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Temporal Dissociation of Late Events in *Bacillus subtilis* Sporulation from Expression of Genes That Determine Them

HOWARD F. JENKINSON,[1] DESMOND KAY,[2] AND JOEL MANDELSTAM[1]*

Microbiology Unit, Department of Biochemistry,[1] and Sir William Dunn School of Pathology,[2] University of Oxford, Oxford, OX1 3QU, England

During sporulation in replacement medium, resistance to toluene to heating at 65°C, to lysozyme, and to heating at 80°C appeared in sequence between 4 and 8 h after the induction of sporulation (i.e., between t_4 and t_8). The addition of sufficient chloramphenicol at $t_{4.5}$ to prevent protein synthesis nevertheless allowed the emergence of all of these types of resistance except lysozyme resistance. The numbers of spores with these types of resistance (lysozyme resistance again excepted) increased about fourfold when phenylmethylsulfonyl fluoride (an inhibitor of serine protease activity) was also present. Thus, the observed increases in resistance in the 2 h after the addition of chloramphenicol resulted from the utilization of preformed protein elements. Dipicolinate did not seem to be a determining factor in the development of any of these forms of resistance. Electron micrographs showed that inhibition of protein synthesis did not prevent deposition of the outer layers of the spores. Lysozyme resistance developed differently; synthesis of the relevant proteins began later (t_5), and continued synthesis was necessary up to t_8. Some processing of proteins made earlier was a prerequisite for lysozyme resistance. Therefore, it appears that from the viewpoint of regulation, the expression of the genes and the production of the proteins for resistance to toluene, heating at 65°C, and heating at 80°C are all stage IV sporulation events, although the resistance properties themselves appear only during stages V and VI. Lysozyme resistance is the only real late event among those examined. The germination characteristics of the spores, which are also late events, are discussed in this context, as they too are dependent on proteins that are synthesized much earlier.

Sporulation in *Bacillus subtilis* is controlled by specific genes lying in a number of clusters, (estimated at between 40 and 60) (11). Of these, more than 30 have now been identified (19). As a first approximation, these clusters can be regarded as regulating a "dependent sequence" of events, that is a sequence in which any one of them fails to occur unless those preceding it have occurred (14, 15). This assumption is in keeping with the biochemical and morphological properties of strains containing single mutations and also with the properties of the double mutants constructed by Coote and Mandelstam (8). However, as these authors pointed out, the idea of a linear dependent sequence was an oversimplification that conflicted with at least two pieces of evidence. The first was provided by the properties of at least one of the double mutants. Its phenotypic behavior could be explained only by assuming that two separate gene clusters, each causing blockage at stage IV in sporulation, were expressed simultaneously (8). The second piece of evidence was more clear-cut and arose from the work of Wood on the alkali-soluble protein of the spore coat (25). Under the standard conditions of sporulation used in this laboratory, he showed, using immunological techniques, that this protein is synthesized during stage II, although it does not become part of the spore structure until stage V. Somewhat similar observations have been made more recently by Munoz et al. (17).

It is sometimes tacitly assumed that the times at which biochemical and morphological events in sporulation are observed correspond roughly with the times at which the genes responsible for them are transcribed and translated. This is reflected in the characterization of genes *spo0A*, *spoIIB*, etc. The findings with the coat protein are in clear disagreement with this assumption, and so are some earlier experiments which showed that if chloramphenicol was added to sporulating cultures at stage V, the heat resistance which was just becoming manifest would continue to develop (4, 22). These observations have not been followed up in detail.

In this paper we consider a number of properties that are associated with the late stages of

spore development. These are resistance to toluene (Tol^r), to chloroform, to heating at 65°C (H65^r), to heating at 80°C (H80^r), and to lysozyme (Lyso^r). Although these types of resistance develop successively during stages V and VI, experiments with chloramphenicol show that the proteins necessary for all of them except the last are synthesized by the end of stage IV. The proteins responsible for lysozyme resistance appear somewhat later.

MATERIALS AND METHODS

Organism. *B. subtilis* 168 (*trpC2*), which requires indole or tryptophan and sporulates normally, was used. Stocks were kept at 4°C as dilute spore suspensions in distilled water.

Induction of sporulation. Cells in exponential growth in casein hydrolysate medium at 37°C were harvested by centrifugation when the culture contained about 0.25 mg (dry weight) of bacteria per ml. The pellet was suspended to the same density in warm resuspension medium containing glutamate, inorganic salts, and 20 μg of tryptophan per ml (21) and incubated with shaking. About 80% of the cells contained refractile spores at 8 h after resuspension. The time of resuspension is denoted t_0, and subsequent times (in hours) are designated t_1, t_2, etc. Growth was followed spectrophotometrically at 600 nm, and dry weights were estimated from a calibration curve relating dry weight and absorbance.

Determination of spore incidence. Spore inclusions were counted by phase-contrast microscopy and expressed as a percentage of the total number of cells. The term phase bright is used to describe all gradations from dull white to bright spores; the term dark forespore is used to describe earlier visible spore inclusions.

Measurement of resistance to organic solvents and to heat. For the determination of toluene and chloroform resistances, samples from cultures were diluted 100-fold in an inorganic salts solution, the constituents of which were identical to those in the resuspension medium. Organic solvent (0.1 ml) was added to 5 ml of the diluted suspension in a test tube. The contents of the tube were then blended in a Vortex mixer for 1 min, and dilutions were plated onto nutrient agar (Oxoid).

For the measurement of heat resistance, a 0.05 ml of culture was added to 4.95 ml of preheated inorganic salts solution in a test tube, which was three-quarters submerged in a covered water bath. After 15 min at the appropriate temperature, the tube was cooled, and suitable dilutions were plated onto nutrient agar.

Lysozyme sensitivity. Lysozyme sensitivity was determined by adding 0.1 ml of culture to 4.90 ml of inorganic salts solution containing 250 μg of lysozyme (EC 3.2.1.17) per ml. After incubation for 10 min at 37°C, dilutions were plated onto nutrient agar.

Determination of DPA. A portion of culture containing 3 to 6 mg (dry weight) of bacteria was centrifuged (5,000 × *g*, 5 min), and the 2,6-dipicolinic acid (DPA) content of the pellet was determined by the method of Janssen et al. (12).

Radioactive labeling experiments. Incorporation of [^{35}S]methionine during sporulation was measured by resuspending bacteria in sporulation medium containing L-[^{35}S]methionine (0.1 μCi/ml, with 50 μg of carrier L-methionine per ml). Duplicate 1-ml samples were removed at intervals and assayed for radioactivity as described below. Rates of L-[^{35}S]cysteine and L-[^{3}H]leucine incorporation during sporulation were measured as follows. At intervals a 5-ml portion of culture was removed into a flask containing 0.1 ml of a solution of radioactive amino acid and carrier amino acid (final concentrations, 0.1 μCi/ml and 20 μg/ml, respectively). After 15 min at 37°C, duplicate 1-ml samples were removed for determination of incorporated radioactivity. In some experiments, 1-ml samples were also added to tubes, each containing 1 ml of 50 mM dithiothreitol (DTT) in 0.05 M $NaHCO_3$–Na_2CO_3 buffer (pH 10) and unlabeled amino acid (0.5 mg/ml), and incubated for 30 min at 37°C before being assayed for radioactivity.

Assay for radioactivity. Each sample to be assayed was added to an equal volume of ice-cold 10% (wt/vol) trichloroacetic acid containing the corresponding unlabeled amino acid (0.5 mg/ml). The contents were mixed, and the tube was chilled in ice for 10 min. The suspension was then filtered on a Whatman GF/C glass fiber filter (diameter, 25 mm), and the precipitate was washed with 10 ml of 10% (wt/vol) trichloroacetic acid and 10 ml of 95% (vol/vol) ethanol. The filters were dried under a vacuum at 85°C for 1 h, and radioactivity was measured in 5 ml of a toluene-based scintillation fluid containing 5 g of 2-(4′-*tert*-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole per liter.

Electron microscopy. Samples (about 10 ml) were removed from sporulating cultures and filtered under a vacuum through a coarse glass sinter. This initial step was essential to remove any crystals of phenylmethylsulfonyl fluoride (PMSF) in cultures to which this inhibitor had been added. The filtrate was centrifuged (5,000 × *g*, 5 min), and the bacterial pellet was washed once with 1 M NaCl and prepared for electron microscopy as described elsewhere (13).

Preparation of spores at t_9. A volume of culture was centrifuged (5,000 × *g*, 10 min), and the pellet was washed once with 1 M NaCl and then twice with deionized water. The spores were resuspended in 0.05 M K_2HPO_4–KH_2PO_4 buffer (pH 7) (buffer K) containing lysozyme (50 μg/ml) and 0.1% (wt/vol) Sarkosyl (Ciba-Geigy) and incubated at 37°C for 10 min. The suspension was then centrifuged (5,000 × *g*, 10 min), and the pellet was washed twice with 1 M NaCl and four times with distilled water. All washing solutions contained 0.01% (vol/vol) Tween 80 to reduce clumping of spores. When examined by phase-contrast microscopy, the preparations of phase-bright spores were free of mother cell debris.

Preparation of spores at t_7. Because a proportion of the phase-bright spores in cultures at t_7 was still sensitive to lysozyme, the lysozyme treatment in the cleaning procedure (see above) was omitted. Instead, the bacterial pellet was resuspended in buffer K containing 0.1% (wt/vol) Sarkosyl and sonicated four times at 60 W with a 1-cm-diameter probe (40 s each time, 4°C). This resulted in the release of more than

70% of the spores from the mother cells. The spores were repeatedly washed with distilled water to remove mother cell debris.

Chemicals. Egg white lysozyme, chloramphenicol, PMSF, DTT, and amino acids were from Sigma Chemical Co., St. Louis, Mo. L-[^{35}S]methionine, L[^{35}S]cysteine hydrochloride, L-[^{3}H]leucine, and [^{35}S]sulfate were purchased from the Radiochemical Centre Ltd., Amersham, England. All other materials were of analytical grade quality.

RESULTS

Development of phase brightness and various types of resistance during sporulation of *B. subtilis*. The progress of sporulation in glutamate minimal resuspension medium under our conditions is shown in Fig. 1. The time of the first appearance of spore inclusions was about 3.5 h after resuspension. At $t_{4.5}$ about 10% of the cells contained phase-bright spores. The onset of Tol^r coincided roughly with the first appearance of dark forespores, and the onset of H65^r coincided with the first appearance of phase-bright spores.

The cultures developed resistance sequentially to toluene, chloroform, 65°C, and then lysozyme and 80°C (Fig. 2). The number of spores resistant to each of these forms of treatment increased from 10^7 colony-forming units per ml or less to about 3×10^8 colony-forming units per ml in 2 to 3 h. The Lyso^r counts were usually higher because lysozyme released spores from cell pairs and consequently increased the number of colony-forming units.

By t_8, about 3×10^8 spores per ml were resistant to all of the separate treatments, and the number increased somewhat (to 3.5×10^8 spores per ml) by t_{20}. This was again attributable to the release of spores from mother cell pairs.

Effect of chloramphenicol on the development of different types of spore resistance. In a preliminary experiment we confirmed the observations of other workers (4, 20) that the addition of chloramphenicol at stage V did not prevent the development of heat resistance. We then added 100 μg of chloramphenicol per ml earlier, at $t_{4.5}$, when most of the developing prespores were at late stage III (Fig. 3A). Even with this much earlier addition the number of heat-resistant spores found in the culture at t_8 was 15% of the number present in an untreated culture (Fig. 4A).

When chloramphenicol was added at $t_{4.5}$, the culture contained about 10^8 Tol^r spores per ml, of which 1.5×10^7 spores per ml were also H65^r. At t_8 the H65^r and H80^r counts had increased to about 5×10^7 spores per ml. However, there was no increase in Lyso^r (Fig. 4A).

In summary, it seemed that about one-half of those spores which were Tol^r at $t_{4.5}$ had the capacity to develop the H65^r and H80^r properties. The other half of the spores were apparently unstable and reverted to toluene sensitivity by t_8. Electron micrographs showed that the spores that were produced in the presence of chloramphenicol were morphologically similar to those obtained from an untreated culture except for some details in the outer coat layers (Fig. 3B and D).

Effect of PMSF on the development of different types of spore resistance in the presence of chloramphenicol. It was shown previously that when PMSF, an inhibitor of

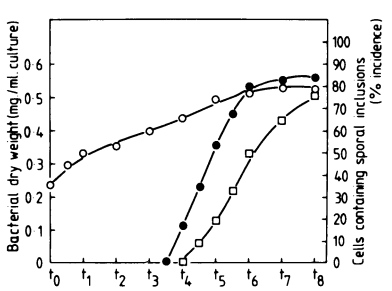

FIG. 1. *Time course of sporulation in resuspension medium. At t_0 exponentially growing cells in casein hydrolysate medium were transferred to resuspension medium as described in the text. At intervals after resuspension samples were taken for determination of bacterial dry weight (○) and the proportions of cells containing dark forespores (●) and phase-bright spores (□).*

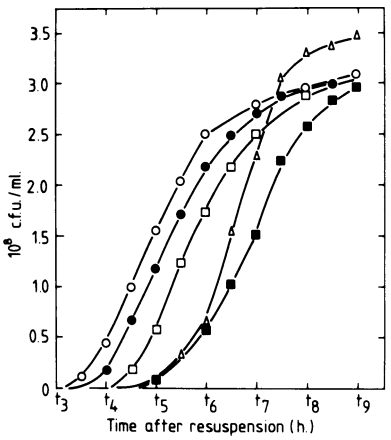

FIG. 2. *Development of resistance to organic solvents, to heat, and to lysozyme during sporulation. At intervals after resuspension samples were removed from the culture and tested for resistance as described in the text. Symbols: ○, toluene; ●, chloroform; □, 65°C for 15 min; ■, 80°C for 15 min; △, lysozyme. At $t_{4.5}$ about 10% of the cells contained phase-bright spores. Vegetative cells were sensitive to all forms of treatment. c.f.u., Colony-forming units.*

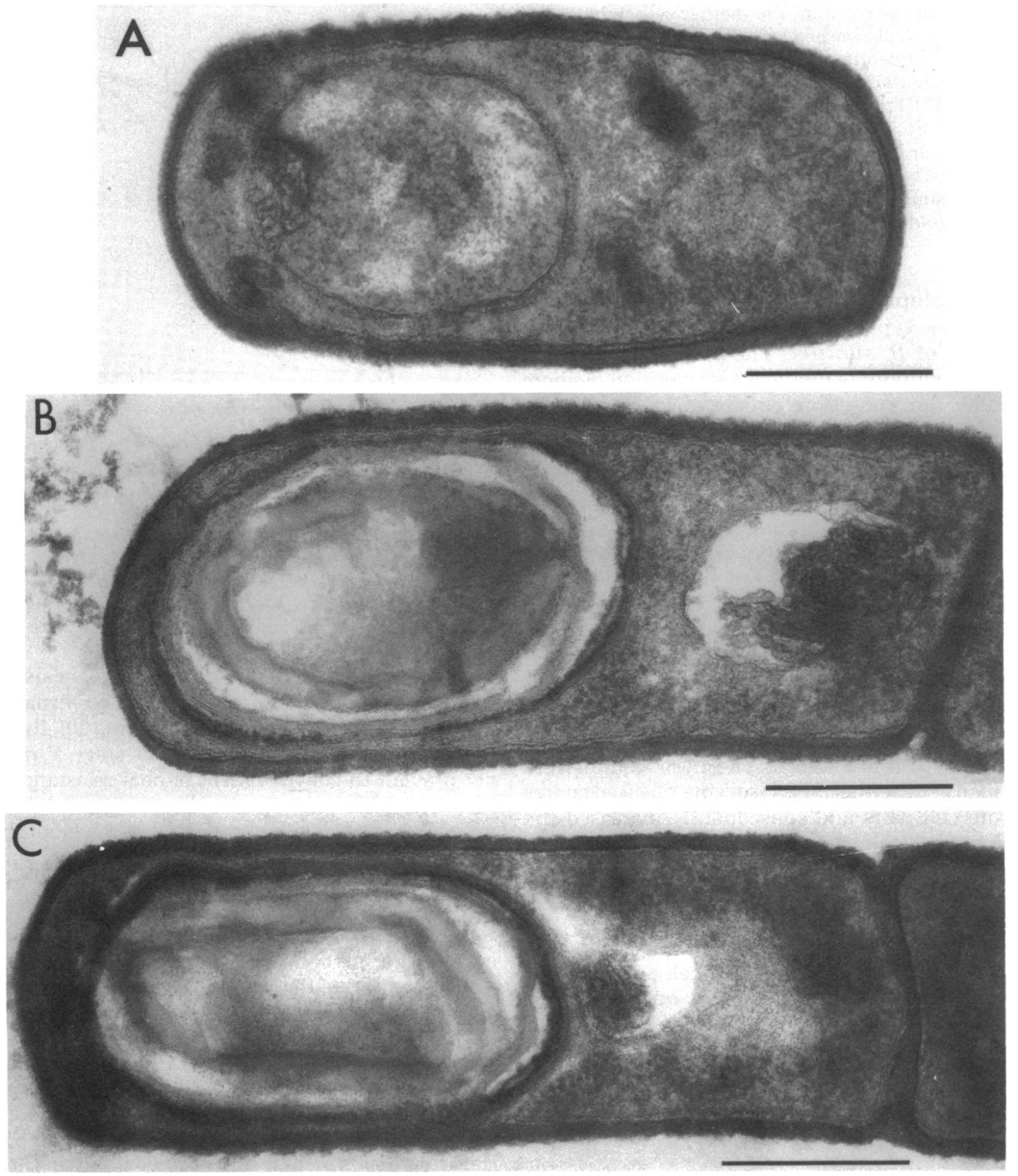

FIG. 3. *Electron micrographs of thin sections of sporulating cells in cultures to which chloramphenicol and PMSF were added during sporulation. (A) Sporulating cell at $t_{4.5}$, before the addition of the inhibitors, which was at late stage III. (B) Normal sporulating cell at t_8, which has reached stage VI. (C) Sporulating cell at t_8 in a culture to which PMSF was added at $t_{4.5}$. The spore has advanced to a stage similar to that in (B). (D) Sporulating cell at t_8 in a culture to which chloramphenicol was added at $t_{4.5}$. The spore has also advanced to a late stage, but the outer densely stained coat is somewhat less well developed. In addition, the inner lamellar structure adjacent to the outer coat in (B) and (C) is not visible. (E) Sporulating cell at t_8 in a culture to which chloramphenicol and PMSF were added at $t_{4.5}$. The spore resembles that developed with chloramphenicol alone (D). (F) Sporulating cell at t_8 in a culture to which chloramphenicol was added at t_5. The spore is not significantly different from that shown in (E). Bars = 1 μm.*

serine proteases, was added to a sporulating culture at t_4, the development of heat resistance was not affected (9). However, we noted that when PMSF (4 mg/ml) was added at $t_{4.5}$ about 50% of the H80^r spores recovered at t_8 were sensitive to lysozyme. Thus, it appeared that protease activity might play a part at a fairly late stage in sporulation, and experiments were

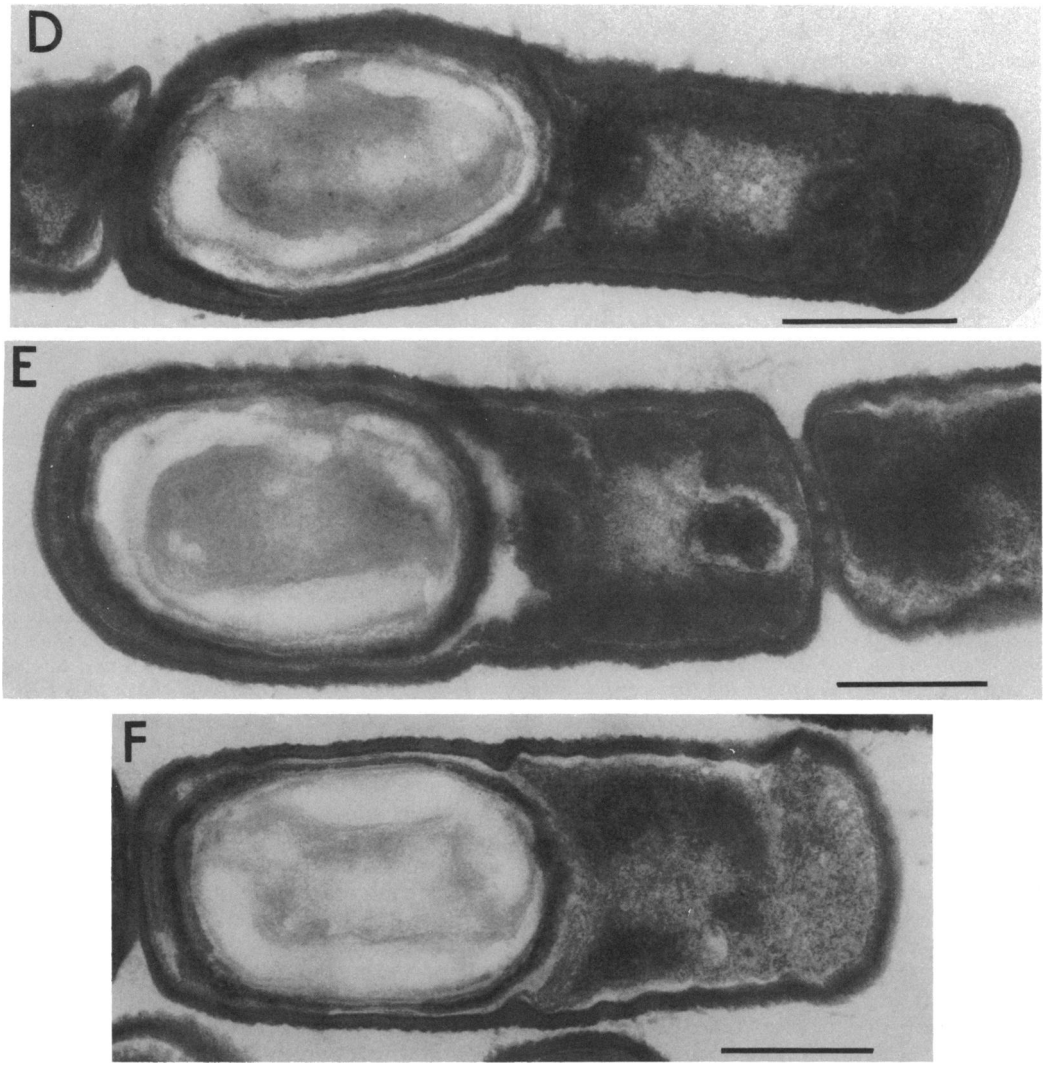

FIG. 3—continued.

carried out to determine whether PMSF would modify the effects of chloramphenicol.

A sporulating culture was treated with PMSF (4 mg/ml) together with chloramphenicol (100 μg/ml) at $t_{4.5}$. This prevented the reversion of Tol^r spores to sensitivity that occurred when chloramphenicol alone was added (Fig. 4B). In fact, the number of Tol^r spores continued to increase for a much longer period, and most of these spores went on to become heat resistant (Fig. 4A and B).

At t_8 the culture treated with both inhibitors contained about 2×10^8 spores per ml that were both Tol^r and H65^r. Most of them were also H80^r, but only 5% were Lyso^r. Thus, by $t_{4.5}$ about 2×10^8 sporangia per ml appeared to have made the proteins needed for heat resistance which was then developed, provided that incubation was continued with PMSF present. The number of H80^r spores was about four times as many as in the culture with chloramphenicol alone. At t_8, the spores produced in all cultures were morphologically similar (Fig. 3B, E, and F).

Effects of chloramphenicol and PMSF on the synthesis and breakdown of proteins during later stages of sporulation. The validity of these experiments depends on the assumption that chloramphenicol and PMSF can enter the sporangia at $t_{4.5}$ and later.

A sporulating culture was treated with L-[^{35}S]-methionine at $t_{4.7}$ and with chloramphenicol shortly thereafter. This stopped further incorporation of label (Fig. 5A), and a similar result was obtained with [^{3}H]leucine. Figure 5 shows that methionine and chloramphenicol reached the sites of protein synthesis until t_8.

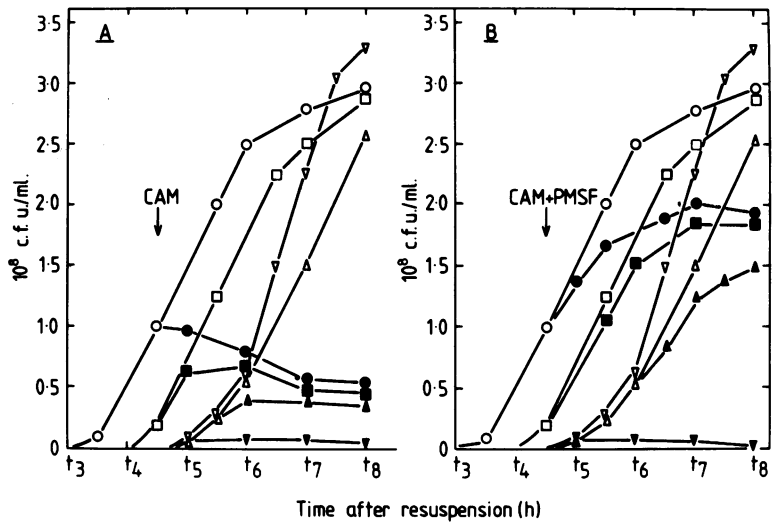

FIG. 4. *Effect of adding chloramphenicol (CAM) (A) or chloramphenicol together with PMSF (B) on the development of Tol^r, H65^r, H80^r, and Lyso^r. At $t_{4.5}$ a sporulating culture was divided into two portions. One was treated with chloramphenicol (100 μg/ml), and the other was treated with chloramphenicol and PMSF (4 mg/ml). Samples were removed at intervals from the cultures and tested for resistance as described in the text. Open symbols show the numbers of resistant spores in an untreated culture, and closed symbols show the numbers in the cultures containing the inhibitors. Symbols: ○ and ●, Tol^r; □ and ■, H65^r; △ and ▲, H80^r; ▽ and ▼, Lyso^r. Arrows mark the times of addition of chloramphenicol and chloramphenicol plus PMSF. c.f.u., Colony-forming units.*

The stabilization of sporangial proteins by PMSF was measured as follows. Bacteria were resuspended in sporulation medium containing L-[^{35}S]methionine. Samples (1 ml) were removed at intervals, and the radioactivities were determined. Incorporation was linear to t_3 and reached a maximum at about t_5 (data not shown). At $t_{4.5}$ a 10-fold excess of L-methionine (0.5 mg/ml) was added to reduce any further incorporation and to trap labeled methionine released by proteolysis. Three portions were immediately removed from the culture and incubated with chloramphenicol, PMSF, or both inhibitors. In the control culture the cells lost about 13% of their radioactivity between $t_{4.5}$ and $t_{5.5}$; with chloramphenicol this was reduced to about 5%. PMSF reduced proteolytic degradation to <0.5%/h (Fig. 5B).

Effects of PMSF and chloramphenicol separately on the development of Lyso^r. Portions (5 ml) of a sporulating culture were removed at intervals and treated with either chloramphenicol or PMSF. A sample was taken immediately for determination of the Lyso^r count. The portions were shaken until t_8 and then sampled again for Lyso^r counts.

The experiment showed that chloramphenicol added at any time prevented further development of Lyso^r and that continued protein synthesis was needed until t_7 (Fig. 6); this was in contrast to the other types of resistance. Furthermore, the proteins specifically necessary for the development of Lyso^r were synthesized mainly after t_5, since by that time PMSF had almost ceased to have any inhibitory effect. It follows that the inhibition of Lyso^r in experiments with both inhibitors was primarily due to chloramphenicol.

Proportions of total spore protein synthesized at various times during normal sporulation. The proportions of total spore protein which were synthesized both before and after $t_{4.5}$ were measured as follows. Bacteria were resuspended in sporulation medium, and at intervals 10-ml portions were removed into L-[^{35}S]-methionine (0.1 μCi/ml, with 50 μg of carrier L-methionine per ml) and incubated to t_9. The cultures were harvested, the spores were separated and washed, and the specific radioactivities were determined. Table 1 shows that in the course of normal sporulation about 70% of the proteins finally found in the spores had already been synthesized by $t_{4.5}$. Since the electron micrographs (Fig. 3) showed that these proteins had not been laid down at that stage, they must have been present in the cytoplasm of the other cells, awaiting processing or assembly as envisaged, for example, by Pandey and Aronson (18).

Effect of chloramphenicol and PMSF on the synthesis of DPA. Since chloramphenicol inhibits DPA synthesis (20), presumably by preventing formation of DPA synthetase (6), and

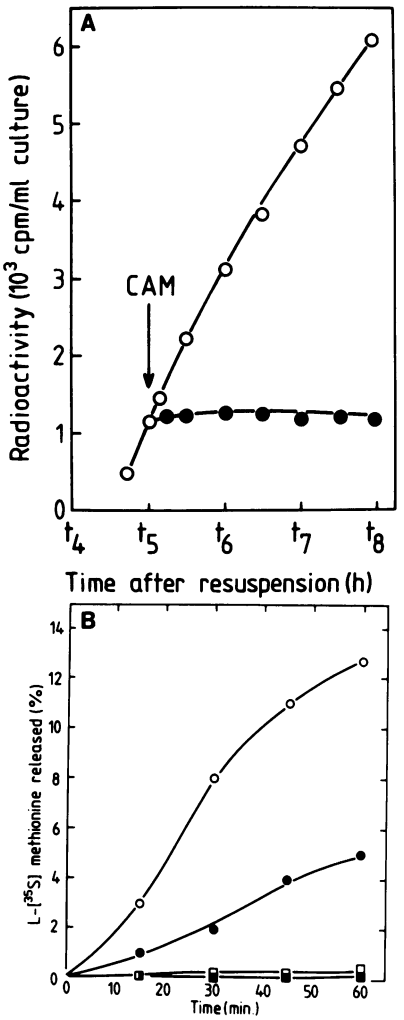

FIG. 5. *Effects of chloramphenicol (CAM) and PMSF on protein synthesis and breakdown during later stages of sporulation. (A) Sporulating bacteria were treated at $t_{4.7}$ with L-[^{35}S]methionine (0.1 μCi/ml, with 50 μg of carrier L-methionine per ml). At t_5 the culture was divided, and chloramphenicol (100 μg/ml) was added to one portion. Duplicate samples were taken up to t_8 for measurement of incorporated radioactivity. Symbols: ○, no addition; ●, chloramphenicol. (B) Sporulating bacteria were labeled from t_0 by incubating them in resuspension medium with [^{35}S]methionine (see above). At $t_{4.5}$ an excess of unlabeled L-methionine (final concentration, 0.5 mg/ml) was added. Three portions (20 ml each) were immediately transferred into three flasks containing chloramphenicol (final concentration, 100 μg/ml), PMSF (final concentration, 4 mg/ml), or both inhibitors. Duplicate 1-ml samples were withdrawn at intervals from each culture, and the radioactivities remaining in the acid-insoluble fractions were measured as described in the text. Symbols: ○, no addition; ●, chloramphenicol alone; □, chloramphenicol plus PMSF; ■, PMSF alone. A value of 100% was equivalent to 9,280 cpm/ml.*

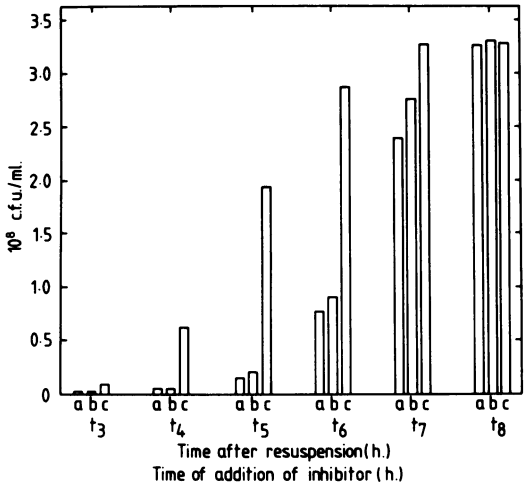

FIG. 6. *Comparison of the effects of chloramphenicol and PMSF added separately at different times during sporulation on the development of Lyso^r. At intervals after resuspension, 10-ml portions of a sporulating culture were exposed to chloramphenicol (100 μg/ml) or PMSF (4 mg/ml). The Lyso^r count was measured immediately and again after incubation until t_8. (a) Lyso^r count at time of addition of inhibitor. (b) Lyso^r count at t_8 when chloramphenicol was added at the times indicated. (c) Lyso^r count at t_8 when PMSF was added at the times indicated. c.f.u., Colony-forming units.*

TABLE 1. *Proportions of spore proteins synthesized at various times during sporulation*[a]

Period of labeling	Radioactivity in spores at t_9 (cpm/mg (dry wt) of spores)	Radioactivity (% of fully labeled spores)
t_0–t_9	2,650	100
t_1–t_9	2,250	85
t_2–t_9	1,750	66
t_3–t_9	1,400	53
t_4–t_9	950	36
$t_{4.5}$–t_9	780	29
t_5–t_9	650	25
t_6–t_9	450	17
t_7–t_9	250	9
t_8–t_9	90	3

[a] Spores from cells that had been exposed to L-[^{35}S]-methionine from t_0 when sporulation was induced were considered fully labeled. If the parent cells had been grown in the presence of labeled methionine, the specific activities would have been about 5% higher (16).

this is likely to affect heat resistance (5), it was necessary to determine the effect of chloramphenicol on DPA synthesis under our conditions.

Chloramphenicol was added at $t_{4.5}$ to a sporulating culture, and the DPA in the cells was measured up to t_9. The DPA content increased for about 60 min after the addition and then declined (Fig. 7). This decline was probably due

to the germination of sensitive spores, and it appears to show that the DPA that was present was insufficient to stabilize them. Somewhat more DPA was synthesized when chloramphenicol and PMSF were added together than when chloramphenicol alone was added (Fig. 7).

With chloramphenicol alone, DPA synthesis until $t_{5.5}$ was virtually the same as DPA synthesis in the presence of PMSF, and even at t_8, after some loss, the DPA content was still over 70% of that in the culture containing PMSF. Nevertheless, the numbers of heat-resistant spores were very different. With chloramphenicol alone the $H80^r$ count was only 5×10^7 spores per ml. In the other two treated cultures the counts were at least fourfold higher, and in the control the count was about 3×10^8 spores per ml.

We added DPA (100 μg/ml) together with chloramphenicol to a sporulating culture at $t_{4.5}$. However, this did not increase the number of heat-resistant spores formed (about 5×10^7 spores per ml) at t_8. When PMSF alone was added at $t_{4.5}$, the amount of DPA synthesized was about 50% of that in the control culture (Fig. 7).

The lack of a relationship between DPA content and $H80^r$ count, in conjunction with the fact that adding DPA externally made no difference, suggested strongly that the reduced heat resistance measured after chloramphenicol treatment did not result primarily from a deficit of DPA.

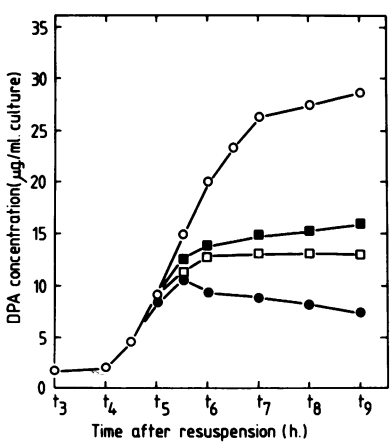

FIG. 7. *Effect of chloramphenicol and PMSF on the synthesis of DPA during sporulation. At $t_{4.5}$ three 100-ml portions of a sporulating culture were transferred into flasks containing chloramphenicol (final concentration, 100 μg/ml), PMSF (final concentration, 4 mg/ml), or both inhibitors. Samples were withdrawn from each culture at intervals and centrifuged, and the DPA contents of the cells were determined as described in the text. Symbols: ○, no addition; ●, chloramphenicol alone; □, chloramphenicol plus PMSF; ■, PMSF alone. At $t_{4.5}$ about 10% of the cells contained phase-bright spores.*

Relative rates of incorporation of L-cysteine by peptide bonding and by disulfide interchange during sporulation. Cysteine-rich proteins are synthesized during sporulation. In free spores they are located in the coat fraction (24), but the time of appearance of these proteins in relation to other late sporulation-specific events is not clear (23).

At 0.5-h intervals after resuspension, 5-ml portions of a sporulating culture were removed and incubated with L-[^{35}S]cysteine for 15 min. Samples from each portion were then assayed for radioactivity. There was a high initial rate of cysteine uptake, which fell considerably by t_2 (Fig. 8A). This was followed by a continuous increase in the rates of incorporation from t_4 to t_7 (Fig. 8A).

There is evidence to suggest that much of the late incorporation of cysteine during sporulation in *B. cereus* occurs by disulfide interchange (3), and this can be selectively inhibited by reagents which compete for sulfhydryl groups (1). However, the timing of this reaction in relation to the development of different types of spore resistance had not been determined previously, nor apparently had this reaction been studied in *B. subtilis*. Accordingly, at intervals after resuspension, 5-ml portions of culture were incubated with L-[^{35}S]cysteine for 15 min as described above. Duplicate samples were then extracted with 25 mM DTT at pH 10 as described above before the radioactivity incorporated was determined. Initially, (t_0 to t_1) when most of the cells were at stage 0/I, the proportion of label removed by DTT was about 15 to 20% (Fig. 8A). After $t_{1.5}$ (about stage II), total incorporation was less, but the extractable fraction increased to about 30% at t_4. In the later stages of sporulation (t_6 to t_8) the rate of incorporation increased and about 70% was extracted by DTT (Fig. 8A).

Effect of chloramphenicol on relative rates of incorporation of L-cysteine by peptide bonding and by disulfide interchange during sporulation. To determine whether the cysteine incorporated in the 15-min pulse and subsequently removed by DTT was involved in exchange with residues in preformed proteins or in nascent proteins, protein synthesis was stopped with chloramphenicol just before the addition of L-[^{35}S]cysteine. At intervals after resuspension in sporulation medium, 5-ml portions of culture were removed into chloramphenicol (100 μg/ml). After 30 s L-[^{35}S]cysteine was added, and incubation was continued for 15 min. The amounts of radioactivity incorporated in the 15-min pulse periods were, as expected, much lower in the presence of chloramphenicol (Fig. 8B). Most of the counts (>90%) in each

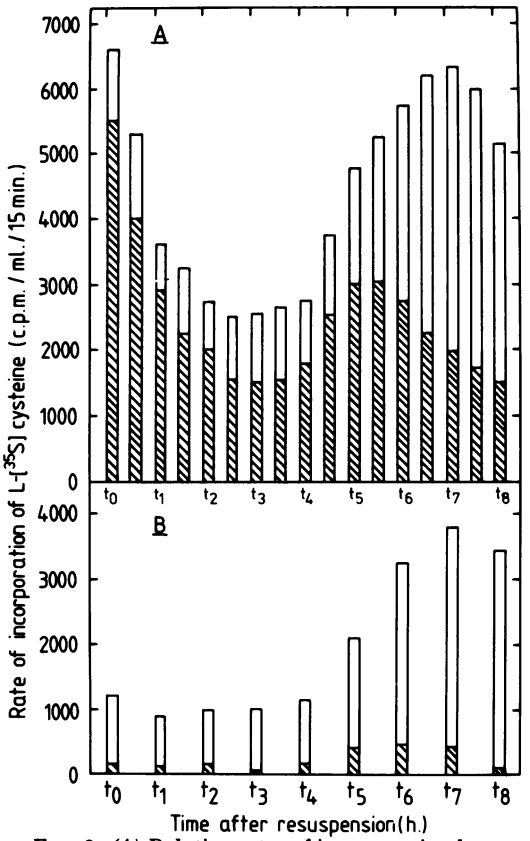

FIG. 8. *(A) Relative rates of incorporation by sporangia of L-[^{35}S]cysteine by peptide bonding and by disulfide interchange during sporulation. At 0.5-h intervals after resuspension, 5-ml portions of culture were removed and exposed to L-[^{35}S]cysteine (0.1 μCi/ml, with 20 μg of carrier L-cysteine hydrochloride per ml) for 15 min. Radioactivities were measured in duplicate 1-ml samples, as described in the text (open bars). Additional duplicate 1-ml samples were added to two tubes, each containing 1 ml of 50 mM DTT in 0.05 M $NaHCO_3$-Na_2CO_3 buffer (pH 10) and L-cysteine hydrochloride (0.5 mg/ml) and incubated for 30 min at 37°C. To each of the tubes was added 2 ml of 10% (wt/vol) ice-cold trichloroacetic acid, and the radioactivities were measured as described in the text (hatched bars). (B) Rates of L-[^{35}S]cysteine incorporation during sporulation in the presence of chloramphenicol. Portions of a sporulating culture were removed at 1-h intervals and exposed to chloramphenicol (100 μg/ml) and L-[^{35}S]cysteine for 15 min, as described above. Radioactivities were then determined in duplicate samples before and after treatment of the cells with DTT as described above in (A). The bars show total radioactivity incorporated in 15 min; the unhatched portions represent the amounts of radioactivity removed by treatment with DTT.*

sample were extracted with DTT. A comparison of Fig. 8A and B shows that the amounts of ^{35}S extractable by DTT (i.e., taken up by exchange in the presence of chloramphenicol) were very similar to the amounts in the control. This result indicates that the exchange reaction takes place with preformed proteins and that it increases markedly between t_4 and t_7.

Effects of chloramphenicol and PMSF on rates of cysteine uptake after $t_{4.5}$. Portions of a sporulating culture were treated at $t_{4.5}$ with chloramphenicol or PMSF. Samples were removed from all of the cultures at intervals and used for measuring the rates of incorporation of L-[^{35}S]cysteine and the fractions that were extractable with DTT.

PMSF added at $t_{4.5}$ inhibited the uptake of cysteine by interchange from t_5 onward, but it did not inhibit uptake by peptide bond formation (Fig. 9A and C). Chloramphenicol had the opposite effect; it inhibited the peptide bond incorporation but allowed disulfide interchange to proceed (Fig. 9B). When both inhibitors were added, both types of incorporation were reduced (Fig. 9D).

Thus, it is clear that PMSF, by inhibiting proteolysis or otherwise, radically reduced the number of sites available for disulfide interchange.

Extractability of incorporated leucine by DTT. The rates of incorporation of L-[^{3}H]leucine were also measured during sporulation, and the cells were extracted with DTT to check that the procedure was not causing loss of counts for some other reason. The rates of L-[^{3}H]leucine incorporation were measured by 15-min pulse-labeling at 1-h intervals from t_0 to t_8. Less than 5% of the radioactivity incorporated into each sample was removed by DTT.

Comparison of cysteine incorporation into spores and sporangia at t_6. The previous experiments did not distinguish between cysteine incorporated by interchange into the mother cells and that incorporated into the spores. Accordingly, L-[^{35}S]cysteine (0.1 μCi/ml, with 20 μg of carrier L-cysteine hydrochloride per ml) was added to a sporulating culture at t_6, and the amounts of radioactivity in the sporangia and the spores (isolated at t_7) were measured before and after extraction with 25 mM DTT.

About 40% of the radioactivity was removed from the sporangia by DTT (Fig. 10A). Although the amount removed from the isolated spores was somewhat less (27%), it was clear that interchange was taking place with both sporangial and spore proteins. When PMSF was present, the fraction removed by DTT was substantially reduced (Fig. 10A), both in the sporangia and in the spores (15 and 5.6%, respectively).

The results obtained from labeling with L-[^{35}S]cysteine from t_6 to t_7 were then compared

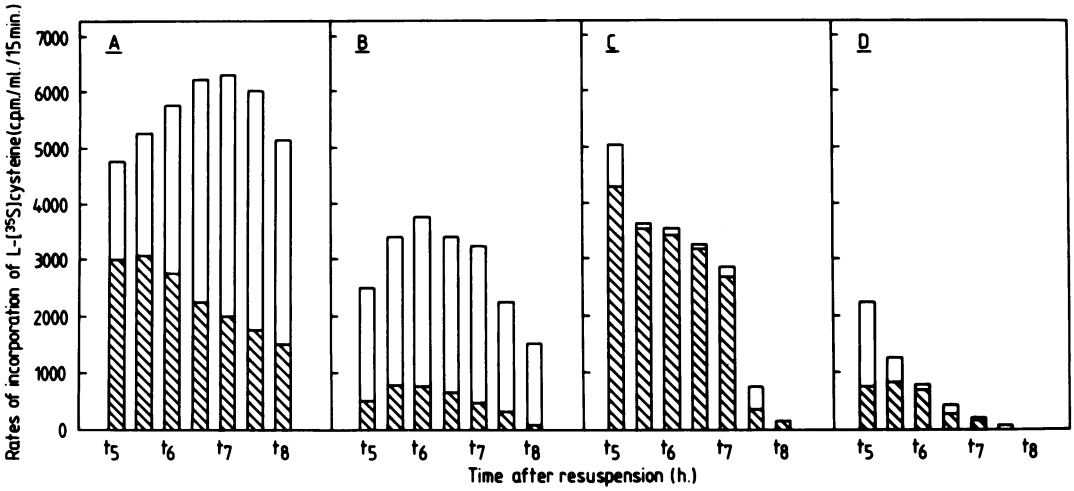

FIG. 9. *Effect of chloramphenicol and PMSF added at $t_{4.5}$ on the subsequent rates of L-[^{35}S]cysteine incorporation during sporulation. At $t_{4.5}$ three 50-ml portions of a sporulating culture were transferred into three flasks containing chloramphenicol (final concentration, 100 μg/ml), PMSF (final concentration, 4 mg/ml), or both inhibitors. Total radioactivity and the fraction extractable with DTT were measured in duplicate samples (see legend to Fig. 8). The bars show total radioactivities incorporated in 15 min; the unhatched portions represent the amounts of radioactivity removed by treatment with DTT. (A) Untreated culture. (B) Chloramphenicol. (C) PMSF. (D) Chloramphenicol plus PMSF.*

with those obtained from labeling with [^{35}S]sulfate. The reason for this experiment was that adding an excess of cysteine might have promoted more interchange than that which would have been obtained with endogenously synthesized cysteine. Labeling with [^{35}S]sulfate would reflect more accurately what occurs in normal sporulation.

In the following experiment, 0.04 M $MgSO_4 \cdot 7H_2O$ in the sporulation medium was replaced by 0.04 M $MgCl_2 \cdot 6H_2O$, and this did not affect the rate of sporulation. At t_6, $^{35}SO_4^{2-}$ (0.5 μCi/ml, with 40 μg of carrier per ml) was added to a sporulating culture; L-methionine (50 μg/ml) was also added, so that the radioactive methionine formed from $^{35}SO_4^{2-}$ would be heavily diluted with carrier and therefore not contribute appreciably to the measured incorporation.

In the control culture the ratio of peptide bond label to extractable label in the sporangia was somewhat higher when sulfate was used for labeling instead of cysteine (Fig. 10A and B). With spore proteins both methods of labeling gave a similar proportion of extractable radioactivity. Again, PMSF reduced the extractable component, but the effect in the spores was much less marked (Fig. 10B).

DISCUSSION

The spore properties examined in this paper are Tol^r, H65^r, H80^r, and Lyso^r. During normal sporulation these forms of resistance develop consecutively and appear graphically as a series of roughly parallel curves. Of these, the first (Tol^r) begins after $t_{3.5}$ (stage III), and the last (H80^r and Lyso^r) begin after t_5 (stage V) and reach their full values 3 or 4 h later at the end of stage VI.

When protein synthesis is inhibited by chloramphenicol, two of these types of resistance (H65^r and H80^r) nevertheless increase several-fold and reach similar values (about 5×10^7 spores per ml) (Fig. 4A). Apparently then, at $t_{4.5}$ these sporangia have already synthesized the proteins needed for the development of heat resistance. In addition, the proteins that later constitute the outer layers of the spore had also been made (Fig. 3), since they too appeared despite the presence of chloramphenicol.

Tol^r developed differently. At $t_{4.5}$ the Tol^r count was already 10^8 spores/ml. After chloramphenicol was added, the count fell progressively, until it reached the same value as the H65^r and H80^r counts. The implication is that about one-half of those sporangia that were Tol^r at $t_{4.5}$ already possessed the proteins needed to develop heat resistance in the ensuing period. The other half did not possess these proteins, and their prespores lost their Tol^r. A relevant factor is the series of changes in germination properties that take place at about the same time (10). Up to $t_{4.5}$, prespores can germinate spontaneously (that is, without any externally added germinants), whereas a little later they no longer do so unless L-alanine or some other

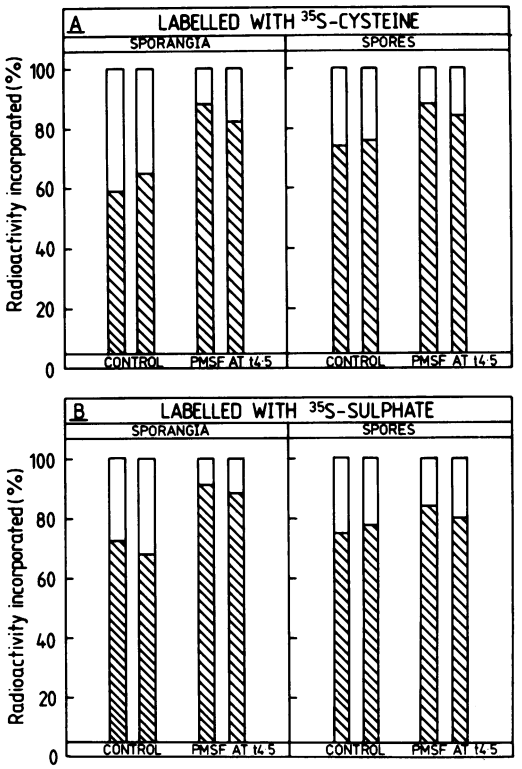

FIG. 10. *Incorporation of L-[^{35}S]cysteine (A) or [^{35}S]sulfate (B) into total sporangial protein and into spore protein during sporulation in the presence or absence of PMSF. At $t_{4.5}$ a 100-ml sporulating culture was divided into two equal portions, one of which was incubated with PMSF (4 mg/ml) and the other of which was left untreated. At t_6 each culture was further divided into two portions, one of which was exposed to L-[^{35}S]cysteine (0.1 μCi/ml, with 20 μg of carrier L-cysteine hydrochloride per ml) and the other of which was exposed to [^{35}S]sulfate (0.5 μCi/ml, with 40 μg of carrier SO_4^{2-} per ml and 50 μg of L-methionine per ml). At t_7 the bacteria from each culture were harvested by centrifugation (5,000 × g, 5 min) and resuspended to their original volumes in buffer K (see text). Radioactivities were determined in duplicate 1-ml samples before and after treatment with DTT, as described in the legend to Fig. 8. The remaining bacteria (about 23 ml in buffer K) from each culture were recentrifuged and resuspended in 2 ml of buffer K. The spores were released from their mother cells (see text), and radioactivities were determined before and after treatment with DTT. The results of two separate experiments are shown. Since the actual amounts of radioactivity incorporated varied between the experiments, for ease of comparison the total radioactivities in sporangial and spore fractions were designated 100%. The unhatched portions then represent the percentages of the total radioactivities removed by DTT. The 100% values were as follows (in counts per minute per milliliter): (A) control, sporangia, 11,036 and 8,523; control, spores, 2,909 and 1,968; With PMSF, sporangia, 4,856 and 6,324; with PMSF, spores, 2,703 and 2,943. (B) Control, sporangia, 5,050 and 6,432; control, spores, 2,703 and 1,945. With PMSF, sporangia, 4,893 and 3,677; with PMSF, spores, 2,312 and 1,998.*

germinant is present. Thus, the alternative explanation is that about one-half of the Tol^r spores at $t_{4.5}$ are lost by spontaneous germination during the subsequent incubation with chloramphenicol, whereas the remainder go on to develop heat resistance.

Whatever the explanation may be for the decline in Tol^r counts, it is clear that a significant proportion of the sporangia at $t_{4.5}$ possess the proteins needed for all of the types of resistance measured, except those for lysozyme.

The experiments with PMSF show that the number of sporangia that have made their "late" proteins is greater than would appear from the results in Fig. 4A. Thus, when PMSF is present, about four times as many spores develop heat resistance, and the number reaches about 60% of the value achieved in the untreated control. However, Lyso^r is not enhanced, and it is clear that the proteins needed for developing this property have still not been synthesized.

The enhancement of heat resistance by PMSF could be reasonably attributed to its action as an inhibitor of intracellular proteolysis (Fig. 5B), although we cannot exclude the possibility that it might act in some other way. Under the conditions of our experiments, intracellular protein degradation has been shown to proceed at rates of about 10%/h (16). It is therefore conceivable that those cells which have synthesized just enough of the critical proteins at $t_{4.5}$ degrade some of them during the ensuing incubation and so fail to achieve resistance. When PMSF is present, the proteins are stabilized and the resistance properties develop.

These interpretations of the chloramphenicol experiments are supported by the evidence of the electron micrographs and by the labeling experiments (Table 1). The former show that, although the prespores at $t_{4.5}$ contain little coat material, the coat layers are deposited more or less normally by t_8, even in the presence of chloramphenicol (Fig. 3). The labeling experiments show that at $t_{4.5}$ the sporangia have already synthesized 70% of all of the protein that will ultimately be assembled in the spores.

Since chloramphenicol acts indiscriminately on protein synthesis, in addition to inhibiting the structural proteins of the spores, it also inhibits production of some enzymes and therefore the small-molecule products of these enzymes. In this context DPA is particularly relevant because it has been implicated in the de-

velopment of heat resistance (5). However, the results shown in Fig. 7 and described in the text make it very unlikely that the reduced heat resistance counts resulting from chloramphenicol treatment or the increases obtained in the presence of PMSF are consequences of changes in DPA content.

Another secondary reaction that might be affected by the inhibitors we used is the incorporation of cysteine into protein. When sporulating cells are pulsed for 15 min with labeled cysteine, the total incorporation measured has two components: first, cysteine taken up by disulfide interchange and hence extractable with such reagents as DTT; and second, cysteine incorporated by peptide bond formation and therefore not extractable. Both types of uptake occur throughout sporulation (1, 3), although the relative proportions differ from t_0 to t_8. If chloramphenicol is added to a sporulating culture at the same time as the cysteine, it largely abolishes the peptide-bound component, but has little effect on the incorporation of extractable cysteine. This makes it clear that disulfide interchange occurs with proteins that have already been formed rather than with nascent proteins. This was confirmed by the experiment in which a single addition of chloramphenicol or PMSF or both was made at $t_{4.5}$ and the capacities of the cultures to incorporate cysteine were measured from t_5 onward. With chloramphenicol alone the peptide-bound component was, as expected, much reduced (Fig. 9B). The fact that chloramphenicol apparently inhibited the incorporation of cysteine less than the incorporation of methionine or leucine can be attributed to incomplete extraction of disulfide-bound label with DTT. However, extractable cysteine continued to be incorporated up to t_8 in substantial amounts. PMSF had the effect of progressively diminishing the number of sites available for disulfide interchange from t_5 onward. When both inhibitors were added, both peptide-bound and extractable cysteine were diminished.

The effects of PMSF can be summarized as follows. When protein synthesis is inhibited at $t_{4.5}$, PMSF greatly enhances the development of resistance to toluene and heat. It also reduces the fraction of cysteine incorporated into protein by disulfide interchange. The manner in which it does so is less obvious, but the experiments shown in Fig. 9 suggest that if proteolysis is inhibited, —SH groups that would otherwise have become available for disulfide interchange remain unavailable. The capacity for disulfide interchange therefore appears to be the result of some sort of processing of proteins in the manner suggested by, for instance, Cheng and Aronson (7) and Pandey and Aronson (18). The physiological function of such processing is not very apparent; preventing it by the addition of PMSF does not seriously affect either the deposition of spore coat layers (Fig. 3) or the development of heat resistance in normal cultures. It does, however, enhance the number of heat-resistant spores produced in cultures treated with chloramphenicol. Although we have attributed this action to the protective effect of PMSF on proteins, it remains possible that a secondary effect on the number of residues available for disulfide interchange is a contributory factor.

Lyso^r differs from the other forms of resistance in several ways: the proteins necessary for its development appear later, mainly after t_5 (Fig. 6); full development of resistance depends on continued protein synthesis right up to t_7; and no enhancement of the property is obtained when PMSF is present. On the contrary, if PMSF is added at any time up to t_5 subsequent development of Lyso^r is reduced. The simplest explanation is that some earlier proteolytic step has to occur, without which the structural components necessary for Lyso^r cannot be assembled.

The object of this study was to determine the times at which the genes for a number of late events in sporulation are transcribed and translated. In summary, the results presented in this paper and in the accompanying paper (10) show that the proteins for all of the types of resistance which we considered, those for the coat proteins and those for the development of the germination properties of the spore, are all synthesized by the end of stage IV.

One might assume that the sequential appearance of various sporulation characteristics reflects the order of sequential expression of the corresponding genes. Our results show that this is incorrect. Instead, it has to be accepted that the major sporulation events occurring in stages V and VI are secondary consequences. The primary events (gene expression and protein synthesis) occurred before the end of stage IV and, in the case of some coat proteins, in stage II (see above).

It follows that the development of the observed succession of spore properties reflects a succession of events involving some form of processing and perhaps self-assembly. In fact, Aronson and Fitz-James (2) have shown that the deposition of the outer coat material of *B. cereus* spores can occur spontaneously. Although an attempt to demonstrate self-assembly during sporulation in *B. subtilis* after t_5 was unsuccessful (10), it is, nevertheless, reasonable to suppose that a more extensive occurrence of self-assem-

bly characterizes sporulation during stages V and VI.

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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. **Aronson, A. I., and P. C. Fitz-James.** 1971. Reconstitution of bacterial spore coat layers in vitro. J. Bacteriol. **108**:571–578.
3. **Aronson, A. I., and P. Fitz-James.** 1976. Structure and morphogenesis of the bacterial spore coat. Bacteriol. Rev. **40**:360–402.
4. **Balassa, G.** 1966. Synthèse et fonction des ARN messagers au cours de la sporulation de *Bacillus subtilis*. Ann. Inst. Pasteur Paris **110**:175–191.
5. **Balassa, G., P. Milhaud, E. Raulet, M. T. Silva, and J. C. F. Sousa.** 1979. A *Bacillus subtilis* mutant requiring dipicolinic acid for the development of heat-resistant spores. J. Gen. Microbiol. **110**:365–379.
6. **Chasin, L. A., and J. Szulmajster.** 1969. Enzymes of dipicolinic acid biosynthesis in *Bacillus subtilis*, p. 133–147. *In* L. L. Campbell (ed.), Spores IV. American Society for Microbiology, Bethesda, Md.
7. **Cheng, Y. E., and A. I. Aronson.** 1977. Alterations of spore coat processing and protein turnover in a *Bacillus cereus* mutant with a defective postexponential intracellular protease. Proc. Natl. Acad. Sci. U.S.A. **74**:1254–1258.
8. **Coote, J. G., and J. Mandelstam.** 1973. Use of constructed double mutants for determining the temporal order of expression of sporulation genes in *Bacillus subtilus*. J. Bacteriol. **114**:1254–1263.
9. **Dancer, B. N., and J. Mandelstam.** 1975. Production and possible function of serine protease during sporulation of *Bacillus subtilis*. J. Bacteriol. **121**:406–410.
10. **Dion, P., and J. Mandelstam.** 1980. Germination properties as marker events characterizing later stages of *Bacillus subtilis* spore formation. J. Bacteriol. **141**:786–792.
11. **Hranueli, D., P. J. Piggot, and J. Mandelstam.** 1974. Statistical estimate of the total number of operons specific for *Bacillus subtilis* sporulation. J. Bacteriol. **119**:684–690.
12. **Janssen, F. W., A. J. Lund, and L. E. Anderson.** 1958. Colorimetric assay for dipicolinic acid in bacterial spores. Science **127**:26–27.
13. **Kay, D., and S. C. Warren.** 1968. Sporulation in *Bacillus subtilis*. Morphological changes. Biochem. J. **109**:819–824.
14. **Mandelstam, J.** 1969. Regulation of bacterial spore formation. Symp. Soc. Gen. Microbiol. **19**:377–404.
15. **Mandelstam, J.** 1976. Bacterial sporulation: a problem in the biochemistry and genetics of a primitive developmental system. Proc. R. Soc. London Ser. B **193**:89–106.
16. **Mandelstam, J., and W. M. Waites.** 1968. Sporulation in *Bacillus subtilis*. The role of exoprotease. Biochem. J. **109**:793–801.
17. **Munoz, L. E., T. Nakayama, and R. H. Doi.** 1978. Expression of spore coat protein gene, an "early sporulation gene," and its relationship to RNA polymerase modification, p. 213–219. *In* G. Chambliss and J. C. Vary (ed.), Spores VII. American Society for Microbiology, Washington, D.C.
18. **Pandey, N. K., and A. I. Aronson.** 1979. Properties of the *Bacillus subtilis* spore coat. J. Bacteriol. **137**:1208–1218.
19. **Piggot, P. J., and J. G. Coote.** 1976. Genetic aspects of bacterial endospore formation. Bacteriol. Rev. **40**:908–962.
20. **Ryter, A., and J. Szulmajster.** 1965. Action du chloramphénicol sur la sporogenèse de *B. subtilis*. Ann. Inst. Pasteur Paris **110**:640–651.
21. **Sterlini, J. M., and J. Mandelstam.** 1969. Commitment to sporulation in *Bacillus subtilis* and its relationship to development of actinomycin resistance. Biochem. J. **113**:29–37.
22. **Szulmajster, J., and R. E. Canfield.** 1965. Changements biochimiques associés a la sporulation de *B. subtilis*. Coloq. Int. CNRS **124**:587–596.
23. **Vinter, V.** 1959. Sporulation of bacilli. VII. The participation of cysteine and cystine in spore formation by *Bacillus megatherium*. Folia Microbiol. (Prague) **4**: 216–221.
24. **Vinter, V.** 1969. Physiology and biochemistry of sporulation, p. 73–123. *In* G. W. Gould and A. Hurst (ed.), The bacterial spore. Academic Press Inc., London.
25. **Wood, D. A.** 1972. Sporulation in *Bacillus subtilis*. Properties and time of synthesis of alkali-soluble protein of the spore coat. Biochem. J. **130**:505–514.