January 1975

# Lipid Metabolism During Bacterial Growth, Sporulation, and Germination: Kinetics of Fatty Acid and Macromolecular Synthesis During Spore Germination and Outgrowth of *Bacillus thuringiensis*

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JOURNAL OF BACTERIOLOGY, Jan. 1975, p. 227-233

Vol. 121, No. 1
Printed in U.S.A.

# Lipid Metabolism During Bacterial Growth, Sporulation, and Germination: Kinetics of Fatty Acid and Macromolecular Synthesis During Spore Germination and Outgrowth of *Bacillus thuringiensis*

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Received for publication 25 September 1974

The timing and kinetics of fatty acid synthesis are delineated for *Bacillus thuringiensis* spore germination and outgrowth by analyzing [U-$^{14}$C]acetate and [2-$^{3}$H]glycerol incorporation into chloroform-methanol-extractable and trichloroacetic acid-precipitable lipids. In addition to measurement of pulsed and continuous labeling of fatty acids, monitoring the incorporation of radioactive phenylalanine, thymidine, and uridine from the onset of germination through first cell division provides a profile of biochemical activities related to membrane differentiation and cellular development. Upon germination, ribonucleic acid synthesis is initiated, immediately followed by rapid and extensive fatty acid synthesis that in turn precedes protein, deoxyribonucleic acid and triglyceride synthesis. Significantly, formation of fatty acids from acetate exhibits further developmental periodicity in which a large transient increase in fatty acid synthetic activity coincides with the approach of cell division. Radiorespirometric analyses indicates only slight oxidative decarboxylation of acetate and corroborates the extreme involvement of acetate in specific fatty acid biosynthetic reactions throughout cellular modification. These findings graphically demonstrate an intimate association of fatty acid metabolism with commitment to spore outgrowth and subsequent cell division.

Bacterial spore germination has frequently been employed as a model system to study cellular differentiation (13, 14). Because spores germinate synchronously under appropriate cultural conditions, the timing of various biochemical events can be compared as the cells progress through outgrowth. Though the temporal relationship of protein, ribonucleic acid, and deoxyribonucleic acid synthesis to cellular modification has been studied in several germination systems (1, 12, 20, 23), little information is known about the timing of membrane synthesis. Invariably, it has been found that ribonucleic acid is the first macromolecule synthesized after germination (1, 12, 14, 20, 21, 23). Most studies to date on membrane development (2, 10, 11, 15, 18) have been concerned with spore formation rather than with germination. Pitel and Gilvarg (18) used [$^{14}$C]ethanolamine and [$^{14}$C]acetate to demonstrate that phospholipid synthesis continues unabated during septation and forespore envelopment, whereas peptidoglycan synthesis is halted. We recently demonstrated changes in membrane phospholipid (5) and fatty acid metabolism (L. A. Bulla, Jr., and T. L. Mounts, Fed. Proc. **32**:634, 1973) during the developmental stages of *Bacillus thuringiensis* spore formation. Because alterations in metabolism of membrane lipids exhibit a temporal relationship to the developmental shift from vegetative growth to spore formation, it is probable that similar changes accompany the emergence from spore dormancy. Therefore, we wanted to investigate membrane fatty acid composition and synthesis during spore germination and outgrowth of *B. thuringiensis* to correlate certain biochemical events with morphological changes. To do so, knowledge of the timing of new membrane synthesis during germination is essential.

Dawes and Halvorson (8) described four phases for the incorporation of [2-$^{3}$H]glycerol into total lipid during outgrowth of *B. cereus*. They showed that an initial burst of lipid synthesis was followed by a prolonged lag period during which no radioactive glycerol was incor-

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porated. Lipid synthesis resumed at $T_{110}$, concomitant with the onset of net DNA synthesis, and increased to a higher rate at $T_{180}$ when the cells started to divide. If these four phases could be demonstrated in *B. thuringiensis*, they would delineate the most appropriate periods for comparison of fatty acids during outgrowth. Accordingly, we used [$^{14}$C]acetate and [2-$^{3}$H]glycerol to follow the kinetics of lipid synthesis in outgrowing spores of *B. thuringiensis*. Both total trichloroacetic acid-precipitable and chloroform-methanol-extractable lipids were determined. The timing of fatty acid synthesis is seen in better perspective if it is observed within the framework of other macromolecular syntheses. Therefore, we examined the timing of protein, ribonucleic acid, and deoxyribonucleic acid synthesis as well. Scandella and Kornberg (22) described the fatty acid composition of *B. megaterium* cells after germination. However, no previous work has been reported on the kinetics of fatty acid synthesis or on the incorporation of acetate into trichloroacetic acid-precipitable material during spore germination and outgrowth.

*B. thuringiensis* differs from other sporeformers in that it produces a discrete, parasporal protein crystal during sporogenesis. The germination properties of spores formed by cells with crystal-forming ability have not been sufficiently studied to determine whether germination is influenced by the presence of the crystal during sporulation. Our survey of macromolecular and membrane fatty acid synthesis during germination and outgrowth of *B. thuringiensis* is the first to be reported with a crystal-forming bacterium. It is reasonable to expect that spore structure is slightly different in crystal formers than noncrystal formers because Delafield et al. (9) showed that the crystal of *B. thuringiensis* arises in part from excess production of a spore coat protein. (Presented at the 74th annual meeting of the American Society for Microbiology, Chicago, Illinois, 12 to 17 May 1974.)

## MATERIALS AND METHODS

**Organism and cultural conditions.** *B. thuringiensis* NRRL B-4027 was used throughout this study. The strain was originally provided by A. A. Yousten, Department of Biology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia. Spores were prepared by growing the organism in 8 liters of a modified GYS medium: $(NH)_2SO_4$, 0.2%; yeast extract, 0.2%; and $K_2HPO_4$, 0.05% (adjusted to pH 7.3). After autoclaving, the following ingredients were added aseptically: glucose, 0.1%; $MgSO_4$, 0.02%; $CaCl_2 \cdot 2H_2O$, 0.008%; and $MnSO_2 \cdot H_2O$, 0.005%. The spores were harvested, washed once in distilled water and once in 0.05 M phosphate buffer (pH 7.2), and suspended in 100 ml of the phosphate buffer. Spores were heat-activated before germination by homogenizing for 2 min in a Potter-Elvehjem hand homogenizer, heat-shocking for 30 min at 80 C, and homogenizing an additional 2 min. The spores were germinated in the modified GYS medium to which L-alanine (100 μg/ml), adenosine (500 μg/ml), and ethylenediaminetetraacetic acid (25 μg/ml) had been added. We found that the rate of germination, as determined by the decreased optical density at 600 nm (see Fig. 1), was enhanced when ethylenediaminetetraacetic acid was present.

**Incorporation experiments.** The procedures used were basically those of Rodenberg et al. (21). Heat-activated spores were transferred at $T_0$ into flasks containing modified GYS medium, germinating agents, and the appropriate radioactive substrate. We employed L-[2,3-$^{3}$H]phenylalanine to follow protein synthesis, [5,6-$^{3}$H]uridine for ribonucleic acid, [6-$^{3}$H]thymidine for deoxyribonucleic acid, and both [2-$^{3}$H]glycerol and [U-$^{14}$C]acetate for lipid synthesis. Whenever [6-$^{3}$H]thymidine was used, the medium was supplemented with 250 μg of 2-deoxyadenosine, uracil, cytosine, and guanosine (19) per ml to ensure incorporation of radioactive thymidine into deoxyribonucleic acid rather than into ribonucleic acid. The flasks were rotated in a water bath at 28 C and 1 ml-samples were taken at intervals of either 2 or 5 min. Samples were added to 1 ml of 10% trichloroacetic acid containing carrier substrate (100 μg/ml) and filtered on 25-mm membrane filters (0.45 μm; Millipore Corp., Bedford, Mass.). The filters were washed four times with 5 ml of 5% trichloroacetic acid containing carrier substrate (50 μg/ml), placed in 10 ml of the scintillation mixture of Winkler and Wilson (24), and counted in a liquid scintillation spectrometer.

**Chloroform-methanol extraction.** The synthesis of chloroform-methanol-extractable material was studied by procedures identical to those used for total incorporation except that the precipitates were collected on Whatman GP83 glass fiber filters. These filters were extracted for 24 h in scintillation vials containing 10 ml of chloroform-methanol (2:1, vol/vol). The filters were removed, dried in an oven at 80 C, and counted in 10 ml of toluene containing 6 mg of 2,5-diphenyloxazole per ml and 0.1 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene per ml. The chloroform-methanol from which the filters had been removed was evaporated to dryness and counted by adding 10 ml of the toluene–2,5-diphenyloxazole–1,4-bis-2-(5-phenyloxazolyl)-benzene mixture to the scintillation vials.

**Methyl ester preparation.** Methyl esters were prepared as reported previously (4). Cells were extracted for 72 h in 20 ml of chloroform-methanol (2:1, vol/vol), filtered on methanol-washed Whatman no. 1 paper, and washed with 10 ml of chloroform-methanol. The filtrates were combined, dried under a nitrogen stream, and dissolved in 10 ml of chloroform-methanol. This solution was filtered through a Swinny syringe filter holder (13 mm), washed with 5 ml of chloroform-methanol, dried under a nitrogen stream, and redissolved in 0.5 ml of ether. The methyl

esters were formed by the addition of 1 ml of 0.5 N KOH in methanol. After 30 min, the solution was neutralized with 1 N HCl. The methyl esters were removed by extracting three separate times with 1 ml of hexane. The hexane solution was dried under a nitrogen stream and finally resuspended in 0.5 ml of hexane.

**Radiorespirometry during germination and outgrowth.** Radiorespirometry was normally conducted on resting cells. We modified our usual radiorespirometric techniques (6) in this situation by germinating the spores within the respirometric flasks and continuously monitoring the effluent carbon dioxide during germination and outgrowth. At $T_0$, 10 mg of heat-activated spores were injected into respirometer flasks containing 30 ml of modified GYS medium supplemented with ethylenediaminetetraacetic acid (25 $\mu$g/ml), L-alanine (100 $\mu$g/ml), adenosine (500 $\mu$g/ml), and radioactive substrate. [1-$^{14}$C]acetate and [2-$^{14}$C]acetate were added at a specific activity of 2.5 $\mu$Ci per mg per 30 ml; [2-$^{14}$C]glutamate, [3,4-$^{14}$C]glutamate, and [5-$^{14}$C]glutamate were present at 1 $\mu$Ci per mg per 30 ml. Metabolic $CO_2$ was trapped in 10 ml of ethanol-ethanolamine (2:1, vol/vol) which was replenished at 15-min intervals throughout outgrowth. Subsequent procedures were identical to those already described (6).

**Materials.** The specifically labeled acetate and glutamate were purchased from New England Nuclear Corp., Boston, Mass., the 2-deoxyadenosine from Sigma Chemical Co., Boston, Mass., and the L-[2,3-$^3$H]phenylalanine, [5,6-$^3$H]uridine, [6-$^3$H]thymidine, and [2-$^3$H]glycerol from International Chemical & Nuclear Corp., Irvine, Calif.

## RESULTS

**Macromolecular synthesis during germination and outgrowth.** Spore germination and outgrowth were monitored by phase-contrast microscopy during the incorporation experiments. All spores lost their refractility by $T_{15}$, at which time the optical density at 600 nm was near minimum (Fig. 1). After this time, the spores swelled, elongated, and underwent septum formation at about $T_{110}$; first cell division occurred at $T_{130}$. Figure 1 shows the rate at which [2-$^3$H]glycerol, [2,3-$^3$H]phenylalanine, [6-$^3$H]thymidine, [U-$^{14}$C]acetate, and [5,6-$^3$H]uridine were incorporated into trichloroacetic acid-precipitable material. The cells incorporated uridine and acetate more rapidly than the other precursors. Uracil, cytosine, guanosine, and 2-deoxyadenosine (all 250 $\mu$g/ml) were added to each flask. Using this procedure, Rana and Halvorson (19) found that 97% of the [6-$^3$H]thymidine was incorporated into deoxyribonucleic acid. Approximately three times more [6-$^3$H]thymidine was incorporated in the presence of 2-deoxyadenosine than in its absence. A similar effect has been observed in *B. megaterium* (23).

The onset of macromolecular synthesis during spore germination and outgrowth has been studied in several other bacterial systems (1, 12, 21, 23) with the uniform result that ribonucleic acid is the first macromolecule to be synthesized. This phenomenon is also true for *B. thuringiensis* spores (Fig. 1). Acetate incorporation commenced rapidly, but not as rapidly as uridine incorporation. The exact time at which incorporation of the respective precursors begins can be seen more clearly on a logarithmic scale (Fig. 2). The linear scale in Fig. 1 tends to obscure small increases at the onset of glycerol, phenylalanine, and thymidine incorporation. Uridine incorporation started at about $T_{10}$ and acetate incorporation at $T_{20}$, whereas glycerol, phenylalanine, and thymidine incorporation began at about $T_{30\text{-}40}$ (Fig. 2). The rates of incorporation of the latter three precursors appear linear when plotted logarithmically (Fig. 2). This is surprising because the cells do not increase in number during outgrowth; also, germination is complete by $T_{15}$. No discontinuities in total lipid synthesis were seen.

**Chloroform-methanol extraction.** [U-$^{14}$C]acetate and [2-$^3$H]glycerol are not incorporated solely into lipid. Total trichloroacetic acid-precipitable [U-$^{14}$C]acetate and [2-$^3$H]glycerol were fractionated into chloroform-methanol-extractable and nonextractable portions. This procedure was done to determine whether the chloroform-methanol-extractable lipid exhibited any developmental changes not evident in the total incorporation data. Throughout outgrowth, 40 to 50% of the [U-$^{14}$C]acetate and [2-$^3$H]glycerol was incorporated into the chloroform-methanol-extractable fraction. A burst in extractable acetate incorporation was evident at $T_{110}$ (Fig. 3) and a build-up in nonextractable glycerol at $T_{90\text{-}100}$ preceded the rapid [2-$^3$H]glycerol incorporation at $T_{110}$ (Fig. 4).

**Radiorespirometry of outgrowing spores.** Because germinating spores actively incorporate acetate into trichloroacetic acid-precipitable material, we were able to determine whether acetate metabolism during outgrowth included oxidation to $CO_2$ via the tricarboxylic acid cycle. Spores were germinated in respirometer flasks containing specifically labeled acetate and glutamate, and the release of $^{14}CO_2$ was followed continuously throughout outgrowth. Both acetate and glutamate were incorporated extensively into the cells but less than 0.1% of the radioactivity was recovered as $^{14}CO_2$, irrespective of the substrate or the position of the radioisotope. Neither acetate nor glutamate was oxidized via the tricarboxylic

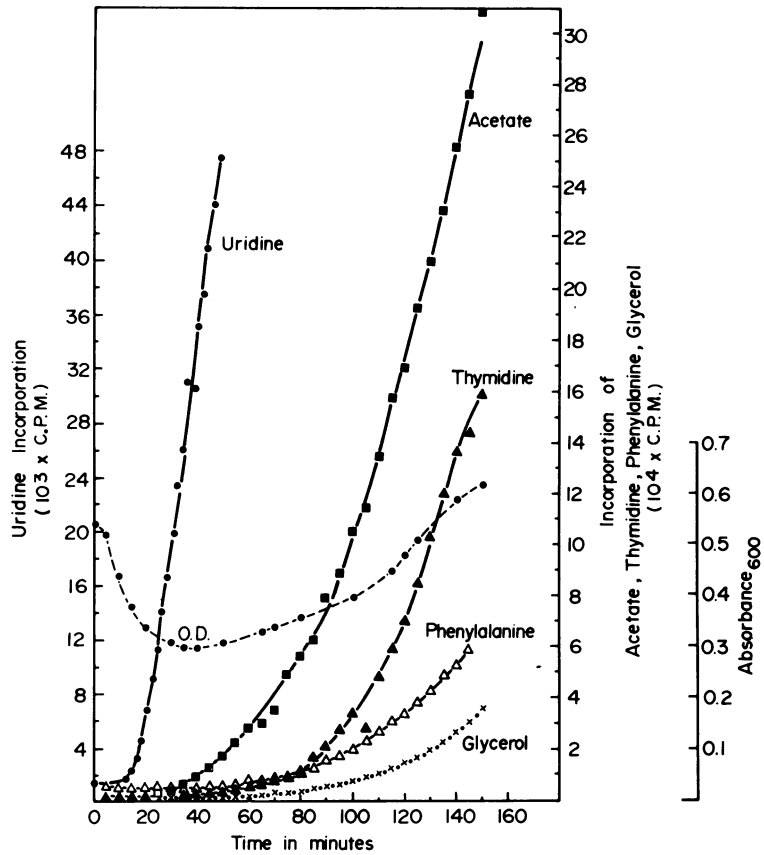


FIG. 1. *Kinetics of lipid, protein, ribonucleic acid, and deoxyribonucleic acid synthesis during outgrowth of B. thuringiensis. Heat-activated spores were germinated at 600 μg/ml in GYS medium supplemented with ethylenediaminetetraacetic acid (25 μg/ml), L-alanine (100 μg/ml), and adenosine (500 μg/ml) containing either [U-$^{14}$C]acetate (2.5 μCi per 20 μg per ml), [2-$^{3}$H]glycerol (5 μCi per 5 μg per ml), L-[2,3-$^{3}$H]phenylalanine (5 μCi per 20 μg per ml), [5,6-$^{3}$H]uridine (8 μCi per 40 μg per ml), or [6-$^{3}$H]thymidine (5 μCi per 5 μg per ml). In addition, all cultures contained 250 μg each of uracil, cytosine, guanosine, and 2-deoxyadenosine per ml (20). Flasks were incubated with shaking at 28 C, and 1-ml samples were transferred at 5-min intervals into 1 ml of trichloroacetic acid (10%) containing 100 μg of carrier substrate per ml. The phenylalanine samples were heated at 100 C for 15 min. All samples were filtered on 25-mm membrane filters (0.45 μm; Millipore) and washed four times with 5 ml of trichloroacetic acid (5%) containing 50 μg of carrier substrate per ml. Germination was measured by following the decrease in optical density (O.D.) at 600 nm in a Bausch & Lomb Spectronic 20 spectrophotometer. Symbols: ×, trichloroacetic acid-precipitable; [2-$^{3}$H]glycerol; Δ, L-[2,3-$^{3}$H]phenylalanine; ▲, [6-$^{3}$H]thymidine; ■, [U-$^{14}$C]acetate; ●--●, O.D. of culture at 600 nm; and ●——●, uridine.*

acid cycle during outgrowth. This finding is in agreement with previous work on germination of *B. cereus* spores (3).

**Conversion of acetate to fatty acids.** The chloroform-methanol extraction procedure allowed determination of total lipid synthesis. In addition, the specific incorporation of acetate into fatty acids was investigated by purifying the fatty acid methyl esters (4) before the extent of acetate incorporation was measured. Because no dramatic shifts in the rate of acetate incorporation during outgrowth were detected (Fig. 2 and 3), we examined fatty acid synthesis in four successive half-hour intervals covering the duration of outgrowth. The extent of acetate incorporation into fatty acids as indicated by continuous labeling (Fig. 5) closely approximated the patterns previously observed (Fig. 1-3); that with pulse labeling did not. When the radioactive acetate was added at $T_{60}$ and the cells harvested at $T_{90}$, eight times more radioactivity was found in the fatty acid fraction than when the acetate was added at $T_0$. This phenomenon is not the result of prior utilization of the acetate added at $T_0$ for purposes other than fatty acid synthesis (it is not oxidized to $CO_2$)

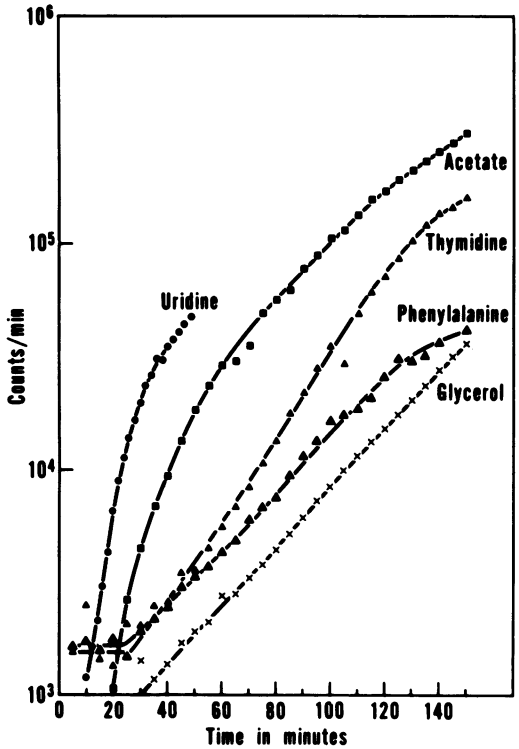


FIG. 2. *Kinetics of uridine, acetate, thymidine, phenylalanine, and glycerol incorporation during outgrowth of B. thuringiensis. Data of Fig. 1 are plotted on logarithmic scale. L-Phenylalanine data is from a separate experiment identical to Fig. 1 except that the precipitates were also washed with two 5-ml portions of ethanol. Symbols: ■, trichloroacetic acid-precipitable [U-$^{14}$C]acetate; ●, [5,6-$^{3}$H]uridine; ▲, [6-$^{3}$H] thymidine; Δ, L-[2,3-$^{3}$H]phenylalanine; and ×, [2-$^{3}$H]glycerol.*

because, in the continuous labeling experiment (Fig. 5), high levels of radioactivity did appear in the fatty acid fraction when the cells were harvested at $T_{120}$. These results seem to indicate initial use of acetate to form a precursor, or storage compound, that is only transformed to fatty acids at the onset of septum formation.

## DISCUSSION

The major finding reported in this paper is that acetate is incorporated rapidly and extensively into trichloroacetic acid-precipitable material and that much of this incorporation constitutes fatty acid synthesis. Whether the appearance of fatty acid synthesis immediately after the onset of ribonucleic acid synthesis is characteristic of other spore germination systems is not yet known. No developmental pattern was observed for total glycerol incorporation. Indeed, the rate of glycerol incorporation was linear when plotted on a logarithmic scale (Fig. 2). Acetate incorporation, however, did show some developmental periodicity in the chloroform-methanol-extractable fraction. The rapid increase at $T_{110}$ coincides with the onset of septum formation. Incorporation of both acetate (Fig. 3) and glycerol (Fig. 4) increases dramatically with the approach of cell division. Daniels (7) found that vegetative cells of *B. megaterium* and *Escherichia coli* exhibited large transient increases in lipid synthesis as they entered cell division.

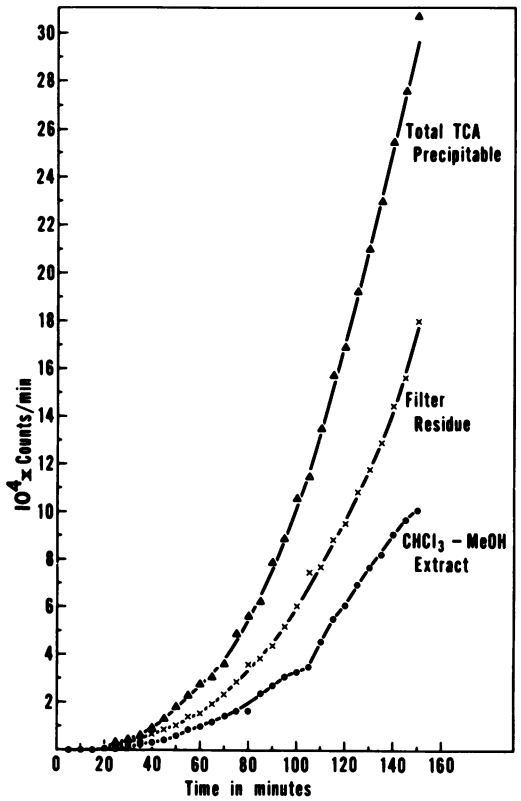


FIG. 3. *Distribution of acetate incorporated during outgrowth of B. thuringiensis. Conditions were identical to those of Fig. 1 except that the trichloroacetic acid precipitates were filtered on Whatman GP83 glass fiber filters. After washing, these filters were placed in scintillation vials containing 10 ml of chloroform-methanol (2:1, vol/vol) and allowed to stand for 24 h. Radioactivity in the filter residues was counted after the filters were removed from the chloroform-methanol, dried in an oven at 80 C and counted. The chloroform-methanol from which the filters were removed was evaporated for 72 h in a hood. Radioactivity in the dry chloroform-methanol extract was counted after adding 10 ml of scintillation fluid to the vials and gently swirling. Symbols: ×, filter residue; ●, chloroform-methanol extract; and Δ, total trichloroacetic acid-precipitable [U-$^{14}$C]acetate (data of Fig. 1).*

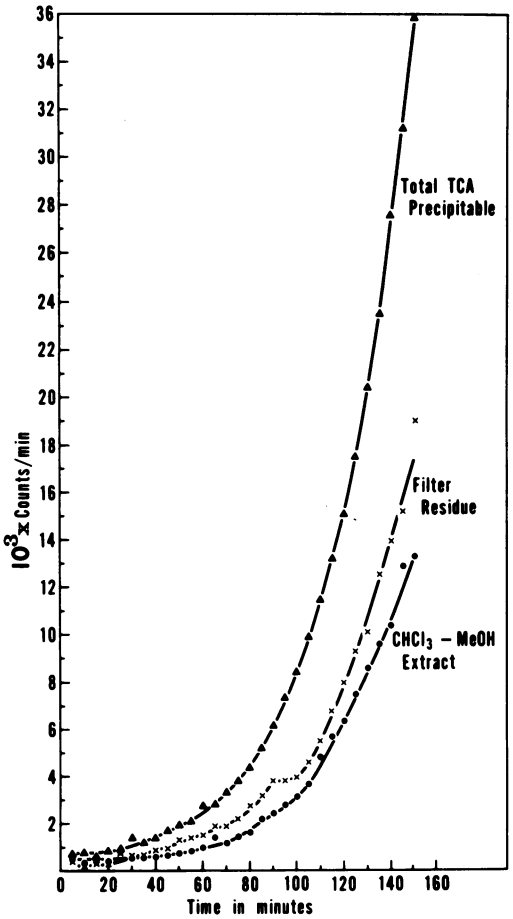


FIG. 4. *Distribution of glycerol incorporated during outgrowth of B. thuringiensis. Conditions were identical to those of Fig. 1. Filter residues and chloroform-methanol extracts were obtained as in Fig. 3. Symbols as in Fig. 3 (data of Fig. 1, note change in scale).*

The onset of DNA synthesis in *B. thuringiensis* at $T_{40}$ (Fig. 2) is in agreement with values found in other germination systems. Replicative DNA synthesis was found to begin at $T_{30}$ for outgrowth of *B. cereus* (20), $T_{45}$ for *B. subtilis* (1), and $T_{55}$ for *B. megaterium* (23). These times cannot be compared precisely because the strains of *B. cereus* and *B. subtilis* used (1, 20) germinate more rapidly than does *B. thuringiensis*. Setlow's value (23) of $T_{55}$ probably reflects his choice of a minimal germination medium, whereas our use of a GYS medium resulted in a rapid increase in absorbance at 600 nm (Fig. 1) after germination.

Dawes and Halvorson (8) reported that lipid synthesis could proceed without prior protein synthesis. We found that acetate incorporation into chloroform-methanol-extractable material precedes that of exogenous phenylalanine for protein synthesis (Fig. 1-3). Net protein synthesis started only after $T_{20}$ (Fig. 2). This time is later than has been observed in other systems (1, 12, 21). We did not conduct protein inhibitor studies and so cannot exclude the possibility of cryptic protein synthesis prior to $T_{40}$. There are indications from other systems that failure to incorporate exogenous phenylalanine does not necessarily indicate complete absence of protein synthesis (1, 12). Cryptic protein synthesis could result from use of nonradioactive precursor pools or from yeast extract in the germina-

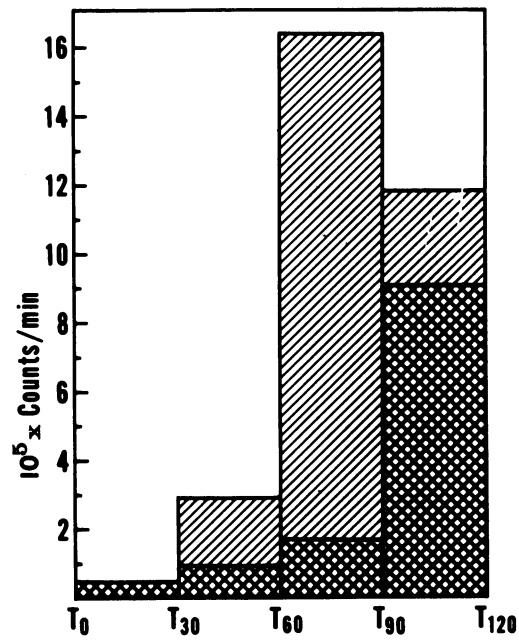


FIG. 5. *Acetate incorporation into fatty acids: pulse versus continuous labeling. Heat-activated spores (1.68 mg/ml, dry weight) were germinated in GYS medium supplemented with ethylenediaminetetraacetic acid (25 μg/ml), L-alanine (100 μg/ml), and adenosine (500 μg/ml). Flasks were incubated at 28 C and aerated by rotary agitation at 200 rpm. Spores were inoculated at $T_0$. [1-$^{14}$C]acetate (1.2 μCi per 20 μg per ml) was also added at $T_0$ in the continuous-labeling experiment; 25-ml samples were taken at 30-min intervals. [1-$^{14}$C]acetate (1.2 μCi per 20 μg per ml) was added at $T_{30}$, $T_{60}$, and $T_{90}$ to individual flasks containing spore suspensions identical to those used for the continuous-labeling experiment. Samples (25 ml) were taken 30 min after [1-$^{14}$C]acetate addition. All samples were immediately chilled, centrifuged at 4 C, and washed with 25 ml of 0.02 M phosphate buffer (pH 7.4). The cells were extracted for 72 h in 20 ml of chloroform-methanol (2:1, vol/vol) under a nitrogen atmosphere. Fatty acid methyl esters were prepared as previously described (4), suspended in 0.5 ml of hexane, and counted for radioactivity. Symbols: slant hatching, pulse labels; cross hatching, continuous label.*

tion medium. The presence of intact parasporal protein crystals throughout outgrowth as observed via phase-contrast microscopy precludes dilution of the radioactive phenylalanine by hydrolyzed protein crystal.

Radiorespirometric experiments reveal that no $^{14}CO_2$ is released from specifically labeled acetate or glutamate. This means that there is extensive incorporation of acetate into lipid material in the absence of a functional tricarboxylic acid cyclic. Absence of the tricarboxylic acid cycle has previously been observed during germination of *B. cereus* and *B. subtilis* (3). Acetate incorporation observed in our experiments is not simply part of an extensive oxidative acetate metabolism, and, consequently, enhances the significance of specific conversion of acetate to fatty acids. The pulse label data in Fig. 5 are consistent with rapid incorporation of acetate into a storage compound or fatty acid precursor, followed by more extensive fatty acid synthesis later in outgrowth. If a fatty acid precursor is present, polyhydroxybutyrate is a likely candidate. During sporulation, 70% of the acetate is converted to polyhydroxybutyrate (16) and very little of the polyhydroxybutyrate is released as $CO_2$ when it is later converted to permanent cell components (17).

## ACKNOWLEDGMENTS

We gratefully acknowledge the capable technical assistance of Gordon L. Adams.

K.W.N. was a recipient of a postdoctoral resident research associateship established by the Agricultural Research Service, U.S. Department of Agriculture, in association with the National Academy of Sciences, National Research Council, 1971-1973.

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