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Protein Synthesis and Breakdown in the Mother-Cell and Forespore Compartments during Spore Morphogenesis in *Bacillus megaterium*

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Recently developed techniques for isolating forespores from bacilli at all stages of spore morphogenesis have been exploited to investigate the contribution of each of the two compartments of the sporulating cell to the overall pattern of protein synthesis and degradation during sporulation in *Bacillus megaterium*. These studies have shown: (1) that protein synthesis continues in both compartments throughout spore morphogenesis; (2) that the degradation of proteins made at all times during vegetative growth and sporulation is confined to the mother-cell compartment; (3) that proteins synthesized in the mother-cell compartment during sporulation are subsequently degraded more rapidly than proteins synthesized during vegetative growth. This rate of degradation increases the later the proteins are synthesized in the sporulation sequence. Mature spores were disrupted, and the percentage of the total protein in soluble and particulate fractions was determined. Pulse-labelling experiments were performed to investigate the extent to which the proteins of these two fractions are newly synthesized during sporulation. These data were used to calculate the extent of capture of vegetative cell protein at the time of formation of the forespore septum. The value obtained is consistent with evidence from electron micrographs and supports a model for the origin of spore protein in which there is no protein turnover in the developing forespore.

Certain bacteria respond to depletion of metabolites in the culture environment by the development of dormant endospores. This process of sporulation requires an ordered sequence of biochemical and morphological changes which have been reviewed (Dawes & Hansen, 1972). In one of the first of these morphological changes the vegetative cell is asymmetrically partitioned into two compartments by the formation of a transverse double-membrane septum. The smaller of these two compartments (forespore), which is destined to become the mature spore, is next engulfed within the larger compartment (mother cell) by a continued invagination of the transverse septum. With the completion of this engulfment, the forespore exists within the mother-cell cytoplasm as a discrete cell, containing a complete genome, and bounded by a double membrane. Continued development of the forespore leads to the production of the mature dormant spore, which is eventually released on lysis of the mother cell.

Many important questions about the biochemistry and control of sporulation have remained unanswered owing to the absence of techniques for separating the developing forespore from the mother cell at all stages of its development and hence to permit their separate analysis (Kornberg *et al.*, 1968). At least 70% of the spore protein in *Bacillus thuringiensis* and *Bacillus subtilis* has been shown to be newly synthesized during sporulation (Monro, 1961; Mandelstam & Waites, 1968; Spudich & Kornberg, 1968*a*) and many enzymes either appear for the first time, or increase markedly in inactivity during spore morphogenesis (Kornberg *et al.*, 1968). Until now, however, it has not been possible to examine the location and distribution of these events within the two cell compartments and the factors controlling this distribution (Kornberg *et al.*, 1968).

A number of workers have succeeded in isolating forespores in the final stage of development when they are relatively resistant to lytic and mechanical treatments (Szulmajster & Hanson, 1965; Spudich & Kornberg, 1968*b*). Attempts to isolate forespores at earlier stages have until recently been less successful. Kawasaki *et al.* (1967) described some properties of forespores isolated from a single unidentified sporulation stage of *B. subtilis*. These forespores were not heat-resistant, but could form colonies on nutrient agar plates. Observations on the development of resistance to osmotic lysis during sporulation (Ellar & Posgate, 1974) suggest that these forespores had probably reached stage V before isolation. Lundgren *et al.* (1969) described the isolation of forespores from *Bacillus cereus* in early stage IV, but concluded that the yields obtained were inadequate for biochemical work (Felix & Lundgren, 1972). However, two reports have described the isolation of forespores at all stages of development and in quantities sufficient for biochemical analysis (Andreoli *et al.*, 1973;

Ellar & Posgate, 1974). Ellar & Posgate (1974) used a strain of *Bacillus megaterium* which remains lysozyme-sensitive throughout sporulation. It was thus possible to prepare protoplasts from sporulating cells and to release forespores by selective disruption of the mother-cell plasma membrane. Using this system Ellar & Posgate (1974) have shown that the spore-specific compound dipicolinic acid is found exclusively in the forespore compartment. They have also prepared mother-cell and forespore membrane fractions and have studied differences between the two membranes, particularly with respect to changes in composition during sporulation.

La Nauze *et al.* (1974) have used the system of Ellar & Posgate (1974) to study the metabolic capabilities of protoplasts and forespores formed from sporulating *B. megaterium* cells. Their results indicate that although sporangial protoplasts at stage IV resemble whole cells in their ability to accumulate calcium, isolated forespores do not, indicating a requirement for the structural and functional integrity of the mother cell in this process.

One important question which has remained unanswered because of the lack of a suitable method for isolating forespores, concerns the location of the extensive protein turnover which has been shown to occur during sporulation (Monro, 1961; Foster & Perry, 1954; Mandelstam & Waites, 1968; Spudich & Kornberg, 1968*b*). It is not known whether this turnover is confined to the mother cell, the forespore, or is a feature of metabolism in both compartments. Clearly protein turnover appears to be an important biochemical event in sporulation, since mutants which do not show turnover are almost invariably asporogenous (Mandelstam, 1971). It was originally suggested that protein turnover is necessary to ensure a steady supply of pool amino acids from which the new proteins needed for sporulation can be synthesized (Mandelstam, 1960). Experiments of Mandelstam & Waites (1968), however, suggest that this may not be the primary role of protein turnover. It seems more likely that the importance of turnover during sporulation lies in the requirement of the cell to change significantly its complement of proteins. In such a system protein breakdown may be as important as protein synthesis as a means of metabolic control (Schimke, 1969).

The aim of these experiments was therefore to investigate the location and timing of protein synthesis and breakdown in the two cell compartments during sporulation, in the system for forespore isolation developed by Ellar & Posgate (1974).

Experimental

Materials

The organism used was a sporogenic strain of *B. megaterium* KM. This organism has the advantage of remaining lysozyme-sensitive throughout sporulation and it is therefore possible to prepare protoplasts and isolate the developing forespores, at all stages of their development, by selectively rupturing the mother-cell plasma membrane (Ellar & Posgate, 1974).

Radioactively labelled phenylalanine (L-3-[U-^{14}C]-phenylalanine; 513mCi/mmol) and leucine (L-[U-^{14}C]leucine; 342mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

All other chemicals used were of analytical reagent grade where these were available.

Methods

Growth and sporulation. To obtain synchronous sporulation, the method of transferring exponentially growing cells from a rich to a minimal salts medium was used. Synchronous sporulation is used in these studies to describe a system in which the population of cells in any given sporulation stage rises from 0 to 90% within 90min.

Cells growing exponentially in a peptone–yeast extract medium (PWYE medium) were then transferred to pre-warmed minimal salts sporulation medium (CCY medium) as previously described (Ellar & Posgate, 1974). All cultures were grown at 30°C in a Gallenkamp orbital incubator shaking at 200rev./min. Growth, sporulation and morphological synchrony were monitored as previously described (Ellar & Posgate, 1974).

Measurement of the degradation of pre-existing vegetative cell protein in whole cells and in separated mother-cell and forespore compartments during sporulation. The following procedure, based on that of Spudich & Kornberg (1968*b*), was used to investigate the degradation of pre-existing vegetative-cell protein during subsequent sporulation. Cells growing exponentially in peptone–yeast extract medium were used to inoculate 100ml of labelling medium to a density of approx. 10μg dry weight of cells per ml of culture. The labelling medium, based on the CCY sporulation medium, contained $MgSO_4,7H_2O$ (0.5mM), $MnSO_4,4H_2O$ (0.01mM), $Fe_2(SO_4)_3,9H_2O$ (0.05mM), $ZnSO_4,7H_2O$ (0.05mM), K_2SO_4 (1.0mM), $CaCl_2,6H_2O$ (0.1mM), KH_2PO_4 (50.0mM) and KOH (42.5mM). For every 100ml of this salts solution, 0.5ml of the following nutrient stock solution was added: casein hydrolysate (British Drug Houses, Poole, Dorset, U.K.) (10%)–enzymic yeast extract (Difco, Detroit, Mich. 48201, U.S.A.) (2%). Either [^{14}C]phenylalanine (final concentration 0.1μCi/ml) or [^{14}C]leucine (final concentration 0.1μCi/ml) was added. The cells from this culture were harvested while still in the exponential phase of growth (at

approx. 150μg cell dry weight per ml), washed with CCY medium (pre-warmed to 30°C) and used as an inoculum for 500ml of CCY medium to give a suspension density of approx. 30μg cell dry weight per ml.

Duplicate samples were removed initially and at intervals thereafter, for the determination of total cell protein and incorporated radioactivity as described below. After 4h a chase of unlabelled phenylalanine or leucine (6mmol) was added. Once the cells were observed to contain a fully engulfed forespore, samples (50ml) were removed at intervals and fractionated to give mother-cell and forespore preparations as described by Ellar & Posgate (1974), with small modifications. Cells were harvested from the CCY medium by centrifugation at 8000*g* for 3min at 4°C and resuspended in sucrose – salts (SPM) buffer [0.6M-sucrose (pH6.3) – 0.1M-potassium phosphate buffer–0.016M-$MgSO_4$]. Protoplasts were formed by incubating this suspension with lysozyme (200μg/ml) for up to 30min at 37°C. The suspension was cooled to 4°C in ice and the forespores were released from the protoplasts by sonication for up to six 1s pulses with a 1.2cm (0.5 inch) sonic probe (Dawe Instruments Ltd., London W.3, U.K.) operating at maximum output. The isolated forespores were separated from the mother-cell material by centrifugation at 11600*g* for 3min at 4°C. The supernatant was retained as the mother-cell fraction and the forespore pellet was washed once with 20ml of sucrose–salts buffer and finally resuspended in 10ml of phosphate–magnesium (PM) buffer [0.1M-potassium phosphate buffer (pH6.3)–0.016M-$MgSO_4$].

Preparation of whole cell samples for total protein determination. Duplicate 5ml samples of culture were taken at intervals and the cells sedimented by centrifugation, washed once with 5ml of distilled water and resuspended in 5ml of distilled water. Lysozyme was added to give a final concentration of 200μg/ml and the suspension incubated at 37°C for 5–15min. Sodium dodecyl sulphate [0.1ml of a 10% (w/v) solution] was added, whereupon the suspension became clear. This solution was assayed for protein by the method of Lowry *et al.* (1951). The total protein in a sample of 5ml of distilled water containing lysozyme (200μg/ml) and sodium dodecyl sulphate assayed in a similar manner, was subtracted from each value for the cell suspensions.

Assay of total protein in the isolated mother-cell and forespore fractions. Total protein in the isolated fractions was assayed directly by the method of Lowry *et al.* (1951). Sodium dodecyl sulphate (0.1ml; 10% solution) was added to each sample to aid solubilization of the protein. The same quantity was added to the blanks.

Assay for radioactivity. Ice-cold 50% (w/v) trichloroacetic acid (1ml) was added to duplicate samples of the original culture (2.5ml) and to each of the isolated fractions (2ml). Each sample was shaken and held in ice for 10min. Samples were then filtered on Whatman GF/C 25mm diameter glass-fibre filters and the precipitate was washed with 6vol. of distilled water. The filters were dried at 105°C for 1h and counted for radioactivity in a toluene-based scintillation fluid containing 4g of 2,5-bis-(5-t-butylbenzoxazol-2-yl)thiophen scintillant/litre.

Fractionation of the cells to determine the nature of the incorporated radioactivity. A sample of cell suspension taken at stage II of sporulation was fractionated into pool, lipid, protein and nucleic acid fractions (Roberts *et al.*, 1955) and each fraction assayed for radioactivity.

Investigation of protein synthesis and multiple turnover during sporulation. To investigate the extent and cellular location of protein synthesis and degradation during sporulation, the following procedure was adopted, based on that of Spudich & Kornberg (1968*b*). Cultures (500ml) of the organism in CCY medium were grown to a given stage of sporulation. [^{14}C]Phenylalanine (20μCi; 513mCi/mmol) was then introduced into the culture and after growth for a further hour a chase of 6mmol of unlabelled L-phenylalanine was added. At subsequent intervals, samples were removed for assay of trichloroacetic acid-precipitable radioactivity in whole cells and in mother-cell and forespore preparations after fractionation as described above. This experiment was carried out six times with the isotope being added at $t_{2.7}$, t_3, t_5, $t_{6.5}$, t_7 and $t_{8.25}$ (t_n is used to denote the time in hours after the end of exponential growth).

Fractionation of forespores and mature spores into soluble and particulate fractions. Three different methods were used to disrupt forespores. (1) Forespores at stage III and IV could be lysed by suspension in the PM buffer. (2) Forespores at stage V and VI and mature spores could be disrupted by sonication for 30min in a Mullard type SL 82 ultrasonic chamber. (3) Forespores at stage V and VI and mature spores could be disrupted by homogenization for 30s in a Braun cell homogenizer (model MSK) with glass beads (0.10–0.11mm diam.); 10ml of the forespore preparation was homogenized with 50g of glass beads plus 5ml of deionized water. The forespore homogenate was separated from the beads by filtration through a coarse sintered-glass filter. The beads were then washed with approx. 40ml of deionized water. The filtrate and washings were pooled.

Disrupted forespore preparations were centrifuged at 165000*g* for 1h in a Beckman L2-50 ultracentrifuge in a type 50 fixed-angle rotor. The supernatant was designated the soluble fraction and the pellet the particulate fraction.

Unless otherwise stated, total protein in these fractions was assayed by the method of Lowry *et al.* (1951) with 0.1ml of 10% sodium dodecyl sulphate solution added to each assay to aid solubilization. In

some samples total protein was also assayed by the ninhydrin reaction (Moore & Stein, 1948) after total hydrolysis of the protein with 6M-HCl for 20h at 105°C.

Investigation of the extent to which soluble and particulate fractions of forespores and mature spores are newly synthesized during sporulation. CCY medium (750ml) containing 30μCi of [^{14}C]phenylalanine (513mCi/mmol) was inoculated to a suspension density of approx. 20μg cell dry weight per ml with cells from an exponential-phase culture in PWYE medium and incubated as described. At t_1 8mmol of unlabelled L-phenylalanine was added and a sample of culture was removed for assay of total protein and acid-precipitable radioactivity as previously described.

At subsequent intervals 50ml samples of culture were removed in duplicate and fractioned into mother-cell and forespore fractions as previously described. Forespore fractions were then disrupted by one of the above methods and centrifuged to yield soluble and particulate fractions. Both fractions were assayed for total protein and for radioactivity.

Results

Progress of growth and sporulation

Fig. 1 shows the progress of growth in the minimal-salts sporulation medium expressed in terms of extinction and bacterial dry weight, together with the time of appearance of the morphological stages of sporulation as originally defined by Schaeffer *et al.* (1965). The organism grew exponentially in this medium for two to three generations and the time at which exponential growth ceased is designated t_0. The first stage of sporulation detectable in the phase-contrast microscope (stage II) occurred at approximately t_2. All subsequent stages of sporulation followed in a reproducibly synchronous manner. At t_{11} more than 90% of the population were free spores.

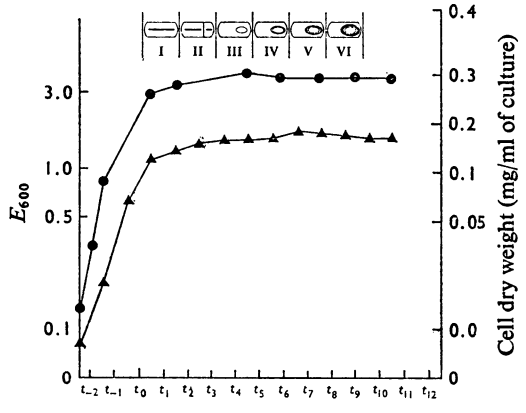

Fig. 1. *Characteristics of growth and sporulation of the organism in CCY medium*

Samples were taken at regular intervals for the determination of absorbance (▲) and cell dry weight (●). Sporulation stages were characterized by observation of the cells in the phase-contrast microscope.

Degradation during sporulation of proteins synthesized during vegetative growth

Fig. 2 shows the changes in the amount of acid-precipitable radioactivity in whole cells and in the isolated mother-cell and forespore fractions during sporulation in the presence of [^{14}C]phenylalanine (Figs. 2*a* and 2*b*), and [^{14}C]leucine (Fig. 2*c*). Before t_0 there is no appreciable change in the acid-precipitable radioactivity in the whole cells. After t_0 the amount in whole cells begins to decrease, reaching a mean maximum rate between t_2 and t_7 of 10.2% (S.D.± 0.5%)/h in the three experiments of Fig. 2. There appears to be a slight decrease in the rate of loss of radioactivity between t_7 and t_8. (Fractionation of a sample of cell suspension taken at stage II from a culture grown in the presence of [^{14}C]phenylalanine showed that 97% of the recovered counts were in the protein fraction and that 97% of these co-chromatographed with a phenylalanine marker.)

Since the total cell protein remains relatively constant after t_0 (Fig. 2*a*) and since there is no cell lysis before t_8 (as measured by the turbidity of the culture), this observed loss of acid-precipitable counts must represent turnover of intracellular protein. In the three experiments described in Fig. 2 the mean percentage loss of total counts over the period t_0–t_8 was 68.3±5.2%, indicating that this percentage of the total sporangial protein must be newly synthesized during this period.

It was important to determine whether this breakdown of protein was occurring in both compartments of the sporulating cell or only one. Fig. 2 also shows therefore the variation in acid-precipitable radioactivity in the isolated mother-cell and forespore fractions in the three experiments. No change in the amount of acid-precipitable radioactivity is observed in the forespore fractions, except for small fluctuations reasonably attributable to experimental error. This implies that there is no breakdown of protein in this compartment after t_3. In the mother-cell fractions, however, from t_3 to t_7 there is a mean decrease in the amount of acid-precipitable radioactivity of 12.2± 2.6%/h in the experiments described in Fig. 2. This can be seen to parallel the loss of radioactivity from the whole cells. After t_7 the experiments described in

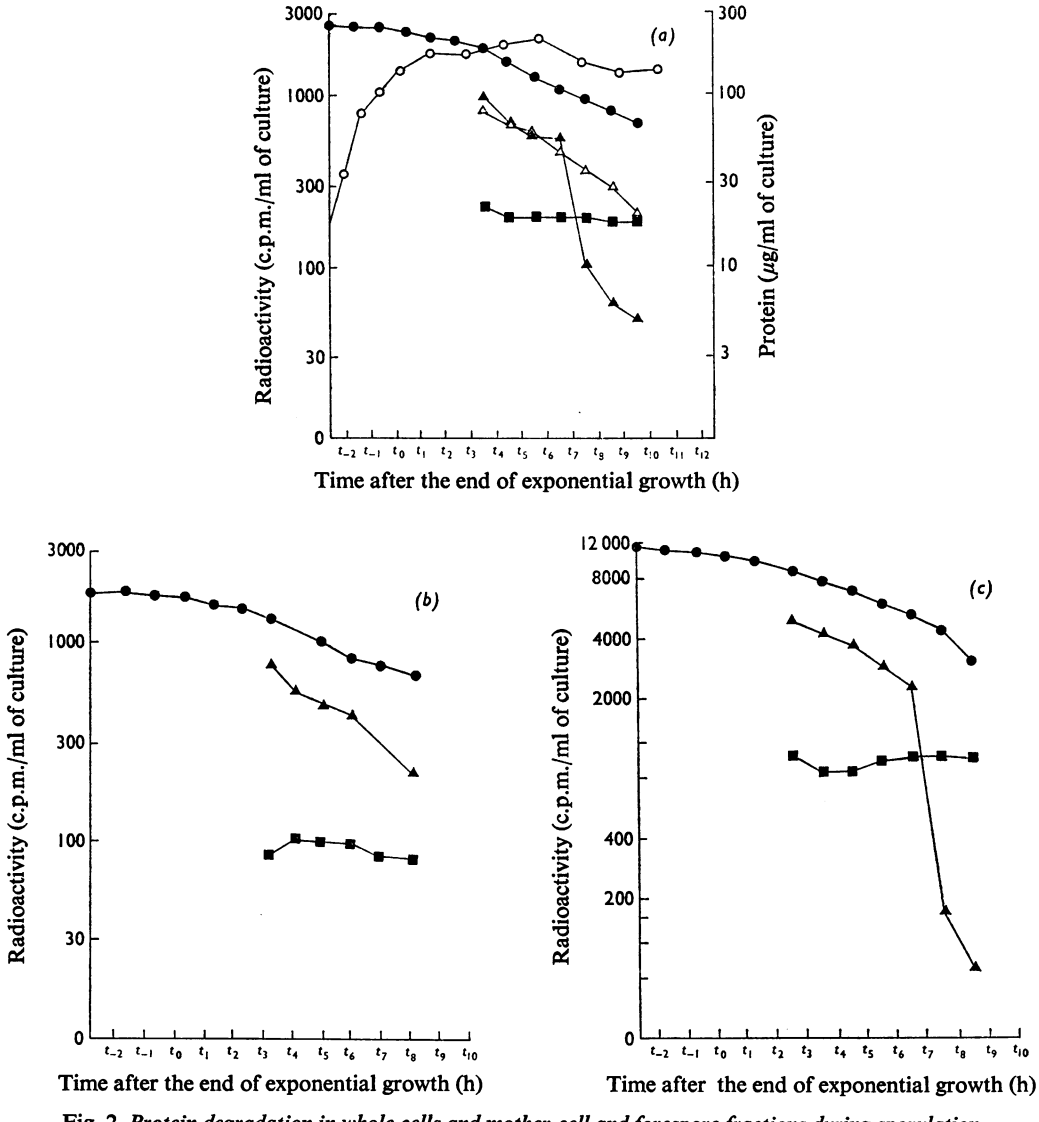

Fig. 2. *Protein degradation in whole cells and mother-cell and forespore fractions during sporulation*

Cells were grown in 100ml of labelling medium containing either [^{14}C]phenylalanine at 0.1 μCi/ml (*a* and *b*) or [^{14}C]leucine at 0.1 μCi/ml (*c*), and the cells from this culture were harvested in exponential phase used as an inoculum for 500ml of CCY medium. After 4h of growth 6mmol of the appropriate unlabelled amino acid was added to the culture. Samples were removed at intervals for determination of acid-precipitable radioactivity and total protein in the whole cells, and for fractionation into forespore and mother-cell fractions which were also assayed for acid-precipitable radioactivity. ○, Total protein in whole cells; ●, c.p.m./ml of culture in whole cells; ▲, c.p.m./ml of culture, filtered to collect the trichloroacetic acid precipitate, mother-cell fractions; △, c.p.m./ml of culture centrifuged to collect the trichloroacetic acid precipitate, mother-cell fractions; ■, c.p.m. /ml of culture, filtered to collect the trichloroacetic acid precipitate, forespore fractions.

Figs. 2*a* and 2*c* show a sharp fall in the amount of acid-precipitable radioactivity in the mother-cell fractions. Although this may represent the lytic activity associated with the release of the mature spores, it is more likely an artifact of the assay procedure, since it occurs before any release of mature spores can be observed and since it is not paralleled in the whole-cell samples. The trichloroacetic acid

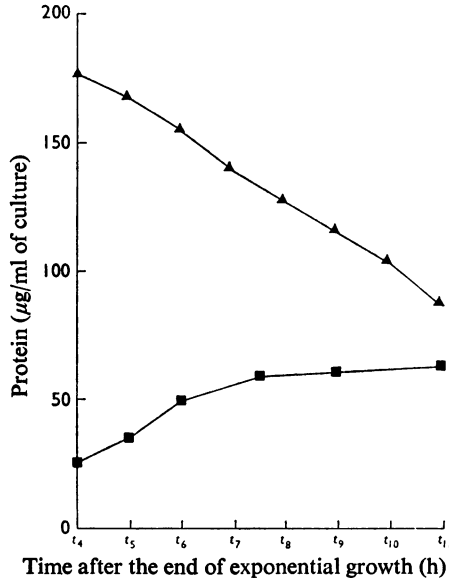

Fig. 3. *Changes in the concentrations of total protein in the isolated mother-cell and forespore fractions during sporulation*

▲, Mother-cell fractions; ■, forespore fractions.

precipitate in these samples is colloidal in nature and it appears that it may not be retained by the glass-fibre filters used to collect the precipitates, since if the samples are centrifuged to sediment the precipitate before filtering, then this sharp fall is not observed (Fig. 2*a*) and the results for the mother-cell fraction closely parallel those for the whole cells.

Although there is no change in total cell protein during sporulation, there is a steady fall in the amount of protein in the mother-cell fraction and a concomitant rise in total protein in the forespore fraction (Fig. 3).

Protein synthesis and multiple turnover during sporulation

The previous experiments leave two important questions unanswered: first, does protein synthesis continue in both compartments throughout sporulation and, if so, what is the relative contribution of each compartment to the total synthesis; secondly, are proteins degraded selectively with respect say to their time of synthesis during exponential growth and sporulation. The second type of pulse-labelling experiment in which proteins were labelled at different times during sporulation and their subsequent metabolism, followed both in whole cells and the isolated fractions, attempts to answer these questions.

Fig. 4 shows the results from six experiments in which proteins were labelled by incorporation of [^{14}C]-phenylalanine for periods of 1 h at different stages during sporulation. The results for the whole cells (Fig. 4*a*) show that proteins synthesized after the end of exponential growth do undergo subsequent degradation, but that the overall rate of loss of counts from these proteins decreases the later the proteins are synthesized in the developmental sequence before t_8 (Table 1). After t_8 there is a rise in the overall rate of degradation. The results for the mother-cell fraction (Fig. 4*b*) show that at all times during sporulation, proteins synthesized in this compartment are subject to extensive subsequent breakdown, and that the overall rate of loss of counts from these proteins increases the later they are synthesized in the sporulation sequence (Table 1). There appears to be a temporary decrease in the rate of loss of counts from all proteins for approx. 1 h between t_7 and t_9. A similar effect was observed by Spudich & Kornberg (1968*b*), who suggested several possible explanations for this observation. In the forespore compartment there is no appreciable loss of radioactivity from proteins synthesized at any time during sporulation (Fig. 4*c*).

Since [^{14}C]phenylalanine can be incorporated into acid-precipitable material up to and including the hour $t_{8.25}$–$t_{9.25}$, protein synthesis appears to continue in both compartments up to the point at which the mature spore is released from the sporangium. The percentage of the total protein synthesis taking place in each compartment over the time-periods studied is shown in Table 2. This was calculated by expressing the incorporation into each compartment over the hour as a percentage of the total incorporation over this period.

Examination of Fig. 4(*b*) and 4(*c*) suggests that the fall in the percentage protein synthesis in the mother-cell compartment from $t_{2.7}$ to $t_{7.5}$ is due to a fall in the total synthesis in this compartment whereas that in the forespore compartment remains relatively constant. The subsequent increase in the percentage synthesis in the mother-cell compartment from t_7 to $t_{9.25}$ is due to both a rise in the total synthesis in this compartment and a fall in the total synthesis in the forespore compartment. This discussion assumes that no proteins are synthesized in one compartment and subsequently transferred to the other.

Spore disruption and determination of the proportion of the total protein in soluble and particulate fractions of the spore

In three experiments assay of the total protein in the soluble and particulate fractions recovered after the disruption of spores in the Braun homogenizer showed that 52–54% of the total spore protein was contained in the particulate fraction. The overall recovery of spore protein in these two fractions was,

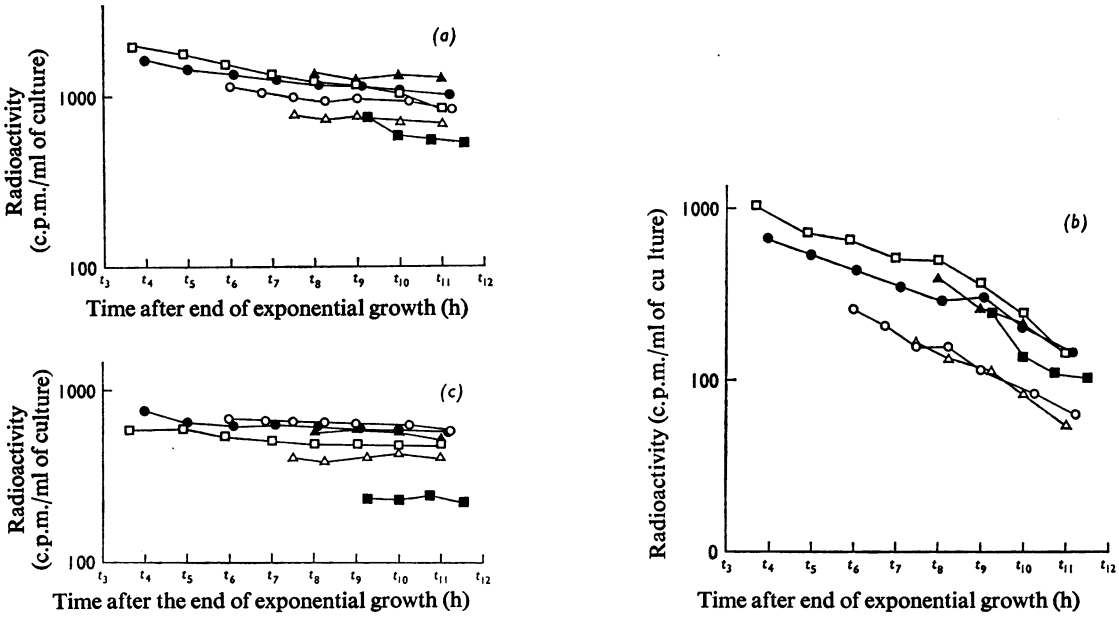

Fig. 4. *Degradation of proteins synthesized at different times during sporulation*

Each of six cultures of the organism was grown in 500ml of CCY medium under similar conditions. [^{14}C]Phenylalanine (20μCi) was added 1 h before the first time-point shown for each experiment, and after 1 h 6 mmol of unlabelled phenylalanine was added. Acid-precipitable radioactivity was assayed at subsequent intervals in whole cells and in isolated mother-cell and forespore fractions. Time of [^{14}C]phenylalanine addition in each case: □, $t_{2.7}$; ●, t_3; ○, t_5; △, $t_{6.5}$; ▲, t_7; ■, $t_{8.25}$.

Table 1. *Rates of loss of counts from proteins made at specified times during sporulation*

Values represent the rate of loss of counts averaged over the remainder of the sporulation sequence after addition of the chase of unlabelled phenylalanine 1 h after addition of the [^{14}C]phenylalanine. t_0 is the time at which exponential growth ceases; at t_{11} more than 90% of the population consists of free spores.

Time of addition of [^{14}C]phenylalanine	Average rate of protein degradation (%/h)	
	Whole cell	Mother-cell compartment
$t_{2.7}$	8.1	9.1
t_3	7.2	10.8
t_5	4.9	14.5
$t_{6.5}$	2.9	19.0
t_7	1.5	20.9
$t_{8.25}$	6.2	30.6

Table 2. *Partition of total protein synthesis between the two compartments of the developing sporangium at various times during development*

Values were obtained by expressing the total incorporation of [^{14}C]phenylalanine into acid-precipitable material in each compartment over the hour after addition of the isotope and expressing this as a percentage of the total incorporation into the whole cell over this period.

Time of addition of [^{14}C]phenylalanine	Partition of total protein synthesis occurring in the subsequent hour	
	% in mother-cell compartment	% in forespore compartment
$t_{2.7}$	64	36
t_3	46	53
t_5	28	72
$t_{6.5}$	28	72
t_7	41	59
$t_{8.25}$	50	50

however, only 50–60% in the three experiments. This was somewhat disappointing since the Braun homogenizer had a number of important advantages over other methods of disruption; first, spore disruption was rapid [30s compared with 15–20min required for homogenization with plastic beads (Ellar & Posgate, 1974) or 35min for disruption by sonication]; secondly, since the temperature was readily maintained below 4°C throughout disruption, any proteolysis was minimized; and finally, the method yielded large

Table 3. *Origin of protein bound to the glass beads used in the Braun homogenizer*

Spores were disrupted by sonication and the sonicates mixed with a sample of glass beads. After mixing, the broken-spore preparation was separated into soluble and particulate fractions. Values were obtained by comparison of the concentrations of protein in the two fractions before and after mixing with the glass beads.

Experiment	% Total spore protein bound to the beads	Origin of the bound protein	
		% Particulate	% Soluble
1	42	63	36
2	52	65	35
3	47	69	31
Mean ± S.D.		66 ± 3	34 ± 3

recognizable fragments of spore integuments, indicating that little particulate material would be extensively 'solubilized' during the disruption process.

Assay of a sample of the glass beads recovered after Braun homogenization showed that the remaining 40–50% of the spore protein was, in fact, bound to the beads and was not readily removed by further washing. (Washing the beads with a further 20ml of deionized water removed only 5% of the bound protein.)

Continued use of the Braun homogenizer for spore disruption required some characterization of this protein bound to the beads. In this experiment samples of the spore suspensions were first disrupted in the Mullard sonicator. A sample of the sonicate was removed and centrifuged as before to yield soluble and particulate fractions. The remainder of the sonicate was mixed with a sample of the glass beads used in the Braun homogenizer, in equivalent proportions to those used in the disruption experiments. The sonicate was shaken with the glass beads for 1–2min and the beads were then removed by filtration and washed as before. The filtrate was centrifuged as previously described to yield soluble and particulate fractions and the total protein in the two fractions and that remaining bound to the beads was assayed. By comparing the amount of protein in the two fractions before and after mixing with the beads it was possible to obtain values for the composition of the bound protein. The results of these experiments are shown in Table 3.

Although this is not a strict control, since pre-broken cells are being mixed with the beads and the homogenization step is omitted, the consistency of the results was such as to justify their use to apportion that fraction of spore protein found bound to the beads to the final soluble and particulate fractions. When this is done a mean value of 59.2±2.7% is obtained for the percentage of the spore protein in the particulate fraction. A similar experiment was performed to characterize radioactivity bound to the beads. To check that the sodium dodecyl sulphate–Folin assay is in fact measuring the total protein in the two fractions, parallel assays were carried out by the ninhydrin reaction. The result obtained (59.5±2%) is in good agreement with that above and indicates that the bulk of the spore protein is assayable with sodium dodecyl sulphate at alkaline pH. This is consistent with findings of Fitz-James (1971) for spores of *B. cereus* and *B. megaterium*. In contrast, in spores of *B. subtilis*, the spore coat accounts for a much higher proportion of the spore protein (80% compared with 40–60%) and only about one-half of the spore-coat protein appears to be solubilized by such treatments (Spudich & Kornberg, 1968*a*).

Table 4. *Protein concentrations in soluble and particulate fractions of isolated forespores expressed as a percentage of the total protein in the stationary-phase cell*

For details see the text.

Stage of forespore development	Protein expressed as a percentage of that in the stationary-phase cell	
	Forespore soluble fraction	Forespore particulate fraction
III	7.05	7.14
IV	8.95	10.5
VI	13.02	17.8
Mature spore	13.26	21.6

Table 5. *Extent to which proteins of the soluble and particulate fractions of the mature spore are newly synthesized during sporulation*

For details see the text.

Experiment	Method of spore disruption	Percentage of the spore protein newly synthesized after t_1	
		Soluble	Particulate
1	Braun homogenizer	68	67
	Mullard sonicator	80.5	66
2	Braun homogenizer	74	70
	Mullard sonicator	74	64
3	Braun homogenizer	74	65
	Mullard sonicator	80.5	68
Mean ± S.D.		75 ± 5	67 ± 2

Protein concentrations in sporulating cells and forespores at different stages of development

Table 4 shows the concentrations of protein in soluble and particulate fractions of forespores at various stages, expressed as a percentage of the total protein in a stationary-phase cell. The forespores at

stages III and IV were lysed by suspension in PM buffer. Those at stage VI and the mature spores were disrupted in a Braun homogenizer and corrections were applied for the protein bound to the beads.

Determination of the extent to which the proteins of the soluble and particulate fractions of the mature spore are newly synthesized during sporulation

Table 5 shows the results of three separate experiments for the determination of the extent to which soluble and particulate spore proteins are newly synthesized during sporulation. The results are given for parallel experiments in which the spores were disrupted in either the Mullard sonication apparatus or the Braun homogenizer. In each case the results are obtained by taking the specific radioactivity of the protein in the fraction, subtracting this from the specific radioactivity of stationary-phase cell protein at t_1 and expressing the result as a percentage of the specific radioactivity at t_1. The results for samples prepared by disruption in the Braun homogenizer are corrected for both the protein and the radioactivity bound to the glass beads. (In three separate experiments, measurements of the radioactivity bound to the glass beads showed that $58\pm2\%$ was derived from the particulate fraction and $42\pm2\%$ was derived from the soluble fraction of the spore homogenate.)

Discussion

The first type of experiment (Fig. 2) demonstrates that protein labelled during exponential growth is degraded during sporulation at rates similar to those observed in other micro-organisms. Spudich & Kornberg (1968*a*) report a value of 18%/h for protein degraded during sporulation in *B. subtilis*, but this is obtained by considering each period of 1h separately, and expressing the amount of acid-precipitable radioactivity at the end of each hour as a percentage of that at the beginning of the hour. Treatment of their data in the same way as that presented here gives a value of 10.1%/h, which is consistent with the values of 8–10%/h reported by Mandelstam & Waites (1968) in *B. subtilis* and with the rates presented here for *B. megaterium*. Similar rates were also observed during sporulation of *B. thuringiensis* by Monro (1961) and of *Bacillus mycoides* by Foster & Perry (1954).

The new information revealed by the first experiment is that this protein breakdown is not occurring in the forespore, but is confined exclusively to the mother-cell compartment during development.

One possible criticism of the first type of experiment would be that the nutritional limitations which induce sporulation are alleviated by the addition of the large amount of unlabelled amino acid only 1h after the end of exponential growth and some 2h before the cells become committed to sporulation at stage III (Szulmajster, 1973). Nevertheless, in all experiments of this type no significant perturbation of the sporulation sequence was observed. When in a similar experiment the unlabelled amino acid chase was added at t_0, the appearance of each sporulation stage was delayed by approx. 2h. A similar effect was observed by Spudich & Kornberg (1968*b*). In the second type of experiment this effect is minimized since the chase was always added at stage III or later, and therefore after the cells have become committed to sporulation. Since in the first experiment similar results were obtained with both [^{14}C]phenylalanine and [^{14}C]leucine, it can be assumed that the effects observed represent metabolism of total protein and not that of the individual amino acids.

The results of the second pulse-labelling experiment reveal: (1) that protein synthesis is continuing in both compartments up to the time of release of the mature spore; (2) there is no degradation of protein in the forespore compartment; (3) that proteins synthesized in the mother-cell compartment after t_3 are subsequently degraded at rates increasingly faster than that for proteins synthesized during exponential growth. The incorporation of [^{14}C]phenylalanine into acid-precipitable material in both compartments throughout sporulation indicates that both compartments remain permeable to externally added phenylalanine during spore morphogenesis and that the absence of protein turnover in the forespore compartment cannot be an artifact arising from the inability of the unlabelled chase to penetrate the forespore. This assumes, however, that protein is not being synthesized in the mother-cell compartment and subsequently exported into the forespore. Although it seems unlikely that proteins of the spore core are synthesized outside the forespore, it has been suggested by a number of workers that spore-coat protein may be synthesized in the mother-cell compartment and built up on the outside of the forespore protoplast (Murrell, 1967). There is, however, no direct evidence at present for the site of synthesis of spore-coat precursors. The observation that although protein synthesis continues until t_8 the overall rate of degradation in the whole cell is lowered, is consistent with the finding that there is no degradation of protein in the forespore compartment and that up to t_7 an increasingly greater proportion of the total protein synthesis is taking place in this compartment. It is interesting, however, that proteins synthesized in the mother-cell compartment after t_3 are degraded at an increasing faster rate than proteins before t_3. This agrees with the general observation that proteins synthesized in starving cells are much more unstable than proteins synthesized under more favourable conditions (Pine, 1972). The increase in the rate of degradation of proteins synthesized after $t_{8.25}$ measured in whole cells is due to the fact that at

this time there is an increase in the extent of synthesis in the mother-cell compartment, where protein degradation is then at a rate of 30%/h.

Our results indicate a model for the origin of spore protein in which there is no protein degradation in the forespore compartment. This model was not favoured by Spudich & Kornberg (1968*a*). The justification for their conclusion was based on calculations of the extent to which proteins of the mature spore were newly synthesized during sporulation in *B. subtilis*. These calculations indicated that in a model where there was no turnover in the forespore, only 0.7–1.8% of the stationary-phase cell protein was captured in the newly formed forespore compartment at the time of septation, whereas electron-microscopic evidence suggested that 16–20% of the volume of the stationary-phase cell is captured in this compartment.

We therefore repeated Spudich & Kornberg's (1968*a*) calculation, substituting values obtained in the present investigation for the concentrations of protein in soluble and particulate fractions of the mature spore and for the extent to which the protein in these fractions is newly synthesized during sporulation. Our results reveal that in *B. megaterium* the total protein content of the mature spore represents 35% of the total protein in the stationary-phase vegatative cell, and that the concentration of protein in the soluble and particulate fractions of the mature spore represents 13.3 and 21.6% respectively of the stationary-phase cell protein (Table 4). These compare with values of 37, 7 and 30% respectively for *B. subtilis* obtained by Spudich & Kornberg (1968*a*) and are consistent with the report by Aronson & Fitz-James (1968) that in *B. megaterium* and *B. cereus* a much lower percentage of the spore protein is in the coat fraction than in *B. subtilis* (40–60% compared with 80%).

Our results also show that 75% of the spore soluble protein and 66.7% of the particulate protein were newly synthesized during sporulation (Table 5). These values are significantly different from the findings of Spudich & Kornberg (1968*a*) that in *B. subtilis*, 75–90% of spore-core protein and 95% of coat protein was newly synthesized.

We can now proceed to calculate the extent of capture of stationary-phase cell protein using the values obtained in this investigation. Since from our results, the spore soluble fraction comprises 13.3% of the early stationary-phase cell protein and since 75% of this is newly synthesized, 3.75% must be captured from the stationary-phase vegetative cell. Similarly, the spore particulate fraction comprises 21.6% of the stationary-phase cell protein and since 67% of this is newly synthesized, 7% must be derived from the stationary-phase cell. It is notable that this latter value corresponds almost exactly to the amount of particulate (i.e. membrane) material in the stage III forespore (Table 4). Spudich & Kornberg (1968*a*) do not include the contribution of the membrane in their calculation.

Thus the extent of capture of vegetative-cell protein is 3.75% plus 7% to give a total of 10.75%. On several grounds this is probably a more realistic value than that of 0.7–1.8% obtained by Spudich & Kornberg (1968*a*). Estimation of the volume of the stationary-phase cell which is captured at the time of septation, by means of electron micrographs is difficult and although a value of 16–20% seem reasonable, examination of some sections (Ellar *et al.*, 1967; Kay & Warren, 1968) suggest it could well be lower. It is also probably unwise to assume that the entire volume of the newly formed forespore is occupied by protein. For instance, it is known that the forespore contains at least one complete genome (Doi, 1969), and this might occupy a considerable proportion of the early forespore volume.

The values for the extent of capture of stationary-phase cell material in the forespore calculated from our experiments are consistent with a model for the origin of spore proteins in which no breakdown of protein occurs in the forespore compartment. There is therefore no theoretical objection to the acceptance of the experimental results showing no breakdown of protein in the forespore compartment during sporulation.

The fact that degradation of proteins made at any time during exponential growth and sporulation appears to be confined to the mother-cell compartment is not unexpected. Since sporulation is taking place when the external medium is depleted of metabolites, it would be uneconomic for the cell to degrade those new proteins which are required for the conversion step, namely the proteins of the developing forespore. By concentrating proteolytic activity in the compartment which contains redundant vegetative-cell proteins, the cell can make the fullest use of endogenous material and at the same time maintain a concentration gradient of precursors for protein synthesis across the double membrane surrounding the forespore. As the experiments show, it is in this compartment that the bulk of the protein synthesis is taking place in the later stages of development. On other grounds, some protein degradation might have been expected in the forespore compartment if post-synthetic proteolytic modification (Sadoff *et al.*, 1970) is a factor in the synthesis of a significant number of spore-specific proteins. Low amounts of this type of proteolysis might still be occurring, but would not be detectable in these experiments.

The protein synthesis which occurs in the mother cell throughout the developmental sequence can be accounted for in several ways. (1) It represents residual synthesis of vegetative protein, not transported into developing spore but involved in the production of essential precursors which are required to maintain

the integrity of the mother-cell compartment. This model implies that all proteins which occur in the mature spore are synthesized in the forespore compartment, but continued protein synthesis in the mother-cell compartment fulfills an essential supportive role. (2) It may reflect the inability of the cell to achieve an immediate and rapid cessation of vegetative-cell-protein synthesis. This explanation implies that proteins synthesized during the resulting gradual decline are in no way connected with spore morphogenesis. (3) Some proteins which are found in the mature spore are synthesized in the mother-cell compartment, and are subsequently translocated as such into the forespore core, or are laid down in the form of surface structures outside the forespore membranes. The methods described here for isolating the forespore throughout development will permit these possibilities to be examined in detail.

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