNA is partially denatured.[2] Sakabe and Okazaki have shown that newly synthesized DNA has a slower sedimentation rate compared to the bulk DNA in alkaline sucrose-gradient centrifugation.[3]

The major difficulties encountered in this investigation of the molecular events at the DNA replicating point were (1) lack of a convenient method for fractionating DNA according to its secondary structure, (2) the high speed of the replication process, and (3) poor recovery of newly synthesized DNA.[1, 4]

This report concerns the structure of newly synthesized DNA and the kinetics of its production. The above-described difficulties were at least partially overcome by the application of hydroxyapatite chromatography plus variations on other procedures used in studying DNA replication. The results indicate that the earliest detectable intermediate of DNA replication, in both *Bacillus subtilis* and *Escherichia coli*, upon isolation has all the characteristics of single-stranded DNA. This first intermediate has a very transient existence and is rapidly converted to a double-stranded form of DNA *in vivo*.

Materials and Methods.—Bacterial strains: A thymine-requiring *B. subtilis* strain (168-*thy*)[5] and *E. coli* B3 (*thy*), kindly provided by Dr. E. M. Witkin, were used for the isolation of labeled DNA. *B. subtilis*, MU8U5U16 (168-*leu-met-ade*, from Dr. N. Sueoka), was used as a recipient for transformation.

Media: For *B. subtilis*, medium Y consisted of Spizizen's medium supplemented with casamino acid (200 μg/ml), thymine (20 μg/ml), L-tryptophan (50 μg/ml), and L-methionine (50 μg/ml). Medium Y − T was medium Y without thymine. For *E. coli*, modified TCG medium[6] was supplemented with thymine (40 μg/ml).

Labeling of DNA: B. subtilis: One ml of overnight culture of *B. subtilis* (168-*thy*) in medium Y was transferred to 50 ml of the same medium containing 0.04 μc/ml of C^{14}-thymine (2-C^{14}-thymine, sp. act. 57 μc/μM, New England Nuclear Corp.). The cells were grown at 30°C with constant rotary shaking. At the middle of the exponential phase (1–2 × 10^8/ml viable cells), cells were collected and washed with prewarmed (30°) medium Y − T on a membrane filter. The cells were immediately transferred to 50 ml of medium Y − T and incubated at 30°C to deplete the cellular precursor pool. At 8–10 min, 5–10 ml was transferred to a beaker and stirred by a magnetic stirrer. Pulse labeling was carried out by adding one-fifth vol of medium Y-T, containing H^3-thymidine (methyl-H^3-thymidine, sp. act. 16.7 c/mM, New England Nuclear Corp.), to give a final radioactivity of 10 μc/ml. Chase experiments were done by adding one-fifth vol of medium Y containing 2 mg/ml of thymidine. To isolate DNA, the process was stopped at the desired time by pouring approximately 100 ml of cold buffer B (0.01 *M* Tris, pH 7.4,

0.015 M NaCl, 0.0015 M Na_3-citrate, 0.01 M EDTA, and 0.02 M NaN_3) with crushed ice into the cell suspension. To assay total incorporated radioactivity, 1- to 2-ml cell suspensions were withdrawn at various time intervals, and the incorporation was stopped by adding an equal volume of cold TCA (20%). *E. coli:* The same procedures were used as for *B. subtilis*, except that (1) cells were grown in modified TCG medium and (2) the reaction was stopped by cold buffer B supplemented with sodium fluoride (0.02 M).

Isolation of DNA: B. subtilis: After the reaction was stopped by cold buffer B, the cells were collected by centrifugation in the cold. They were washed once and resuspended in 1–2 ml of cold buffer B. Lysozyme (500 μg/ml) was added, and the mixture was incubated at 37°C for 10 min. One-tenth vol of SLS-Tris solution (5% SLS, 1 M Tris, pH 9.0) and 2 mg/ml of pronase (Cal biochem, previously heat-treated at 80°C for 10 min in $^1/_{10}$ SSC) were added and incubated at 42°C for 3 hr. Fifty mg/ml of sodium p-amino salicylate was added and the mixture was shaken gently with an equal volume of 88% phenol–12% m-cresol–0.1% -8-hydroxyquinoline[2, 7] for 15 min. DNA was precipitated from the aqueous layer with ethanol and kept overnight at −20°C. *E. coli:* The same procedures were employed as with *B. subtilis* except that (1) cells were not washed but resuspended in buffer B (plus sodium fluoride, 0.02 M) after centrifugation, and (2) lysozyme treatment was carried out at 0°C for 20 min.

Hydroxyapatite chromatography: Hydroxyapatite[8] was prepared according to Miyazawa and Thomas.[9] In some preliminary experiments commercial hydroxyapatite (Calbiochem) was used. Chromatography was carried out essentially according to Bernardi[10] to separate single- and double-stranded DNA. Elution was done by a linear sodium phosphate gradient ranging from 0.07 M to 0.35 M, pH 7.0. A total of 40 ml eluent was collected in 2-ml fractions. For some purposes, a batch method was devised as follows. DNA samples (2 ml) in 0.16 M sodium phosphate buffer (pH 7.0) were mixed with approximately 200 mg of hydroxyapatite, and shaken for 5 min. After centrifugation, the supernatant was collected and the hydroxyapatite pellet washed with 1.0 ml of 0.16 M phosphate buffer and centrifuged, and the supernatants were combined. These contained the single-stranded DNA. The hydroxyapatite pellet was resuspended in 2 ml of 0.30 M sodium phosphate buffer (pH 7.0), and the suspension was then shaken and centrifuged. This procedure was repeated again with another 1.0 ml of 0.30 M sodium phosphate buffer and both supernatants were combined. The double-stranded DNA was eluted with the second pair of supernatants. Exact concentration of phosphate buffers must be determined for each batch of hydroxyapatite.

Isolation of standard DNA: DNA used as standard was isolated from *B. subtilis* (168W) or *B. subtilis* (168-*thy*) essentially according to the procedure of Saito and Miura.[11] A slightly modified phenol procedure[12] was used to isolate P^{32}-labeled T7 phage DNA (kindly provided by Dr. E. Burgi).

Results and Discussion.—Kinetics of H^3-thymidine incorporation and isolation of DNA: Exponentially growing thymine-requiring cells (*B. subtilis* and *E. coli*) were washed and incubated in a medium without thymidine for eight to ten minutes to deplete the precursor pool and then pulsed with H^3-thymidine for short periods (5 sec to 10 min). As shown in Figure 1*A*, incorporation of H^3-thymidine into DNA occurs linearly without any appreciable lag period. When cold thymidine (330 μg/ml) was added five seconds after addition of H^3-thymidine, the incorporation ceased in approximately two minutes, suggesting that most of the H^3-thymidine in the pool was chased out during this period (Fig. 1*B*). DNA was isolated following such pulse and pulse-plus-chase conditions by stopping DNA replication and using the modified phenol procedure[7] after treating the cells with lysozyme, SLS, and pronase. Under these conditions, at least 80 per cent of newly synthesized DNA (*B. subtilis*) was extracted into the aqueous phase after phenol treatment. Usually bulk DNA had been prelabeled

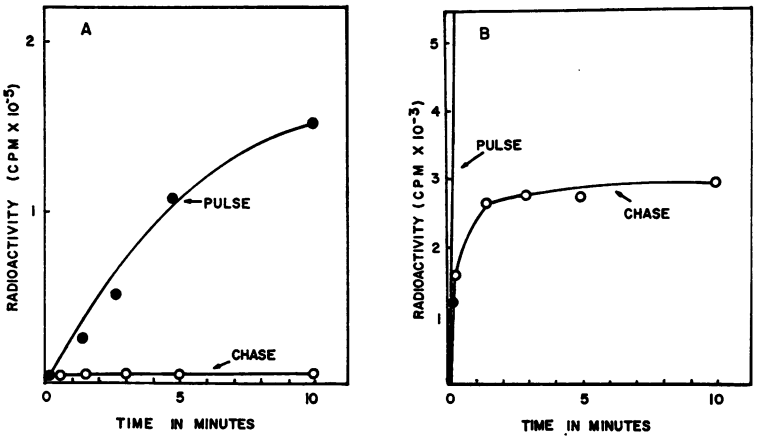


FIG. 1.—The incorporation of H^3-thymidine into DNA in *B. subtilis*. (*A*) Pulse experiment: (●—●), H^3-thymidine (10 μc/ml) was added at zero time. Chase experiment: (O—O), 5 sec after the addition of H^3-thymidine (10 μc/ml), cold thymidine (final concentration of 300 μg/ml) was added. Samples (0.5 ml) were assayed. (*B*) The same as (*A*), except that the ordinate is expanded to show the details of the chase experiment.

with C^{14}-thymine during several prior generations to distinguish it from tritium-labeled newly synthesized DNA.

Hydroxyapatite chromatography of newly synthesized DNA: The DNA samples were subjected to hydroxyapatite columns to fractionate the DNA according to its secondary structure.[10] As seen in Figures 2 (*B. subtilis*) and 3 (*E. coli*), most of the H^3-DNA receiving the shortest pulse (5 sec and 6 sec) was eluted earlier (fraction I) than the C^{14}-labeled double-stranded bulk DNA (fraction II). This suggests that the first intermediate of DNA replication differs in its structure from double-stranded DNA. After a 1-minute (*B. subtilis*) and a 40-second (*E. coli*) pulse, the H^3-radioactivities can be seen to be distributed more or less equally between the two fractions. After a 4-minute pulse (*B. subtilis*), most of the H^3-radioactivity coincides with the peak of C^{14}-bulk DNA. The first peak (fraction I) was eluted from the hydroxyapatite columns at phosphate buffer concentrations ranging from 0.13 to 0.16 *M*.

Essentially the same profiles were obtained with DNA (1) that was pulsed without thymine starvation, (2) that was pulsed with either diluted H^3-thymidine (10-fold dilution with cold thymidine) or 2-C^{14}-thymidine (sp. act. was approximately 1/300 of the radioactivity used in the main H^3-thymidine experiments), (3) that was present in a preparation which did not undergo phenol extraction, and (4) that was isolated in buffer with a higher salt concentration (NaCl at 0.15 *M*). These results argue against the possibility that the fraction I DNA is a product of abnormal physiological conditions, of radioisotope effects, or of denaturation during isolation. Ribonuclease treatment also did not affect the profile.

If fraction I is an intermediate state of DNA in its replication, then the radioactivity of fraction I should be chased out by cold thymidine. A large amount

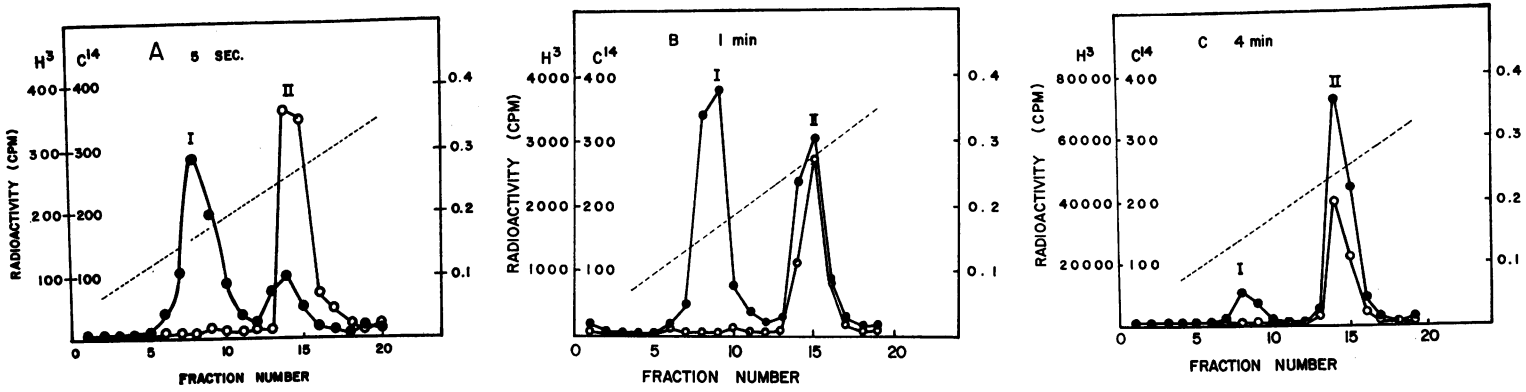


FIG. 2.—Hydroxyapatite chromatography of pulse-labeled DNA from *B. subtilis*. Exponentially growing *B. subtilis* (168-*thy*) cells, prelabeled with C^{14}-thymine (0.04 μc/ml), were pulsed with H^3-thymidine (10 μc/ml) (*A*) for 5 sec; (*B*) for 1 min; and (*C*) for 4 min. Samples of (*A*), (*B*) (6 ml), and *C*) (12 ml) were withdrawn, and the DNA was isolated and fractionated as described in *Materials and Methods*. All the isolated DNA was applied on the columns except that of sample (*C*), where only half of the fraction was assayed for radioactivity, and the rest of it was saved for rechromatography.

●—●, H^3-radioactivity; ○—○, C^{14}-radioactivity; - - - - -, phosphate buffer concentration.

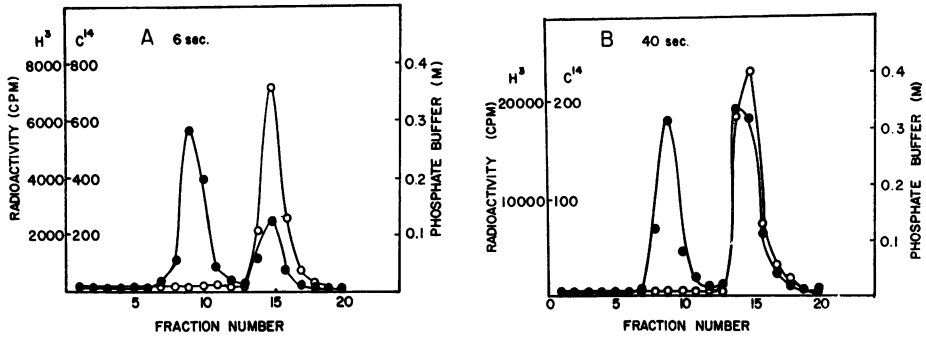


FIG. 3.—Hydroxyapatite chromatography of pulse-labeled DNA from *E. coli*. Exponentially growing *E. coli* B_3 (*thy*) cells, prelabeled with C^{14}-thymine (0.04 μc/ml), were pulsed with H^3-thymidine (10 μc/ml) for (*A*) 6 sec and (*B*) 40 sec. From a 6-ml sample of (*A*) and a 2-ml sample of (*B*), DNA was isolated and fractionated as described in *Materials and Methods*.

●—●, H^3-radioactivity; ○—○, C^{14}-radioactivity; - - - -, phosphate buffer concentration.

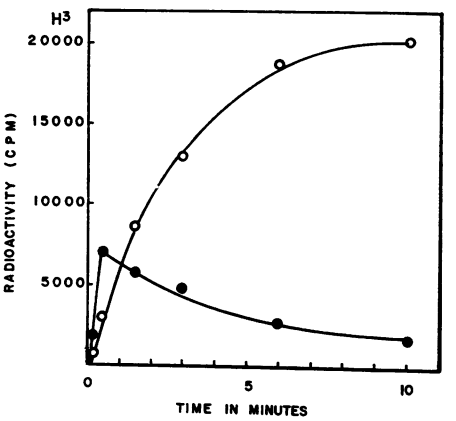


FIG. 4.—The incorporation in *B. subtilis* (168-*thy*) of H^3-thymidine into fraction I and fraction II after chasing with cold thymidine. Cells prelabeled with C^{14}-thymine (0.04 μc/ml) were pulsed for 5 sec with H^3-thymidine (10 μc/ml) and chased with cold thymidine. From each 5 ml of cell suspension, DNA was isolated and fractionated with hydroxyapatite by the batch procedure. Since there was some difference in recovery of DNA from the different time samples, all radioactivity was calibrated on the basis of recovery of C^{14}-DNA.
●—●, Radioactivity in fraction I; O—O, radioactivity in fraction II.

of cold thymidine was added at five seconds after H^3-thymidine addition, and DNA was isolated at various time intervals and separated by hydroxyapatite into fraction I and fraction II (double-stranded DNA). Figure 4 shows the distribution of radioactivity between the two fractions as a function of duration of the chase. This figure shows that labeled fraction I DNA was chased by cold thymidine and apparently converted into fraction II, suggesting that indeed fraction I DNA is an intermediate of DNA replication.

Characterization of fraction I DNA: Since single-stranded DNA is eluted at lower phosphate buffer concentration than is double-stranded DNA, the fraction I DNA (from the 4-min pulse, Fig. 2*C*) was mixed with C^{14}-heat-denatured DNA and rechromatographed. As shown in Figure 5, both C^{14} and H^3 radioactivities were eluted at the same position, suggesting that fraction I DNA is single-stranded. As confirmation, the same fraction was centrifuged in CsCl with C^{14}-heat-denatured DNA and cold native DNA (Fig. 6). The H^3-DNA had essentially the same density as the artificially prepared single-stranded DNA (C^{14}-labeled) and was separated from native DNA (identified by transforming activity). The reason for the slight skew of the H^3-DNA peak toward higher density has not yet been determined. The broadness of the H^3-DNA peak is probably due to smaller molecular weight.[13]

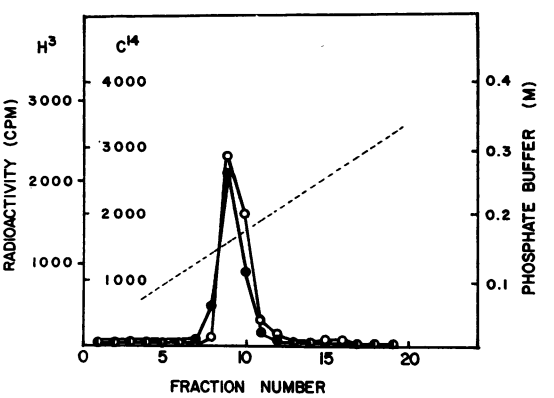


FIG. 5.—Rechromatography of fraction I. An aliquot of fraction I from sample (*C*) of Fig. 2 (0.2 ml each from tubes 8 and 9 with a total radioactivity of about 4000 cpm) was mixed with 1 μg of alkaline-denatured standard *B. subtilis* C^{14}-DNA (total radioactivity approximately 5000 cpm), and was fractionated by a hydroxyapatite column under tne same conditions as described for Fig. 2.
●—●, H^3-radioactivity; O—O, C^{14}-radioactivity; ----, phosphate buffer concentration.

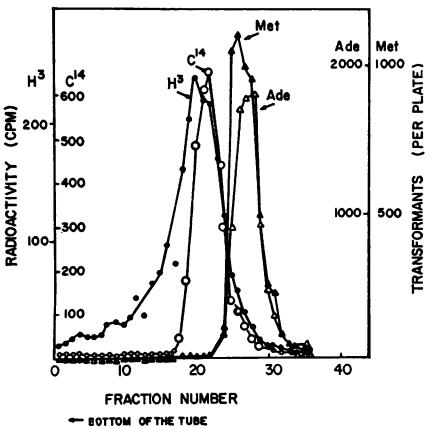


FIG. 6.—CsCl density-gradient centrifugation of fraction I DNA. A portion of fraction I DNA (0.6 ml, total H^3-radioactivity approximately 9000 cpm), which was isolated and fractionated under the same conditions as shown for Fig. 2, sample (*C*), was mixed with 1.5 μg of C^{14}-labeled heat-denatured *B. subtilis* (168-*thy*) DNA (sp. act. approximately 5000 cpm/μg) plus 2.5 μg of native *B. subtilis* (168W) DNA. The density was adjusted to 1.700 with CsCl (total volume 2.5 ml) and centrifuged at 36,000 rpm in a SW39 rotor for 48 hr at 22°C. Three-drop fractions were collected from the bottom of the tubes and diluted with 1.0 ml of $^1/_{10}$ SSC. Of each fraction, 0.6 ml was analyzed for radioactivity and 0.1 ml for transforming activity[14] (*leu-met-ade*).

●—●, H^3-radioactivity; ○—○, C^{14}-radioactivity; △—△, adenine transformants; ▲—▲, methionine transformants.

Although these results suggest that fraction I is a single-stranded DNA, it is possible that the newly formed H^3-labeled DNA is associated by hydrogen bonds with a much longer single-stranded template DNA existing in cells as prereplicative DNA (suggested by Rolfe[15] as well as Rosenberg and Cavalieri[16]). Such a complex might behave as single-stranded DNA. This possibility was excluded by the following experiments. Fraction I DNA was treated with alkali to destroy any hydrogen bonds and then compared as to molecular size with untreated fraction I DNA by neutral sucrose-gradient centrifugation. As shown in Figure 7, no significant difference was observed in their molecular size. Furthermore, fraction I DNA was treated with exonuclease I, which is known to attack only single-stranded DNA.[17] Figure 8 shows that more than 99 per cent of fraction I DNA became acid-soluble following incubation with exonuclease I, whereas C^{14}-labeled double-stranded DNA remained intact. From these results it can be concluded that fraction I DNA is single-stranded. As a preliminary determination by alkaline and neutral sucrose-gradient centrifugation,[19] the molecular weight of this single-stranded DNA ranges from approximately a quarter million (5-sec pulse sample) to about two to three million (1-min pulse sample).

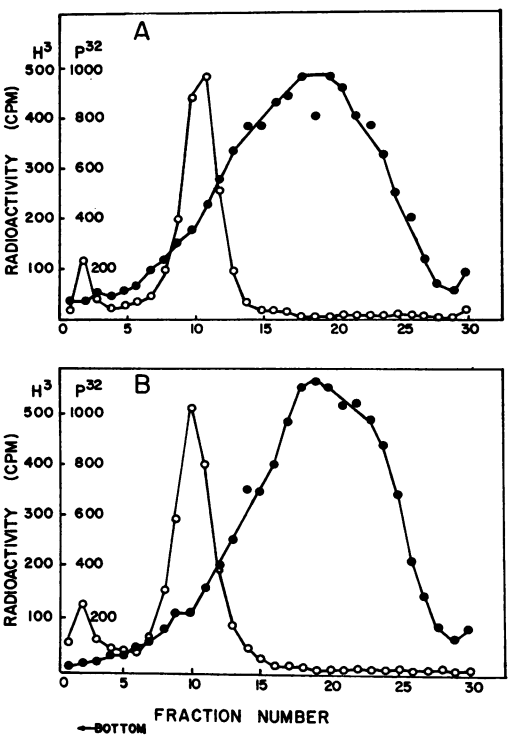
The fact that the earliest-detectable product of DNA replication is isolated from cells as single-stranded DNA does not prove that it exists in a single-stranded form in the cells. However, it seems reasonable to conclude that the newly synthesized DNA at this initial stage of replication does not exist in the normal, stable double-helical form that characterizes the bulk of the DNA in these cells.

Several models of DNA replication *in vivo* seem worth considering. One obvious model assumes that the single-stranded fraction I DNA is actually the form in which newly synthesized DNA is initially produced in the cell. This implies that direct base pairing by hydrogen bonds[20] is not the primary factor in tem-

FIG. 7(*below*).—Effect of alkaline treatment of fraction I DNA. Fraction I DNA, from the *B. subtilis* cells subjected to a 3-min pulse, was fractionated by the batch procedure, dialyzed against $^1/_{10}$ SSC, and concentrated to $^1/_{16}$ of the original volume under vacuum. A portion of it was brought to 0.2 *M* NaOH concentration, kept for 5 min at room temperature, and neutralized with NaH_2PO_4 solution. After being mixed with P^{32}-labeled T7 phage DNA as a reference marker, the sample (approximately 0.2 ml) was layered on 4.8 ml of sucrose gradient solution (5–20% in 0.1 *M* NaCl, 0.01 *M* Tris buffer, pH 7.8) and centrifuged 36,000 rpm for 4 hr at 5°C in a SW50 rotor. Each group of seven drops was collected. A control sample was treated in the same way but without alkaline treatment.

(*A*) No alkaline treatment (control); (*B*) alkaline-treated.

●—●, H^3-radioactivity (fraction I DNA); O—O, P^{32}-radioactivity (T7 phage DNA).



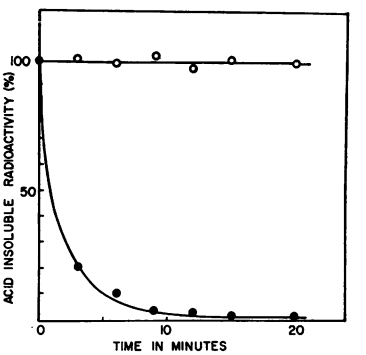


FIG. 8.—Exonuclease I treatment of fraction I DNA. Fraction I DNA, from *B. subtilis* (following a 1-min pulse), was dialyzed against $^1/_{10}$ SSC overnight. A portion of it (approximately 3000 cpm) was mixed with C^{14}-native *B. subtilis* DNA (approximately 0.3 μg, 1500 cpm) and then treated with exonuclease I (3 units/tube) as described by Lehman.[18] At the indicated time intervals, TCA (10%)-insoluble radioactivities were assayed. Radioactivities are shown as relative values compared to the value at zero-time incubation.

●—●, H^3-radioactivity; O—O, C^{14}-radioactivity.

plate copying. Template copying may be mediated by some protein entity, perhaps DNA polymerase, which could recognize the bases at its allosteric sites. Subsequently the newly synthesized single-stranded DNA would form a stable double helix with its template. Evidence that alleles of the DNA polymerase gene of T4 bacteriophage act as mutator genes[21] is consistent with this model.

A more intriguing model would assume that the prereplicative single-stranded template DNA exists in a distorted or stretched form, perhaps fixed upon a structural element of the cell membrane. Newly synthesized DNA, even though produced by direct hydrogen-bonding between base pairs, would be initially unable to form a normal double helix with template DNA. As a result, the stacking forces, which are major stabilizing forces of the normal double-helical

DNA, would be absent or greatly reduced. *In vivo*, some cellular mechanism may help to stabilize this distorted double-stranded DNA. However, during isolation procedures, single-stranded DNA (fraction I DNA) might be readily released from such a form of double-stranded DNA.

A third model assumes that copying of the template occurs on the double-stranded DNA, rather than a single-stranded DNA, to form a transient, unstable triple-stranded DNA. If so, the DNA polymerase must be able to recognize the bases which are inside the DNA structure.

Other models could explain the results presented here. In any event, it is quite clear that the initial product of DNA synthesis is transient and is not a normal, stable double-helical DNA. A most important question still remaining is whether single-stranded fraction I DNA exists as such in the cell.

Summary.—Single-stranded DNA was isolated from *B. subtilis* and *E. coli* as the initial product of DNA synthesis in cells pulse-labeled with H^3-thymidine. This first intermediate of DNA replication was found to be transient by pulse and chase experiments. Some possible models of the DNA replication mechanism are considered.

Note added in proof: After this manuscript was submitted, Okazaki *et al.* (these PROCEEDINGS, **59,** 598 (1968)) reported that an appreciable fraction of newly synthesized DNA as isolated appeared to be single-stranded.

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The following abbreviations are used: Tris, tris(hydroxymethyl)aminomethane; SSC, standard saline citrate (0.15 *M* NaCl, 0.015 *M* Na_3-citrate); $^1/_{10}$ SSC, 10 times diluted SSC; SLS, sodium lauryl sulfate; EDTA, ethylenediaminetetraacetate; TCA, trichloroacetic acid.

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