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Release and Recovery of Forespores from *Bacillus cereus*

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A method is described which makes possible the release of immature forespores from sporulating cells at specific stages of development, from the completion of stage III through to mature spore formation. With the aid of zonal density gradient centrifugation, the method makes possible the recovery of quantities of forespores ample for biochemical and physical studies. With the capability to examine forespores and some mother cell components independently, we have established that several enzymes associated with the sporulation process are localized in the newly developed forespores. Studies showed that aspartate aminotransferase and alanine aminotransferase are associated with the forespores, whereas L-alanine dehydrogenase is found only in the mother cell cytoplasm.

Various studies have made it apparent that the physiological and developmental changes which occur during vegetative growth and subsequent endospore formation in *Bacillus* species involve a series of integrated biochemical events (12). Consequently, the differentiation evident in bacterial sporulation provides a particularly useful tool for the study of morphogenic and biochemical relationships.

Electron microscope studies (5, 13, 21) have shown that stage I of sporulation in which a single axial nuclear thread is present in the mother cell is followed by the invagination of the cytoplasmic membrane to form a spore septum (stage II), separating the mother cell from forespore compartment. During stage III, the forespore compartment itself becomes engulfed by the rapid growth of folded mother cell cytoplasmic membrane. At the completion of stage III, the forespore appears as a phase-contrast dark body, containing two distinct membranes, and is apparently free floating in the mother cell cytoplasm. The forespore subsequently develops a mucopeptide-containing (peptidoglycan) cortex region and germ cell wall between the outer and inner forespore membranes (OFSM and IFSM, respectively). The cortex continues to grow while the membranous, loose-fitting exosporium which engulfs the developing forespore is formed during stage IV. Spore coat(s) synthesis is initiated on the exterior surface of the OFSM at stage V. Maturation and release of the heat- and chemical-resistant spores occurs during stages VI and VII.

Despite the brevity of this description, it is evident that the rapidly developing forespore membranes and other integuments, and their developmental properties, play an important role in the differentiation process. Most of the fragmentary information concerning spore integuments has been obtained in studies employing relatively harsh methods for the disruption of sporulating cells and mature spores (9, 12). Consequently, it has not been possible to effectively separate the mother cell from forespore components, or to obtain homogeneous preparations of spore integuments. In particular, the lack of an experimentally practical and selective method for the release of abundant quantities of forespores from sporulating cells during all stages of development has severely limited understanding of this complex differentiation process.

This paper reports the development of a method which provides for the efficient release of intact forespores at specific stages of sporulation. In addition, the recovery of ample quantities of the differentiated structures permits their biochemical evaluation by conventional means. (This work was presented in part at the annual meeting of the Pacific Slope Biochemical Conference, Davis, California, June, 1972.)

MATERIALS AND METHODS

Growth of cells. A laboratory strain of *Bacillus cereus*, with the same bacteriological properties as *B.*

cereus strain 9373 (18), was grown in 12-liter volumes of modified G medium (7) containing 0.1% sucrose and 0.1% yeast extract (Difco) in a New Brunswick laboratory fermentor, vigorously aerated with 5 liters of air per min, agitated at 150 rpm, and maintained at 30 C. The inoculum for large-scale growth was prepared with the active culture technique of Collier (3), utilizing a minimum of five serial transfers at 3-h intervals, grown on the same medium. Growth was measured turbidimetrically at 540 nm.

Electron microscopy. Forespores and whole sporulating cells were fixed with 2% glutaraldehyde in 50 mM T-buffer [tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.4, containing 10 mM $MgCl_2$ and 10 mM $CaCl_2$] by a modification of the method of Sabatini et al. (17), followed by postfixation in 1% osmium tetroxide, agar embedding, and uranyl acetate washes as described by Ryter and Kellenberger (14). Since young forespores are highly susceptible to disruption by osmotic shock, in the fixation of forespore preparations the T-buffer also contained 30% (wt/wt) sucrose, to minimize disruption. Dehydration and embedding of specimens was as described by Spurr (18).

Enzyme assays. Reduced nicotinamide adenine dinucleotide (NADH) oxidase (NADH-O_2 oxidoreductase) (EC 1.6.3.1) (4) and L-alanine dehydrogenase (EC 1.4.11) (19) were measured spectrophotometrically by following the rate of NADH oxidation at 340 nm by methods described elsewhere, with the exception that, in the case of alanine dehydrogenase (19), 0.1 M Tris-hydrochloride, pH 7.7, was used instead of triethanolamine buffer. NADH-DCIP oxidoreductase (EC 1.6.99.1) was measured spectrophotometrically at 600 nm (4). Aspartate aminotransferase (GOT) (EC 2.6.1.1) (8) and alanine aminotransferase (GPT) (EC 2.6.1.2) (20) were measured with assay kits obtained from Calbiochem, La Jolla, Calif. Protein was determined by the method of Lowry et al. (11). Enzyme activity was proportional to enzyme concentration and is expressed as micromoles of substrate converted or product formed per hour.

RESULTS

Growth and sporulation of cells. When the conditions specified for the preparation of inoculum and the growth and sporulation of cells were strictly observed, growth curves (Fig. 1) could consistently be reproduced in repetitive experiments. Thus, the termination of exponential growth (T_0), the formation of forespores at the completion of stage III ($T_{3½}$) (which appear as phase-dark bodies), and the whitening (not refractile) (1, 12) of the forespore body in more than 90% of the cells at $T_{4½}$ (1) were predictable and reproducible (to within 15 min) in individual experiments. The morphological stages of sporulation and the subscripts T_0, $T_{3½}$, and $T_{4½}$ are defined and employed as previously described (12, 15). Individual cultures were periodically examined with a phase-contrast microscope to evaluate the degree of synchrony, and subsequent examination with an electron microscope served to confirm that, typically, more than 90% of the cells were at the same stage of sporulation simultaneously. The high degree of synchrony observed under these controlled conditions for growth and sporulation is in agreement with that previously reported for strains of *B. cereus* (1, 10).

Release and recovery of forespores. Initially, studies were conducted to evaluate the feasibility of accomplishing the release of forespores from sporulating cells at various stages of development with the aid of specific enzymes, e.g., lysozyme, deoxyribonuclease (DNase) I, and nonionic surfactants. Although some modest success was achieved, all of the methods evaluated suffered the following shortcomings and were therefore rejected: (i) efficacy of mother cell disruption was frequently low, and when the method was effective, stability of the released forespores was poor; (ii) recovery of forespores at the end of stage III or early stage IV gave extremely poor yields of damaged forespores, and relatively modest yields of intact forespores were achieved only from cells at late stage IV or later; (iii) several hours were required to accomplish the release and recovery of forespores, and consequently some forespores continued to develop, resulting in lack of uniformity in those recovered.

An alternative method for cell disruption and forespore release was sought and developed. Twenty grams of sporulating cells, harvested

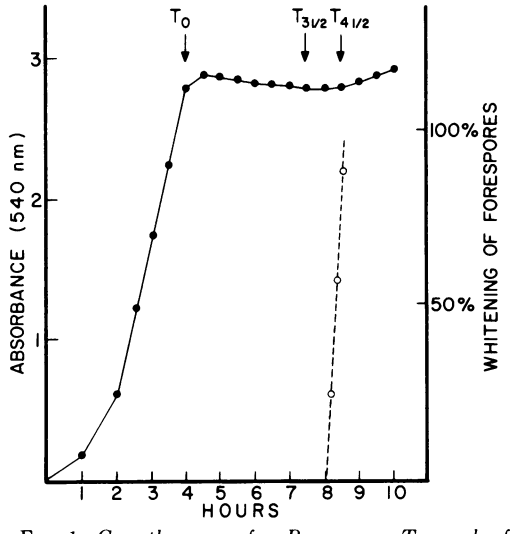

FIG. 1. *Growth curve for B. cereus. T_0, end of exponential growth; $T_{3½}$ and $T_{4½}$, points in time at which cells were harvested and forespores released. Symbols: absorbance, ●; whitening of forespores, O.*

after 7.5 h of growth ($T_{3\frac{1}{2}}$) and, at the completion of stage III of development (Fig. 1), were suspended in 100 ml of 20 mM T-buffer containing 30% (wt/wt) sucrose. The suspended cells were disrupted with either a model RM-1 or RF-1 Ribi cell fractionator (Ivan Sorvall Inc., Norwalk, Conn.) at 30,000 lb/in^2 and at 5 C. Over 97% of the cells were disrupted, releasing intact forespores, as determined by phase-contrast microscopy and subsequently confirmed by electron microscopy. The viscous preparation recovered was treated with 100 μg of bovine pancreatic DNase I (Calbiochem) for 5 min at 25 C and centrifuged at 1,000 $\times$ g for 3 min. The supernatant fraction was carefully decanted and saved; the pellet was suspended in sucrose T-buffer and centrifuged as above. The pooled supernatant fractions contained primarily forespores, less than 0.3% whole cells (as determined by phase-contrast microscopy), membrane fragments, and mother cell cytoplasm.

The forespore suspension was dynamically loaded into a model SZ-14 reorienting density gradient zonal rotor (Ivan Sorvall Inc.) containing a linear sucrose density gradient ranging from 30% (wt/wt) to 65% in 20 mM T-buffer. The gradient was fractionated after 25 min of centrifugation at 25,000 $\times$ g and 0 C, and the 20-ml fractions were collected and monitored turbidimetrically at 540 nm (Fig. 2). Under the conditions employed, the small number of cells present were found in fraction number 3, followed by forespores (peak A) in fractions 11 to 19, large membrane fragments (peak B) in fractions 34 to 40, and small elements such as

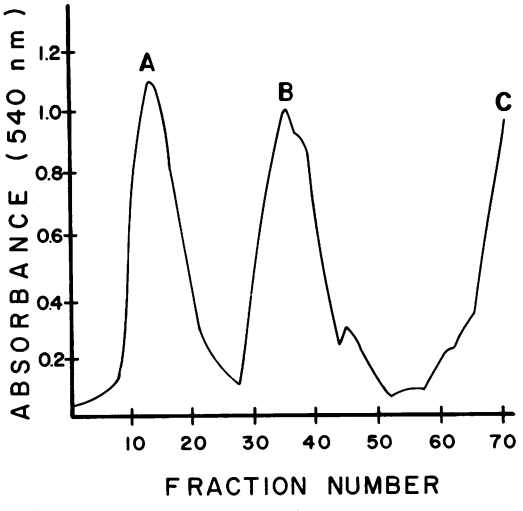

FIG. 2. *Zonal sucrose density gradient pattern of released forespores (peak A), mother cell membranes (peak B), and cytoplasmic components (peak C).*

small membrane fragments, ribosomes, and soluble proteins (peak C) in fractions 62 to 70. The forespore fractions were examined with phase-contrast microscopy and typically showed no contamination by whole cells or detectable cell components. In a series of 10 typical experiments, it was demonstrated that from the equivalent of 4 g (dry weight) of $T_{3\frac{1}{2}}$ cells (Fig. 1) the dry weight yield of released forespores ranged from 0.52 to 0.76 g, i.e., 13 to 19% of the total sporulating cell mass. The dry weight yield of forespores isolated from sporulating cells at late stage IV or early stage V ($T_{4\frac{1}{2}}$) of development (Fig. 1) ranged from 15.5% to 24% of the dry cell mass.

When the forespore release and recovery procedure described was modified by deleting the step involving the preliminary centrifugation (1,000 $\times$ g for 3 min) of the released forespore preparation, for the purpose of removing undisrupted whole cells, dry weight yields of forespores increased by 15 to 20%. Thus, instead of a forespore recovery of 13 to 19% (dry weight) of the total sporulating cell mass from cells harvested at $T_{3\frac{1}{2}}$ and 15.5 to 24% from cells harvested at $T_{4\frac{1}{2}}$, 15 to 23% and 18 to 29%, respectively, were recoverable. However, the density gradient forespore fraction corresponding to peak A (Fig. 2) showed a 2 to 4% contamination with whole sporulating cells.

Electron microscopy of released forespores. Figure 3 shows a group of forespores released at the completion of stage III ($T_{3\frac{1}{2}}$) and isolated by the method described. The intact forespores show the presence of both IFSM and OFSM, the typical granular appearance of the cytoplasm, and no detectable cortex material between IFSM and OFSM. Since young forespores in particular are highly susceptible to osmotic shock, it was consistently observed during these studies that the mildest of fixation methods used in the preparation of electron microscope specimens resulted in significant damage to the structures. This was due primarily to the need to markedly reduce the sucrose concentration during fixation and the apparent inability to fix the structures rapidly and effectively, resulting in a very significant decrease in the stability of the forespores. The disruptive effects of the fixation process were readily observable with phase-contrast microscopy, with the occurrence of pronounced swelling of the structures and an increasing percentage of the forespores undergoing disruption in parallel with the fixation process. The limited definition evidenced by the two forespore membranes (Fig. 3), although both membranes are demonstrated, is presumably due to the damage in-

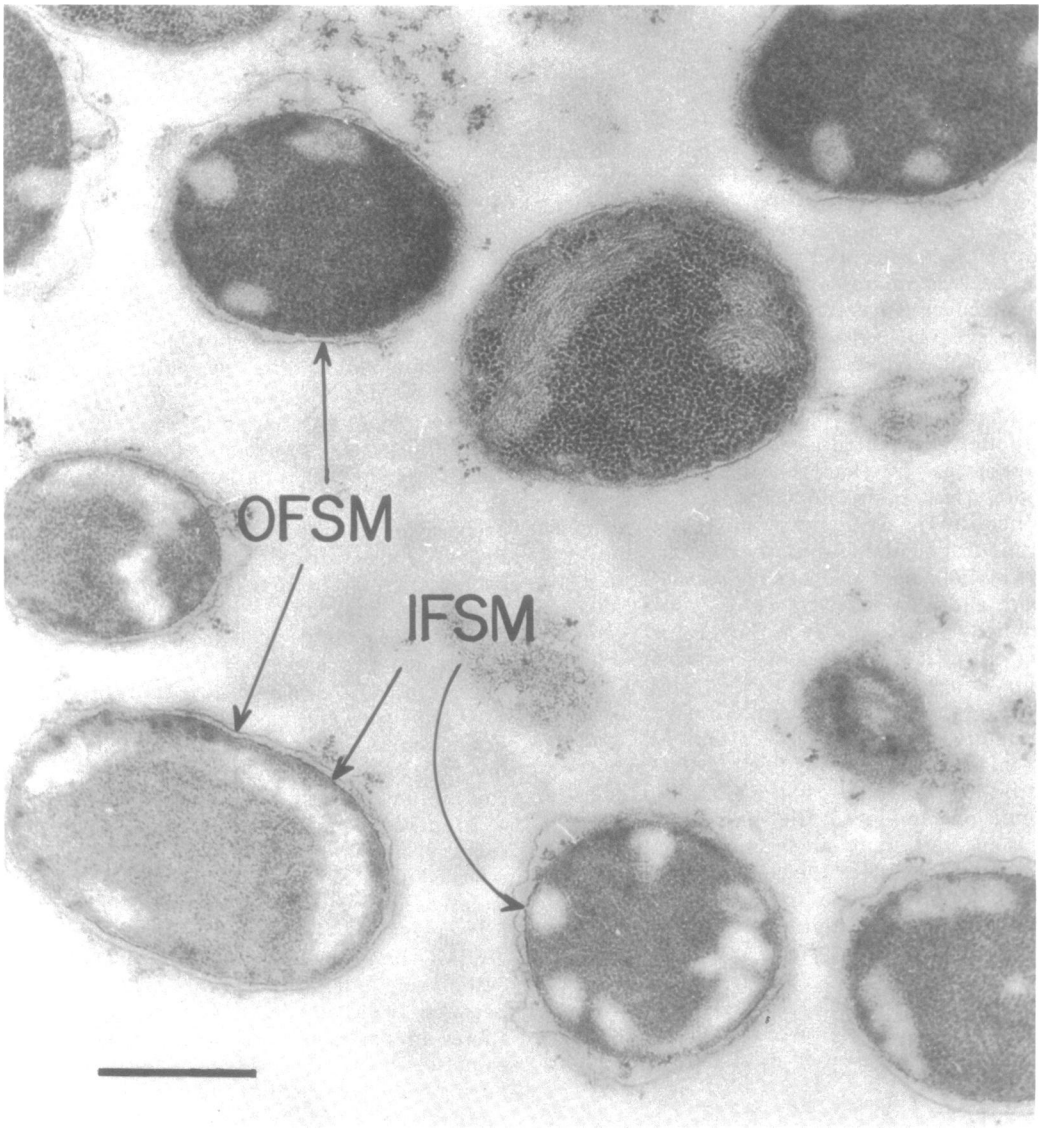

FIG. 3. *Thin section of a group of forespores released from sporulating B. cereus cells at the completion of stage III ($T_{3\frac{1}{2}}$) and recovered by zonal density gradient centrifugation. Note the presence of outer forespore membrane (OFSM), inner forespore membrane (IFSM), and the absence of cortex. The scale bar represents 0.5 μm.*

curred during the partially effective fixation procedure. In marked contrast, young forespores at the same stage of development were quite stable for several hours in T-buffer containing 20 to 30% (wt/wt) sucrose. Figure 4 shows, at a higher magnification (×270,000), adjacent young forespores isolated at the completion of stage III ($T_{3\frac{1}{2}}$). Although a significant degree of resolution is lost at this magnification, the presence of IFSM and OFSM is demonstrated and the absence of cortex is evident. The characteristic appearance of the cytoplasm and nuclear material are also shown.

In Fig. 5 a forespore typical of those released at very early stage V ($T_{4\frac{1}{2}}$), an intact exosporium, engulfed cytoplasm, OFSM, developing cortex, IFSM, and forespore cytoplasm are all clearly seen. Close examination of the outer edge of the OFSM indicates that spore coat synthesis has been initiated (deposition of spore coat proteins), which is typical of early stage V in *B. cereus* (1). Also, it is quite evident that the

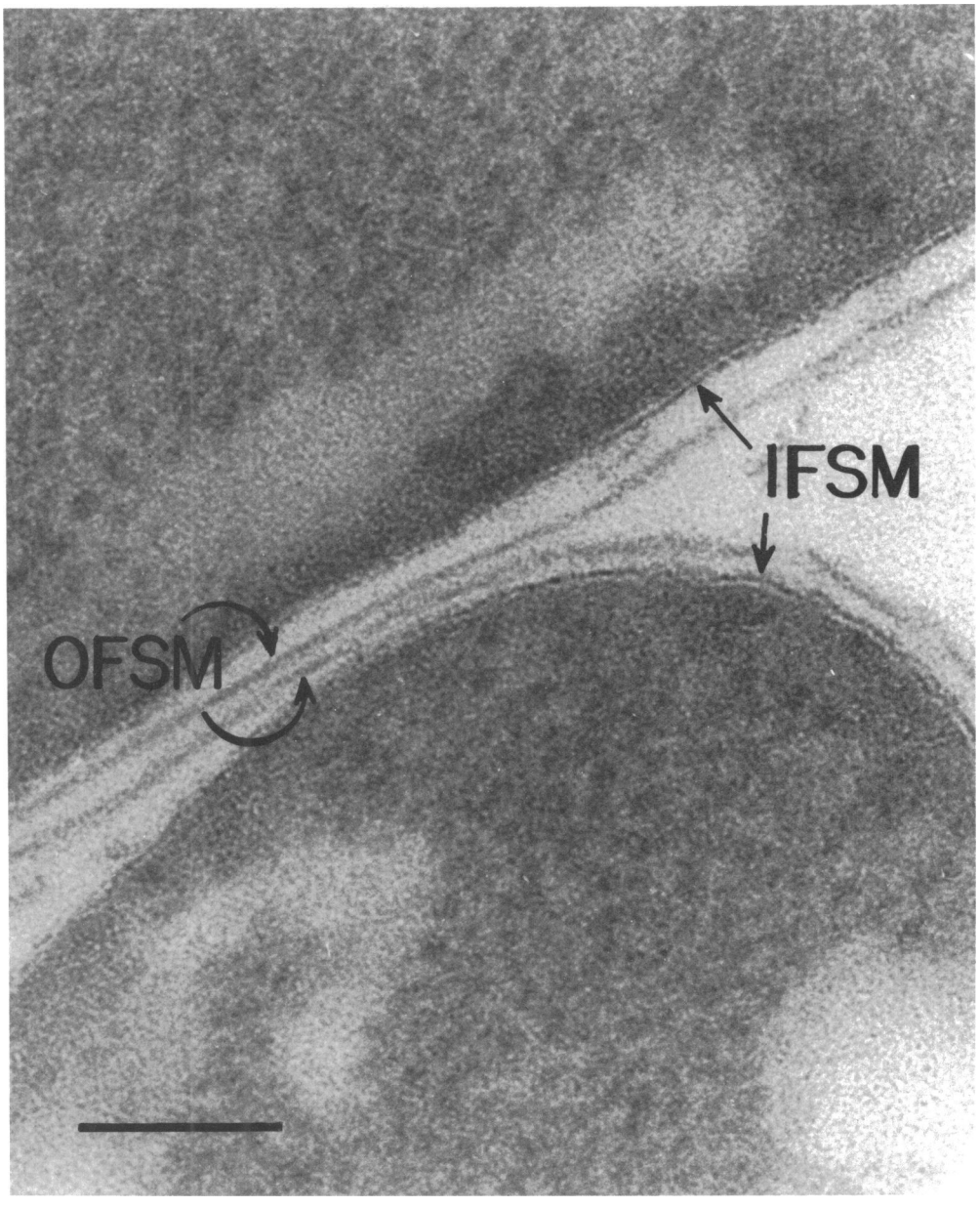

FIG. 4. *Thin section of adjacent B. cereus forespores (same stage as in Fig. 3). Note the presence of OFSM, IFSM, and absence of cortex in each forespore. The scale bar represents 0.1 μm.*

exosporium is fully developed and was preserved during the isolation procedure. It should be noted that forespores at approximately this stage of development, i.e., early stage V or late stage IV, are somewhat less subject to osmotic shock, more amenable to fixation and thin sectioning, and recoverable as homogenous preparations in yields of 15.5 to 24% (dry weight) of the total sporulating cell mass.

Enzyme activity of forespores. With the availability of a method which makes possible the recovery of gram quantities of forespores at specific stages of development, assays were conducted for the presence in isolated forespores and mother cell components of enzymes known to occur during sporulation in *B. cereus*. The levels of GOT, GPT, NADH oxidase, NADH-DCIP oxidoreductase, and alanine de-

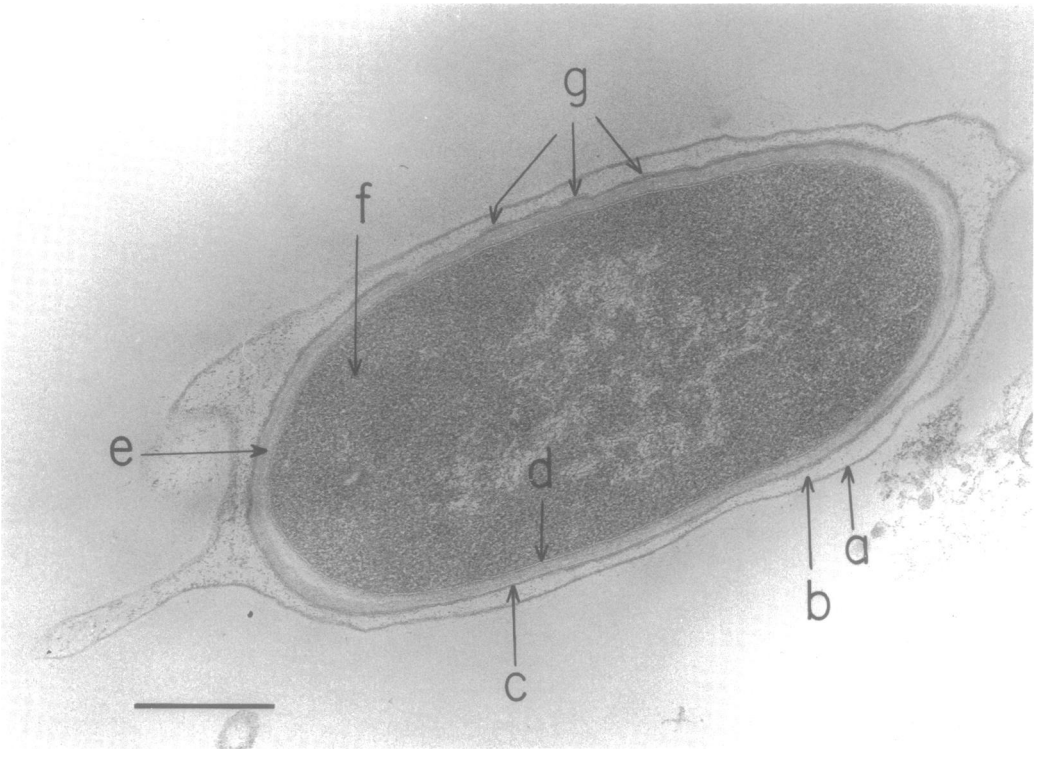

FIG. 5. *Thin section of B. cereus forespore released at very early stage V of development ($T_{4½}$). Note preservation of exosporium (a); exosporium-engulfed cytoplasm (b); outer forespore membrane (c); inner forespore membrane (d); cortex (e); and forespore cytoplasm (f). Initial stages of early spore coat(s) deposition are evident (g). The scale bar represents 0.25 μm.*

hydrogenase were measured in whole sporulating cells disrupted at the completion of stage III ($T_{3½}$), forespores released and recovered at the same stage of development (Fig. 2, peak A), and mother cell membrane (peak B) and cytoplasmic (peak C) preparations. The results presented in Table 1 show that the specific activity of the GPT and GOT present in isolated forespores is 8 to 12 times greater than that in cell-free extracts from whole sporulating cells and 30 to 50 times greater than in mother cell membrane and cytoplasmic preparations. Of the total GPT and GOT activity present in sporulating cells, 50% and 70%, respectively,

TABLE 1. *Specific and total enzyme levels in sporulating cell preparations and in forespores and mother cell components after recovery by zonal density gradient centrifugation*

Enzyme	Enzyme activity[a]							
	Sporulating cells[b]		Forespores		Mother cell membranes		Mother cell cytoplasm	
	Specific	Total	Specific	Total	Specific	Total	Specific	Total
Alanine aminotransferase	1.0	950	8.4	470	0.2		<0.4	
Aspartate aminotransferase	0.9	855	10.9	600	0.2		<0.4	
NADH oxidase	6.8	6,530	15.9	875	1.2			
NADH-DCIP oxidoreductase	11.2	10,640	11.1	610	1.9			
Alanine dehydrogenase	24	22,000	<0.2	11	0		30	

[a] Specific enzyme activity is expressed as micromoles of substrate converted or product formed per hour per milligram of protein.

[b] Whole sporulating cells at the completion of stage III of development ($T_{3½}$) were disrupted in the model RM Ribi cell fractionator at 30,000 lb/in², suspended in 20 mM T-buffer.

was recovered in the isolated forespores. In marked contrast, alanine dehydrogenase was found at high levels in whole-cell and mother-cell cytoplasmic preparations, and was almost undetectable in forespores. NADH oxidase and NADH-DCIP oxidoreductase activities are present at comparable specific activity in forespores and whole cells.

DISCUSSION

An efficient and highly reproducible method for the release and recovery of forespores has been developed. The method described has the following important attributes. (i) The yield of forespores recovered ranges from 13 to 24% of the total sporulating cell mass (dry weight) treated. (ii) Forespores are efficiently released and recovered at all stages of development from the completion of stage III on. (iii) Uniform preparations of forespores are recovered (see Fig. 3). (iv) The method is quite direct and rapid, requiring well under 2 h overall to recover the purified forespores. (v) The method yields intact forespores, with the preservation of OFSM, IFSM, exosporium, and engulfed cytoplasm at appropriate stages (see Fig. 3, 4, and 5). (vi) Preliminary studies show that it can be used with effectiveness for the recovery of forespores from sporulating cells of *B. megaterium* and *B. subtilis*, as well as *B. cereus*. For several of these reasons, the method described is clearly superior to the enzymatic method of forespore release evaluated early in our studies and that utilizing sonic oscillation which was recently reported (6, 12). It should also be noted that, although several grams of forespores are available with the method in the unmodified form presented, quantities can be increased by 15 to 20% by eliminating the preliminary slow-speed centrifugation of the crude forespore preparation. Alternatively, quantities of homogeneous preparations of forespores can readily be increased severalfold simply by acquiring sufficient additional instrumentation for zonal density gradient centrifugation.

The availability of gram quantities of isolated forespores offers an opportunity for the biochemical study of this developmental form as an independent entity. Thus, our preliminary studies, presented here by way of example, show that two amino acid transaminases, GPT and GOT, are present in forespores at relatively high levels of specific and total activity, as compared to "total" sporulating cell preparations. The data suggest that the bulk of the transaminases present may be associated with the developing forespores. Studies currently in progress are intended to more clearly establish the localization of the two transaminases in the sporulating cell. GPT and GOT have previously been associated with the sporulation process in *B. cereus* (2), and their presence in sporulating cells of *B. cereus* and *B. megaterium* was subsequently confirmed in this laboratory (J. Takemoto, M.S. thesis, California State University, Los Angeles, 1969). Although the specific activities and total activities of NADH oxidase and NADH-DCIP oxidoreductase detected in forespores and sporulating cells (Table 1) are somewhat higher than those previously reported in vegetative and sporulating cell preparations (4, 6), their detection is in keeping with their known presence during sporulation. In marked contrast to the readily detectable levels of GPT, GOT, NADH oxidase, and NADH-DCIP oxidoreductase in forespores, alanine dehydrogenase was barely detectable. However, high levels of the latter were very evident in the disrupted sporulating cell preparation and mother cell cytoplasm fraction (Table 1). These findings suggest that little if any of the high level of alanine dehydrogenase present in the mother cell during the early stages of forespore formation through the completion of stage III survives in the forespore, presumably as a consequence of accelerated protein turnover (9). The data also suggest that the synthesis of alanine dehydrogenase does not continue at a significant rate in the forespore. However, GOT, GPT, NADH oxidase, and NADH-DCIP oxidoreductase activity persist and may in fact increase during forespore formation. Whatever the ultimate explanation for these marked differences in forespore enzyme content, it is quite evident that the method now available will provide these unique developmental structures in quantities sufficient to permit more comprehensive biochemical studies.

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LITERATURE CITED

1. Aronson, A. I., and P. C. Fitz-James. 1968. Biosynthesis of bacterial spore coats. J. Mol. Biol. **33**:199–212.
2. Buono, F., R. Testa, and D. G. Lundgren. 1966. Physiology of growth and sporulation in *Bacillus cereus*. I. Effect of glutamic and other amino acids. J. Bacteriol. **91**:2291–2299.
3. Collier, R. E. 1957. An approach to synchronous growth for spore formation in *Clostridium roseum*, p. 10–17. *In* H. O. Halvorson (ed.), Spores. Amer. Inst. Biol. Sci., Washington, D.C.
4. Eisenberg, R. C., L. Yu, and M. J. Wolin. 1970. Masking of *Bacillus megaterium* KM membrane reduced nicotinamide adenine dinucleotide oxidase and solubilization studies. J. Bacteriol. **102**:161–171.
5. Ellar, D. J., and D. G. Lundgren. 1966. Fine structure of

sporulation in *Bacillus cereus* grown in a chemically defined medium. J. Bacteriol. **92:**1748-1764.
6. Felix, J., and D. G. Lundgren. 1972. Some membrane characteristics of *Bacillus cereus* during growth and sporulation, p. 35-44. *In* H. O. Halvorson, R. Hanson, and L. L. Campbell (ed.), Spores V. American Society for Microbiology, Bethesda, Md.
7. Hanson, R. S., V. R. Srinivasan, and H. O. Halvorson. 1963. Biochemistry of sporulation. I. Metabolism of acetate by vegetative and sporulating cells. J. Bacteriol. **85:**451-460.
8. Karmen, A. 1955. A note on the spectrophotometric assay of glutamic-oxalacetic transaminase in human blood serum. J. Clin. Invest. **34:**131-133.
9. Kornberg, A., J. A. Spudich, D. L. Nelson, and M. P. Deutscher. 1968. Origin of proteins in sporulation. Annu. Rev. Biochem. **37:**51-78.
10. Lang, D. R., and D. G. Lundgren. 1970. Lipid composition of *Bacillus cereus* during growth and sporulation. J. Bacteriol. **101:**483-489.
11. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265-275.
12. Lundgren, D. G., D. F. Karp, and D. R. Lang. 1969. Structure-function relationships in bacterial sporulation, p. 20-38. *In* L. L. Campbell (ed.), Spores IV. American Society for Microbiology, Bethesda, Md.
13. Murrell, W. G. 1967. The biochemistry of the bacterial endospore. Advan. Microb. Physiol. **1:**133-251.
14. Ryter, A. 1965. Etude morphologique de la sporulation de *Bacillus subtilis*. Ann. Inst. Pasteur **108:**40-60.
15. Ryter, A., E. Kellenberger, A. Birch-Andersen, and O. Maaløe. 1958. Etude au microscope electronique de plasmas contenant de l'acide desoxyribonucleique. Z. Naturforsch. **136:**597-605.
16. Ryter, A., P. Schaeffer, and H. Ionesco. 1966. Classification cytologique, par leur stade de blocage, des mutants de sporulation de *Bacillus subtilis* Marburg. Ann. Inst. Pasteur **110:**305-315.
17. Sabatini, D. D., K. Bensch, and R. J. Barrnett. 1963. Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. J. Cell Biol. **17:**19-58.
18. Spurr, A. R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26:**31-43.
19. White, P. J. 1972. The nutrition of *Bacillus megaterium* and *Bacillus cereus*. J. Gen. Microbiol. **71:**505-514.
20. Wiame, J. M., A. Pierard, and F. Ramos. 1962. L-Alanine dehydrogenase from *Bacillus subtilis*, p. 673-676. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 5. Academic Press Inc, New York.
21. Wroblewski, F., and J. S. LaDue. 1955. Serum glutamic-oxalacetic transaminase activity as index of liver cell injury: preliminary report. Ann. Intern. Med. **43:** 345-360.
22. Young, E. I., and P. C. Fitz-James. 1959. Chemical and morphological studies of bacterial spore formation. I. The formation of spores in *Bacillus cereus*. J. Biophys. Biochem. Cytol. **6:**467-481.