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# Timing of Enzyme Synthesis During Outgrowth of Spores of *Bacillus cereus*

## II. Relationship Between Ordered Enzyme Synthesis and Deoxyribonucleic Acid Replication

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Experiments were carried out to determine whether, during outgrowth of bacterial spores, deoxyribonucleic acid (DNA) replication provided the basis by which ordered transcription was controlled. During outgrowth, significant DNA synthesis does not occur until just prior to the onset of cell division. However, incorporation of radioactive DNA precursors into DNA is observed within 5 to 10 min after the initiation of germination. By employing a thymine-requiring auxotroph and $^3$H-bromodeoxyuridine, this incorporation appears to be a result of DNA replication and not repair synthesis. For the following reasons it was concluded that, during outgrowth, transcriptional processes were not ordered by DNA replication. (i) In a thymine auxotroph, thymine addition did not alter the periodicity of induced $\alpha$-glucosidase and histidase synthesis during outgrowth. (ii) DNA synthesis was inhibited 80% by 5-fluoro-2'-deoxyuridine (FUdR), and, after a 5-min lag, completely by mitomycin C, but these inhibitors exerted a differential effect on induced histidase synthesis. Enzyme synthesis was insensitive to FUdR but was inhibited by mitomycin C, presumably as a result of cross-linking of the complementary DNA strands.

Masters and Pardee (16) demonstrated that in synchronized vegetative cells of *Bacillus subtilis* the timing of basal enzyme synthesis can be correlated with the time of replication of the corresponding portion of the genome. It has also been shown that the periodic steps of enzyme synthesis occurring during cell division in yeast are directly related to structural gene positions on the chromosome (Tauro and Halvorson, *in press*). Finally, induced enzymes of the *lac* operon of *Escherichia coli* appear in the same sequence as the gene order found within this operon (1).

The simplest model which one could invoke to explain these results is that transcription for these enzymes follows, or is governed by, deoxyribonucleic acid (DNA) replication. Several attempts have been made to clarify this relationship. In synchronous cultures of yeast (9) and bacteria (4, 19), it has been observed that species of ribonucleic acid (RNA) having different nucleotide composition are synthesized at different intervals of the cell cycle. However, these results do not permit a decision to be made as to whether RNA transcription is ordered *by* or *during* DNA replication.

Studies on the relationship between DNA replication and enzyme synthesis have yielded conflicting results. By employing thymine-requiring auxotrophs of *E. coli*, Hanawalt and Wax (10) and Overath and Stange (17) concluded that DNA synthesis was not necessary for enzyme induction. Experiments with the fission yeast *Schizosaccharomyces pombe* have demonstrated that initiation of enzyme steps in synchronous cultures occurs during periods when there is no net DNA synthesis (2). In contrast with these results, Knutsen (13) observed that the inducibility of nitrate reductase during the cell cycle of *Chlorella* showed a direct relationship to DNA replication. It has also been suggested that, in *E. coli*, DNA replication is required for the transcription of repressed genes (10).

As yet, no experimental system has permitted a direct test to be made to discover whether ordered transcription is governed by DNA replication. The ordered appearance of enzymes

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during outgrowth of bacterial spores (21, 22) provides a unique system with which this model may be tested. Since the initial phase of outgrowth occurs synchronously in the absence of *net* DNA synthesis (28) and is directly dependent on new messenger RNA transcription (14, 21), the question can be posed directly.

The results presented in this paper show that the timing of enzyme synthesis during outgrowth is not ordered by DNA replication. However, it appears that a "physical modification" in the DNA (such as that produced by mitomycin cross-links) can impair those transcriptional processes which are necessary for sustaining order.

## MATERIALS AND METHODS

*Organism. B. cereus* strain T, hereafter referred to as the wild type, was used in this study. A thymine-requiring auxotroph of this strain was isolated by modifying the methods of Wachsman et al. (25) and Farmer and Rothman (7).

*Growth and preparation of spores.* Spores of the wild type were obtained as previously described (24). Spores of the thymine-requiring strain were prepared in the Thy-NB medium described below. The mutant required a high aeration rate in order to sporulate, and, for growth on a rotary shaker, a maximum of 250 ml of culture was used in a 2-liter Erlenmeyer flask. For larger volumes, the aeration rate was 1.3 to 1.5 liters of air per min per liter of medium. A 5% inoculum of a 30-hr growth was employed so that by 33 to 36 hr almost 95% of the culture consisted of cells in the late stages of sporulation. The final spore crops were lyophilized and stored at −20 C. All outgrowth experiments with these spores were carried out at 30 C.

The procedures for heat-activation and initiation of germination have been described (24).

*Media.* The Thy-NB medium for sporulation of the thymine-requiring auxotroph contained (in grams per liter): nutrient broth, 8; glucose, 4; thymine, 0.2; and the same minerals as in the modified G medium (24) except that $(NH_4)_2SO_4$ was reduced to 2 g per liter. G-Peptone broth used in the isolation of the thymine-requiring auxotroph contained (in grams per liter): yeast extract, 4; Difco peptone, 5; glucose, 4; 0.01 M potassium phosphate buffer, *p*H 7.0; and the same minerals as in the chemically defined growth and sporulation medium with glucose (CDGS-Glu; 22). All other media have been described (22).

*Isolation of thymine-requiring auxotroph.* A heat-activated spore suspension (2 mg/ml) was prepared, and 0.1-ml samples were inoculated into tubes containing 4.9 ml of CDGS-Glu plus 200 μg of aminopterin per ml. To initiate germination, the medium was supplemented with 50 μg of L-alanine per ml. The cultures were incubated on a rotary shaker (30 C) for several days. From tubes that showed growth, colonies were picked and subcultured twice in the same medium. When the second subculture had reached log-phase growth, it was diluted 100-fold into G-Peptone broth. This culture was grown to an optical density at 600 mμ of 0.4. A 1,000-fold dilution was made and suitable samples were spread on CDGS-Glu agar containing aminopterin and thymine, each at 200 μg/ml. The plates were incubated for 3 days and the largest colonies were picked and tested for their thymine or thymidine requirements.

*DNA isolation.* The procedure used combined the methods of Marmur (15) and Douthit and Halvorson (5). Germinated cells were collected by centrifugation and were washed twice with a modified SCET buffer (5) containing: 1 M NaCl, 1% cetyltrimethylammonium bromide (w/v), $10^{-2}$ M ethylenediaminetetraacetate, and $10^{-2}$ M tris(hydroxymethyl)aminomethane-chloride (*p*H 8.0). The cells were frozen at −20 C, lyophilized, and ground (10 min at room temperature) with a mortar and pestle, employing a 1:2 (w/w) ratio of cells to glass beads (20-μ diameter; Minnesota Mining and Manufacturing Co., St. Paul, Minn.). The cells and beads were added to a solution of hot SCET buffer (65 C) and were incubated for 10 min. (A ratio of 20 ml of buffer per gram of the lyophilized cell preparation was employed.) Solid pronase (B grade, Calbiochem, Los Angeles, Calif.) was added (1 mg/ml, final concentration), and the incubation was continued for an additional 4 hr. The solution was cooled to 37 C, powdered pronase was added again (1 mg/ml), and incubation was continued for an additional 12 hr. The remainder of the procedure was described by Douthit and Halvorson (5).

*Measurement of incorporation of radioactive precursors.* The procedures for measuring protein synthesis have been described (22). For measuring nucleic acid synthesis, two procedures were used. (i) The incorporation of saturating concentrations of $^{14}$C-uracil or $^{3}$H-thymidine into cold trichloroacetic acid precipitates was employed as an indication of RNA or DNA synthesis. Samples of known volume were removed at intervals after the addition of the radioactive isotope and immediately added to an equal volume of cold 10% trichloroacetic acid containing the pyrimidine or nucleoside (100 to 200 μg/ml). The samples were stored at 0 C for 1 to 2 hr, and the precipitates were collected on membrane filters (0.45-μ pore size, 25 mm in diameter; Millipore Corp., Bedford, Mass.), washed two times with 3 ml of cold 5% trichloroacetic acid containing the nonradioactive pyrimidine or nucleoside (50 μg/ml), and then washed two times with 3 ml of 95% ethyl alcohol to remove trichloroacetic acid. The filter papers were dried and monitored for radioactivity as previously described (22). (ii) $^{14}$C-adenine incorporation into alkali-stable materials was used to estimate DNA synthesis. In these experiments, germination was initiated by the addition of the spore suspension to a medium containing 15 μg of inosine plus 270 μg of L-alanine per ml (W-T. Hsu, Ph.D. Thesis, University of Illinois, 1963). A known volume of the culture was removed at intervals after the addition of the isotope and immediately added to an equal volume of cold 2 N NaOH containing $^{12}$C-adenine (200 μg/ml). The samples were allowed to stand on ice for 1 hr; then

they were placed in a water bath at 35 C and incubated for 20 to 24 hr. Samples were subsequently chilled on ice and neutralized by the addition of cold 2 N HCl. Salmon sperm DNA (75 μg) was added as carrier material and the DNA was precipitated by the addition of cold trichloroacetic acid (5% final concentration). Samples were allowed to stand at 0 C for 1 to 2 hr; the precipitates were collected, washed, and dried, and their radioactivity was measured as described above. Proof that adenine incorporation represents DNA synthesis will be given later (Fig. 5 and Table 1).

*Recovery and measurement of radioactivity in DNA after CsCl density gradient centrifugation.* Fractions of the CsCl density gradient were collected from the bottom by using a commercial piercing unit (Buchler Instruments, Inc., Fort Lee, N.J.). Salmon sperm DNA (100 μg) was added to each fraction and the samples were chilled on ice. Cold trichloroacetic acid was added to a final concentration of 5%, the samples were stored at 0 C for 1 to 2 hr, and the radioactivity of the precipitates was determined as described.

*Enzyme determinations.* The procedures for enzyme assays have been described (22).

*Materials.* Maltose was obtained from Matheson, Coleman & Bell (East Rutherford, N.J.) and was purified as previously described (22). Mitomycin C was purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). Stock solutions (0.5 mg/ml) were prepared in 0.067 M phosphate buffer, *p*H 6.8. All experiments with mitomycin C were carried out in subdued light. Chloramphenicol was a gift of Parke, Davis and Co. (Detroit, Mich.). Aminopterin was obtained from K & K Laboratories (Jamaica, N.Y.). It was prepared as a stock solution of 2 mg/ml and was sterilized by filtration. 5-Fluoro-2'-deoxyuridine (FUdR) was a gift of Hoffmann-La Roche, Inc. (Nutley, N.J.). Cetyltrimethyammonium bromide and *p*-nitrophenyl-α-D-glucoside were purchased from General Biochemicals (Chagrin Falls, Ohio). Salmon sperm DNA was obtained from Calbiochem (Los Angeles, Calif.). $^{3}$H-thymidine, $^{14}$C-adenine, and $^{14}$C-uracil were purchased from New England Nuclear Corp. (Boston, Mass.), $^{3}$H-adenine from Schwarz Bio Research, Inc. (Orangeburg, N.Y.), and $^{3}$H-bromodeoxyuridine ($^{3}$H-BUdR) was obtained from Nuclear-Chicago Corp. (Des Plaines, Ill.).

## RESULTS

When an estimation of DNA synthesis was made during outgrowth by employing a colorimetric assay, no net increase in DNA was observed until 150 min (Fig. 1). The first signs of cell division occurred at 220 to 230 min. By this criterion, outgrowth appeared to take place in the absence of net DNA synthesis. However, if radioactive thymidine was used to estimate DNA synthesis (Fig. 2), a significant incorporation was observed at any time during outgrowth; this could be inhibited by mitomycin C, a potent inhibitor of DNA synthesis (23). In contrast to *B. subtilis* (26, 30), this incorporation was not inhibited by chloramphenicol (W. Steinberg, M.S. Thesis, University of Wisconsin, 1965), thus indicating that these spores contain a DNA polymerase and proteins essential for the initiation of replication.

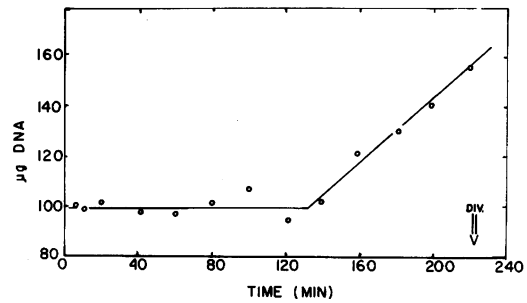


FIG. 1. *Colorimetric assay of net DNA synthesis in outgrowth. Spores were heat-activated and germinated at 100 μg/ml in CDGS-Glu (22) supplemented with adenosine (0.5 mg/ml). At the indicated intervals, 100-ml samples were collected and chilled in an ice-salt bath and trichloroacetic acid was then added to a final concentration of 5%. The samples were centrifuged (10 min at 10,000 × g) and resuspended in 15 ml of 5% trichloroacetic acid. After 2 hr at 0 C, the cells were collected by centrifugation and resuspended in 1 ml of 5% trichloroacetic acid. The preparation was heated at 100 C for 25 min and the precipitate was removed by centrifugation. DNA was estimated on a sample of the supernatant fraction by the diphenylamine reaction (3). Salmon sperm DNA was used as a standard.*

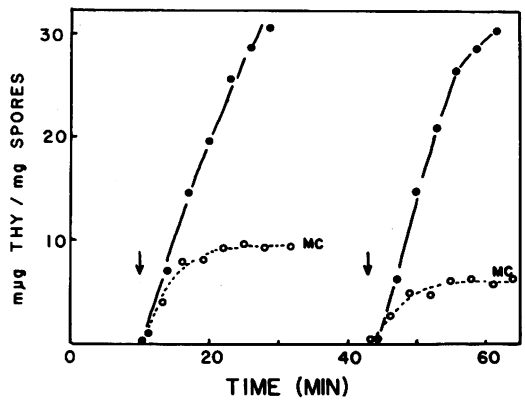


FIG. 2. *Effect of mitomycin C on DNA synthesis during outgrowth. Heat-activated spores were germinated at 500 μg/ml in CDGS-His (22) containing 0.5 mg of adenosine per ml and 100 μg of L-alanine per ml as germinating agents. At the points indicated (10 and 43 min), samples were withdrawn from the main growth flask and added to flasks containing either $^{3}$H-thymidine, 1.43 μc per 2.9 μg per ml (●), or $^{3}$H-thymidine plus mitomycin C, 10 μg/ml (○). Samples (0.6 ml) were removed at the indicated intervals and the radioactivity incorporated into cold trichloroacetic acid precipitates was measured.*

To characterize further the nature of the material into which tritiated thymidine was incorporated, the following experiment was carried out. Heat-activated spores were germinated in the presence of $^3$H-thymidine and incorporation was stopped at the end of 30 min. The DNA isolated from the germinated spores was analyzed for radioactivity distribution by equilibrium sedimentation in CsCl (Fig. 3). The results show that all the radioactivity coincides with the optical density (at 260 m$\mu$) profile. This material was alkali-stable, deoxyribonuclease-sensitive, and ribonuclease-resistant, indicating that $^3$H-thymidine incorporation was a result of DNA synthesis. However, since the amount of DNA synthesized within this period represented only a few per cent of the total DNA present in the spore (8), it was important to know whether or not this synthesis represented DNA replication or DNA synthesis caused by extensive repair processes (20). To resolve this question, and at the same time establish a system with which the model for ordered enzyme synthesis could be tested, a thymine-requiring auxotroph of *B. cereus* T was isolated by using aminopterin as a selective agent.

Figure 4 shows the effect of thymine concentration on the capacity of spores of the thymine-requiring strain to form visible colonies. After 3 days of incubation, only 1 of $10^4$ spores was able to form a colony in the absence of added thymine. Since wild-type spores formed colonies on the minimal medium within 24 hr, the mutant

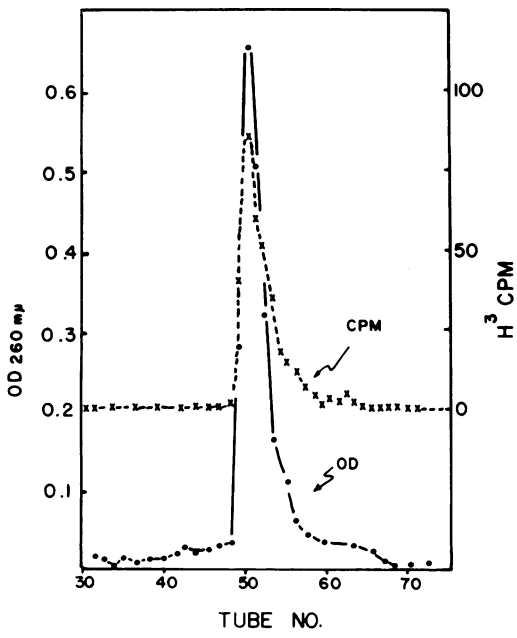


FIG. 3. *CsCl density gradient of DNA synthesized during outgrowth. Spores (300 mg) were heat-activated and germinated at 3 mg/ml in CDGS-Glu (22) containing 0.5 mg of adenosine per ml and $^3$H-thymidine (6 μc per 3 μg per ml). Incorporation was stopped at 30 min by pouring the culture over crushed ice maintained at −20 C. Cells were collected by centrifugation and the DNA was isolated (see Materials and Methods). A total of 200 μg of a mixture of $^3$H- and $^1$H-thymidine DNA was added to a CsCl solution (1.71 g/ml) to a final volume of 3 ml. After centrifugation in the Spinco (model L) ultracentrifuge for 65 hr at 33,000 rev/min at 25 C, with the SW-39 swinging-bucket rotor, fractions of 5 drops each were collected into tubes (12 by 75-mm) containing 0.8 ml of dilute saline citrate (15). The radioactivity and optical density (260 mμ) were measured for each tube.*

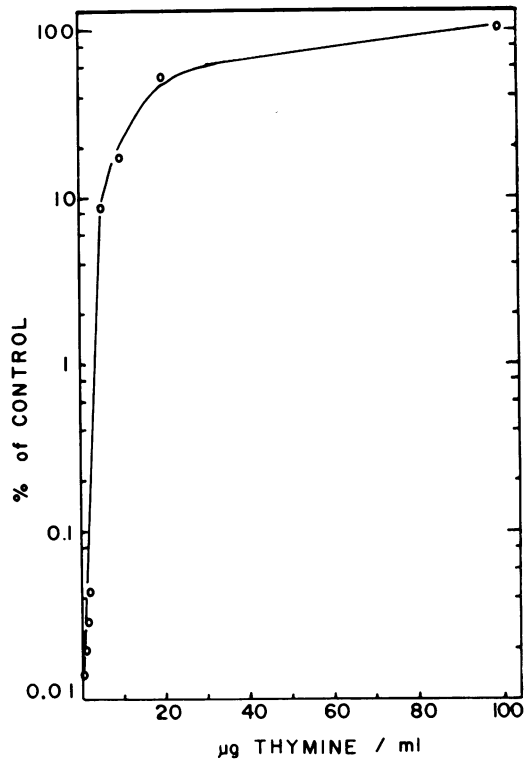


FIG. 4. *Effect of thymine concentration on the growth of the thymine-requiring auxotroph. Appropriate dilutions of heat-activated spores of the thymine-requiring auxotroph were plated on CDGS-Glu (22)-agar containing the indicated concentrations of thymine. The medium was supplemented with L-alanine and adenosine (each at 100 μg/ml), and the plates were incubated at 30 C for 3 days. Each point represents an average value of the number of colonies formed on five replicate plates prepared for each thymine concentration. The control contained 200 μg of thymine per ml.*

was classified as phenotypically *thy*$^-$. However, the possibility existed that the mutant might be "leaky," by having some alternate pathway for thymidylate synthesis, an endogenous supply of thymine, or some system of continuous DNA breakdown and reutilization of end products (12, 27). To test for the extent of "leakiness," the following experiment was carried out (Table 1 and Fig. 5). Germinated spores of the mutant were inoculated into a minimal medium containing $^3$H-adenine. The culture was divided into two portions and thymidine was added to only one. $^3$H-adenine incorporation was stopped at the end of 6 hr of growth. The DNA was isolated from both cultures and analyzed for its specific activity both before and after CsCl density gradient centrifugation. Table 1 shows that the specific activity of the two DNA samples differed by a factor of 11 to 14, thus indicating a significant difference in the amount of DNA synthesized in the presence and absence of exogenous thymidine. The results also suggest that the mutant might be "leaky." However, it is possible that cells undergoing thymineless death will degrade their DNA, and that the surviving cells would give the appearance of "leaky" mutants by feeding on these products.

The extent of DNA synthesis in mutant and wild-type spores was estimated by $^{14}$C-adenine incorporation at saturating concentrations (Fig. 6). In the absence of added thymidine, the rate of DNA synthesis in the mutant was only 4% of the control value (*thy*$^-$ spores plus exogenous thymidine), and only 6% of the wild-type rate (no thymidine added). Although these are minimal estimates, we believe that they approximate net DNA synthesis, since saturating levels of isotope were employed and significant amounts of DNA synthesis (10%) would have been seen in the chemical assays of DNA. In the presence of thymidine, the total amount of DNA synthesized after 60 min of outgrowth represented only 8% of that initially present in the spore. These results accounted for the failure of the colorimetric assay to detect net DNA synthesis.

*Is DNA synthesis during early outgrowth repair or replication?* After we showed that spores of the thymine-requiring strain synthesized DNA at only 4% of the control rate, it was possible to test whether the incorporation of DNA precursors during outgrowth represented repair (turnover) synthesis or replication. When a culture of spores was labeled during outgrowth with $^3$H-BUdR, then, after the DNA was isolated and sheared, the following patterns were seen. DNA synthesis resulting from repair had all the radioactivity associated with the normal density (light-light DNA) as analyzed by equilibrium sedimentation in CsCl, whereas DNA synthesis resulting from replication had all the radioactivity associated with the hybrid density (light-heavy) DNA (18).

To test this model, spores of the thymine-requiring auxotroph were germinated and incubated for 60 min in the presence of $^3$H-BUdR. The DNA was isolated and centrifuged to equilibrium in a CsCl density gradient (Fig. 7). Essentially all of the radioactivity is associated with the hybrid region, and this region represents only 6% of the total DNA. Thus, during out-

TABLE 1. *Test of thymine mutant for "leakiness"*

| Time | DNA source | $OD_{260}$ units | Treatment | Counts/min | Counts/min to OD |
|---|---|---|---|---|---|
| Before CsCl density gradient centrifugation[a] | +Thymidine | 0.426 | $OH^-$ hydrolysis[b] | 29,200 | 6,850 |
| | | | Cold trichloroacetic acid | 28,400 | — |
| | | | Hot trichloroacetic acid[c] | 8 | — |
| | −Thymidine | 0.145 | $OH^-$ hydrolysis | 545 | 482 |
| | | | Cold trichloroacetic acid | 700 | — |
| | | | Hot trichloroacetic acid | 11 | — |
| After CsCl density gradient centrifugation[d] | +Thymidine | 0.112 | Cold trichloroacetic acid | 7,880 | 70,600 |
| | −Thymidine | 0.156 | Cold trichloroacetic acid | 1.018 | 6,510 |

[a] The DNA was prepared as described in Fig. 5 and was dialyzed against 2,000 volumes of standard saline-citrate (15) for 2 hr with three buffer changes.

[b] A sample of the dialyzed preparation was added to NaOH (1 N, final concentration) and incubated for 24 hr at 30 C. The sample was cooled to 4 C and then neutralized with cold 1 N HCl. Salmon sperm DNA (100 μg) and 5% trichloroacetic acid were added, and the radioactivity was recovered and assayed.

[c] A sample of the dialyzed preparation was added to an equal volume of 5% trichloroacetic acid and heated at 95 C for 30 min. The sample was cooled to 4 C, and 100 μg of salmon sperm DNA was added. The solution was adjusted to 5% trichloroacetic acid (final concentration), and the radioactivity was recovered and assayed.

[d] See legend to Fig. 5. Fractions containing DNA were pooled.

growth, the low rate of DNA synthesis represented replication and not repair synthesis.

*Relationship between DNA replication and RNA transcription.* Thymine-mutant spores were germinated in the presence and absence of exogenous thymine, and RNA synthesis was estimated

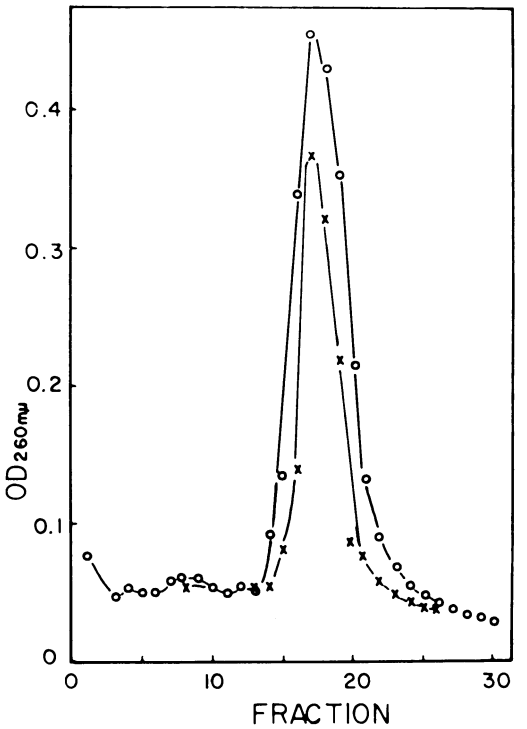


FIG. 5. *CsCl density gradient of $^{3}$H-adenine-labeled DNA made in the presence of and absence of exogenous thymine. Thymine mutant spores (600 mg) were heat-activated and germinated in 600 ml of 0.05 M phosphate buffer, pH 7.0, containing 0.5 mg of adenosine and 100 μg of L-alanine per ml. After 10 min, the germinated spores were collected by centrifugation and were washed twice at room temperature with phosphate buffer (0.06 M, pH 7.0). Spores were resuspended in CDGS-Glu (22), and the culture was divided into 200- and 400-mg portions. $^{3}$H-adenine (2 μc per 20 μg per ml) was added to both flasks, and thymidine (200 μg/ml) was added only to the flask containing 200 mg of spores (○). Growth was stopped after 6 hr by immersing the culture flasks in an ice-salt bath. Cells were harvested by centrifugation and their DNA was isolated (see text). Appropriate portions of the two DNA samples (60 to 80 μg of DNA) were added to a solution of CsCl (1.70 g/ml, final volume 3 ml). After centrifugation in a Spinco (model L2-65) ultracentrifuge for 17 hr at 42,000 rev/min at 25 C, with the SW-50L swinging-bucket rotor, fractions of 10 drops each were collected in tubes (12 by 75 mm) containing 0.4 ml of dilute saline-citrate (14), and the optical density (260 mμ) was determined for each tube.*

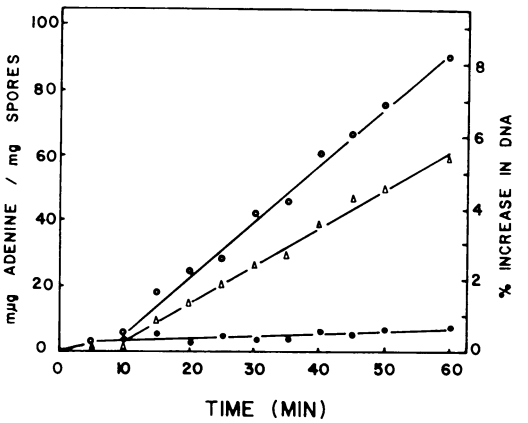


FIG. 6. *DNA synthesis during outgrowth of wild-type and thymine mutant spores. Heat-activated spores of the thymine-requiring auxotroph and wild-type strains were germinated at 500 μg/ml in CDGS-Glu (22) supplemented with L-alanine (270 μg/ml), inosine (15 μg/ml), and saturating levels of $^{14}$C-adenine (0.63 μc per 34.5 μg per ml). Samples (0.6 ml) were removed at the indicated intervals during outgrowth. The radioactivity incorporated into alkali-stable material in the absence and presence of thymidine (200 μg/ml) was measured as described in the text. The results are plotted as millimicrograms of adenine incorporation per milligram of spores and as the percentage increase in total DNA (7). (○) Thymine mutant spores plus thymidine; (△) wild-type spores, no thymidine; (●) thymine mutant spores, no thymidine.*

by the incorporation of $^{14}$C-uracil. Figure 8 shows that uracil incorporation into RNA is not affected by the absence of thymine. The level of RNA synthesized during the first 40 min is the same as that for wild-type spores germinated in the absence of exogenous thymine (Steinberg, M.S. Thesis). Since there was a 25-fold difference in the rate of DNA synthesis when the mutant was germinated in the absence and presence of thymine (Fig. 6), it was concluded that the absence of DNA replication has no effect on RNA synthesis. The majority of the RNA synthesized during the early stages of outgrowth is labile to actinomycin D (22). Details of these experiments will be published elsewhere.

*Ordered transcription and DNA replication.* Outgrowth of spores of *B. cereus* T is characterized by an ordered synthesis of both basal and induced enzymes, which is transcriptionally controlled (20, 22). This raises the question of whether DNA replication serves as the mechanism for ordering transcription. An examination of the timing of induced enzyme synthesis in the thymine mutant eliminates this possibility (Fig. 9). Both α-glucosidase and histidase have the same periodicity of enzyme synthesis in the

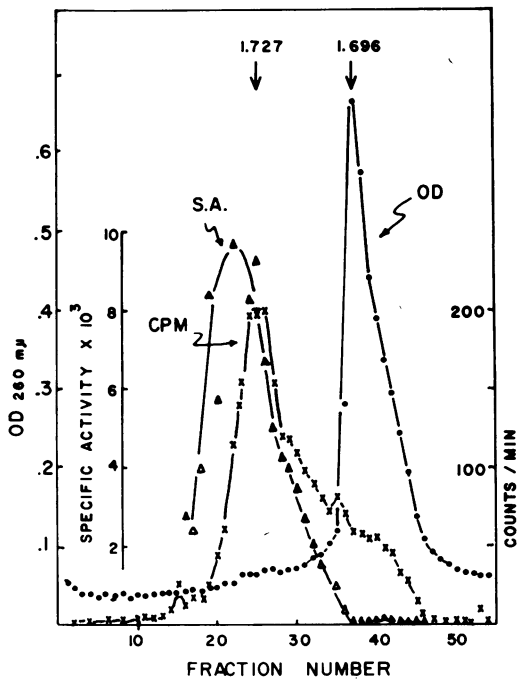


FIG. 7. *CsCl density gradient centrifugation of $^3$H-bromodeoxyuridine ($^3$H-BUdR) DNA synthesized during outgrowth. Thymine mutant spores (500 mg) were heat-activated and germinated in 500 ml of CDGS-Glu (22) containing adenosine (0.5 mg/ml), L-alanine (100 μg/ml), and saturating concentrations of $^3$H-BUdR (0.95 μc per 15 μg per ml). Growth was arrested after 60 min by immersing the culture flask in an ice-salt bath. Cells were collected by centrifugation and DNA was isolated as described in the text. Approximately 100 μg of the $^3$H-BUdR-labeled DNA was added to 3 ml of CsCl (starting density of 1.70 g/cc). The density gradient was formed as described for Fig. 5, except that after 17 hr at 42,000 rev/min the speed was reduced to 32,000 rev/min, and centrifugation was continued for an additional 14 hr. Fractions of 5 drops each were collected and the radioactivity and optical density (260 mμ) were measured for each tube. The density gradient was determined by measuring the refractive index of several undiluted fractions with a refractometer (Bausch & Lomb, Inc., Rochester, N.Y.*

presence and absence of added thymine. The timing of enzyme synthesis is the same as that observed for wild-type spores, even though dormant spores of the mutant have a six- to sevenfold higher level of α-glucosidase. Similarly, thymidine addition does not alter the time in which repressed α-glucosidase appeared (Fig. 10). From these results, it is clear that ordered transcription is not dependent upon DNA replication.

An attempt was made to ascertain how ordering might be sustained in the absence of DNA replication. The DNA inhibitors, mitomycin C, which crosslinks complementary DNA strands (21), and FUdR, a competitive inhibitor of thymidylate synthetase (10), were employed in this study. Figure 11 shows that when mitomycin C was added at zero-time, it prevented the induction of the second step of α-glucosidase, yet had little or no inhibitory effect on the initial period of enzyme synthesis. In contrast, when mitomycin C was added at 29 min, or at intervals thereafter, induced histidase synthesis was terminated within a period of 4 to 5 min (Fig. 11 and 12). The time required for its effect on enzyme synthesis was the same as that previously found for inhibition of DNA synthesis (Fig. 2). On the other hand, the initial rates of RNA and protein synthesis were not inhibited (W. Steinberg, Ph.D. Thesis, University of Wisconsin, 1967).

The effect of mitomycin C on induced histidase and the second burst of α-glucosidase appeared to be in conflict with the conclusions reached with the thymine auxotroph; i.e., DNA replication was not necessary for enzyme timing. The use of FUdR, however, provided an experimental sys-

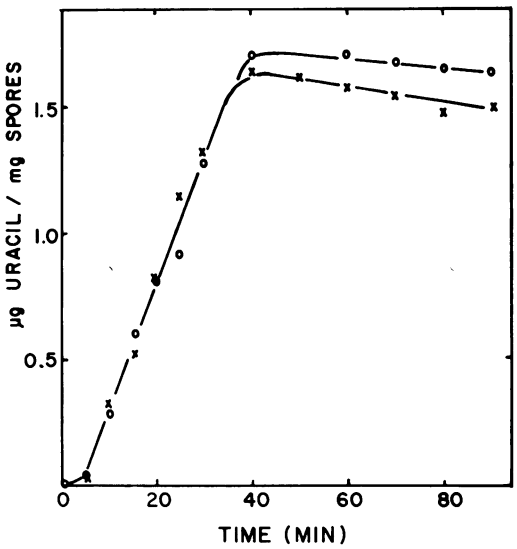


FIG. 8. *RNA synthesis during outgrowth of spores of the thymine mutant. Heat-activated spores were germinated at 0.5 mg/ml in CDGS-His-Mal (22) containing L-alanine (100 μg/ml), adenosine (0.5 mg/ml), and $^{14}$C-uracil (0.5 μc per 5.19 μg per ml). At the indicated intervals, 0.6-ml samples were removed and added to an equal volume of cold 10% trichloroacetic acid containing $^{12}$C-uracil (100 μg/ml), and the radioactivity incorporated into cold trichloroacetic acid precipitates in the presence (○) or absence (×) of exogenous thymine (100 μg/ml) was determined.*

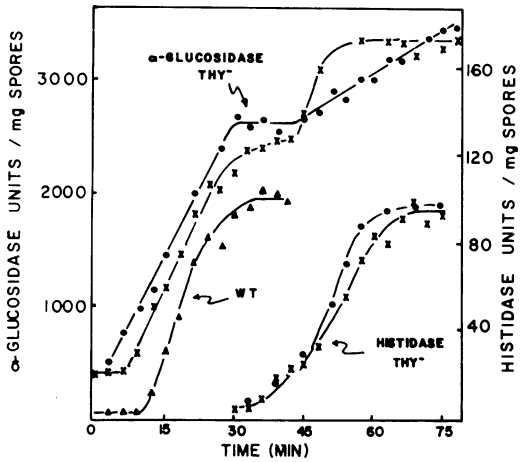


FIG. 9. *Effect of thymine addition on the timing of induced enzyme synthesis during outgrowth of spores of the thymine auxotroph. Spores of the wild-type and thymine mutant were heat-activated and germinated at 500 μg/ml in CDGS-His-Mal (22) containing L-alanine (100 μg/ml) and adenosine (0.5 mg/ml). At the indicated intervals during outgrowth, 5-ml samples were removed and assayed for α-glucosidase and histidase. Thymine-requiring mutant: (×) induced α-glucosidase and histidase, thymine (200 μg/ml) added at zero-time; (●) induced α-glucosidase and histidase, no thymine added. Wild type: (▲) induced α-glucosidase, no thymine added.*

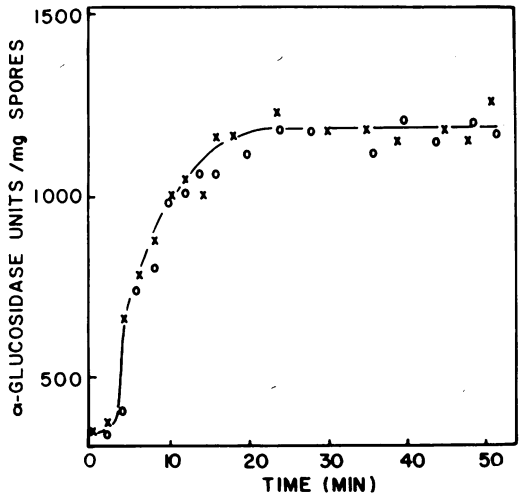


FIG. 10. *Effect of thymidine addition on the timing of repressed α-glucosidase synthesis during outgrowth of spores of the thymine auxotroph. Spores of the thymine mutant were heat-activated and germinated at 500 μg/ml in CDGS-Glu (22) containing L-alanine (100 μg/ml) and adenosine (0.5 mg/ml). At the indicated intervals during outgrowth, 5-ml samples were removed and assayed for α-glucosidase. (×) Repressed α-glucosidase, thymidine (200 μg/ml) added at zero-time; (○) repressed α-glucosidase, no thymidine addition.*

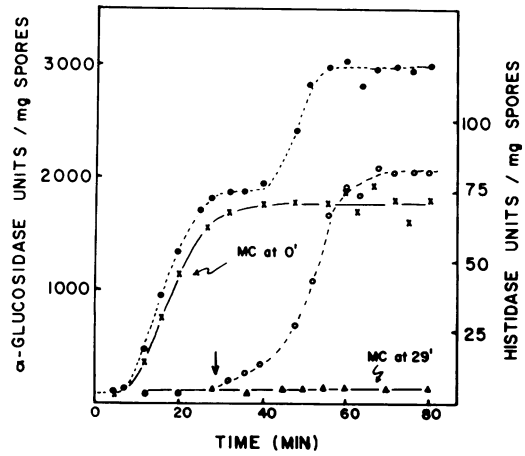


FIG. 11. *Effect of mitomycin C on the timing of enzyme synthesis. Heat-activated spores were germinated at 0.5 mg/ml in CDGS-His-Mal (22) containing adenosine (0.5 mg/ml) and L-alanine (100 μg/ml). At intervals during outgrowth, 5-ml samples were removed and assayed for α-glucosidase and histidase. (●) Induced α-glucosidase, control; (×) induced α-glucosidase, mitomycin (10 μg/ml) added at zero-time; (○) maltose-repressed histidase, control; (▲) maltose-repressed histidase, mitomycin (10 μg/ml) added at 29 min.*

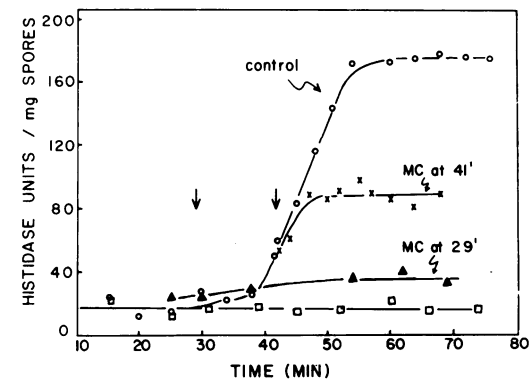


FIG. 12. *Effect of the time of addition of mitomycin C on histidase induction. Heat-activated spores were germinated at 0.5 mg/ml in CDGS-His or CDGS-His-Mal (22). Both media contained adenosine (0.5 mg/ml) and L-alanine (100 μg/ml). At the points indicated (29 and 41 min), a portion of the control culture was added to a flask containing mitomycin C (10 μg/ml), and, at intervals thereafter, 5-ml samples were removed and assayed for histidase. (○) Induced histidase, control; (□) maltose-repressed histidase, mitomycin C added at 29 min; (▲) induced histidase, mitomycin C added at 29 min; (×) induced histidase, mitomycin C added at 41 min.*

tem which would mimic the conditions employed with the *thy*⁻ strain (i.e., inhibition of DNA synthesis at the enzymatic level). The effect of FUdR on DNA synthesis is shown in Fig. 13. The rate of DNA synthesis in the presence of FUdR was only 20% of the control value. However, under these conditions, histidase could be induced to the control level (Fig. 14). Apparently, the effect of mitomycin C on enzyme synthesis was the result of some "physical modification" in the DNA (presumably cross-linking), while inhibition of DNA synthesis by interfering with an enzymatic mechanism (FUdR) had no effect on enzyme timing.

## DISCUSSION

It is concluded from the present evidence that the initiation and maintenance of ordered transcription during outgrowth is not governed by DNA replication. However, whether the repeated rounds of enzyme synthesis are influenced by dichotomous or late DNA replication has not yet been established.

The previous paper (22) demonstrated that enzyme inducibility during outgrowth was limited to specific time intervals. It therefore appears that a mechanism which alters the capacity of DNA to serve as a template for RNA transcription may be involved in the ordering process. In line with this, the effect of cross-linking DNA with mitomycin C indicates that transcription requires an unmodified, native DNA structure. Since the chromosome contained within the bacterial spore may be in the completed form (29), two simple models can be proposed by which ordered transcription can be controlled. First, the state of the DNA within the spore may change in some polarized manner during outgrowth. This could serve as a "signal" for initiating transcription. The alteration may be polarized in one direction, or several initiation points can be present. A second possibility is that the order of transcription is dictated by some temporal (clock) mechanism.

In *B. subtilis*, DNA synthesis is not observed until 120 to 150 min after the initiation of germination (26, 28), even though DNA polymerase and enzymes involved in the synthesis of deoxyribonucleotides are present in the dormant spore (6). In addition to these enzymes, the possibility exists that DNA repair enzymes are also present (30). However, repair synthesis may be below the threshold level necessary for detection (26). Since enzymes required for DNA synthesis are initially present in the spore, it is of interest that during early outgrowth of *B. subtilis* no replication occurs, and in *B. cereus* T there is only a slow rate

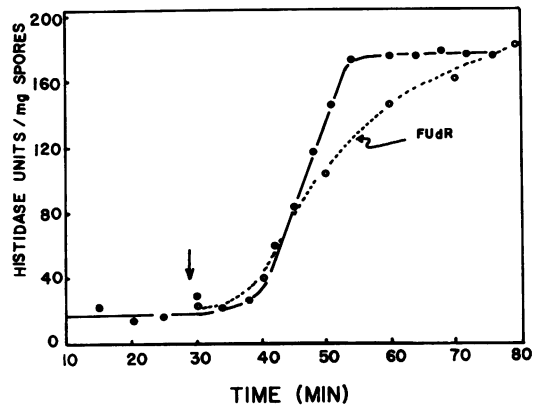


FIG. 14. *Effect of 5-fluoro-2'-deoxyuridine (FUdR) on histidase induction during outgrowth. Heat-activated spores were germinated at 0.5 mg/ml in CDGS-His (22) containing adenosine (0.5 mg/ml) and L-alanine (100 μg/ml). Uridine (100 μg/ml) was added to the culture at 28 min after the initiation of germination (●). At 29 min, part of this control culture was added to a flask containing FUdR (10 μg/ml) (○). Samples (5 ml) were removed from both flasks at the indicated intervals and assayed for histidase.*

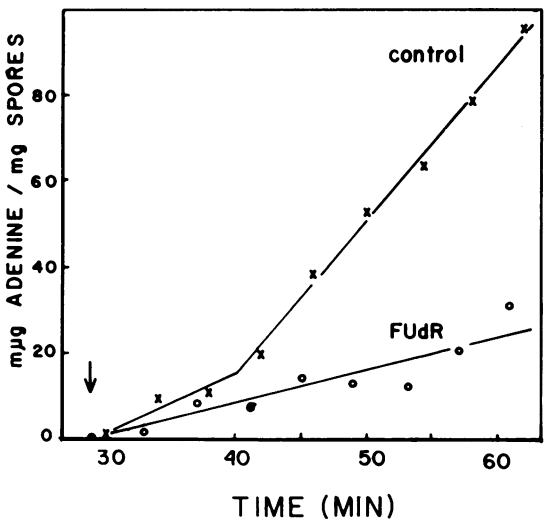


FIG. 13. *Effect of 5-fluoro-2'-deoxyuridine (FUdR) on $^{14}$C-adenine incorporation into DNA. Heat-activated spores were germinated at 500 μg/ml in CDGS-His (22) supplemented with L-alanine (270 μg/ml) and inosine (15 μg/ml) as germinating agents. At 28 min after the initiation of germination, uridine (100 μg/ml) was added to the culture. At 29 min, portions of the culture were added to flasks containing either $^{14}$C-adenine (0.9 μc per 50 μg per ml) (×) or $^{14}$C-adenine plus FUdR (10 μg/ml) (○). Samples (0.6 ml) were removed at the indicated intervals during outgrowth, and the radioactivity incorporated into alkali-stable materials was determined.*

of DNA replication until a period just prior to the first cell division. At present, the factors limiting DNA synthesis during outgrowth are unknown; however, since we are dealing with a system of ordered transcription, it is not unlikely that some protein essential for DNA replication is synthesized in outgrowth.

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