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Macromolecular Synthesis During Microcycle Sporogenesis of *Bacillus cereus* T

IAN MACKECHNIE[1] AND R. S. HANSON
Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

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Microcycle sporogenesis induced in *Bacillus cereus* T by phosphate limitation occurs over a narrow range of phosphate to spore inoculum ratios. Sufficient phosphate is required to satisfy the demands for a twofold increase in deoxyribonucleic acid; net ribonucleic acid synthesis is not required. The total ribonucleic acid content of the culture was variable, and deoxyribonucleic acid synthesis was restricted to a twofold increase. Developmental changes during outgrowth occurred synchronously, whereas enzyme synthesis was periodic. The timing of the synthesis of tricarboxylic cycle enzymes, extracellular protease, arginase, histidase, and alkaline phosphatase was measured. Histidase could be induced after 2.5 hr throughout microcycle sporogenesis. Several other features of macromolecular synthesis during microcycle sporogenesis are described. Differences between this pattern and those observed during outgrowth leading to cell division are discussed. A technique for accurately estimating the levels and time of synthesis of incompletely extractable, labile enzymes is also presented.

The developmental changes accompanying outgrowth of germinated spores and sporogenesis in *Bacillus* species provide a convenient experimental system for studies related to a description of the biochemical basis for morphogenesis in sporeforming bacteria. Microcycle sporogenesis involves the conversion of a dormant spore to a cell to a spore without intervening cell division (9, 13, 27). Microcycle sporogenesis of *B. cereus* T (13) is useful in studies of the type described above because the initial events occur in a relatively synchronous fashion in the entire population (10, 13, 22, 23). Protein synthesis is nonrandom and ordered during outgrowth of *B. cereus* T under conditions leading to outgrowth or microcycle sporogenesis, and the proteins synthesized are different in the two systems (10). All messenger ribonucleic acid (RNA) synthesis must occur after germination, and the time of appearance of those enzymes assayed is believed to be controlled by transcription, according to Steinberg and Halvorson (22) and Rodenberg et al. (18).

Microcycle sporogenesis of *B. cereus* T can be affected by limiting phosphate and occurs over a narrow range of phosphate to spore inoculum ratios (10). A careful comparison of the pattern of macromolecular synthesis during microcycle sporogenesis with that observed during outgrowth leading to cell division should in the future yield valuable information concerning the effect of the derepression of sporulation-related functions on the order of gene expression and should contribute to a better understanding of the regulation of functions related to differentiation. It is also feasible to test the possibility that some division-related functions need not be expressed during microcycle sporogenesis.

This manuscript describes the order of phenotypic expression of some gene functions and the pattern of RNA and deoxyribonucleic acid (DNA) synthesis during microcycle sporogenesis induced by phosphate deprivation.

MATERIALS AND METHODS

Microcycle sporogenesis. Details of the method of preparation of spores of *B. cereus* T and a description of the technique and characteristics of microcycle sporogenesis were given earlier (13). For the studies described here, the microcycle medium was modified by addition of L-arginine (10 mM), because it was found to increase the rate of microcycle sporogenesis. For some experiments, spore suspensions were heat-activated for 20 min at 75 C, instead of for 2 hr at 65 C as previously described. In both cases, heat-activated spores were washed twice with distilled water before inoculation of microcycle cultures. Both heat-activation procedures gave equally rapid spore germination in microcycle medium (98 to 100% in 8 min at 30 C). The microcycle medium was originally

[1] Present address: Bacteriology Section, Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford, England.

described for spore concentrations of 75 μg (dry weight)/ml, and it was shown that the phosphate content of the medium was the sole determinant of whether the cells divided or underwent microcycle sporogenesis. In the experiments reported here, the spore concentration was varied from 75 to 500 μg (dry weight)/ml, and the phosphate concentration was varied in strict proportion except when the effect of different phosphate concentrations on nucleic acid synthesis was studied. No significant changes in the rate of completion of microcycle sporogenesis were observed over a range of spore concentrations from 75 to 500 μg (dry weight)/ml.

Sporulation in media that will not support growth. Samples from microcycle cultures were removed at intervals, centrifuged for 5 min at 25 C, and washed once with and then suspended in an equal volume of the sporulation medium of Srinivasan (21). This medium contains G-salts, 0.05% K_2HPO_4 and 0.05% (w/v) each of glutamate, acetate, and citrate. Cultures were incubated at 30 C and were periodically examined under phase-contrast microscopy for development of refractile spores. This medium does not support cell division because of the absence of essential amino acids.

Preparation of extracts. A modification of the method of Steinberg and Halvorson (22) was used. Samples (9 ml for experiments of 75 μg of spores/ml and 3 or 4 ml for experiments at 500 μg of spores/ml) were removed and added to 1 ml of cold tris(hydroxymethyl)aminomethane (Tris) buffer (0.1 M, *p*H 7.4) containing chloramphenicol (final concentration, 100 μg/ml). The cells were harvested by centrifugation at 12,000 × *g* for 10 min at 4 C, washed once with 4 ml of Tris buffer, and centrifuged. The pellets were frozen at −20 C, lyophilized for 3 to 5 hr, and suspended in 1 ml of Tris buffer. The suspensions were frozen at −20 C, thawed at 4 C, and assayed for alkaline phosphatase, histidase, and arginase. Maximal activities of all three enzymes were detected after two freeze-thaw cycles. There was no loss in activity of arginase, histidase, or alkaline phosphatase after a third freeze-thaw cycle.

To measure the timing of synthesis of enzymes which were not stable to the freeze-thaw technique, the following breakage procedures were employed. A series of 300-ml Florence flasks containing 200 ml of medium was inoculated with spores at 500 μg/ml (phosphate concentration, 5.3×10^{-5} M). At intervals, 90-ml samples were taken by removing small amounts of culture from each flask and pipetting them into 10 ml of cold Tris buffer (0.02 M, *p*H 7.5) containing chloramphenicol (1 mg/ml) in a centrifuge bottle. The cells were harvested by centrifugation at 10,000 × *g* for 10 min at 4 C, washed once with 20 ml of Tris buffer, centrifuged, and frozen overnight at −20 C. The next day, the frozen cells were thawed and broken, either by shaking with acid-washed glass beads (200 mesh, Fisher Scientific Co., Pittsburgh, Pa.) in a Nossal disintegrator or by passage through a French pressure cell. For optimal breakage in the Nossal disintegrator, the cells were suspended in 12 ml of cold Tris buffer (0.02 M, *p*H 7.5) containing 10^{-4} M dithiothreitol (DTT), and 9 ml of glass beads was added. The capsule was shaken for 5 min with continuous cooling in a stream of carbon dioxide. The disrupted cells were centrifuged at 41,000 × *g* for 1 hr at 4 C in a Spinco model L ultracentrifuge. The high-speed supernatant fractions were devoid of reduced nicotinamide adenine dinucleotide (NADH) oxidase activity, and were assayed for malic dehydrogenase and glucose dehydrogenase. The sediments from the high-speed centrifugation were suspended in 1 ml of Tris buffer (0.02 M, *p*H 7.5) and assayed for NADH oxidase. The supernatant fractions were assayed for malic dehydrogenase and glucose dehydrogenase.

For breakage in the French pressure cell, the cells were suspended in 10 ml of cold Tris buffer (0.02 M, *p*H 7.5) containing 10^{-4} M DTT and were passed twice through the pressure cell at 4 C. The disrupted suspensions were treated as described for cells broken in the Nossal disintegrator. The 3,000 × *g* supernatant fraction was assayed as rapidly as possible for both aconitase and fumarase.

Since alkaline phosphatase was found to be partially particulate in extracts prepared in the Nossal disintegrator, it was always assayed in the crude 3,000 × *g* supernatant fractions.

Enzyme assays. Wherever possible, enzyme activities were measured on a Gilford 2000 or a Bausch & Lomb Spectronic-600 recording spectrophotometer. Unless otherwise stated, all enzyme assays were performed in 3-ml reaction volumes in 1-cm glass or quartz cuvettes. Two temperatures (25 and 35 C) were used to assay enzymes, for the following reason. In initial experiments on the timing of enzyme synthesis with freeze-thaw extracts, assays were always run at 35 C. However, for extracts prepared by Nossal disintegration or in the French pressure cell, a large number of assays had to be performed and two spectrophotometers were used, only one of which had temperature control. Thus, all enzymes, apart from alkaline phosphatase, were assayed at 25 C in these extracts.

Aconitase and fumarase were measured by the method of Racker (15). For both enzymes, a unit of activity is defined as that amount of enzyme which catalyzes the formation of 1 μmole of product per min at 25 C.

Alkaline phosphatase was measured by a modification of the method of Torriani (26), with *p*-nitrophenylphosphate used as substrate. The reaction mixture contained extract, 300 μmoles of Tris (*p*H 8.5), 8.85 μmoles of Sigma "104" phosphatase substrate, and 3 mmoles of $MgSO_4 \cdot 7H_2O$. The hydrolysis of *p*-nitrophenylphosphate to *p*-nitrophenol was followed at 400 nm. A unit of activity is defined as that amount of enzyme which produces 1 μmole of *p*-nitrophenol per hr at 35 C. The high concentration of Mg^{++} (1 M) employed stimulated enzyme activity fourfold compared with an assay medium from which Mg^{++} was omitted. Concentrations of Mg^{++} less than 1 M gave less stimulation of enzyme activity.

Arginase was measured as described by Ramaley and Bernlohr (16). Ornithine production was estimated by the method of Chinard as modified by Ratner (17). A unit of activity is defined as that

amount of enzyme which produces 1 μmole of ornithine per hr at 35 C.

Glucose dehydrogenase was measured at 340 nm with glucose and nicotinamide adenine dinucleotide (NAD) as substrates. The reaction mixture contained extract, 300 μmoles of Tris (*p*H 8.0), 100 μmoles of glucose, and 0.55 μmoles of NAD. A unit of activity is defined as that amount of enzyme which produces 1 μmole of NADH per min at 25 C.

Histidase was measured by a modification of the methods of Tabor and Mehler (24) and Hartwell and Magasanik (8). The reaction mixture contained extract, 300 μmoles of diethanolamine (*p*H 9.3), and 30 μmoles of L-histidine (*p*H 9.3). The deamination of histidine to urocanic acid was followed at 277 nm.

In some cases, an end-point assay was run. In these instances, the 1 ml of reaction mixture contained extract, 100 μmoles of diethanolamine (*p*H 9.3), and 10 μmoles of L-histidine (*p*H 9.3). Assays were incubated in tubes for 30 min, and the reaction was terminated by the addition of 0.1 ml of 35% (v/v) perchloric acid (PCA). After addition of 1.9 ml of water, the precipitated protein was removed by centrifugation, and the presence of urocanic acid in the PCA supernatant fraction was monitored by its absorption at 268 nm. A unit of activity is defined as that amount of enzyme which produces 1 μmole of urocanic acid per hr at 35 C.

Malic dehydrogenase was measured by a modification of the method of Ochoa (14). The reaction mixture contained extract, 300 μmoles of Tris (*p*H 7.6), 3 μmoles of oxalacetic acid (neutralized with $KHCO_3$), and 0.4 μmole of NADH. A unit of activity is defined as that amount of enzyme which oxidizes 1 μmole of NADH per min at 25 C.

NADH oxidase was measured by following the decrease in absorption at 340 nm with NADH as substrate. The reaction mixture contained extract, 300 μmoles of Tris (*p*H 7.6), and 0.4 μmole of NADH. A unit of activity is defined as that amount of enzyme which oxidizes 1 μmole of NADH per min at 25 C.

Extracellular protease was measured by a modification of the method of Tomarelli, Charney, and Harding (25), with the use of the proteolytic substrate azoalbumin. The 2 ml of reaction mixture contained a suitable volume of culture supernatant fluid (obtained by centrifuging cell samples at 12,000 × *g* for 10 min at 4 C), 10 mg of azoalbumin, and 100 μmoles of Tris (*p*H 7.5). Tubes were incubated for periods of up to 1 hr, and the reaction was terminated by addition of 2 ml of 8% (w/v) trichloroacetic acid. The precipitated protein was removed by centrifugation, and a sample of the supernatant fraction was diluted with an equal volume of 0.5 N NaOH. The extinction of the resulting yellow solution was measured at 440 nm. A unit of protease is defined as that amount of enzyme which catalyzes a change in optical density of 0.01 per hr at 35 C.

Chemical estimations. Inorganic phosphate was estimated by the method of Chen et al. (4) as modified by Ames and Dubin (1).

RNA was estimated by the method of Schneider (19), with 2.4% (v/v) PCA for hydrolysis, and DNA was estimated by the method of Burton (3). Yeast RNA and herring DNA were used as standards. At intervals, samples were pipetted into a small volume of 50% (w/v) trichloroacetic acid (chilled to −5 C in an ice-salt bath). The final concentration of trichloroacetic acid was 5%. Bovine serum albumin (5 mg) was added to each sample, and the precipitated cells plus protein were allowed to stand at 0 C for at least 1 hr before centrifugation at 12,000 × *g* for 10 min at 4 C. The sediments were washed once with 1.5 ml of cold 5% trichloroacetic acid, centrifuged, and frozen. For complete hydrolysis of DNA and RNA, the cells were extracted twice for 30 min at 80 C with 1 ml of 2.4% PCA. In this procedure, all of the RNA and 70% of the DNA were solubilized by the first extraction. The remaining DNA was solubilized by the second extraction. Only 60% of the DNA from spores could be solubilized by extracting twice with 5% trichloroacetic acid for 30 min at 80 or 100 C.

Protein was estimated by the method of Lowry et al. (12), with bovine serum albumin as standard. Glucose was estimated by the Glucostat method (Worthington Biochemical Corp., Freehold, N.J.).

Measurement of incorporation of radioactive precursors. The incorporation of ^{3}H-uracil into the cold trichloroacetic acid-precipitable fraction and ^{14}C-phenylalanine into the hot trichloroacetic acid-precipitable fraction were used as indicators of RNA and protein synthesis, respectively. The radioactive isotope was added to the medium before inoculation, and at various times samples were removed and immediately added to an equal volume of 10% (w/v) trichloroacetic acid containing nonradioactive precursor (100 μg/ml). Precipitated cells which had incorporated ^{3}H-uracil were allowed to stand at 0 C for at least 30 min, collected by filtration on membrane filters (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.), and washed four times with 4 ml of cold 5% trichloroacetic acid containing unlabeled precursor (50 μg/ml). The filters were dried and counted as previously described (13). Greater than 95% of the label incorporated by the cells during exposure to ^{3}H-uracil was solubilized by heating with 5% trichloroacetic acid for 30 min at 95 C in a water bath. Less than 5% of the ^{14}C-phenylalanine incorporated by the cells was solubilized by the same procedure.

Chemicals. L-Histidine hydrochloride monohydrate, L-chloramphenicol, Sigma 104 phosphatase substrate, *p*-nitrophenol, L-ornithine hydrochloride, ninhydrin, hydrindantin, NAD, NADH, oxalacetic acid, azoalbumin, uracil, L-phenylalanine, and L-malic acid were obtained from Sigma Chemical Co., St. Louis, Mo. Tris was obtained from either Sigma Chemical Co. or Schwarz BioResearch, Inc., Orangeburg, N.Y. Dithiothreitol and bovine serum albumin were obtained from Calbiochem, Los Angeles, Calif.; diethanolamine, from Eastman Organic Chemicals, and yeast RNA and herring DNA, from Mann Research Laboratories, Inc. Uracil-5-^{3}*H* (specific activity, 6.0 c/mmole) and ^{14}C-L-phenylalanine (specific activity, 325 mc/mmole) were obtained from Schwarz BioResearch, Inc.

RESULTS

The time course for development of refractile spores in microcycle medium is shown in Fig. 1.

Approximately 50% sporulation occurred by 12 hr and more than 90% occurred by 15 hr, at which time free spores began to appear. The timing of formation of refractile spores was 2.5 to 3 hr earlier than in medium from which arginine was omitted. The reason for the stimulation of the rate of completion of microcycle sporogenesis is not known. Arginase is induced by the cells, and it seems possible that arginine may serve as an energy source and in this way stimulate the rate of development. The time of development of refractile spores did vary slightly with the spore batch and was, on occasion, 1 to 1.5 hr earlier than shown in Fig. 1.

Total DNA and RNA syntheses during microcycle sporogenesis and, for comparison, during cell division are shown in Fig. 2 and 3. Good microcycle sporogenesis and less than 3% cell division occurred over a twofold range of phosphate concentrations (between 5×10^{-5} M and 10^{-4} M when 500 μg of spores/ml was used). At the lower phosphate concentrations, very little net RNA synthesis occurred, whereas at higher phosphate levels a 50% increase in total RNA was observed over a 2.5-hr period. Net DNA synthesis was observed during the first 4.5 hr of microcycle sporogenesis under these conditions. The total amount of DNA and the pattern of synthesis remained unaffected by changes in the phosphate concentration of the medium. At lower spore concentrations (250 μg/ml, 2.5×10^{-5} M PO_4), the pattern of DNA synthesis was essentially the same, but the initial lag was shorter (less than 45 min), the first burst was completed by 1.5 hr, and a second burst of DNA synthesis occurred between 3.5 and 4 hr. At 500 μg/ml, the initial period of DNA synthesis continued for 2.5 hr and a second period occurred between 3.5 and 4.5 hr.

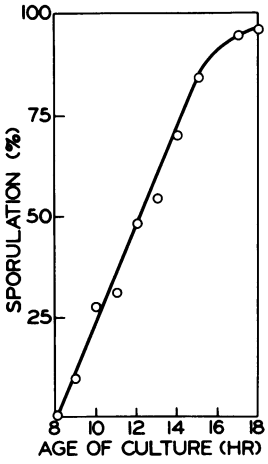

FIG. 1. *Development of refractile spores during microcycle sporogenesis of Bacillus cereus T in modified microcycle medium. The medium was inoculated at 75 μg of spores/ml.*

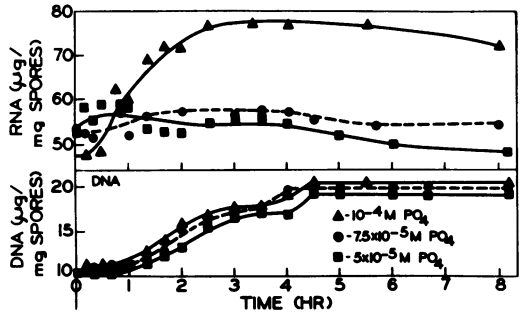

FIG. 2. *DNA and RNA synthesis during microcycle sporogenesis at 50 μg of spores/ml of medium.*

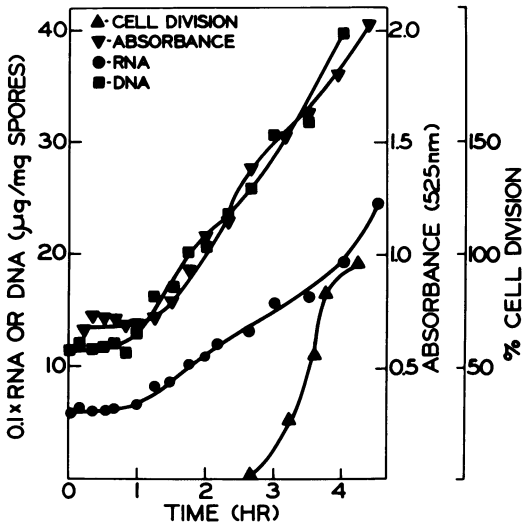

FIG. 3. *DNA and RNA synthesis during outgrowth and cell division. The spore concentration was 250 μg/ml and the microcycle medium was modified by addition of phosphate at 5×10^{-4} M.*

At a spore concentration of 250 μg/ml, an increase in the phosphate level of the medium to 5×10^{-4} M caused almost complete division to occur (Fig. 3). Cell division was measured by observation of a sample with a phase-contrast microscope. The fraction of the cells with division septa was recorded. At higher phosphate levels, 5×10^{-2} M, division was less synchronous. Detectable net DNA synthesis commenced at approximately 40 min and continued until 4 hr in the presence of 5×10^{-4} M phosphate. Net RNA synthesis occurred at the same time (Fig. 3).

Figure 4 shows the rates of RNA synthesis and uptake of inorganic phosphate from the

medium during microcycle sporogenesis. Phosphate uptake was rapid, and none remained in the medium after 45 min of incubation (within the limits of the method of estimation, which would not have detected less than 2×10^{-6} M). The total RNA content of the cells increased by about one-third (from 75 to 100 μg/mg of spores) within 1 hr, and no further increase was detectable to 4.5 hr. This amount of RNA synthesis would consume 0.035 μmole of phosphate/ml of medium (70% of that available). The data on phosphate uptake and total RNA synthesis were obtained from the same experiment. In a separate experiment, the kinetics of incorporation of tritiated uracil by the cells was followed. Uracil uptake was rapid for approximately 30 min, after which time it ceased and the total label in the cellular RNA remained essentially constant to 5 hr. The uracil incorporation at 30 min corresponded to approximately 14% of the total label added to the medium at zero time.

A comparison of the kinetics of incorporation of ^{14}C-L-phenylalanine during microcycle sporogenesis and cell division is presented in Fig. 5. For the initial 1 to 1.5 hr, cells undergoing microcycle sporogenesis incorporated the isotope at a slightly faster rate than cells which eventually divided. After this time, however, the division culture assimilated the isotope at a much faster rate. Division occurred at about 4 hr. At 5 hr, the microcycle and division cultures had assimilated 17 and 50%, respectively, of the label present in the medium at zero time.

Having established these basic points concerning macromolecular synthesis during microcycle sporogenesis, the timing of synthesis of

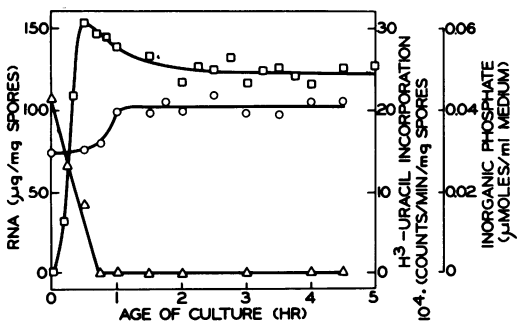

FIG. 4. *RNA synthesis and inorganic phosphate uptake during microcycle sporogenesis. The medium was inoculated at 500 μg of spores/ml. Symbols: ○, total RNA; △, inorganic phosphate; □, ^{3}H-uracil incorporation. In the experiment measuring uracil incorporation, the medium contained ^{3}H-uracil (4.4 μg/ml) of specific activity 18.6 μc/μmole (28,200 counts per min per nmole).*

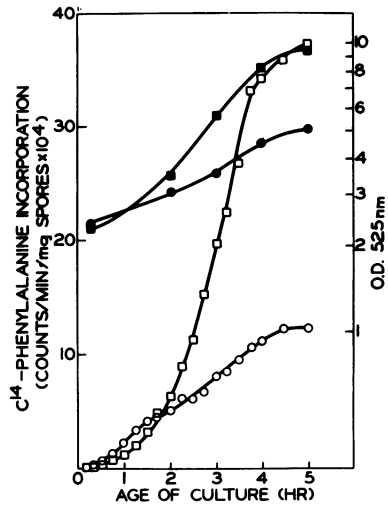

FIG. 5. *Protein synthesis during microcycle sporogenesis and cell division. The medium was inoculated at 500 μg of spores/ml. The media contained ^{14}C-L-phenylalanine (30 μg/ml) of specific activity 0.75 μc/μmole (2,000 counts per min per nmole). ^{14}C-phenylalanine incorporation by microcycle culture (○) and by division culture (□); optical density at 525 nm of microcycle culture (●) and division culture (■). Samples for optical density measurements were taken at intervals beginning 10 min after inoculation, when germination was essentially complete. The microcycle medium was modified by omission of the Tris buffer and addition of phosphate buffer (0.1 M, pH 7.0) and glucose (0.2%, w/v) in order to obtain division.*

several enzymes was examined. Initially, extracts were prepared by the freeze-thaw technique, and the enzymes studied were alkaline phosphatase, arginase, and histidase. Alkaline phosphatase and histidase were already known to be stable in extracts prepared by this technique (22). Extracellular protease was also studied because it is known to be produced by sporulating cells (1), and it has the added advantages of easy assay and stability to repeated freezing and thawing.

The timing of synthesis of alkaline phosphatase, arginase, and extracellular protease is shown in Fig. 6. Alkaline phosphatase and arginase were produced at approximately the same time. The most rapid synthetic period was between 2.5 and 3.5 hr. Alkaline phosphatase activity declined slowly after this time, whereas arginase remained constant. The timing of synthesis of histidase is not shown in Fig. 6, but the active synthetic period was indistinguishable from the time of production of the above two enzymes (Fig. 7). When induced in the presence of arginine, the amount of histidase formed was only 40% of the level obtained when arginine was absent (*unpublished ob-*

servations). In the absence of arginine, the timing of synthesis of both histidase and alkaline phosphatase was delayed 1 to 1.5 hr, owing to the slower rate of development of the cells. At the spore concentration employed in this experiment (75 μg/ml), protease synthesis occurred most rapidly between 4.5 and 5.5 hr. At higher spore concentrations (500 μg/ml), extracellular protease appeared between 3 and 5 hr (*unpublished observations*).

Originally, protease activity was assayed with azocoll (Calbiochem) as a substrate. Azocoll, unlike azoalbumin, is fibrous and insoluble. It was found to be unsuitable, however, because the amount of proteolysis obtained depended on whether assays were performed statically or with vigorous shaking (the latter method gave a 40% stimulation of activity). Furthermore, it was found that the azocoll concentration could be raised to very high levels (30 mg/ml) without reaching enzyme saturating concentrations; e.g., an increase in substrate concentration from 6 to 30 mg/ml gave a two- to fivefold stimulation of activity. Levisohn and Aronson (11) had studied the protease produced by *B. cereus* T during the normal sporulation cycle with azocoll as substrate and had found the enzyme to require Ca^{++} (2 mM) for maximal activity. The extracellular protease produced by *B. cereus* T during microcycle sporogenesis was found to be at least 50% inhibited by 2 mM Ca^{++} if azocoll was used as substrate but not if azoalbumin was employed. The time of appearance of proteolytic activity was the same when azoalbumin and azocoll were used as substrates.

Cells undergoing microcycle sporogenesis retained the ability to induce histidase until at least 10 hr (Fig. 7), by which time refractile spores were beginning to appear (Fig. 1). It is clear, however,

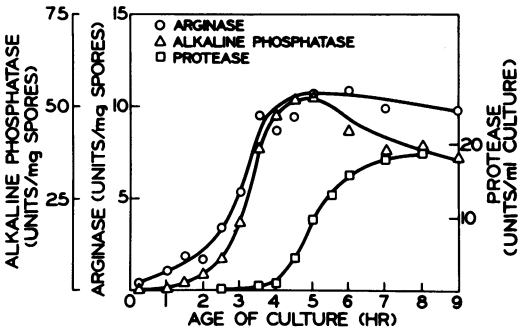

FIG. 6. *Synthesis of alkaline phosphatase, arginase, and extracellular protease during microcycle sporogenesis. The medium was inoculated at 75 μg of spores/ml. Symbols: Δ, alkaline phosphatase; O, arginase; □, extracellular protease.*

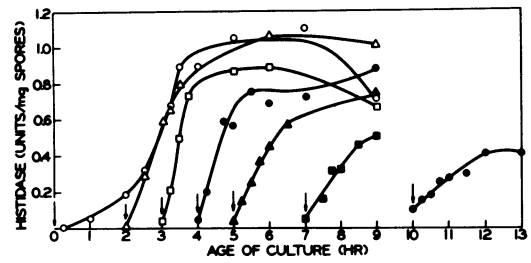

FIG. 7. *Histidase inducibility during microcycle sporogenesis. The medium was inoculated at 75 μg of spores/ml. At the times indicated by the arrows, samples were removed from cultures undergoing microcycle sporogenesis and exposed to histidine (6.5 mM). Culture exposed at zero time (O), 2 hr (Δ), 3 hr (□), 4 hr (●), 5 hr (▲), 7 hr (■), and 10 hr (⊙).*

that the amount and rate of synthesis do vary with the time of induction. The relative rates of synthesis in cultures induced at 0, 2, 3, 4, 5, 7, and 10 hr, arbitrarily defining the rate of synthesis in the culture induced at zero time as unity, were 1, 1, 1.8, 1.1, 0.8, 0.5, and 0.36, respectively. It could be argued that the enzyme synthesis observed after 3.5 hr was due to asynchrony and that microcycle sporogenesis after 2 hr is similar to a population of cells at different stages of the division cycle (i.e., continuously subject to induction). In the absence of cell division, synchrony is difficult to assess. However, the results shown in Table 1 argue in favor of synchrony for at least 2.5 hr. Cells undergoing microcycle sporogenesis were removed from the original medium at various times, washed, and resuspended in the medium described by Srinivasan (21), which supports sporulation of cells already committed to sporulation but does not allow further development of vegetative cells. If cells were transferred to the sporulation medium prior to 2 hr, cellular development was arrested and there was no sporulation at 11.5 hr. When transfer was performed at 2 hr 25 min or later, second-stage spore formation was accelerated over the rate observed in the control culture.

Microcycle sporogenesis of *B. cereus* T occurred in the presence of 10 mM glucose. The appearance of refractile spores was slightly later than in control cultures containing no glucose. Microscopic examination of cultures revealed that glucose caused formation of numerous refractile granules, presumably poly-β-hydroxybutyrate, in the cells within 3 to 5 hr. There was no significant fall in *p*H caused by glucose metabolism. Figure 8 summarizes some data on the effect of glucose on the timing of synthesis of alkaline phosphatase and histidase. The rate of glucose utilization by the cells, which is also shown, remained constant

TABLE 1. *Ability of cells of B. cereus T undergoing microcycle sporogenesis to complete endotropic sporulation*

Age of culture at time of transfer	Resporulation at 11.5 hr
	%
1 hr	None[a]
1 hr 25 min	None[a]
1 hr 55 min	None[a]
2 hr 25 min	62
2 hr 45 min	63
3 hr 10 min	55
3 hr 30 min	72
6 hr 5 min	48
Control	30

[a] Cellular development was completely arrested in these cultures after transfer. The experiment was performed at 500 μg of spores/ml of medium as described in the text.

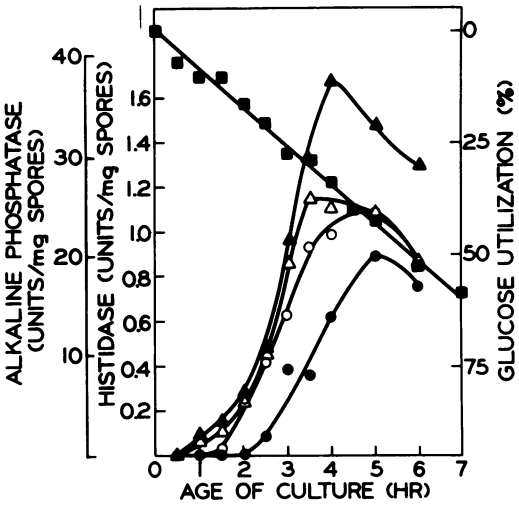

FIG. 8. *Effect of glucose on the timing of synthesis of alkaline phosphatase and histidase during microcycle sporogenesis. The pH of the medium was adjusted to 7.5 and histidine was added (6.5 mM). The spore concentration was 500 μg/ml of medium. The initial glucose concentration in the test culture was 0.194% (w/v). Alkaline phosphatase (△) and histidase (○) in the control culture; alkaline phosphatase (▲) and histidase (●) in the culture containing glucose; (■) glucose utilization (glucose utilized/glucose present at time 0 × 100).*

over the time that measurements were made. At 24 hr, no glucose remained in the medium. The rate of glucose utilization was dependent on the spore concentration, and at 75 μg of spores/ml of medium only 15 to 20% was utilized in 24 hr. Glucose had no effect on the timing of synthesis of alkaline phosphatase, though the amount of enzyme synthesized was increased slightly. In contrast, there was a delay of about 1 hr in the appearance of histidase, though there was little effect on the amount eventually synthesized. The reason for this effect is not clear and requires further investigation. It cannot be explained by complete glucose utilization from the medium nor does it result from a change in the rate of glucose catabolism, according to the data of Fig. 8.

It was of interest to determine the timing of synthesis of the citric acid cycle enzymes. For this purpose, alternative methods of extract preparation had to be found because these enzymes were unstable to the freeze-thaw treatment. Ideally, it is desirable to make quantitative measurements of enzyme activities. The freeze-thaw technique is regarded as meeting this criterion for enzymes stable to this treatment. Specific activities (units per milligram of protein) are meaningless because, while the total number of cells remains constant, the total cellular protein increases. To illustrate the problems involved and the technique which was adopted to circumvent them, Fig. 9 and 10 show some comparative data on the recoveries of alkaline phosphatase and glucose dehydrogenase in extracts prepared by different methods. Cell breakage in the French pressure cell was dependent on the age of cells undergoing microcycle sporogenesis. Microscopic observation showed that germinated spores were very resistant to rupture by this method, but the cells became easier to break as the cycle proceeded. Breakage was not complete at any time. The efficiency of Nossal disintegration was likewise dependent on culture age; germinated spores were the most resistant. However, good breakage of germinated spores could be obtained by shaking for a sufficiently long period. The minimum time required for this was found to be 5 min, and this time was used for extract preparation in all samples. The data in Fig. 9 show that breakage by Nossal disintegration inactivated at least 85% of the cellular alkaline phosphatase. Recovery of alkaline phosphatase at the point of maximal synthesis in extracts prepared by breakage in the French pressure cell was approximately 50% of that obtained in freeze-thaw extracts, and this was evidently the result of incomplete breakage rather than enzyme denaturation.

By comparing the recovery of glucose dehydrogenase in extracts prepared by Nossal disintegration and by passage through the French pressure cell (Fig. 10), it appears, from the efficient breakage effected by Nossal disintegration, that this enzyme, known to be present in spores of *B. cereus* T, remains constant for the first 5 hr of microcycle sporogenesis. The apparent increase in activity of glucose dehydrogenase in extracts

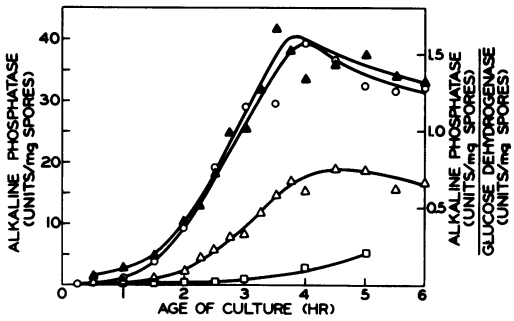

FIG. 9. *Effect of method of cell breakage on the recovery of alkaline phosphatase from extracts of cultures undergoing microcycle sporogenesis. The medium was inoculated at 500 μg of spores/ml. Breakage by (○) freeze-thaw method, (△) French pressure cell, and (□) Nossal disintegration; (▲) activity of alkaline phosphatase in French pressure cell extracts divided by glucose dehydrogenase activity in the same extracts.*

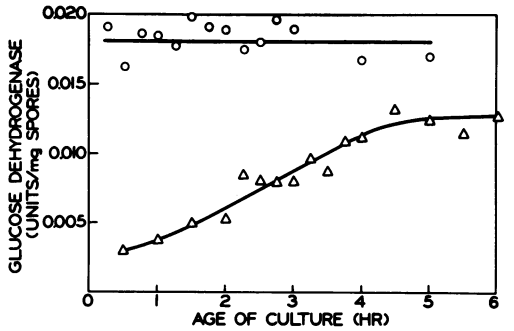

FIG. 10. *Effect of method of cell breakage on the recovery of glucose dehydrogenase from extracts of cultures undergoing microcycle sporogenesis. The medium was inoculated at 500 μg of spores/ml. Breakage by (○) Nossal disintegration and (△) French pressure cell.*

prepared by passage through the French pressure cell was due to the increased efficiency of breakage with time by this method. Even at 5 hr, the activity of glucose dehydrogenase in French pressure cell extracts was less than that in extracts prepared by Nossal disintegration. On the basis of these observations, it seems valid to use the glucose dehydrogenase activity of extracts prepared by passage through a French pressure cell as a criterion of cell breakage and to record all enzyme activities as a function of glucose dehydrogenase activity. When this is done for alkaline phosphatase (Fig. 8), a plot of enzyme activity as a function of the age of the culture is virtually indistinguishable from that obtained by the freeze-thaw technique. Glucose dehydrogenase was stable to repeated freezing and thawing, but attempts to assay it in freeze-thaw extracts were hampered by the presence of an active NADH oxidase and by the tendency of the large amounts of ruptured cells required for the assay to settle in assay cuvettes.

Figures 11 and 12 summarize data on the timing of synthesis of a number of enzymes in extracts prepared by passage through a French pressure cell. For reasons explained above, all enzyme activities are expressed as a function of glucose dehydrogenase activity.

The data from the two experiments agree well and show that the synthesis of the citric acid cycle enzymes was initiated at 1.5 to 2 hr. Malic dehydrogenase synthesis continued until 2.5 hr, and aconitase and fumarase synthesis, until 3 hr. Second periods of synthesis of both malic dehydrogenase and fumarase apparently occurred. Synthesis of particulate NADH oxidase occurred at a slightly later time and, as with aconitase, only one burst was observed.

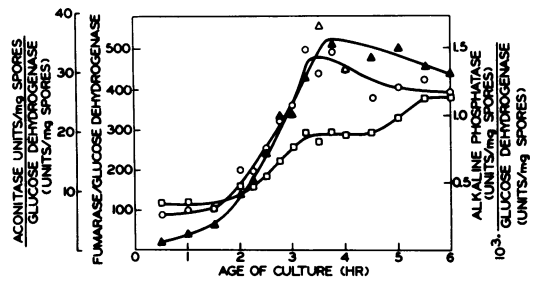

FIG. 11. *Timing of enzyme synthesis during microcycle sporogenesis with the use of extracts prepared by passage through a French pressure cell. The medium was inoculated at 500 μg of spores/ml. Enzyme activities are expressed relative to the glucose dehydrogenase activity of the extracts (see Fig. 8). Symbols: ○, aconitase; □, fumarase; ▲, alkaline phosphatase.*

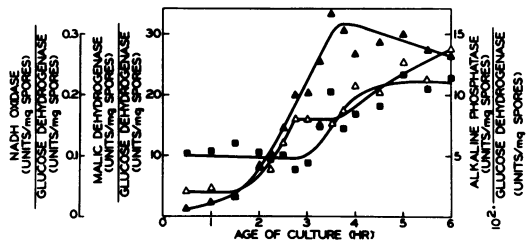

FIG. 12. *Timing of enzyme synthesis during microcycle sporogenesis with the use of extracts prepared by passage through a French pressure cell. The medium was inoculated at 500 μg of spores/ml. Enzyme activities are expressed relative to the glucose dehydrogenase activity (see Fig. 8). Symbols: △, malic dehydrogenase; ■, NADH oxidase; ▲, alkaline phosphatase.*

DISCUSSION

Microcycle sporogenesis, with less than 3% cell division, occurs when the phosphate to spore ratio is between 10^{-4} and 2×10^{-4} moles/g of spores used as inoculum. The amount of phosphate added must be sufficient to allow for a doubling in the DNA content of the inoculum, but very little, if any, net RNA synthesis is required for microcycle sporogenesis. Although the amount of phosphate exceeds that required for DNA synthesis by more than a factor of two, no RNA was synthesized in the experiment described in Fig. 1. Phosphate was always exhausted by 1 hr under our standard conditions, and most of the DNA was synthesized after this time.

RNA synthesis, measured by incorporation of labeled uracil or by chemical estimation, commences shortly after germination and ceases at the time of or shortly after the medium phosphate is depleted. The time of phosphate depletion depends on the phosphate to spore ratio.

In the experiments reported here, DNA synthesis always occurred in two steps. The timing of each step depends primarily on the concentration of the spore inoculum (at constant phosphate to spore ratios) and not on the phosphate to spore ratio. Hoyem et al. (10) reported that rapid synthesis of DNA occurred within 1 hr after initiation of outgrowth and again prior to the appearance of forespores. They also observed lysis of half of the population between 6 and 9 hr of microcycle sporogenesis. We have found thymidine incorporation to be misleading as a means of measuring net DNA synthesis (13). Steinberg and Halvorson (23) also observed rapid incorporation of labeled thymidine but no chemically detectable DNA synthesis shortly after initiation of outgrowth. This burst of incorporation probable represents net DNA synthesis (23), but the quantity is exceedingly small. We have been unable to use thymidine auxotrophs because of their inability to complete microcycle sporogenesis without extensive lysis. In our experiments, we did not observe lysis prior to formation of refractile spores, nor have we detected net DNA synthesis after 5 hr in a number of experiments.

During good microcycle sporogenesis, DNA synthesis always resulted in a doubling of the DNA content of the culture. During outgrowth leading to cell division, DNA synthesis commenced at approximately 1 hr and doubled prior to the first division (Fig. 3). By the completion of the first division, a three- to fourfold increase in DNA over the inoculum level was observed. Steinberg and Halvorson (23) noted a lag of 2 hr in DNA synthesis and a 60% increase at the first division. The RNA content of the culture also doubled prior to the first division and had increased threefold by the completion of division. It is our experience that the RNA content of a culture can be varied under conditions that give good microcycle sporogenesis, whereas DNA synthesis is always restricted to a 100% increase. When sufficient phosphate is added to permit a doubling in the RNA content of the culture, cell division and renewed DNA synthesis occur. This raises some very interesting questions concerning the regulation of sporogenesis. It is important to obtain information on the time of spore septum formation and how it is related to the pattern of DNA synthesis. The ability to complete sporulation on media which will not support growth appears at 2 to 2.5 hr; thus, sporulation-related physiological changes (protein synthesis at the expense of amino acids derived from preexisting protein) occurred prior to the completion of DNA synthesis.

During outgrowth of spores, the rate of protein synthesis appears to be dependent on the amount of actinomycin D-labile RNA (10). During microcycle sporogenesis, protein synthesis is at least as rapid as it is during outgrowth leading to cell division for the first 1.5 hr. After this time, the rate of protein synthesis decreases in the microcycle culture.

We have no reliable information on the nature of proteins made prior to 1.5 hr of microcycle sporogenesis, or that they differ from those synthesized during outgrowth leading to cell division. Hoyem et al. (10) have shown that proteins formed later in microcycle differ from division-related proteins as judged by electrophoretic behavior on polyacrylamide gels. It would be most interesting to discover whether the early gene products differ under division and microcycle conditions, and when the first changes occur.

The enzymes we have measured were selected because, excepting glucose dehydrogenase, they are repressed under conditions which give rapid growth. Conditions which allow rapid synthesis of these enzymes result in increased sporulation. The tricarboxylic acid cycle enzymes, alkaline phosphatase, arginase, and histidase are synthesized most rapidly between 2 and 3.5 hr. Fumarase and malic dehydrogenase are again synthesized in second bursts at 4 to 5 hr. The extracellular alkaline protease, also associated with sporogenesis (11), is synthesized most rapidly at 5 hr. These results and those of Table 1 indicate that sporulation-related metabolism commences at 2 to 2.5 hr (2, 5–7). No true commitment to sporulation occurs prior to 6 hr, because the addition of phosphate and glucose prior to this time results in cell division (*unpublished results*).

The induced synthesis of histidase follows an interestingly different pattern during normal outgrowth and sporogenesis. Steinberg and Halvorson (22) noted that histidase synthesis occurred most rapidly between 50 and 60 min after germination during continuous induction or if inducer was added prior to the time of rapid histidase synthesis. When inducer was added part way through the period of gene expression, the level of enzyme induction was diminished. Addition of inducer after the period of gene expression resulted in a several-fold decrease in the rate of histidase synthesis. In contrast, addition of inducer after the burst time during microcycle sporogenesis results in induction, and histidase can be induced at any time during microcycle sporogenesis up to 10 hr.

Steinberg and Halvorson also observed that addition of actinomycin D shut off induced histidase synthesis in 1 to 2 min. They concluded that periodic synthesis was governed by transcription of a labile messenger, and that the histidase gene was available for transcription only for a limited time interval in synchronously growing cultures.

We have not presented data relating to the time of transcription of the histidase gene after induction, but it is obvious that the gene is continuously open to phenotypic expression during microcycle sporogenesis.

The burst of histidase synthesis is delayed when glucose is present during microcycle sporogenesis, but the amount of enzyme synthesized remains unaffected. The low levels of phosphate used in these experiments limit the rate of glucose oxidation, because addition of 5×10^{-4} M phosphate causes an immediate stimulation of the rate of glucose utilization when added at any time up to 6 hr (I. MacKechnie and R. S. Hanson, *unpublished results*). During outgrowth, at higher phosphate levels, much lower levels of glucose repress histidase synthesis (22).

Our observations also lead to questions concerning the interpretation of the burst time in continually induced cultures. The time at which synthesis ceases during microcycle sporogenesis could very well represent balanced synthesis and turnover. Rapid turnover of vegetative cell protein is associated with sporulation. It is difficult to explain its rapid synthesis when inducer is added later if this is the case. These results demonstrate that the end of a period of synthesis does not necessarily represent the unavailability of the particular cistron to phenotypic expression during microcycle sporogenesis.

In summary, there are many differences between a cytoplasmic partitioning leading to "sporulation division" (20) and cell fission. The map of events on a time scale as employed in this study should lead to a more basic understanding of the nature of these differences and how they are related to cellular differentiation.

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