MEMOIRS OF THE FACULTY OF SCIENCE, KYOTO UNIVERSITY, SERIES OF BIOLOGY
Vol. IV, pp. 116-129, March 1971

DNA Maturation and Protein Synthesis of Bacteriophage T4

By

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(Received January 12, 1971)

ABSTRACT In T4-infected cells of *Escherichia coli*, ^{3}H-DNA synthesized immediately after short pulse of ^{3}H-thymidine was hardly extractable into aqueous layer when pulse labeled culture was lysed with sodium dodecyl sulfate (SDS), but became extractable after chasing by addition of unlabeled thymidine. This conversion of ^{3}H-DNA was prevented by chloramphenicol and did not occur in cells infected with amber mutants defective in some step of head morphogenesis. Sucrose-gradient sedimentation studies showed that the extractable ^{3}H-DNA was the same type as the particle DNA.

When the pulse-labeled culture was lysed with lysozyme-SDS-pronase and the lysates were directly examined by sedimentation, the bulk of ^{3}H-DNA was isolated as a rapidly sedimenting fraction but, after chasing by thymidine ^{3}H-DNA of this fraction (vegetative DNA) was converted into the fraction sedimenting with particle DNA (mature DNA). We call this conversion of ^{3}H-DNA from the vegetative DNA to the mature DNA as DNA maturation. DNA maturation was not blocked by inhibition of DNA by fluoro-deoxyuridine. Inhibitory effect of chloramphenicol on DNA maturation was examined in detail and it is suggested that DNA maturation is limitted by the amount of some stable protein. On addition of chloramphenicol at 5 min, formation of the rapidly sedimenting molecule was blocked.

Introduction

Elaborate investigation of Epstein *et al.* (1) using conditional lethal mutants of bacteriophage T4D has revealed clear evidence that the synthesis of structural components of phage is independently controlled by phage genes. Recently Edgar and Wood (2) have first demonstrated that the structural components of T4 are synthesized independently and assemble effectively *in vitro* to form infective particles. Similar phenomena were reported with other phage (3, 4).

Recent findings indicate that the replicating DNA of T-even phage is structurally different from the DNA in phage particles (5, 6). According to Frankel (5), replicating DNA is distinguished from the DNA in phage particle by

the following major criteria : (i) very rapid sedimentation by neutral sucrose zone centrifugation, (ii) enhanced sensitivity to thermal denaturation and mechanical shear, and (iii) increased susceptibility to exonucleases specific for interruption of the single stranded DNA. He proposed a model for such DNA structure that it is a linear concatenate duplex. Kozinsky *et al.* (6) assumed the replicating DNA was a linear dimer of the particle DNA on the basis of electron micrographic studies. In view of these observations, the replicating DNA, in all likelihood, should be altered to the length and possibly the shape of mature DNA before completion of the assembly of structural components. In pioneering work on morphogenesis of T2 phage, Kellenberger *et al.* (7) observed that the "condensation" of DNA strands was not seen in the electron microgram of thin section of the infected cells, when chloramphenicol (CM) was added to growing culture at 8 min after infection. Addition of the antibiotic under the comparable condition inhibited the formation of mature phage DNA when Frankel (5) examined intracellular DNA by zone centrifugation. These facts suggest that some protein(s) or protein synthesis is necessary for the maturation of phage DNA.

Our results show that the rapidly sedimentable DNA is converted to slowly sedimentable DNA (identical to the particle DNA) with high efficiency. We are tempted to define, at least tentatively, the maturation of T4 DNA as conversion of the rapidly sedimentable type to the slowly sedimentable. Also it is evident in the present study that some protein(s), but not protein synthesis, pertinent to some step(s) in the head assembly process is relevant to the maturation of DNA.

Materials and Methods

(a) *Bacterial strains : Escherichia coli B3* (thymine requiring) and *H* were used from our laboratory stock.

(b) *Phage strains :* T4D and its lysozymeless mutant, *40e*, were used from our laboratory stock. *Amber* mutants of T4D, *amB17* (gene 23) and *amN134* (gene 33) were generous gifts from Dr. Edgar.

(c) *Culture condition :* Cells of *E. coli B3*, grown in TG medium (8) at 37° supplemented with 4μg/ml of thymidine to a density of 5x10^8 cells /ml were centrifuged and suspended in a half volume of the same medium supplemented with 2μg/ml of thymidine. The cells were aerated at 37° and infected with 5 phage particles per bacterium. At 9 min after infection, 1μc/ml of ^{3}H-thymidine (5.0c/m mol) was added, and chased, when necessary, by adding 400μg/ml of thymidine at 10 min. To prepare ^{32}P-phage, *E. coli H* growing in TG medium was infected with T4D at a multiplicity of 5 and labeled with ^{32}P-orthophosphate (0.4μC/μg phosphorus).

(d) *Preparation of lysates :* In Method I, culture of infected cells was mixed with an equal volume of 0.2 M EDTA containing 0.1 M NaCN. Sodium dodecyl sulfate (SDS) was added to make a final concentration of 0.1% and the mixture was incubated for 5 min at 37°. In Method II, culture was lysed by mixing with

an equal volume of 0.2 M EDTA (pH 8.0) containing 0.1 M NaCN, a few drops of chloroform and 200μg/ml of lysozyme. The mixture was incubated at 37° for 5 min, and SDS was added to give a concentration of 0.1%. ^{32}P-labeled purified phage was added as a marker in centrifugal analysis at this step. The lysate was exposed to heat at 70° for 10 min, and further incubated overnight at 30° with 0.2mg/ml of pronase, which had been heat-treated at 70° for 10 min.

(e) *Extraction of DNA:* DNA was extracted from the lysate with an equal volume of water-saturated phenol by rotation (9) for 25 min at room temperature.

(f) *Inhibition of DNA synthesis by fluorodeoxyuridina* (FUDR) : DNA synthesis was determined by measuring the incorporation of ^{3}H-thymidine or ^{14}C-uracil into acid-insoluble fraction. Samples were taken and poured into an equal volume of a mixture containing 1% SDS, 0.2 M EDTA and 0.1 M NaCN at pH 8.0, and aliquotes of lysates were applied on filter paper discs. They were washed with cold 0.3 M trichloracetic acid (TCA) three times, with cold acetone, and then dried in air. In the case of ^{14}C-uracil incorporation, the samples were pretreated with 0.1 M KOH at 37° overnight, before being spotted on the discs. FUDR was added at various times after infection and its effect on incorporation of 2-^{14}C-uracil (2.6μc/μmol) into alkaline resistant fraction was examined (10). As shown in Fig. 1, FUDR inhibited DNA synthesis of infected cells, but the inhibition did not occur immediately when the analog was added earlier than 10 min, while it took place at once when added later than 15 min. These results would be expected because of bacterial contribution to thymine pool at an early stage of phage infection (8). Inhibition of FUDR was especially remarkable when a small amount of ^{3}H-thymidine was used. In Fig. 2, 0.1μc/0.048μg/ml of ^{3}H-thymidine was added at 14 min and 20μg/ml of FUDR and 20 μg/ml of uracil were added at 15 min.

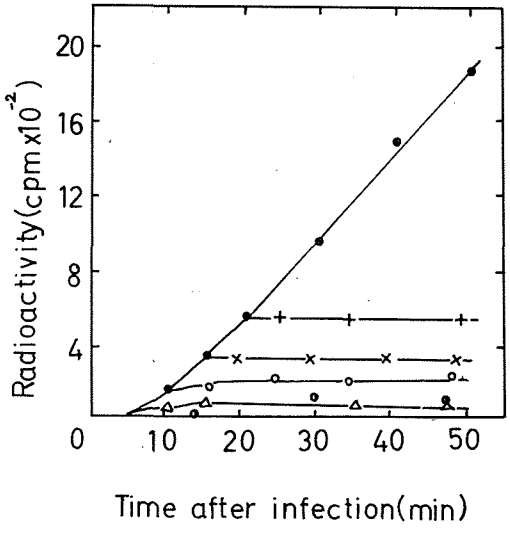

Fig. 1. Effect of FUDR on incorporation of 2-^{14}C-uracil into DNA in phage infected cells of *E. coli H*. Conditions of infection were as described in Materials and Methods. FUDR at a concentration of 20 μg/ml was added at 0 (○), 7 min (△), 10 min (○), 15 min (×) and 20 min (+) after infection. At 1 min after infection 2-^{14}C uracil was added to give a concentration of 0.2 μC (8.61 μg) /ml. At the times noted in the figure, portions of the infected culture were taken and radioactivity was measured in TCA precipitate as described in Materials and Methods.

(g) *Centrifugal analysis.* Sedimen-

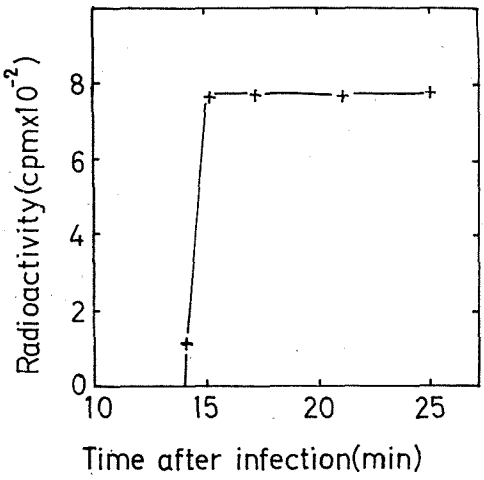

Fig. 2. Effect of FUDR on incorporation of ^{3}H-thymidine into DNA in phage infected cells.
A minute amount (0.1μc/0.0048 μg/ml) of ^{3}H-thymidine was added to the culture of *E. coli H* at 14 min after infection, 20μg/ml of FUDR and 20μg/ml of uracil were added at 15 min, and radioactivity in TCA precipitate was determined.

same gradient made up in 0.1 M NaOH-0.05 M EDTA. After centrifugation, drops were collected from the bottom of the tubes on 23mm filter paper discs (Toyo filter paper No. 2). The discs were washed, after dried, with cold 0.3 M-TCA 3 times, cold acetone and then dried in air. For the sample of alkaline zone centrifugation, 0.05 ml of 1 N HCl was spotted on each paper disc before drying. Recovery of radioactivity after centrifugation was always greater than 90%.

(h) *Measurement of radioactivity*: Radioactivity was measured in a liquid-scintillation counter with 2, 5-diphenyloxazole and 1, 4-bis-2-2 (4-methy 1-5-phenyloxazolyl) -benzene in toluene.

Results

Conversion of replicating DNA to tation analysis by neutral zone centrifugation was performed by layering 0.2 ml of a sample on a 3.9 ml 5% to 20% sucrose gradient made up in 0.1 M NaCl-0.05 M EDTA at pH 8.0, and centrifuging in a Sakuma SW rotor at 30,000 rpm for 30 min at 15°. For alkaline zone centrifugation, NaOH was added to 0.2 M to the overnight lysate, kept for 5 min at room temperature, and its 0.2 ml was centrifuged through the

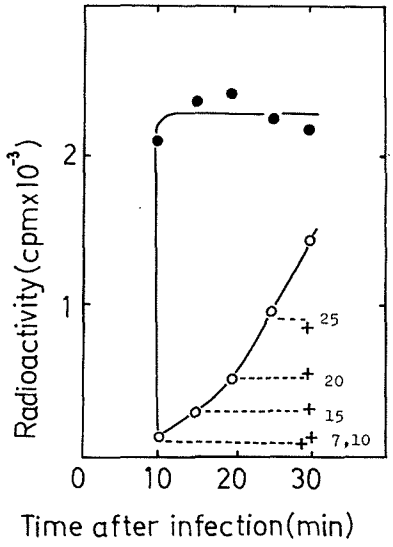

Fig. 3. Extraction of ^{3}H-DNA from phage-infected cells and the effect of CM on the extraction.
^{3}H-thymidine was fed between 9 and 10 min and DNA was extracted with phenol from the lisate prepared by the method I.
—●— Total ^{3}H-DNA,
—○— Phenol-extracted ^{3}H-DNA, and
—+— Phenol-extracted ^{3}H-DNA from infected cells at 30 min, to which CM (50μg/ml) was added at times indicated.

mature DNA

In the experiment, presented in Fig. 3, infected cells were pulse-labeled with ^{3}H-thymidine between 9 and 10 min. At various times samples were removed, divided into three parts: one part was used for measuring incorporation of ^{3}H-thymidine into the DNA, and the second part for extraction of the labeled DNA by Method I. It was noticed that the pulse-labeled DNA was extracted very poorly at the end of labeling but more efficiently at later periods ; more than 80% of the labeled DNA at 30 min. However, when chloramphenicol (CM) was added to the third part shortly before the labeling, little ^{3}H-DNA, if not all, was extractable even at 30 min (Fig. 3) as reported by Smith and Burton (11). Also evident in the figure is that the drug inhibited almost immediately the successive increase of extractable DNA whenever added after the labeling. It was conceivable that phage DNA actively synthesized between 9 and 10 min was subsequently altered in its structure during the course of infection. Accordingly, attempt were made to characterize the extracted DNA by zone centrifugation. As shown in Fig. 4, the sedimentation rate of extracted DNA(more than 90% of the input radioactivity) was indistinguishable from that of particle DNA. These results

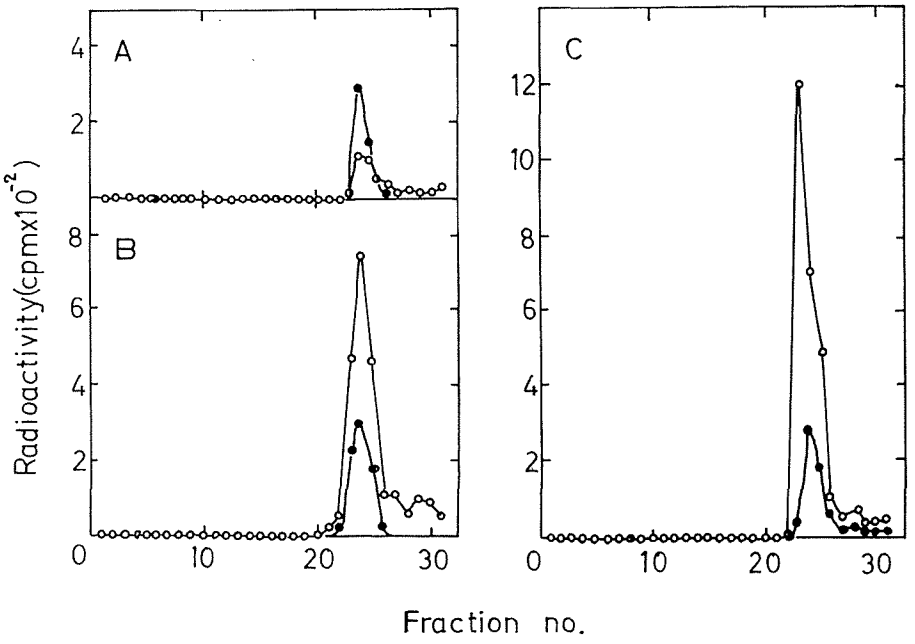

Fig. 4. Sedimentation of SDS-phenol extracts of phage infected cells.
^{3}H-thymidine was fed between 9 and 10 min, samples were taken at 10.5 min (A), 25 min (B) and 40 min (C), lysed by the method I, and ^{3}H-DNA was analyzed after phenol extraction, by neutral sucrose density gradient centrifugation with ^{32}P-labeled phage DNA as a marker as described in Materials and Methods.
—○— ^{3}H-radioactivity, and —●— ^{32}P-radioactivity.

lead us to assume that protein synthesis is needed for conversion of precursor phage DNA which is hardly extractable by Method I to mature phage DNA. Such protein(s) may well be phage specific. It is of note that in cells infected with certain *amber* mutants defective in late protein synthesis (*am N134*) or in head protein formation (*am B17*), there was no enhancement of the extractable ^{3}H-DNA even 30 min after infection (Fig. 5).

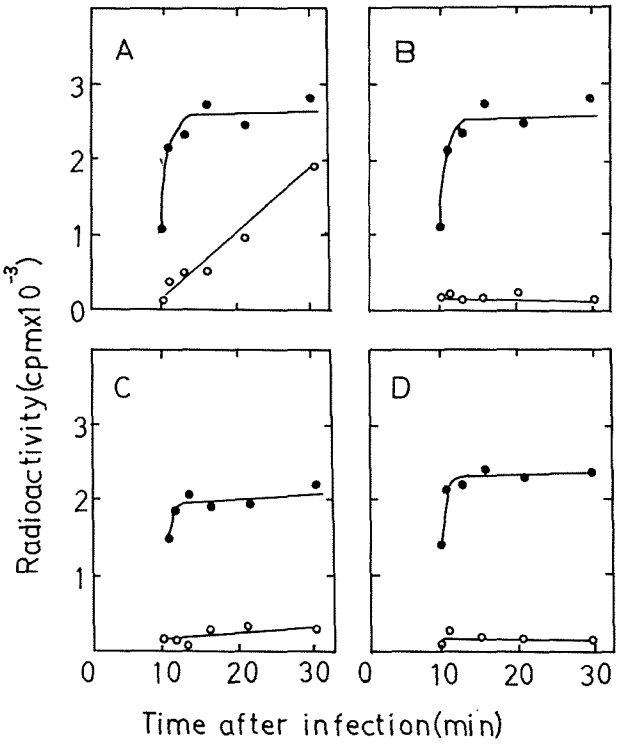

Fig. 5. Extraction of pluse-labeled ^{3}H-DNA from cells infected with *amB17* and *amN134*.
^{3}H-thymidine was fed between 9 and 10 min and ^{3}H-DNA was extracted with phenol from the lysates prepared by the method I.
A ; infection with T4w, B : infection with T4w with addition of 50μg/ml of CM at 7 min, C : infection with *amB17*, D : infection with *amN134*.
—●— total ^{3}H-DNA, and —○— phenol- extracted ^{3}H-DNA from the lysates prepared by the method I.

In contrast with Method I, Method II always gave effective extraction of the ^{3}H-DNA, *i. e.* more than 90% of intracellular ^{3}H-DNA were extractable from any samples taken at any time after the labeling. In the experiment shown in Fig. 6, cells infected with T4w were fed with ^{3}H-thymidine between

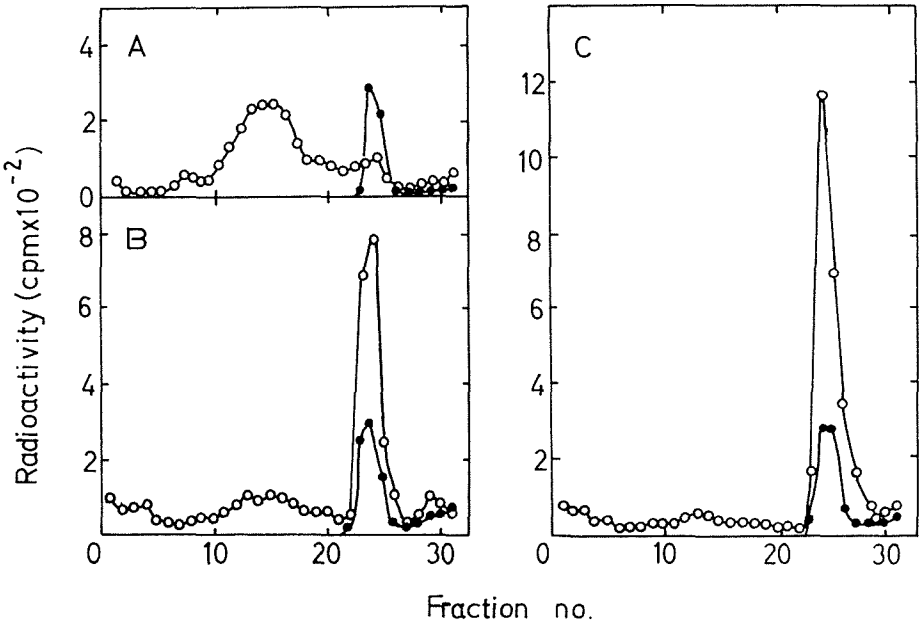

Fig. 6. Sedimentation of ^{3}H-DNA of phage-infected cells.
^{3}H-thymidine was fed between 9 and 10 min. Samples were taken at 10.5 min (A), 25 min (B) and 40 min (C). The lysate (s) were prepared by the method II and analyzed by neutral sucrose density gradient centrifugation.
—○— ^{3}H-radioactivity, and —●— ^{32}P-radioactivity

9 and 10 min and DNA extracted by Method II at 10.5, 25 and 40 min was analyzed by zone centrifugation. At 10.5 min, most of ^{3}H-DNA was in the form that sedimented twice faster than the particle DNA, in agreement with the finding of Frankel (5). At 25 min, fast sedimenting component (s) decreased and the comparable amount of ^{3}H-DNA was recovered at the position of particle DNA. At 40 min, ^{3}H-DNA was almost exclusively present at the position of particle DNA. In all casses, the recovery of ^{3}H-DNA after centrifugation was more than 90%.

From these results, we conclude that the fast sedimenting DNA (or vegetative DNA) is a precursor of the particle (or mature) DNA. We shall refer to the conversion of the fast sedimenting DNA to the showly sedimenting, mature DNA as the DNA maturation of T4 phage.

Effect of inhibitors on maturation of DNA

In conjunction with the previous observations that CM blocked production of particle type of DNA when added at 9 min (5, 12) our results strongly suggest that the drug inhibits the maturation of DNA upon addition. It is of interest to know if simultaneous synthesis of DNA and protein(s) is essential for the maturation of DNA. To explore this problem, *e40*, a lysozymeless mutant of

T4, was employed in the following experiments in order to avoid complication caused by cell lysis during experimental procedures. DNA was labeled with ^{3}H-thymidine at 14 min, its synthesis was arrested at 15 min by FUDR as described in Materials and Methods (Fig. 2), and DNA was extracted at 15 and 25 min for the sedimentation analysis. As shown in Fig. 7, maturation of the DNA occured in the absence of DNA replication. The fact that CM is a powerful inhibitor of DNA maturation was also revealed by sedimentation experiments (Fig. 8), where *e40*-infected cells were added with 50μg/ml of CM at 8 min and fed with ^{3}H-thymidine between 9 and 10 min. In the presence of CM, DNA

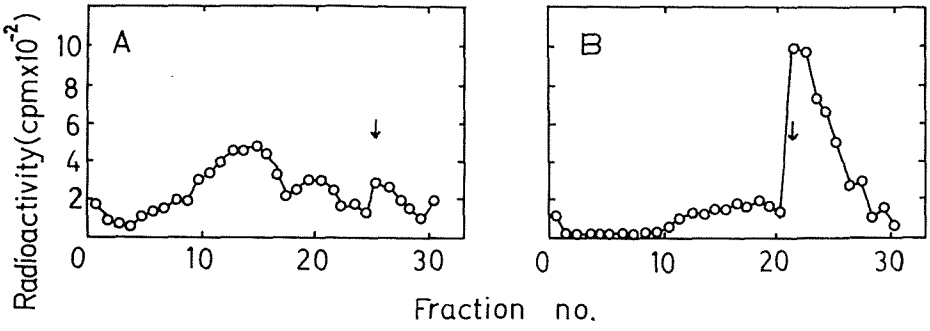

Fig. 7. Sedimentation of DNA of infected cells treated with FUDR.
Cells of *E. coli H* infected with *e40* were labeled with ^{3}H-thymidine at 14 min and DNA synthesis was inhibited by FUDR at 15 min as described in Materials and Methods. Cells were lyzed at 15.5 min (A) and 25 min (B) by the method II, and DNA was analyzed by neutral sucrose density gradient centrifugation. Arrows indicate the position of the maximum peak of ^{32}P-particle DNA.

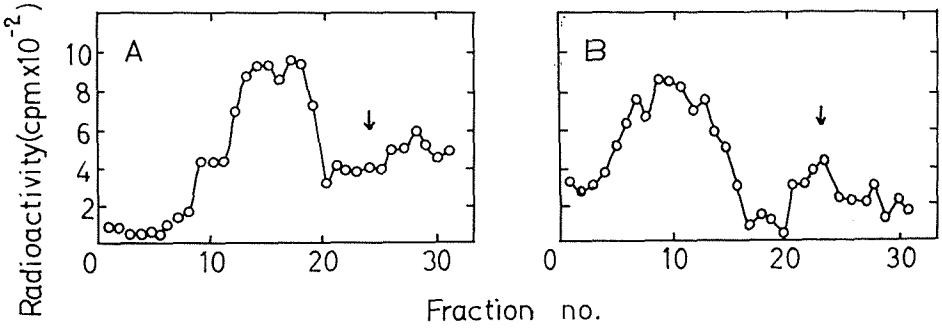

Fig. 8. Sedimentation of DNA of infected cells treated with CM.
Cells of *E. coli B3* were infected with *e40* and 50μg/ml of CM were added at 8 min. ^{3}H-thymidine was fed between 9 and 10 min, samples taken at 10.5 min (A) and 25 min (B) were lyzed by the method II, and analyzed by neutral sucrose density gradient centrifugation. Arrows in the figures indicate the position of the maximum peak of ^{32}P-particle DNA.

then formed was rapidly sedimentable and not converted to the mature DNA (Fig. 8, A and B). Of note is that longer treatment with CM makes the DNA sediment faster. Since CM inhibits the DNA maturation immediately after addition (Fig. 3), a) protein synthesis may couple with the maturation, or b) some specific protein(s) may be necessary for the maturation but extremely unstable, or alternately c) the protein(s) could be stable but may lose activity when functions, so that its intracellular quantity limits the maturation of DNA. If the last hypothesis is the case, and if sufficient amount of the protein(s) accumulates in the infected cells, the DNA maturation should continue even after addition of CM. Such a condition was furnished by inhibiting DNA replication by FUDR, and restoring DNA replication by addition of thymidine together with CM. DNA synthesis of *e40*-infected cells was inhibited by FUDR at 15 min, and restored by thymidine at 25 min. CM was added at 25 min and ^{3}H-thymidine was fed between 26 and 27 min. Comparison of sedimentation pattern of the ^{3}H-DNA taken at 28 min (Fig. 9A) with that taken at 40 min (Fig. 9B) clearly shows the last hypothesis is most likely, that is, rather stable protein is pertinent to the maturation of the precursor DNA, the amount of which possibly limits the maturation.

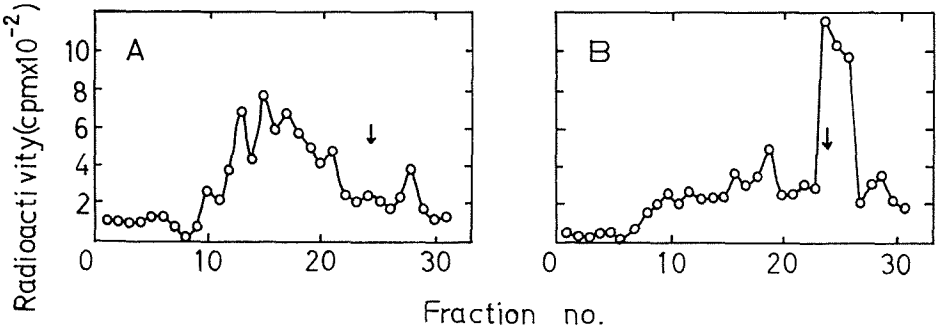

Fig. 9. Sedimentation of DNA of infected cells treated with FUDR and CM. Cells of *E. coli H* was infected with *e40* and 20μ/ml of FUDR and 20μg/ml of uracil were added at 15 min. At 25 min thymidine (2μg/ml) was added together with CM (50μg/ml). ^{3}H-thymidine was fed between 26 and 27 min, samples taken at 28 min (A) and 40 min (B) were lyzed by the method II and analyzed by neutral sucrose density gradient centrifugation. Arrows in figures indicate the position of the maximum peak of ^{32}P-particle DNA.

Alkaline zone sedimentation of DNA

In some of the above experiments DNA was centrifuged in alkaline sucrose density gradient. At an early period after chasing with unlabeled thymidine, single stranded ^{3}H-DNA was smaller than that of the particle DNA (Fig. 10, A), but at later times (after 40 minutes) when most ^{3}H-DNA maturated, the labeled DNA was of the same size with the particle DNA (Fig. 10, B). However, when CM was added at 9 min and the pulse-labeled DNA was examined at 25 min, it was larger than the particle DNA (Fig. 10, C). The size of DNA at the

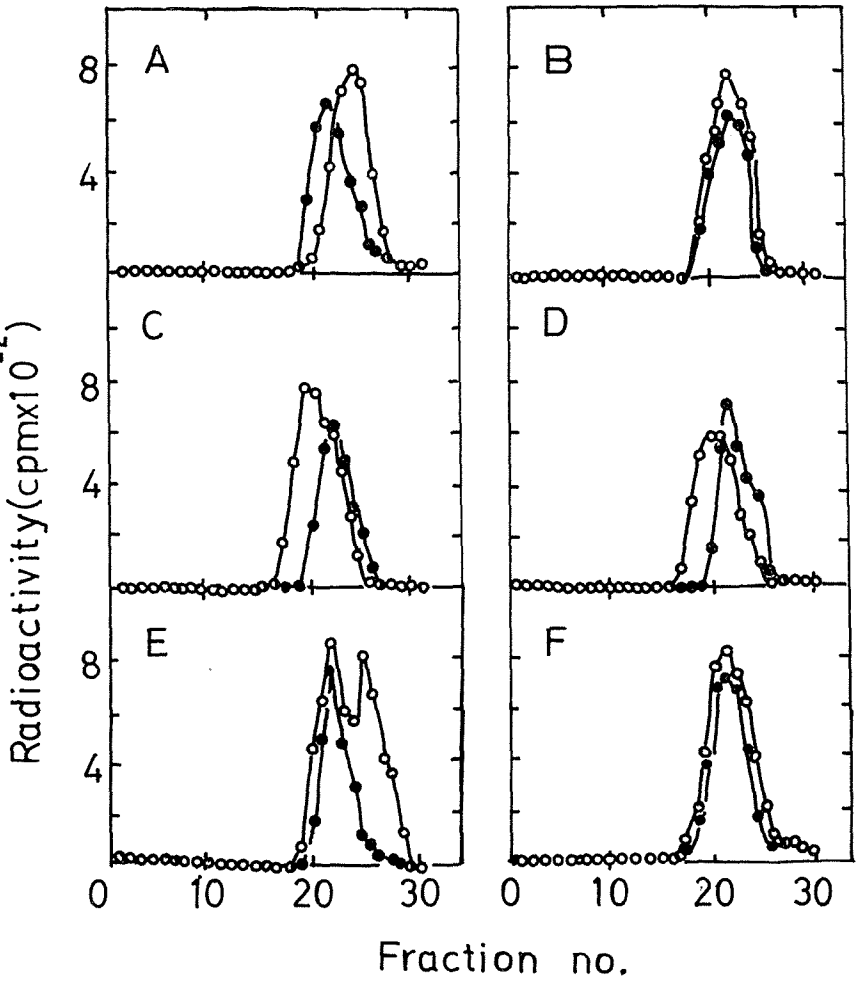

Fig. 10. Sedimentation of DNA of phage-infected cells in alkaline sucrose density gradient centrifugation.

Cells of *E. coli B3* were infected with T4w (A, B, C) or *amB17* (D) and fed with ^{3}H-thymidine between 9 and 10 min. In the case (C) CM (50μg/ml) was added at 8 min. Samples were taken at 10.5 min (A), 25 min (C, D) and 40 min (B). Cells of *E. coli H* infected with T4w were fed with ^{3}H-thymidine at 14 min and DNA synthesis was stoped by FUDR at 15 min as in Fig. 7. Samples were taken at 15.5 min (E) and 25 min (F). The lysates were prepared by the method II and analyzed by alkaline sucrose density gradient centrifugation.

—○— ^{3}H-radioactivity, and —●— ^{32}P-radioactivity

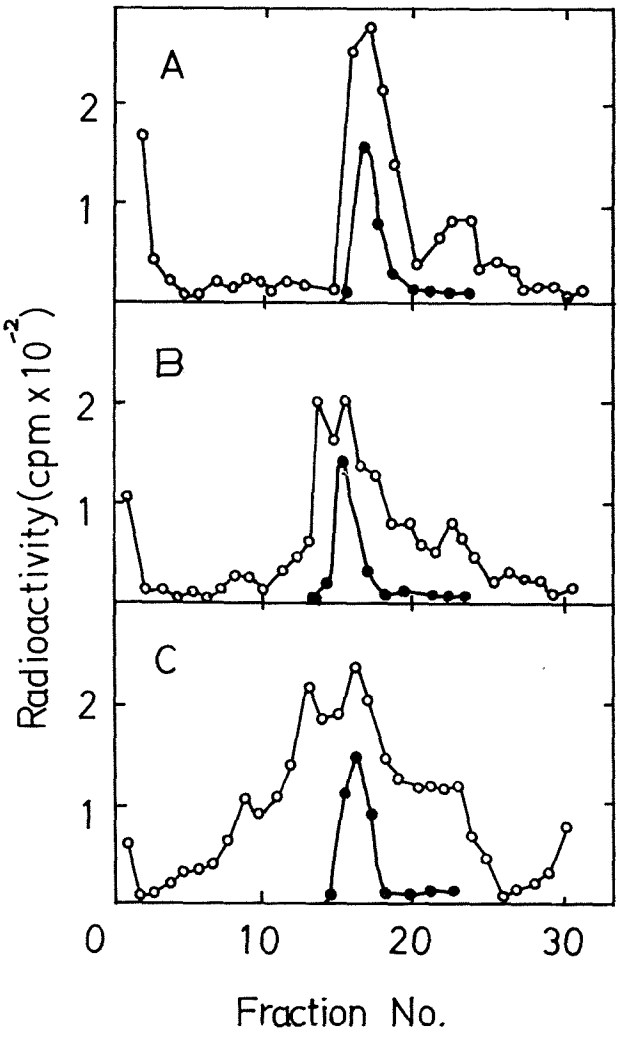

Fig. 11. Effect of CM on the formation of fast sedimenting DNA.

Cells of *E. coli B3* were infected with T4w and CM (50μg/ml) was added at 5 (A), 5.5 (B) and 6 min (C). ^{3}H-thymidine was fed between 9 and 10 min. Samples taken at 10.5 min were lyzed by the method II and analyzed by neutral sucrose density gradient sedimentation.
—○— ^{3}H-radioactivity, and —●— ^{32}P-radioactivity.

maximum peak, as determined by the equation devised by Hershey and Burgi (13), is as twice as larger than particle DNA. Similar results were obtained with a "headless" *am* mutant (Fig. 10, D). In the presence of FUDR, denatured DNA became also longer (Fig. 10, E and F).

Effect of CM on the formation of fast sedimenting DNA

As mentioned above, CM added at 8 min prevented the maturation of the replicating DNA. When the drug was added at 5.5 min, it allowed continuous synthesis of the DNA, *albeit* rather slowly, but blocked the formation of the fast sedimenting DNA ; however addition of CM later than 6 min permitted the formation of the fast sedimenting DNA, as shown in Fig. 11. These data suggest that formation of the fast sedimenting DNA is not ascribed to the continuous replication of T4 DNA and that protein formed or accumulated later than 5.5 min is relevant to the formation. Of interest in this respect is the observation by Kozinsky *et al.* (6), suggesting that recombination mechanism would develop at about 6 min after infection. Alternately, recombination within the molecule may give rise to circular structure of the DNA and continuous synthesis of DNA may occur along the circular template molecules leading to long stranded DNA. However, no one has proved the presence of circular molecule of T4 DNA.

Discussion

As shown in Fig. 6 decrease in the fast sedimenting, replicating DNA synthesized between 9 and 10 min was accompanied with increase of the comparable amount of the particle DNA. This implicates the replicating DNA is a direct precursor of the particle DNA. Polynucleotide chains of newly synthesized DNA revealed by alkaline zone centrifugation (Fig. 10) were not longer than those of the particle DNA, and became to the same size after incubation, even when DNA synthesis was inhibited by FUDR (Fig. 10). These results are not only consistent with the Frankel's (5) that replicating DNA involves regions in which polynucleotide chains are interrupted, but also suggest that such interruptions are joined during the process of maturation. In addition, present studies demonstrate that polynucleotide chains formed in the presence of CM or in restricted cells infected with some of *am* mutants defective in head formation are longer than those of the particle DNA, about twice as long as those of particle DNA, implying that the replicating DNA involves DNA molecules larger than dimer of the particle DNA. Joining of polynucleotide chains was shown to occur in the absence of DNA synthesis. Such natures of polynucleotide chains suggest that the fast sedimenting DNA is longer than the particle DNA, though the presence of DNA with tertiary structure is not excluded at present time. Similar results were obtained by Frankel (14).

DNA maturation process should, therefore, involve two steps ; measuring the length of precursor DNA and cutting the DNA in a definite length. The DNA synthesized in the presence of CM is efficiently incorporated into phage particles, after CM was removed (8, 15). This may support, though not defi-

nitely, above mechanism, since polynucleotide chains of the precursor DNA formed in the presence of CM are longer than those of the particle DNA. In view of these findings, "DNA maturation" detected by the present technique also means cutting of the precursor DNA into a definite length.

Present experiments have shown that the protein pertinent to cutting of the long DNA strand is metabolically rather stable but exhausted for a short time in the course of DNA maturation (Figs. 3 and 9). Synthesis of such protein is controlled by genes 16, 17, and 49 (16, 17). Most possible way to cut the DNA strand is as such that some of them work as enzyme, although other possibilities cannot be ruled out. Even if that is the case, the reaction is not simply catalytic, since before the protein functions components essential for capsid formation should be ready and once it catalyzes it loses activity. This might imply that the protein has enzyme activity when it associates with those components. We assumed previously that intracellular amount of such protein limited the rate of the DNA maturation. In this case any of those components may also limit the maturation, even the "enzyme" is sufficiently synthesized. According to Hosoda & Levinthal (18) half life of major subunit of the capsid from the soluble form to the insoluble is shorter than 2 min.

Since the terminal ends of T4 DNA molecules are undetermined, it must be less specific if an enzyme is involved in cutting of the DNA. It is of interest to know whether DNA maturation occurs with phages containing DNA molecule which is terminally determined. Salzman and Weissbach (19) observed that induction of cells lysogenized with λ induced synthesis of the fast sedimenting DNA molecule, which was then encapsulated into phage particles. In T3-infected cells, also, there occured synthesis of the concatenate form of DNA, which converted to the particle size of DNA (20).

Acknowledgments

We wish to thank Dr. Y. Shimura for his help in preparing this manuscript. This study was supported in part by the grant from the Jane Coffin Childs Memorial Fund for Medical Research and the Research Funds of the Ministry of Education, Japan.

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