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Nature of Deoxyribonucleic Acid Synthesis and Its Relationship to Protein Synthesis During Outgrowth of *Bacillus cereus* T

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Deoxyribonucleic acid (DNA) synthesis during early outgrowth of spores of *Bacillus cereus* T (*thy*$^-$) has been examined. ^{14}C-thymidine incorporated begins 2 to 5 min after germination and continues at a slow rate up to 30 min, after which the rate of ^{14}C-thymidine incorporation increases considerably. Early DNA synthesis up to 30 min after germination is dependent upon simultaneous protein synthesis. The examination of the stability of proteins synthesized soon after germination shows that they are susceptible to intracellular degradation. The evidence provided here indicates that protein degradation is the cause of observed dependence of DNA synthesis on simultaneous protein synthesis. The DNA synthesis occurring soon after germination is primarily a repair type synthesis which is followed by the onset of normal replication approximately 30 min after germination.

After the germination of heat-activated *Bacillus cereus* spores in an appropriate environment, the events leading to vegetative growth occur rapidly and synchronously within the population (7, 23, 29). Protein synthesis during outgrowth is dependent upon new messenger ribonucleic acid (mRNA) transcription and the repair, during germination, of a defective protein synthesizing system (6, 7, 18). The synthesis of RNA is the first to begin after germination and is closely followed by the onset of protein synthesis and by deoxyribonucleic acid (DNA) synthesis at a later time (1, 2, 7, 8, 13). Throughout outgrowth and vegetative growth, the addition of actinomycin D immediately blocks further RNA synthesis and subsequently protein synthesis as well (7, 18).

The process of DNA replication in *Bacillus subtilis* begins at a fixed point on the chromosome and proceeds sequentially along its length (10, 24, 25, 27, 28). Initiation of DNA replication requires an initiator protein, the synthesis of which must be completed before the start of replication (3, 26). However, a round of replication once initiated can be completed in the absence of protein synthesis (3, 26).

Outgrowth is an intermediate step between germination and vegetative growth. Obviously any unique biochemical event(s) that eventually leads to vegetative growth must occur during outgrowth. The present study was undertaken to investigate whether there is any change in the pattern of DNA synthesis during outgrowth or whether there is any change in the unique requirements for DNA synthesis at various stages of outgrowth.

MATERIALS AND METHODS

Organism used. A thymine-requiring auxotroph of *B. cereus* T isolated by Steinberg (17) was used in this study. Three low-caseinase mutants of *B. cereus* T (negligible activity on casein plates) used in some of the experiments were isolated by Ellen Dickinson (M.S. thesis, Univ. of Wisconsin, 1969). These mutants are referred to as mutant number 16, 17, and 18, respectively.

Growth and preparation of spores. Spores of the thymine-requiring strain were prepared as described previously (17). Spores of low protease mutants were obtained by growth and sporulation in modified G medium (4). In all cases the final spore crops were lyophilized and stored at −20 C. All outgrowth experiments with these spores were carried out at 30 C. The composition of modified G medium used for obtaining spores of low caseinase mutants has also been described (4).

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Media. The composition of thymine nutrient broth medium for the growth and sporulation of the thymine auxotroph has been described previously (17). The modified supplemented CDGS medium (11) used in germination and outgrowth experiments had the following composition in grams per liter: L-glutamic acid, 1.8; L-leucine, 0.8; L-valine, 0.3; L-threonine, 0.170; L-methionine, 0.070; L-histidine, 0.050; L-serine, 0.6; L-aspartic acid 0.4; L-argine (free base), 0.28; L-isoleucine, 0.22; L-tryptophan, 0.1; L-glycine, 0.1; L-lysine, 0.08; L-phenylalanine, 0.05; adenosine, 0.5; L-alanine, 0.1; 2-deoxyadenosine, 2.0; uracil, 0.25; guanosine (free base), 0.25; cytosine, 0.25; thymidine, 0.01; $FeSO_4 \cdot 7H_2O$, 0.0005; $CuSO_4 \cdot 5H_2O$, 0.005; $ZnSO_4 \cdot 7H_2O$, 0.005; $MnSO_4 \cdot H_2O$, 0.0305; $MgSO_4 \cdot 7H_2O$, 0.412; $(NH_4)_2SO_4$, 2.0; $CaCl_2 \cdot 2H_2O$, 0.007; glucose, 4.00; and potassium phosphate buffer, *p*H 7.0, 0.1 M final concentration. L-Alanine and adenosine were added for initiating germination, whereas addition of 2-deoxyadenosine, cytosine, guanosine, and uracil was necessary for restricting the thymidine incorporation into DNA only (11).

Measurement of incorporation of radioactive precursors. The incorporation of ^3H- or ^{14}C-thymidine and ^3H-uracil into cold trichloroacetic acid-insoluble material was used for measuring DNA and RNA synthesis, respectively. Protein synthesis was measured by incorporation of ^3H-phenylalanine into hot trichloroacetic acid-insoluble material. The details have been described previously (16, 17).

Isolation of DNA from outgrowing spores. One gram (wet pellet) of outgrowing spores was suspended in 15 ml of 0.05 M tris(hydroxymethyl)aminomethane(Tris)-hydrochloride buffer, *p*H 7.4, containing 0.15% sodium dodecyl sulfate (SDS). The suspension was transferred to a Bronwill bottle containing two volumes of acid-washed 120-μm glass beads. Outgrowing spores were ruptured by use of a Bronwill cell homogenizer. About 70% breakage was obtained after seven treatments of 15 sec each in the Bronwill homogenizer. The contents of the bottle were then transferred to a round-bottom flask. Sodium perchlorate was added to a final concentration of 1.5 M, and the concentration of SDS was raised to 1%. The suspension was heated for 5 to 10 min at 60 C in a water bath. The crude extract was deproteinized four times with equal volume of phenol saturated with 0.05 M Tris buffer, *p*H 7.4. The aqueous phase was extracted five times with ether to remove phenol. The ether was subsequently removed by blowing air over the surface of the solution. Sodium acetate was added to the aqueous phase at the final concentration of 1%, and nucleic acids were precipitated by the addition of two volumes of 95% ethanol at −20 C and storage overnight. The DNA was extracted from the precipitate of nucleic acid by repeated treatments with 0.5 ml of 3 M acetate buffer, *p*H 6.5 (9). The DNA was selectively solubilized by the acetate buffer; RNA was left in the precipitate. The DNA was recovered from the acetate extract by precipitation with two volumes of 95% ethanol at −20 C overnight. The DNA was dissolved in 1 ml of water.

Recovery and measurement of radioactivity and optical density in DNA after CsCl density gradient centrifugation. Fractions (five drops each) of the CsCl density gradient were collected from the bottom of the tube by use of a siphoning device. One milliliter of water was added to each fraction and the optical density at 260 nm (OD_{260}) was measured. Salmon sperm DNA (75 μg) was added to each fraction and the samples were chilled on ice. Cold trichloroacetic acid was added to a final concentration of 5%, and the samples were stored in ice for 1 hr. The precipitates were collected on membrane filters (Millipore, 0.45 μm pore size, 25 mm diameter), washed three times with cold 5% trichloroacetic acid, and then washed three times with 95% ethanol. The filter papers were dried and the radioactivity was monitored in a scintillation counter with 2,5-diphenyloxazole-1, 4-bis-2-(5-phenyloxazolyl)-benzene fluid.

Materials. All radioactive isotopes were obtained from Schwarz BioResearch Laboratories. Actinomycin D was purchased from Merck, Sharp and Dohme. Chloramphenicol was a product of Calbiochem.

RESULTS

Kinetics of DNA synthesis. Steinberg and Halvorson (17) have shown that no net increase in the amount of DNA estimated by colorimetric assay occurs until about 150 min of outgrowth in spores of *B. cereus* T. Thus, outgrowth appeared to take place in the absence of detectable net DNA synthesis. However, if incorporation of radioactive precursors was used as an indication of DNA synthesis, DNA synthesis could be shown to occur as early as 5 min during outgrowth. Since colorimetric assay may not be sensitive enough to detect small increases in the total amount of DNA, incorporation of radioactive precursors into cold trichloroacetic acid-insoluble material was used for measuring DNA synthesis. Figure 1 depicts the kinetics of incorporation of ^{14}C-thymidine, ^3H-thymidine, and ^3H-thymine during 90 min of outgrowth. As can be seen, DNA synthesis started within 5 min after the addition of germination stimulants (zero time) and continued at a slow rate up to 30 min, after which the rate increased by a factor of 2 to 3.

Relationship between DNA synthesis and protein synthesis. The sudden increase in the rate of isotope incorporation after 30 min suggested that certain factors were synthesized soon after germination which eventually triggered the increase in the rate of DNA synthesis. This prompted us to investigate the effect of inhibition of protein synthesis on DNA synthesis at various times during outgrowth.

Chloramphenicol at 20 μg/ml was used for inhibition of protein synthesis, and its effect on DNA synthesis was studied. Two kinds of experiments were done to study this effect. In

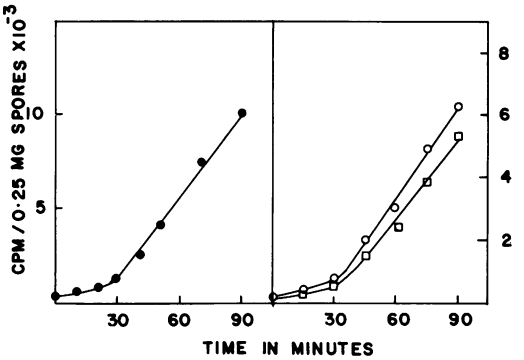

FIG. 1. *Kinetics of thymidine incorporation. Lyophilized spores were suspended in distilled water at a concentration of 2.5 mg/ml and heat-shocked for 20 min at 75 C. The suspension was centrifuged for 10 min at 5,000 × g, and the spores were resuspended in distilled water at 2.5 mg/ml. A 1-ml amount of this suspension was inoculated into modified supplemented CDGS medium (see text) containing either 10 μg of ^{14}C-thymidine (13.6 mCi/mmole), ^{3}H-thymidine (27.3 mCi/mmole), or ^{3}H-thymine (14 mCi/mmole) per ml. Final volume of the culture in each case was 10 ml, containing spores at a concentration of 0.25 mg/ml. The culture was allowed to grow at 30 C in the water bath with mild shaking. At indicated intervals, 1-ml samples were drawn from the culture, and radioactivity incorporated into cold trichloroacetic acid-insoluble material was measured. Radioactivity incorporated as counts per min per 0.25 mg of spores is plotted against time. Symbols: (●) ^{14}C-thymidine incorporation; (○) ^{3}H-thymidine incorporation; (□) ^{3}H-thymine incorporation.*

one case, heat-activated spores were germinated in the medium containing ^{3}H-thymidine and allowed to grow, and, at various times during outgrowth, the sensitivity of incorporation to chloramphenicol was examined. This experiment is referred to as continuous label experiment, and the results are presented in Fig. 2. In another kind of experiment, the spores were allowed to germinate and grow in the absence of ^{3}H-thymidine and, at various times during outgrowth, two separate 1-ml samples were drawn from the culture and labeled for 6 min with 5 μCi of ^{3}H-thymidine per ml in the presence and absence of chloramphenicol. The results are presented in Fig. 3. The results in Fig. 2 and Fig. 3 show that DNA synthesis occurring early in the outgrowth (before 30 min) is sensitive to chloramphenicol, whereas after 30 min of outgrowth DNA synthesis continues even in the presence of chloramphenicol. The time of escape from inhibition by chloramphenicol is not very clear in Fig. 2 but is evident in Fig. 3 and appears to be at approximately 30 min. The purpose here is not to pinpoint the time of escape but only to emphasize that such escape does occur. DNA synthesis during early outgrowth (before 30 min) requires simultaneous protein synthesis, since ^{3}H-thymidine incorporation stopped after transfer of culture to chloramphenicol. The DNA synthesis occurring after 30 min also requires protein synthesis which is completed during the first 30 min. This is shown by the fact that DNA synthesis did not occur even past escape point when chloramphenicol was added before 30 min (Fig. 2). This later DNA synthesis, however, was independent of simultaneous protein synthesis, as it could occur in the presence of chloramphenicol. An examination of Fig. 2 shows that ^{3}H-thymidine is incorporated at a relatively slow rate in the presence of chloramphenicol when compared to control rate (35 min and later). This can be explained by assuming that the event which makes DNA synthesis independent of simultaneous protein synthesis does not occur at the same time in all cells.

The effect of inhibition of protein synthesis on DNA synthesis during outgrowth was also

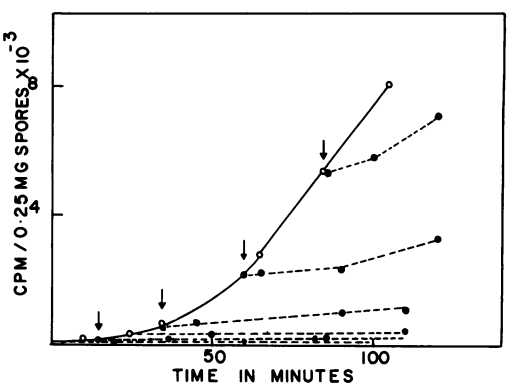

FIG. 2. *Effect of chloramphenicol on DNA synthesis. Heat-activated spores were germinated at 0.25 mg/ml in modified supplemented CDGS medium containing ^{3}H-thymidine at specific activity of 1 μCi per 10 μg per ml (see legend to Fig. 1). Final volume of culture was 50 ml and it was grown at 30 C with mild shaking. At indicated intervals, 5-ml volumes from this control culture were transferred to separate flasks containing chloramphenicol at final concentration of 20 μg/ml, and incubation was continued. At times indicated, 1-ml samples were drawn from the control and chloramphenicol-treated cultures, and incorporation of radioactivity into cold trichloroacetic acid-insoluble material was measured as described in text. The results obtained are plotted as counts/min incorporated per 0.25 mg of spores against time. Symbols: (○) incorporation of ^{3}H-thymidine in untreated culture; (●) effect of chloramphenicol on continuous incorporation when cultures were transferred to flasks containing chloramphenicol.*

examined by using puromycin at 100 μg/ml. The results obtained are identical to the results obtained with chloramphenicol.

Effect of inhibition of RNA synthesis on DNA synthesis. The dormant spore is essentially devoid of mRNA, and protein synthesis during outgrowth is dependent upon new mRNA transcription and the repair of a defective protein synthesizing system (7, 18). Inhibition of RNA synthesis during outgrowth results in the inhibition of protein synthesis 3 to 4 min later (6, 16-18). Thus, an examination of the effect of inhibitors of RNA synthesis on DNA synthesis provides a means not only to study indirectly the relationship of protein synthesis to DNA synthesis, but also to examine the role of transcription per se in DNA replication. Heat-shocked spores were germinated and grown in a medium containing 10 μg of unlabeled thymidine per ml. At various times during outgrowth, two separate 1-ml samples were drawn and pulsed with 5 μCi of ^{3}H-thymidine per ml for 5 min in the presence and absence of actinomycin D. The radioactivity incorporated into cold acid-insoluble material was measured for each sample. Per cent inhibition of DNA synthesis was calculated. The results (Fig. 4) show that in comparison to chloramphenicol and puromycin, which inhibited DNA synthesis completely during early outgrowth (before 30 min), actinomycin D caused only partial inhibition of DNA synthesis.

There are at least two explanations for the partial inhibition of DNA synthesis by actinomycin D immediately after germination. (i) DNA synthesis is dependent on protein synthesis. Spores contain some mRNA which codes for the synthesis of the necessary proteins. This possibility has been the subject of debate in recent years. (ii) There is a delay (a few minutes) in the ability of actinomycin D, added externally at zero time, to stop RNA synthesis. To test the second possibility, the kinetics of ^{3}H-uracil, ^{3}H-thymidine, and ^{3}H-phenylalanine incorporation into RNA, DNA, and protein in the presence of actinomycin D were examined (Fig. 5). DNA synthesis in the presence of actinomycin D continued for 6 min and then stopped. No RNA or protein synthesis was observed in the presence of actinomycin D. The possibility still exists that RNA and protein synthesis may have occurred for a few min from unlabeled precursor pools if we assume: (i) there was a delay of a few minutes in the ability of actinomycin D added at zero time to stop RNA synthesis; (ii) protein synthesis in the presence of actinomycin D at zero time occurred for a few minutes using unlabeled precursors from the pool. This synthesis subsequently stopped due to the degradation of previously synthesized mRNA. Inhibition of DNA synthesis after 6 min by actinomycin D (Fig. 5) would be expected since DNA synthesis during this period requires continuous protein synthesis (Fig. 2).

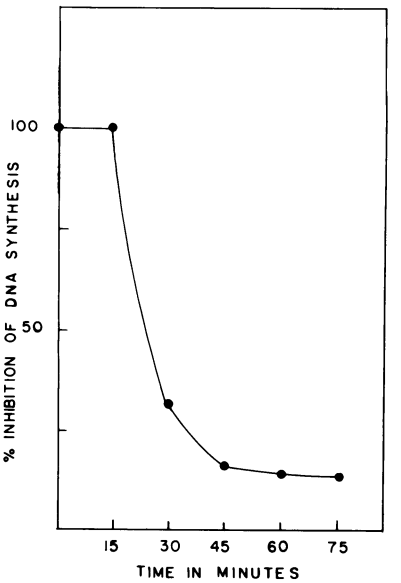

FIG. 3. *Effect of chloramphenicol on DNA synthesis. Heat-activated spores were germinated at 0.25 mg/ml in modified supplemented CDGS medium (see legend to Fig. 1). Final volume of the culture was 20 ml and incubation was at 30 C with mild shaking. At times indicated, two separate 1-ml samples were taken from the culture. One sample was pulsed for 6 min with 5 μCi of ^{3}H-thymidine in a test tube containing chloramphenicol at 20 μg/ml, while the other was pulsed in the absence of chloramphenicol in a similar way. Incorporation at the end of 6 min was stopped by adding an equal volume of cold 10% trichloroacetic acid containing 100 μg of unlabeled thymidine per ml, and radioactivity incorporated into cold acid precipitates was measured by the procedure described in the text. Results are plotted as per cent inhibition of DNA synthesis by chloramphenicol at various times during outgrowth.*

Examination of the stability of proteins synthesized early in the outgrowth of B. cereus T (thy$^-$). The unexpected dependence of DNA synthesis on simultaneous continuous protein synthesis during early outgrowth made it necessary to investigate the stability of the proteins synthesized during the early stages of outgrowth. Heat-activated spores of *B. cereus* T (*thy*$^-$) were germinated for 15 min in the medium containing ^{3}H-phenylalanine. Subsequent protein synthesis was stopped by the addition of chloramphenicol. Stability of the

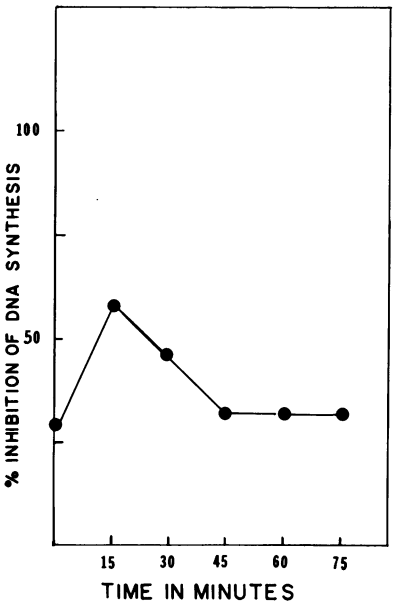

FIG. 4. *Effect of actinomycin D on DNA synthesis. Heat-activated spores were germinated at 0.25 mg/ml in modified supplemented CDGS medium containing unlabeled thymidine (10 μg/ml) and incubated at 30 C. At times indicated, two separate 1-ml samples were taken from the culture. One sample was pulsed for 6 min with 5 μCi of ^{3}H-thymidine in the presence of 15 μg of actinomycin D per ml, while the other was pulsed in the absence of actinomycin D in a similar way. Incorporation at the end of 6 min was stopped by addition of equal volume of cold 10% trichloroacetic acid containing 100 μg of unlabeled thymidine per ml. Acid precipitates were collected on membrane filters, and radioactivity incorporated was monitored as described in the text. Results are plotted as per cent inhibition of DNA synthesis by actinomycin D at various times during outgrowth.*

proteins synthesized before the addition of chloramphenicol could now be tested by allowing the culture to grow and measuring radioactivity remaining in hot acid precipitates at various times. The results of this experiment presented in Fig. 6 (*thy*$^-$) show that a significant fraction of proteins synthesized before 15 min is degraded on further incubation.

Examination of the stability of proteins synthesized early in the outgrowth of low-caseinase mutants of B. cereus T. What is the effect of degradation of newly synthesized proteins on the DNA synthesis early in outgrowth? This question can be conveniently answered by studying the mutants in which the proteins synthesized early in outgrowth are stable. The stability of newly synthesized proteins during outgrowth was examined in three low-caseinase mutants of *B. cereus* T by the procedure already described. (These mutants were isolated by Ellen Dickinson and were characterized as low-caseinase mutants on the basis of negligible proteolytic activity on casein plates.) The results are presented in Fig. 6 and show that, in contrast to the *thy*$^-$ mutant, the proteins synthesized are stable in these mutants during the period examined.

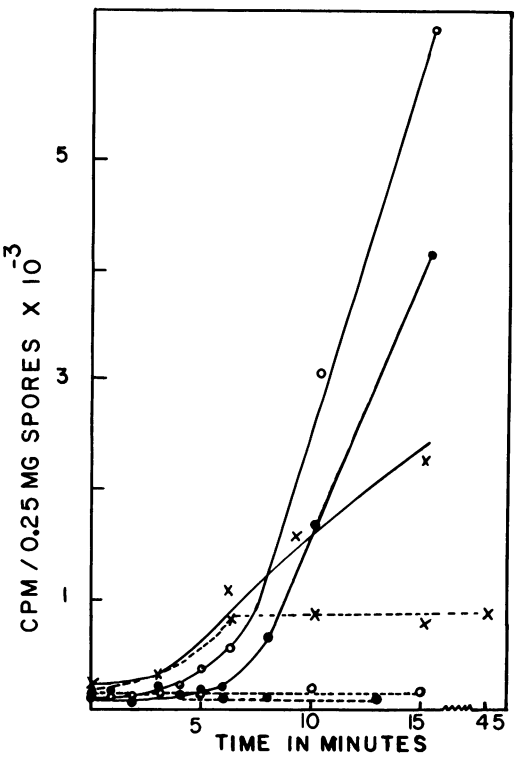

FIG. 5. *Effect of actinomycin D on the kinetics of incorporation of ^{3}H-uracil, ^{3}H-phenylalanine, and ^{3}H-thymidine. Heat-activated spores (20 min at 75 C) were germinated at a final concentration of 0.25 mg/ml in modified supplemented CDGS medium containing ^{3}H-uracil (0.5 μCi per 5 μg per ml), or ^{3}H-phenylalanine (1 μCi per 5 μg per 0.1 ml), or ^{3}H-thymidine (1 μCi per 10 μg per ml). Cultures were incubated at 30 C. At indicated times, 1-ml samples were taken, and radioactivity incorporated was measured as described in the text. Effect of actinomycin D was studied by adding actinomycin D (15 μg/ml) at zero time. Symbols: (O) ^{3}H-uracil incorporation in absence of actinomycin D; (--O--) ^{3}H-uracil incorporation in presence of actinomycin D; (—●—) ^{3}H-phenylalanine incorporation in absence of actinomycin D; (--●--) ^{3}H-phenylalanine incorporation in presence of actinomycin D; (—×—) ^{3}H-thymidine incorporation in absence of actinomycin D; (--×--) ^{3}H-thymidine incorporation in presence of actinomycin D.*

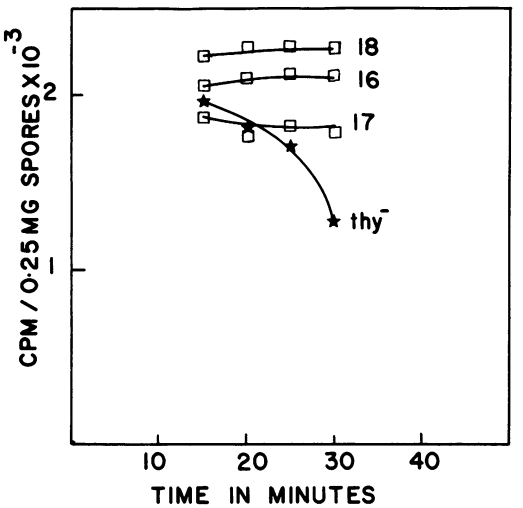

FIG. 6. *Stability of the newly synthesized proteins. Results of four different experiments are compiled in this figure. In each case, heat-activated spores were germinated at 0.25 mg/ml in modified CDGS medium containing ^{3}H-phenylalanine at 8 μCi per 50 μg per ml. Incorporation of ^{3}H-phenylalanine was allowed to continue for 15 min, at which time further incorporation was stopped by adding chloramphenicol (20 μg/ml). At indicated times after addition of chloramphenicol, 1-ml samples were taken and radioactivity incorporated into hot trichloroacetic acid precipitates was measured by procedure described in the text. Results are plotted as counts/min remaining per 0.25 mg of spores at various times after addition of chloramphenicol. Symbols: (★) Bacillus cereus T (thy⁻); (□) low-caseinase mutants number 16, 17, and 18.*

Effect of inhibition of protein synthesis on DNA synthesis during 30 min of outgrowth in low-protease mutants. The effect of inhibition of protein synthesis on DNA synthesis was studied in each of the low-protease mutants and the results obtained were identical. Figure 7 depicts the effects of chloramphenicol on DNA synthesis in mutant 16. It is observed that in this mutant, in which newly synthesized proteins are not degraded, DNA synthesis is not inhibited by chloramphenicol at 10, 20, or 30 min.

Nature of DNA synthesis. Only the initiation of replication is dependent upon prior protein synthesis. Thus, a round of replication, once initiated, will not be inhibited by the subsequent addition of chloramphenicol. On the basis of this criterion, initiation of DNA replication in *B. cereus* T seems to occur approximately 30 min after germination. However, a significant amount of ^{3}H-thymidine incorporation into trichloroacetic acid-insoluble material was observed before 30 min. Furthermore, the DNA synthesis occurring before 30 min had several unusual properties. (i) Thymidine incorporation started almost immediately after germination without any significant lag. (ii) The rate of ^{3}H-thymidine incorporation was lower during the first 30 min than it was after 30 min. (iii) ^{3}H-thymidine incorporation was dependent upon continuous protein synthesis. Thus, it appeared unlikely that the DNA synthesis observed before 30 min represented normal replication. Instead, the possibility emerged of a repair-like DNA synthesis which is completed before the initiation of normal DNA replication. Therefore, the nature of DNA synthesis during outgrowth was examined from the point of view of repair or replication.

Basis of distinction between DNA repair and DNA replication. If a culture of spores were labeled during outgrowth with ^{3}H-thymidine in the presence of an excess of unlabeled bromo-deoxyuridine (BUdR), then after the DNA was isolated, sheared, and centrifuged to equilibrium in a CsCl density gradient, the following patterns would be seen. The DNA syn-

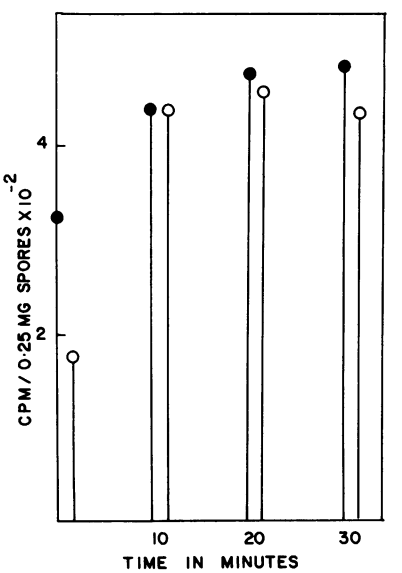

FIG. 7. *DNA synthesis in low-protease mutant in presence of chloramphenicol. Effect of chloramphenicol on DNA synthesis in low-caseinase mutant was examined in a pulse-labeling experiment by procedure described in legend to Fig. 3. Results are plotted as counts/min incorporated per 0.25 mg of spores in a 6-min pulse with 5 μCi of ^{3}H-thymidine at various times during outgrowth (at 0, 10, 20, and 30 min), corrected for zero-time incorporation (150 counts/min per 10.25 mg of spores). Symbols: (●) in the absence of chloramphenicol; (○) in the presence of 20 μg of chloramphenicol per ml.*

thesis resulting from repair would have all the radioactivity associated with only a slightly heavier density (due to limited BUdR incorporation) than the normal density [light-light DNA (LL)], whereas, in the case of replication, the radioactivity would be associated with a true hybrid density [light-heavy (HL)] DNA (25).

Nature of DNA synthesis immediately after germination. The spores of *B. cereus* T (*thy*$^-$) were germinated and incubated for 20 min in the presence of BUdR (20 μg/ml) in medium containing ^{3}H-thymidine at the specific activity of 1 μCi per 2 μg per ml. The DNA was isolated and centrifuged to equilibrium in a CsCl density gradient (9). The OD and radioactivity profile of the gradient was determined as described above. As shown in Fig. 8, all of the radioactivity is either associated with the LL OD peak or displaced slightly to the heavier density as expected for repair synthesis, but considerably lighter than the density (1.727 g cm^{-3}) expected for HL hybrid DNA (17). Thus, although the occurrence of some DNA replication cannot be eliminated, DNA synthesis occurring soon after germination is primarily a repair type synthesis.

Nature of DNA synthesis occurring after 35 min of germination. The incorporation of ^{3}H-thymidine into cold acid-insoluble material is not inhibited by addition of chloramphenicol at or after 30 min during outgrowth of *B. cereus* T (*thy*$^-$) (Fig. 2). The nature of DNA synthesis at 35 min during outgrowth was examined to see if it represented true replication. The spores were germinated and incubated for 35 min in the presence of 2 μg of unlabeled thymidine per ml. At 35 min, unlabeled BUdR (20 μg/ml) and ^{3}H-thymidine (1 μCi/ml) were added to the culture, and incubation was continued until 55 min. The DNA was isolated and analyzed in a CsCl density gradient as described previously. The results obtained are presented in Fig. 9. As can be seen, essentially all the radioactivity is associated with the region of hybrid density. Thus, it can be concluded that DNA synthesis occurring after 35 min of outgrowth is mostly replication.

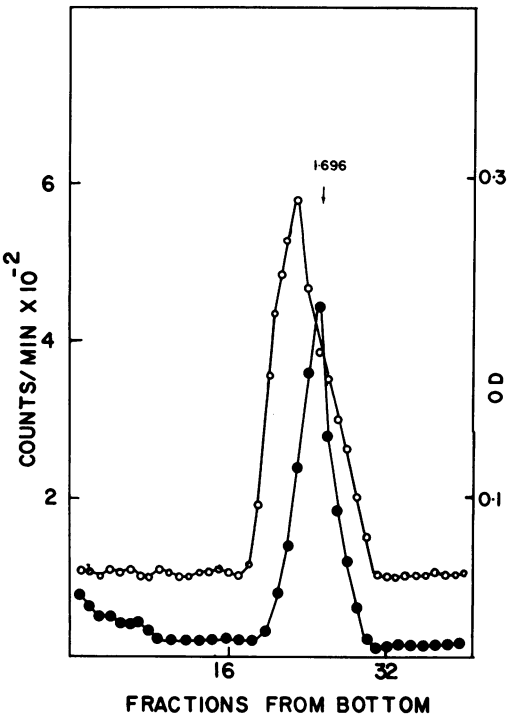

FIG. 8. *CsCl density gradient centrifugation of BUdR DNA synthesized during 20 min of outgrowth. Spores of thymine-requiring mutant (500 mg) were heat-activated and germinated in 300 ml of supplemented CDGS medium containing ^{3}H-thymidine (1 μCi per 2 μg per ml) and 20 μg of unlabeled BUdR per ml. Growth was arrested after 20 min by pouring the culture over −20 C ice. Cells were collected by centrifugation, and DNA was isolated by procedure described in the text. Approximately 80 μg of DNA was added to 3 ml of CsCl (starting density of 1.70 g/ml). The density gradient was formed by centrifugation in a Spinco model L2-65 ultracentrifuge for 50 hr (42,000 rev/min for 17 hr, then reduced to 35,000 rev/min for additional 33 hr) with an SW50L swinging bucket rotor at 25 C. Fractions (five drops) were collected from the bottom of the tube by a siphoning device. Optical density (●) and radioactivity (○) in the fractions were determined as described in the text. Density gradient (position of 1.696 g/ml peak) was determined by measuring the refractive index of several undiluted fractions with a Bausch and Lomb refractometer.*

DISCUSSION

The timing of initiation of DNA synthesis can be determined either by chemical estimation directly or by use of a radioactive isotope. However, significant differences are observed in the timing of initiation of DNA synthesis determined by these separate methods. In general, DNA synthesis can be shown to occur very early in the outgrowth by the use of radioactive isotopes, whereas net increase in the amount of DNA (when estimated chemically) occurs only later (17). The chemical method may not be sensitive enough to detect small increases in the amount of DNA.

When *B. cereus* T (thy$^-$) spores were germinated in medium containing ^{3}H-thymidine in the presence of excess unlabeled BUdR and

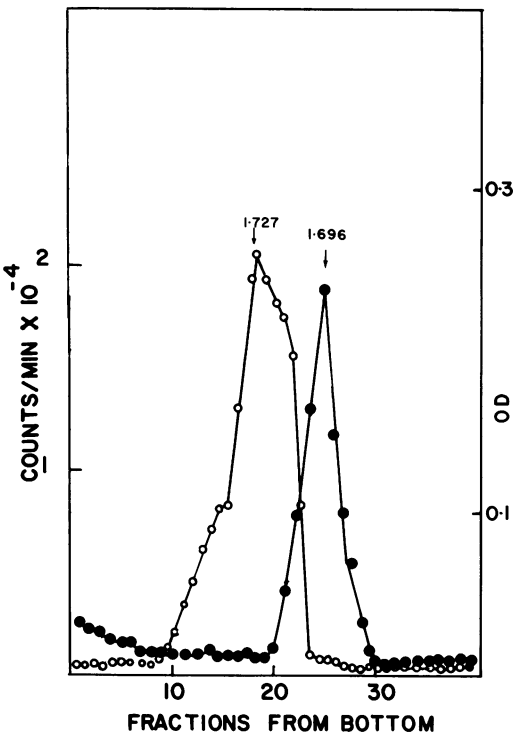

FIG. 9. *CsCl density gradient centrifugation of BUdR DNA synthesized between 35 and 55 min of outgrowth. Procedure used was same as for Fig. 5, except that BUdR (20 μg/ml) and ^{3}H-thymidine (1 μCi/ml) were added at 35 min, and growth was arrested at 55 min. DNA was isolated and analyzed as described for Fig. 5. Symbols:* (●) *optical density;* (○) *radioactivity.*

DNA was isolated after 20 min and analyzed by equilibrium centrifugation in a CsCl density gradient, a significant amount of radioactivity was associated with the DNA of parental density. This DNA synthesis was concluded to have resulted from repair synthesis. Although the possibility that some replication also occurred during this period cannot be ruled out, a small amount of repair synthesis was observed by Yoshikawa during outgrowth of *B. subtilis* (26). However, a detailed study of the extent of repair synthesis during outgrowth of *B. subtilis* led Wake to suggest that repair, if it occurs at all during outgrowth, is too small to be detected successfully (25). A similar observation was made by Steinberg and Halvorson for *B. cereus* T (17). The experiments reported in this paper are not adequate to estimate accurately the extent of DNA repair synthesis as compared to replication during outgrowth of *B. cereus*. However, the results do show that the repair is largely completed before the onset of normal DNA replication. Furthermore, the radioactivity incorporated into LL DNA in the presence of BUdR between 0 and 20 min is only 0.4% of the radioactivity incorporated into hybrid density DNA between 35 and 55 min under similar conditions. Since the first round of replication is completed only at about 180 min (G. Spiegelman, *personal communication*) and repair does not occur after initiation of replication, the total quantity of repair synthesis compared to a round of replication would be insignificant.

It has been shown previously that single-strand breaks in DNA caused by ionizing radiation of *B. subtilis* spores can be repaired during germination, and the repair is completed before the onset of normal DNA replication (22; Tanooka et al., U.S.-Japan Coop. Sci. Prog.: joint meeting on genetic and biochemical dormancy, p. 38, 1970). In this paper we have presented evidence for a small amount of repair (probably different from rejoining of single-strand breaks) which is also largely completed before the onset of normal replication.

The nature of the DNA in dormant spores is as yet unclear. Several workers have shown that DNA in spores has a spore-specific state which makes it resistant to ultraviolet and ionizing radiations (12, 14, 19, 20). It is conceivable that the change of DNA to the spore-specific state during the process of sporulation probably results in some changes in the secondary structure of DNA. These changes in the secondary structure can be recognized by repair enzymes during germination (5). Thus, it appears that the advantages acquired by the spore in terms of resistance to radiation are compensated by the need of repair during outgrowth. The early DNA synthesis occurring soon after germination and characterized as repair was found to be dependent upon continuous protein synthesis. The observed degradation of newly synthesized proteins could explain the dependence of DNA synthesis on continuous protein synthesis, if one or more of the enzymes required for DNA synthesis during early outgrowth is active, only when its concentration is above a critical limit. If we assume that the amount of one or more of these required enzymes normally found in early outgrowth represent the critical limit, then any degradation of this enzyme(s) would make the DNA synthesis dependent on *simultaneous* continuous protein synthesis. In the low-caseinase mutants, in which the newly synthesized protein(s) is not degraded, the amount of this enzyme(s) would remain above some critical level. Under such circumstances DNA synthesis in these mutants should no longer be

dependent on simultaneous continuous protein synthesis, and this is exactly what we find.

On the basis of results obtained in this study we propose that one or more of the proteins required for DNA synthesis during early outgrowth is synthesized soon after germination and is present in some limiting concentrations. Furthermore this protein(s) is subject to intracellular degradation, which in turn results in the observed dependence of DNA synthesis on simultaneous continuous protein synthesis during 30 min of outgrowth. This limiting enzyme(s) may include precursor biosynthetic enzymes, endonuclease, or ligase essential for repair. Recently Tanooka and his coworkers have shown that several kinases involved in DNA metabolism are very low or almost absent in spores of *B. subtilis* and are synthesized during outgrowth, reaching a maximum at the onset of replication (21). It is possible that some of these enzymes may be limiting in the case of *B. cereus* also.

Several workers have shown that initiation of DNA replication in *B. subtilis* requires the synthesis of an initiator protein which is absent in spores (3, 26). The synthesis of initiator protein has been shown to occur at a certain time during outgrowth (26). In the case of *B. cereus* a similar situation exists since addition of chloramphenicol will inhibit DNA synthesis only when added within the first 30 min of outgrowth. Thus, synthesis of initiator protein(s) occurs soon after germination, and replication is initiated at approximately 30 min after germination.

Besides protein synthesis, transcription may also be required for the initiation of DNA replication. Evidence is available which suggests that a physical alteration of DNA at the origin, which occurs during transcription, is required for the initiation of DNA replication in λ (15). Lark has observed a similar situation in *Escherichia coli* (*personal communication*). When the effect of actinomycin D on DNA synthesis during outgrowth of *B. cereus* was studied, it was observed that addition of this antibiotic at zero time will inhibit initiation of DNA replication at 30 min (Fig. 5). However, it is not certain whether the inhibition of DNA synthesis by actinomycin D is due to the inhibition of transcription per se or due to the inhibition of protein synthesis.

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