*STUDIES OF DNA REPLICATION IN VIVO, II. EVIDENCE FOR THE SECOND INTERMEDIATE**

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In the preceding paper,[1] it was demonstrated that the initial product of bacterial DNA synthesis is isolated as single-stranded DNA. The characteristics of this first intermediate of DNA synthesis suggested possible models for the templating process of DNA replication. The isolated single-stranded DNA has a much smaller molecular weight than the bulk DNA, confirming the recent findings of Okazaki *et al.*[2, 3] However, the secondary structure of the replicating point still remains largely unknown despite preliminary indications of some unusual properties of replicating DNA.[2–6] Kidson separated the newly synthesized DNA by countercurrent distribution and suggested that this DNA is partially denatured.[6] It became important to try to trace the fate in the cells of the newly synthesized DNA, which is isolated in a single-stranded form.

This report concerns the isolation of a second intermediate, from *Bacillus subtilis*, which may represent a second stage of DNA replication. This intermediate exhibits many of the characteristics of double-stranded DNA. However, there is evidence that part of it has a single-stranded structure.

Materials and Methods.—Bacterial strains and media: A thymine-requiring *B. subtilis* strain (168-*thy*)[7] was used throughout these studies. Medium Y[1] and medium Y − T[1] were used.

Labeling and isolation of DNA: The same procedures were used as reported previously,[1] except for the following changes. (1) Cells were resuspended in half a volume of Y − T (25 ml) after being washed on Millipore filters. (2) Buffer C (0.01 *M* Tris, pH 7.8, 0.01 *M* EDTA, 0.015 *M* NaCl, 0.02 *M* NaN_3, and 0.02 *M* NaF) was used instead of buffer B. (3) Lysozyme treatment was carried out at 0°C for 40 min and then at 37°C for 10 min.

Hydroxyapatite chromatography of DNA: Chromatography procedures[8] and a batch procedure to fractionate DNA by hydroxyapatite[1] were reported previously.

Nitrocellulose treatment of DNA: Nitrocellulose powder (Hercules Powder Co., kindly provided by Drs. T. Kasai and E. K. F. Bautz) was homogenized in 2 × SSC by a Waring Blendor for 3 min before use. Approximately 100 mg of nitrocellulose was mixed with DNA samples that had been dissolved in 2 × SSC, and the mixture was shaken for 5 min. After centrifugation, the nitrocellulose was shaken with 2 × SSC once more, and both supernatants were combined. To calculate the percentage of DNA bound to nitrocellulose, the supernatant radioactivity was subtracted from the total input-sample radioactivity before treatment. Approximately 95% of heat-denatured DNA and 20–40% of native DNA bind with nitrocellulose under these conditions. In a limited number of experiments, essentially the same results were obtained with commercially available nitrocellulose filters (S & S B6 filters).

Cs_2SO_4-Hg(II) centrifugation: Cs_2SO_4-Hg(II) centrifugation was carried out according to Nandi *et al.*[9] All the DNA samples were dialyzed against 0.02 *M* Na_2SO_4, 0.002 *M* borate buffer at pH 8.7 before being mixed with $HgCl_2$.

Results.—As reported in the preceding paper,[1] under the conditions employed, most of the tritium-labeled DNA from cultures receiving the shortest pulse (5 sec) was isolated as single-stranded DNA for both *B. subtilis* and *Escherichia coli*.

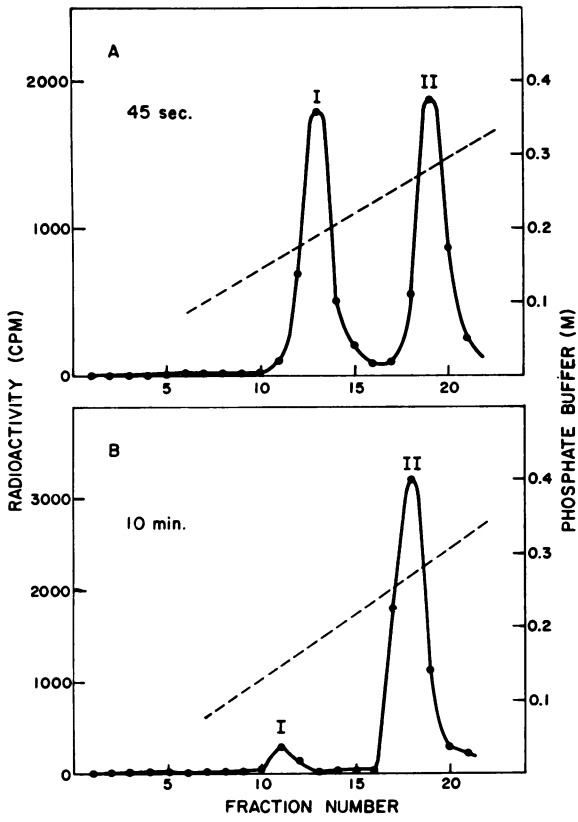

FIG. 1.—Hydroxyapatite chromatography of pulse-labeled DNA from *B. subtilis*. Exponentially growing *B. subtilis* (168-*thy*) cells were pulsed with H^3-thymidine (10 μc/ml) for 45 sec (*A*) and 10 min (*B*). In (B), 1 μg/ml of carrier thymidine was added during the pulse. From samples of *A* (5 ml) and *B* (3 ml), DNA was isolated and fractionated as described in *Materials and Methods* and in the previous paper.[1] From each 2-ml fraction, 0.1 ml was used for assaying radioactivity. (●——●) H^3-radioactivity; (——) phosphate-buffer concentration.

This was demonstrated by fractionation of the labeled products by hydroxyapatite chromatography. With longer pulses (40–60 sec), the labeled DNA was distributed more or less equally between single-stranded and double-stranded DNA, and at ten minutes almost all the radioactivity had accumulated in the double-stranded DNA. The latter two cases are shown in Figure 1*A* and *B*, where pulse-labeled DNA (45 sec and 10 min, respectively) from *B. subtilis* was separated into two fractions, fraction I DNA (single-stranded DNA) and fraction II DNA (double-stranded DNA).

It was shown previously that the double-stranded DNA of fraction II included the bulk DNA of the cells. However, it seemed possible that the component of fraction II that represented newly synthesized DNA, especially from the 45-second pulse sample, might have some unique structure, although it was eluted at the same position as bulk DNA. Therefore, some properties of the two longer pulse samples of fraction II DNA (45-sec and 10-min pulse-labeled) were compared to those of standard native double-stranded DNA and artificially prepared single-stranded DNA.

(1) *Affinity of fraction II DNA for nitrocellulose powder:* Nitrocellulose is known to have a strong affinity for single-stranded DNA in high salt concentration and, to a much lesser extent, for double-stranded DNA.[10, 11] To test the

TABLE 1. *Nitrocellulose binding of fraction II DNA.*

DNA	Experiment	(*A*) cpm in input	(*B*) cpm in supernatant	(*C*) cpm bound (*A* − *B*)	(*D*) Per cent bound
Heat-denatured DNA (C^{14})	1	4,365	134	4,231	97
	2	4,161	62	4,099	99
Native DNA (C^{14})	1	4,098	2,586	1,512	37
	2	4,001	2,380	1,621	41
Fraction II of 45-sec pulse DNA (H^3)	1	1,052	96	956	91
	2	1,058	109	949	90
Fraction II of 10-min pulse DNA (H^3)	1	11,653	7,917	3,736	32
	2	14,120	8,864	5,256	37

Samples of fraction II DNA (isolated by a batch procedure[1]), C^{14}-heat-denatured DNA, and native *B. subtilis* DNA were treated with nitrocellulose powder as described in *Materials and Methods*. After centrifugation, the radioactivity of the supernatant (*B*) was subtracted from the total input radioactivity (*A*) to determine the radioactivity of the nitrocellulose-bound DNA (*C*).

two samples of fraction II DNA (45-sec and 10-min pulse), double-stranded DNA, and heat-denatured DNA for their affinity for nitrocellulose, they were mixed with nitrocellulose powder in saline citrate solution (2× SSC, 0.30 *M* NaCl, 0.03 *M* Na_3-citrate). As is shown in Table 1, in control experiments, more than 95 per cent of heat-denatured DNA binds with nitrocellulose, as compared to only 40 per cent of standard native DNA. The extent of binding of native DNA varies from 20 to 40 per cent for unknown reasons. Approximately 90 per cent of the fraction II DNA from the 45-second pulse sample binds to nitrocellulose, which is almost the same high affinity as that of single-stranded (heat-denatured) DNA. In contrast, only about 35 per cent of the labeled fraction II DNA from the 10-minute pulse sample binds to nitrocellulose, which is essentially the same affinity as that of standard native DNA. These results suggest that the portion of fraction II that consists of the most recently synthesized DNA (labeled DNA from the 45-sec pulse sample) has a partially single-stranded structure, even though it behaves like double-stranded DNA in hydroxyapatite chromatography. Further evidence for this interpretation will be presented later.

If the DNA that binds to the nitrocellulose powder represents a second stage in DNA replication, under conditions of pulse and chase, the radioactivity of this fraction should appear after the radioactivity of the single-stranded DNA fraction is chased out. To check this point, a large amount of cold thymidine was added five seconds after the addition of H^3-thymidine, and DNA was isolated at various time intervals. These DNA samples were first separated by hydroxyapatite into fraction I (single-stranded DNA) and fraction II. Fraction II DNA was further treated with nitrocellulose powder to separate nitrocellulose-bound DNA from nitrocellulose-insensitive DNA. As shown in Figure 2*A*, radioactivity in single-stranded DNA was rapidly chased out after 30 seconds, whereas radioactivity in nitrocellulose-bound DNA in fraction II began to increase at this time and leveled off at three minutes. On the other hand, the radioactivity in the fraction II DNA that does not bind to nitrocellulose powder increased only

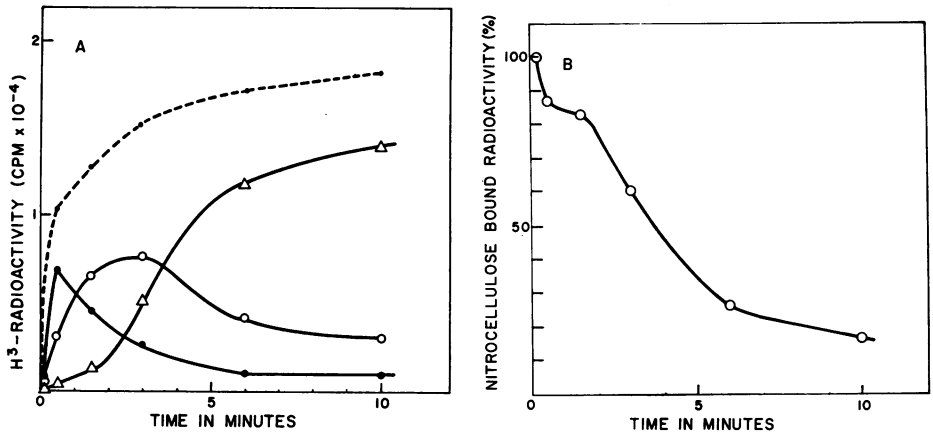

FIG. 2.—The incorporation of H^3-thymidine into DNA that has been chased with cold thymidine.

(*A*) Cells prelabeled with C^{14}-thymidine (0.08 μc/ml) were pulsed for 5 sec with H^3-thymidine (10 μc/ml) and chased with cold thymidine as described previously (generation time of about 80 min).

At indicated times, 5-ml samples were taken, and the DNA was isolated and fractionated with hydroxyapatite by the batch procedure[1] into fraction I DNA (single-stranded DNA) and fraction II DNA.

A portion of fraction II DNA from each sample was further treated with nitrocellulose powder, and the nitrocellulose-bound radioactivity was determined as described in Table 1. 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 of fraction I DNA (single-stranded DNA); (○——○) radioactivity of nitrocellulose-bound DNA from fraction II; (△——△) radioactivity of the supernatant from fraction II DNA after nitrocellulose treatment; (●------●) total radioactivity.

(*B*) The percentage of the radioactivity of nitrocellulose-bound DNA from fraction II recalculated as a function of time of chasing.

after a considerable lag period. Figure 2*B* shows the percentage of labeled fraction II DNA that is nitrocellulose-bound as a function of chase time. At the end of the five-second pulse (zero time of chase), almost 100 per cent of labeled fraction II DNA binds to nitrocellulose. With increasing time of chase, the percentage that binds gradually decreases. All these results support the view that nitrocellulose-bound DNA represents a second stage of DNA during replication. It is worth noting here that most studies of DNA synthesis that follow radioactive labels under chase conditions consider the labeled DNA as a single entity exemplified by the top curve of Figure 2*A*. The fractionation techniques described here reveal that the labeled, newly synthesized DNA is isolated as a mixture of three components varying in proportions as a function of the time of chasing, as shown in Figure 2*A*.

(2) *CsCl and Cs_2SO_4-Hg(II) density gradient centrifugation of fraction II DNA:* If a considerable portion of the second intermediate is single-stranded, the difference between this portion and native double-stranded DNA should be demonstrable by density gradient centrifugation. Tritium-labeled fraction II DNA from the 45-second pulse sample (90% affinity to nitrocellulose) was mixed with C^{14}-heat-denatured *B. subtilis* DNA and cold standard native DNA and was

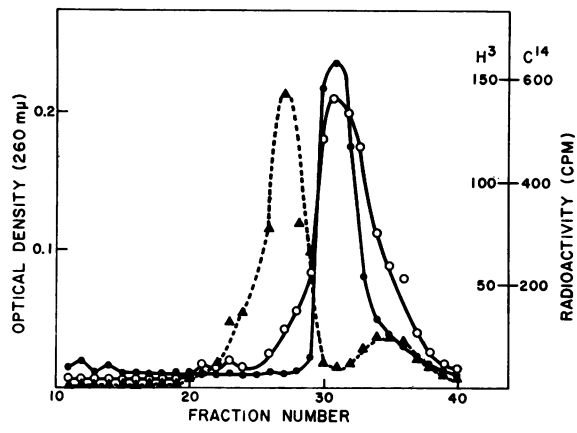

FIG. 3.—CsCl density gradient centrifugation of fraction II DNA. H^3-fraction II DNA (45-sec pulse), native DNA (*B. subtilis*, 33 μg), and C^{14}-heat-denatured DNA (*B. subtilis*, approximately 1.0 μg) were mixed with CsCl, and the density was adjusted to 1.710. The total 2.5 ml was centrifuged in a SW50 rotor at 36,000 rpm for 45 hr at 25°C. Two-drop fractions were collected through a needle.

(●——●) Optical density at 260 mμ; (O——O) H^3-radioactivity; (▲------▲) C^{14}-radioactivity.

centrifuged in CsCl. As shown in Figure 3, the curve for this fraction II DNA shows no significant difference in position or special skewness toward single-stranded DNA density when compared to that of the standard native double-stranded DNA. This suggests that the single-stranded part of the second intermediate is so small a portion of each primarily double-stranded DNA molecule that the usual density gradient technique cannot detect the difference between the single-stranded and the native double-stranded DNA.

Nandi *et al.* reported[9] the separation of single- and double-stranded DNA by Cs_2SO_4 density gradient centrifugation after the DNA was complexed with Hg(II). As is shown in Figure 4*A*, when H^3-labeled fraction II DNA (45-sec pulse sample), C^{14}-heat-denatured DNA, and cold standard native DNA were mixed with Hg(II) and centrifuged in Cs_2SO_4, the curve for H^3-radioactivity shifted sig-

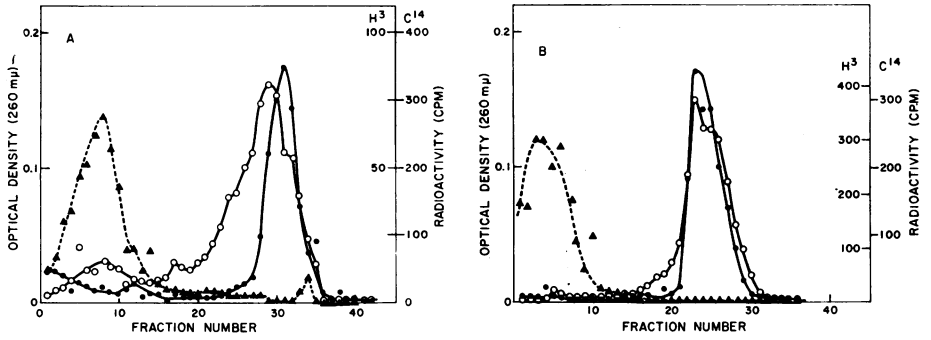

FIG. 4.—Cs_2SO_4-Hg(II) density gradient centrifugation[9] of fraction II DNA. A mixture of H^3-labeled fraction II DNA (*A*, 45-sec pulse, and *B*, 10-min pulse), native DNA (*B. subtilis*, 33 μg), and C^{14}-heat-denatured DNA (*B. subtilis*, approximately 1.0 μg) was mixed with $HgCl_2$ and Cs_2SO_4 solution in a borate buffer (0.01 *M*, pH 8.7) to give r_f (Hg(II)/phosphate) 0.25 and density 1.545. A total of 2.8 ml in polyallomer tubes was centrifuged in a Spinco SW50 rotor at 36,000 rpm for 45 hr at 25°C. Three-drop fractions were collected from the bottom of the tubes through a needle.

(●——●) Optical density at 260 mμ; (O——O) H^3-radioactivity; (▲------▲) C^{14}-radioactivity.

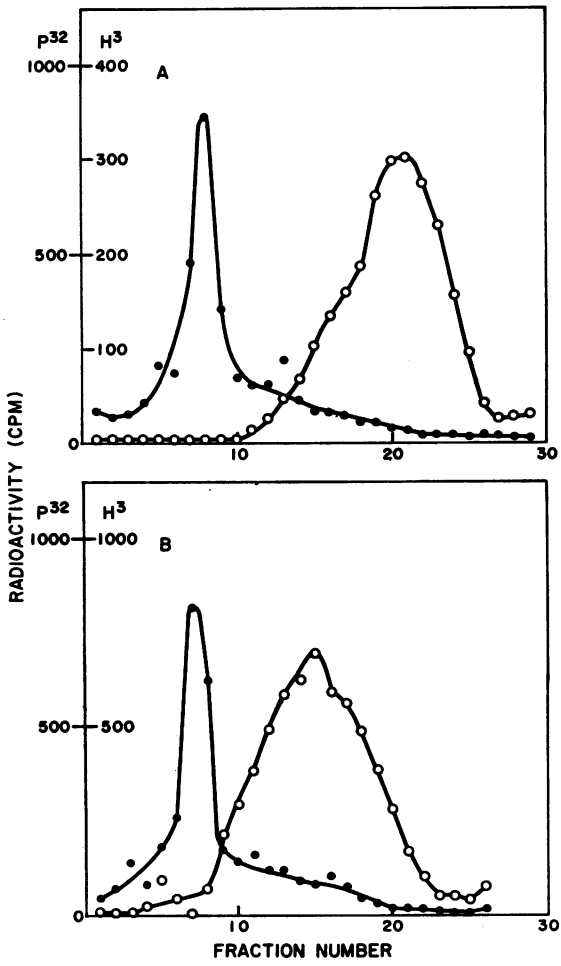

FIG. 5.—Alkaline sucrose gradient centrifugation of fraction II DNA. Approximately 0.4 ml of tritium-labeled fraction II DNA (*A*, 45-sec pulse sample, and *B*, 10-min pulse sample) and P^{32}-T_7 DNA (as a reference) were layered on alkaline sucrose gradient (28 ml, 5-20% sucrose in 0.1 *M* NaOH, 0.9 *M* NaCl, 0.001 *M* EDTA) and centrifuged at 22,000 rpm for 16 hr at 5°C in a Spinco SW 25.1 rotor. Twelve-drop fractions were collected and assayed for radioactivity.
(O——O) H^3-radioactivity;
(●——●) P^{32}-radioactivity.

nificantly from the position of the standard native DNA toward the position of C^{14}-labeled single-stranded DNA. This shift is not found for fraction II DNA of the ten-minute pulse sample, where the H^3-radioactivity profile and the optical-density profile of native DNA coincide almost perfectly (Fig. 4*B*). These results further support the idea that the second intermediate, which is isolated as the labeled component of fraction II DNA from the 45-second pulse sample, consists of double-stranded DNA molecules with single-stranded segments. It is difficult to determine with certainty the percentage of DNA that is single-stranded. However, if the degree of shift of the density toward the position of single-stranded DNA corresponds to the extent of the single-strandedness, an average of approximately 10 per cent of each molecule would be single-stranded. Since the average molecular weight of this fraction was calculated by sucrose gradient centrifugation to be approximately 10 million (10-min sample, 14 million) as double-stranded DNA (according to Studier's equation),[12] the single-

stranded portion of this molecule would consist of 1 million molecular weight equivalents, or an average of 3000 nucleotides.

(3) *Alkaline sucrose gradient centrifugation of fraction II DNA:* The possible existence of gaps in the newly synthesized strand of the second intermediate was tested by alkaline sucrose gradient centrifugation. As is shown in Figure 5, the radioactivity of the fraction II DNA from the 45-second pulse sample sediments much slower (average 14*S*, molecular weight approximately 1.2 million) than that of the ten-minute sample (average 22*S*, molecular weight approximately 3.5 million). This indicates that the newly synthesized DNA of the second intermediate exists as short fragments that increase in length with time. This is consistent with the findings of Okazaki *et al.*[2, 3] When not denatured, the labeled DNA of fraction II from the ten-minute pulse sample has an average molecular weight of about 14 million. Apparently the newly synthesized DNA of even the ten-minute pulse is not a continuous strand (one gap per molecule). This may be because ten minutes represents a somewhat short pulse under the conditions used (about 80-minute generation time), and there may also be some attack by endonuclease during preparation. More extensive studies on the size distribution of the two intermediates as a function of time will be reported later.

Discussion.—Sequential replication of the bacterial chromosome has been well established,[13-17] and experiments by Kornberg and co-workers have revealed the mechanism of action of DNA polymerase *in vitro*.[18,19] When the results reported here are combined with those of others, the following mechanism of DNA replication *in vivo* is suggested. The product of DNA polymerase, isolated as the first intermediate, has a low molecular weight and may exist in the cells as single-stranded DNA, or it may be loosely bound to the template DNA. This first stage of DNA synthesis is converted to a second stage. This second stage is represented by the material isolated as the second intermediate, consisting of double-stranded DNA molecules with single-stranded segments and gaps in the newly synthesized strand. It is not yet clear whether or not the single-stranded segments exist *in vivo* as such. Possibly ligase patches the gaps in the newly synthesized strand.

The changes through the different stages of DNA replication may represent continuous and gradual processes. If so, then the second intermediate may represent a continuous spectrum of states in the conversion of the first into the final stage of DNA replication.

We do not know whether the single-stranded portion of the second intermediate consists of template DNA, newly synthesized DNA, or both. Perhaps the single-stranded portion is the template region for the first intermediate, isolated as single-stranded DNA. Investigations along these lines are now in progress. The structural relationship of the second intermediate to the so-called Y structure of the chromosome at the replication point is not understood. Since the molecules now being studied are not chromosomes but rather isolation products, it is important to relate these molecules to the structure of replicating chromosomes.

Summary.—It was demonstrated that the first intermediate of DNA replication, isolated as single-stranded DNA, is converted to a second intermediate.

The structure of the second intermediate appears to be that of a double-stranded DNA moiety, which has a small but significant single-stranded portion and demonstrable gaps in the newly synthesized strand.

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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); EDTA, ethylenediaminetetraacetate.

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