Sporulation in *Bacillus subtilis*

PROPERTIES AND TIME OF SYNTHESIS OF ALKALI-SOLUBLE PROTEIN OF THE SPORE COAT

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An alkali-extractable protein fraction comprising 6% of the dry weight of the spore can be removed from spores of *Bacillus subtilis* 168. Three different extraction procedures each yield at least one similar protein. Extracted protein behaved as a single species on ion-exchange chromatography or gel filtration, but two polypeptides were found on electrophoresis. Comparison of molecular weights on electrophoresis and by sucrose-gradient analysis suggests that the protein(s) undergo aggregation into multimers. Extracted spores remain viable, but are altered in density and lysozyme sensitivity and they aggregate together. Electron microscopy of extracted spores shows that loss of material seems to occur from the outer coat layers. Extraction therefore probably removes a specific fraction of the spore-coat protein, but without impairment to the spore protoplast. This protein can first be detected immunologically 4h after the initiation of sporulation, and the synthesis of this protein is sensitive to chloramphenicol, actinomycin D and rifamycin. Labelling experiments also show that the protein begins to be synthesized early in sporulation. Examination of the ability of asporogenous mutants to produce cross-reacting material indicates that some event in stage II of sporulation determines its production.

Spore-coat proteins may represent an unique class of spore-specific gene products (Mandelstam, 1969; Hanson *et al.*, 1970). Most of the spore coat in bacilli consists of protein and can comprise up to 80% of the total spore protein (Murrell, 1969). One of the components obtained from spore-coat preparations is an alkali-extractable protein (Kadota *et al.*, 1965; Kondo & Foster, 1967; Gould *et al.*, 1970) constituting 1–6% of the dry weight of the spores.

Morphologically the spore coats appear at stage V of sporulation (Ryter *et al.*, 1966; Kay & Warren, 1968; Fitz-James & Young, 1969). However, 'pulse-chase' labelling experiments with sporulating cultures of *Bacillus cereus* T suggested that synthesis of coat polypeptides begins several hours before the morphological appearance of coats (Aronson & Fitz-James, 1968), whereas in *Bacillus subtilis*, labelling experiments showed that the bulk of coat synthesis took place after the development of refractility, and synthesis may have been concomitant with the deposition of coat protein on the developing spore (Spudich & Kornberg, 1968). Since the overall morphological events of sporulation are very similar in these two bacilli it seemed unlikely that the timing of coat synthesis should be so different.

This present investigation was carried out partly

to determine the time of synthesis of this alkali-extractable protein by employing both radioactive labelling and immunological procedures. In addition some properties of the protein were examined and it was also shown that the extracted spores were still viable and heat-resistant though altered in their physical properties. Finally, asporogenous mutants blocked at various points in the developmental pathway were examined for their ability to produce the protein, since it is known that other biochemical events of sporulation previously examined are connected with morphological development (Waites *et al.*, 1970; Wood, 1971).

Materials and Methods

Organisms and spore preparations

Organisms. *B. subtilis* 168 (Marburg strain) requiring indole or tryptophan for growth was used for most experiments. The asporogenous mutants used in one experiment are derived from *B. subtilis* 168 and have been examined previously for their other biochemical and morphological characteristics (Waites *et al.*, 1970). *B. subtilis* 12A⁻, a mutant blocked at stage 0 of sporulation and lacking protease and antibiotic activity (Rogolsky, 1968), was kindly provided by Dr. M. Rogolsky. This strain is reported as liberating a semi-crystalline protein on lysis in

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liquid medium. This liberated protein can be dissolved only in dilute alkali (0.1M-NaOH). *Bacillus licheniformis* N.T.C.C. 6346, *Bacillus megaterium* N.T.C.C. 7581, *Bacillus sphaericus* N.T.C.C. 7582, *Bacillus cereus* N.T.C.C. 7587, *Bacillus macerans* N.T.C.C. 7588 and *Bacillus pantothenicus* N.T.C.C. 8162 were obtained from the National Type Culture Collection, Colindale, London N.W.9, U.K. A suspension of spores (500mg dry wt.) of *Clostridium roseum* was given by Dr. W. M. Waites.

Growth and sporulation. Organisms were maintained on nutrient-agar plates (Oxoid). To initiate sporulation cells growing exponentially in a hydrolysed casein medium were resuspended at 0.25mg dry wt./ml into a medium containing glutamate and inorganic salts (Sterlini & Mandelstam, 1969). The time of resuspension will be referred to as the t_0 for sporulation and all subsequent times during sporulation as t_1 , t_2 etc. The growth and sporulation temperature was 37°C.

Spore suspensions were produced by growing bacteria for 3–4 days at 37°C on large trays (50cm × 30cm) of nutrient-agar. *B. subtilis* 12A⁻ was grown in glucose-peptone medium and the semi-crystalline protein was harvested and washed as described by Rogolsky (1968).

Purification of spores. Purified spores were prepared by the method of Bonsen *et al.* (1969). The spores were stored at -20°C as frozen suspensions.

Preparation of soluble cell extracts. A culture (50ml) at the appropriate sporulation stage was centrifuged, the cell pellet resuspended in 1.0ml of 0.1M-sodium phosphate buffer, pH7.2, and ultrasonicated for 2min to obtain 99% cell breakage (MSE sonicator, 1.5A). The suspension was centrifuged at 30000g for 30min. Samples (0.1ml) of the supernatant were used for incubation with *B. subtilis* 12A⁻ protein or wild-type extracts.

Determination of spore incidence in a sporulating culture. Refractile spores were counted in the phase-contrast microscope and expressed as a percentage of the total number of cells. For each determination 300 cells were counted.

Spore protein

Preparation of alkali-extractable spore protein. Three procedures (A, B and C) were used to determine whether the products were comparable and also whether the procedures affected the spores differently. The first two procedures are previously published methods: A, method of Gould *et al.* (1970); B, method of Delafield *et al.* (1968).

Procedure C. Spore suspensions (10mg in 2ml of water) were incubated in 10.0ml of 0.1M-NaOH at 4°C for 15min with no pretreatment in mercaptoethanol or urea. The spores were centrifuged down and the alkaline extract was retained.

Spore suspensions (10mg in 2ml of water) were also incubated in either 7M-urea, pH2.8, or 10% (w/v) 2-mercaptoethanol (0.5ml/mg dry wt. of spores) for 1h at 37°C. The spores were then centrifuged down and the supernatant was retained.

The extracts from all procedures were dialysed for 15h against two changes of 0.5M-sodium acetate-acetic acid buffer, pH5.0 (20ml of dialysis buffer/ml of extracted solution). The solution in the dialysis sac usually formed a visible precipitate, which was collected by centrifugation at 30000g for 1h. The pellet was then dissolved in either 0.1M-diethanolamine-HCl buffer, pH10.0, or in 0.1M-NaOH (1.0ml of buffer or alkali/1.0ml of original alkali extract).

Column chromatography. Columns of both Sephadex G-100 and G-200 (110cm × 3.5cm) were equilibrated in 0.1M-diethanolamine-HCl buffer, pH10.0. Column void volumes were determined by elution of Blue Dextran solutions [Pharmacia (G.B.) Ltd., London W.5, U.K.]. Solutions (3ml) of alkali-extractable protein (5mg/ml in 0.1M-diethanolamine-HCl buffer, pH10.0) were layered on and eluted with more of the same buffer solution. Fractions (3ml) were collected and E_{280} was measured.

Columns of DE52 DEAE-cellulose (20cm × 1.5cm) were equilibrated with 0.1M-diethanolamine-HCl buffer, pH10.0. Protein samples (2.0ml of 4mg/ml solution) were run on and then washed with 1 column volume of the same buffer. Protein was eluted with a linear gradient (0–1.0M in 300ml) of NaCl. From each type of column fractions (3ml) were collected and E_{280} was measured. The peak fractions were concentrated in an Amicon ultrafilter with a UM10 membrane.

Protein determination. Protein was determined by the method of Lowry *et al.* (1951). Standards were prepared from bovine plasma albumin, fraction V (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.).

Polyacrylamide-gel electrophoresis. Polyacrylamide-gel columns (7.5%, 10cm × 1.0cm) were prepared by the method of Davis (1964) but with the omission of both spacer and small-pore gel layers.

Sodium dodecyl sulphate-gel electrophoresis was carried out essentially by the method of Dunker & Rueckert (1969), gel concentrations of either 5% or 10% being used.

Amino acid analysis. Protein samples were washed with water or extensively dialysed against water. Protein was determined in the suspension and a sample calculated to contain 1mg of protein was dried in a vacuum desiccator over P₂O₅. The samples were hydrolysed in 6M-HCl (0.5ml) at 110°C in sealed tubes for 24h (Spackman *et al.*, 1958). Hydrolysates were then examined in a Spinco amino acid analyser.

Identification of N-terminal residues. N-Terminal

analysis was performed by using a dansyl chloride procedure (Gray & Hartley, 1963). Dns-amino acids were detected as u.v.-absorbing spots after chromatography on polyamide sheets (Glenn & Mandelstam, 1971).

Serology

Pooled, concentrated peak fractions from the void volume of Sephadex G-100 gel filtration (10mg of protein) were mixed with Freund's adjuvant and the total protein was injected into the foot-pad of two male rabbits. After 6 weeks each rabbit received a further 2mg of protein by the same route and 1 week later serum samples were tested for antibody activity to alkali-extractable protein. Booster injections of 5mg of protein were subsequently given at suitable intervals to maintain antibody titre.

Antiserum to alkali-extractable coat protein of *Bacillus cereus* T, and antiserum to crystal protein of *Bacillus thuringiensis* serotypes 3 and 9, were kindly given by Dr. H. Somerville. Precipitin reactions were examined by the double-diffusion technique (Ouchterlony, 1958).

Time of appearance of protein

For measuring the appearance of alkali-extractable protein during sporulation a 1 litre resuspension experiment was set up, and 100ml samples were taken hourly until t_7 . They were centrifuged and the cell pellets stored at -20°C overnight. They were thawed and then extracted by procedure A (Gould *et al.*, 1970).

Asporogenous mutants were examined for the presence of cross-reacting material as follows. Samples (200ml) of resuspension culture at t_7 were centrifuged and the cell pellet was extracted as described above; the extracts were tested for cross-reaction to antibody prepared against extract from procedure A.

General methods

Density-gradient centrifugation. (i) Protein extracted from spores was analysed on sucrose density gradients to estimate its sedimentation coefficient and hence its molecular weight. Sucrose gradients (5ml; 5–20%, w/v) in either 0.1M-diethanolamine-HCl buffer, pH 10.0, or 0.1M-NaOH were prepared. Protein preparations (0.3ml of 5mg/ml soln.) were layered on them and then centrifuged at 100000g in a MSE 3 × 5ml swing-out rotor. Alkaline phosphatase from *Escherichia coli* (gift of Dr. P. Piggot) was used as an internal marker for calculating approximate sedimentation constants and molecular weights (Martin & Ames, 1961).

(ii) Extracted and non-extracted spore suspensions

were analysed on Urografin gradients (Squibb Inc., New Brunswick, U.S.A.) (Tamir & Gilvarg, 1966).

Electron microscopy. Spore suspensions from each of the extraction procedures, and also untreated spores, were centrifuged to give a small pellet (about 5mg dry wt. of spores). This was then prepared for electron microscopy as described by Kay & Warren (1968).

Heat resistance, lysozyme sensitivity and outgrowth of treated and untreated spores. Spore suspensions were assayed for heat resistance by incubation at 85°C , samples being withdrawn at intervals and suitable dilutions plated on nutrient-agar. Lysozyme sensitivity was examined by incubating the suspensions at 37°C in lysozyme solution (50 $\mu\text{g}/\text{ml}$ in 0.01M-sodium phosphate buffer, pH 7.2) and measuring both the change in E_{600} and also the percentage of phase-bright spores surviving. Outgrowth was measured by following the commencement of exponential growth in the cell suspension when shaken in Penassay broth (Difco, West Molesey, Surrey, U.K.) at 37°C .

Determination of 2,6-dipicolinic acid. Suitable suspensions of treated spores (50ml of 20mg dry wt. of spores/ml) were assayed for dipicolinic acid by using the reagents of Janssen *et al.* (1958).

Alkaline phosphatase activity. Alkaline phosphatase activity was determined as described by Waites *et al.* (1970).

Radioactive labelling experiments

Label 'chase' experiments. A culture (1 litre) was resuspended in sporulation medium containing L-[U- ^3H]leucine (0.075 μCi and 20 $\mu\text{g}/\text{ml}$). At hourly intervals from t_4 , 90ml samples were withdrawn, centrifuged and the pellet was washed at 37°C with 20ml of resuspension medium, centrifuged again, and finally resuspended in 100ml of resuspension medium containing 0.5mg of L-leucine/ml and incubated until t_{24} . The suspension was centrifuged and the spores were purified (see above) and the alkali-extractable protein was removed by procedure A. The final pellet of extracted protein after acid precipitation was dissolved in 0.6ml of 0.1M-NaOH and duplicate samples (0.03ml) were assayed for protein content. Two further duplicate samples (0.2ml) were treated at 4°C with 0.2ml of 10% (w/v) trichloroacetic acid for 1h, and filtered through glass-fibre discs (2.5cm; GF/C grade, Whatman). The filter was washed with cold (4°C) 5% (w/v) trichloroacetic acid (10ml) containing 500 μg of L-leucine/ml. The discs were then dried in a vacuum oven at 80°C for 1h, and the radioactivity was measured by liquid-scintillation counting (Blundell & Wild, 1969). The culture supernatants still contained approx. 30% of the initial quantity of labelled leucine, as determined by high-voltage paper electrophoresis followed by

elution and counting of the leucine spot for radioactivity (Dreyer & Bynum, 1967). Similar experiments were also carried out with L-[³⁵S]methionine by using 100 μ Ci of label and 'chasing' with medium (100 ml) containing unlabelled methionine (0.5 mg/ml).

To determine the stage at which the protein became detectable in the cytoplasm of the mother cells the following procedure was used for label 'chase' experiments. Samples (20 ml) were removed from the same cultures at the same time-intervals. They were centrifuged and the cell pellet was incubated in 1 ml of lysozyme solution (50 μ g/ml in 0.01 M-sodium phosphate, pH 7.2) for 1 h. The lysate was then incubated with 0.1 ml of 1.0 M-NaOH for 1 h at 4°C, centrifuged (6000g for 10 min) and the supernatant retained. Samples of the supernatant (200 μ g of protein) were subjected to electrophoresis on 7.5% polyacrylamide gels (see above) and stained for protein. The band moving with the tracker dye was removed by cutting a 0.5 cm-thick slice and dried overnight at 50°C. The dry gel was then dissolved in 1.0 ml of 30% (v/v) H₂O₂. Dissolved protein was precipitated with 1.0 ml of 10% (w/v) trichloroacetic acid at 4°C for 30 min, filtered, and washed with 20 ml of 5% trichloroacetic acid containing 500 μ g of unlabelled L-leucine/ml. The filter was dried (see above) and the radioactivity determined.

Results

Extracted protein

Removal of alkali-extractable protein from spores. Each of the three procedures used to remove the alkali-extractable protein yielded a fraction containing about 6% of the dry weight of the spores, and in each the spores retained full refractility. Treatment of the spores with 7 M-urea or 10% (w/v) mercapto-ethanol alone failed to remove a significant amount of protein. Extracted spores formed visible clumps and on standing sedimented faster than untreated spores. Repeated extraction of spores with 0.1 M-NaOH after procedure A yielded decreasing amounts of protein of similar electrophoretic and immunological properties, and the spores finally obtained formed clumps, which could not be disaggregated even after vigorous agitation. After precipitation of the protein by dialysis against 0.5 M-acetate buffer, pH 5.0, a waxy pellet was obtained that was difficult to dissolve in 0.1 M-diethanolamine-HCl buffer, pH 10.0. Concentrated solutions (>10 mg/ml) of protein stored at 4°C underwent spontaneous aggregation into visible particles.

Sephadex filtration. Most (>95%) of the protein in solution in 0.1 M-diethanolamine-HCl buffer, pH 10.0 (see the Materials and Methods section), was eluted in the void volume from a Sephadex G-100 column. Some small peaks of lower molecular weight were

also detected, but the material in these was identical with that in the major peak by immunological and electrophoretic criteria. Protein from extraction procedure A dissolved in 0.1 M-diethanolamine-HCl buffer, pH 10.0, was also eluted in the void volume from Sephadex G-200 columns. A single broad peak of lower molecular weight was also detected, but both the species of different molecular weights showed similar immunological and electrophoretic properties. These observations suggest that the extracted protein may be capable of reversible aggregation into a range of sizes. The smallest species detected had a molecular weight of about 1.4×10^4 , judged by its elution volume compared with standard proteins (Andrews, 1964). Extracted protein from procedures B and C showed similar behaviour on both Sephadex G-100 and G-200 columns.

Solubility properties of extracted protein. Soluble protein extracts (2.0 ml; 1.0 mg of protein/ml) from procedures A, B and C were dialysed for 24 h against a series of buffers over the range pH 3.0–10.0. The precipitated protein was removed by centrifugation (20000g for 20 min) and the protein remaining in the supernatant measured. The results showed that each extract had a similar solubility profile, over 50% of the protein precipitating at pH 7.0. At this pH value both the precipitate and the supernatant fraction were identical on electrophoretic and immunological analysis.

DEAE-cellulose chromatography. Chromatography on DE52 cellulose columns showed that most (>95%) of the protein extracted by procedure A and applied to the column was eluted as a single peak with 0.6 M-NaCl. This peak also showed a single band of protein entering polyacrylamide gels, and was equivalent to material from procedure A by immunological criteria. Similarly the protein from the void volume of Sephadex G-100 columns also gave a single peak on elution from DE52 cellulose columns under the same conditions.

Polyacrylamide gel electrophoresis. In 7.5% polyacrylamide gels a single band of protein moving with the tracker dye was observed with extracts from procedures A, B and C. In all three cases a large amount of protein was excluded from the gel, indicating that very large molecules (mol.wt. $>1 \times 10^6$) were present. A similar band was seen with the protein peaks from Sephadex G-100, G-200 and DEAE-cellulose chromatography. Both the excluded protein and the fraction entering the polyacrylamide gel appeared identical when tested against antiserum.

To examine the possibility that the protein consisted of a number of similarly charged polypeptides of differing molecular weight but capable of co-aggregation in the conditions used for chromatographic procedures, samples were analysed in 5% sodium dodecyl sulphate-polyacrylamide-gel columns and compared with marker proteins of

known molecular weight (cytochrome *c*, pepsin and bovine serum albumin). In 5% gels bands of molecular weight 1×10^4 , 5.6×10^4 , 1.22×10^5 , 1.74×10^5 and 2.12×10^5 were obtained from extract A pre-treated at 60°C in 1% sodium dodecyl sulphate, 1% (v/v) β -mercaptoethanol and 4M-urea for 5 min before electrophoresis. Similar results were obtained with extracts B and C. In samples pre-incubated at 100°C for 5 min in the above reagents only two bands were seen, of molecular weight 1×10^4 and 5.6×10^4 .

Sucrose-gradient analysis of soluble protein. Centrifugation of protein direct from procedure A (0.3 ml of a 5 mg/ml soln.), dissolved in 0.1 M-diethanolamine-HCl buffer, pH 10.0, in sucrose-diethanolamine gradients (see the Materials and Methods section) showed that most of the protein sedimented with *s* values greater than 19.0S corresponding to a molecular weight of 4.2×10^5 . Protein dissolved in 0.1 M-NaOH smeared throughout the gradient, but a peak of *s* value 8.9S was detected, corresponding to a molecular weight of 1.3×10^5 . This compares closely with a value of 1.2×10^5 obtained for one of the bands in sodium dodecyl sulphate-polyacrylamide gels.

Amino acid analysis. Table 1 shows the amino acid composition of extracts from procedures A, B and C and also the semi-crystalline protein prepared from *B. subtilis* 12A⁻. The results show that the proteins removed by procedures A and C are very similar in composition, and both lacked detectable cysteic acid. The protein from procedure B (see the Materials and

Methods section) seems distinct from the other two in composition, particularly with respect to lysine, arginine, alanine, valine and tyrosine residues, and also in that a small amount of cysteic acid was detected. In no case was diaminopimelic acid or hexosamine detected, indicating that there was very little contamination from the spore cortex. The protein from *B. subtilis* 12A⁻ seems closest in composition to that from extract B but is still quite distinct in having no cysteic acid and a low amount of histidine.

N-Terminal analysis. N-Terminal analysis by the dansyl chloride method on extracts from procedures A, B or C gave the following Dns-amino acids in approximately equal amounts: Dns-alanine, Dns-leucine, ϵ -Dns-lysine and trace amounts of Dns-glycine and Dns-phenylalanine. The inability to recover larger amounts of Dns-lysine could not be explained. Neither was the inability to detect other N-terminal compounds in extract from procedure B, in view of its differing amino acid composition. It therefore seems likely that the proteins may contain at least two polypeptide chains.

Serology

Protein samples from each extraction procedure (see the Materials and Methods section) were tested against antibody prepared to extract from procedure A. Extracts from procedures A and B gave a single continuous non-overlapping precipitin line, showing

Table 1. *Amino acid analyses of extracts from spores and of Bacillus subtilis* 12A⁻ semi-crystalline protein

Duplicate samples (approx. 1.0 mg) were hydrolysed for 24 h and examined in the amino acid analyser. The values are given as residues of the listed amino acid/100 residues. For extraction procedures A, B, and C see the Materials and Methods section.

Amino acid	Procedure A extract	Procedure B extract	Procedure C extract	<i>B. subtilis</i> 12A ⁻ semi-crystalline protein
Lysine	14.6	6.8	12.6	6.1
Histidine	4.4	6.2	3.7	1.9
Arginine	8.9	3.4	10.4	3.3
Cysteic acid	—	0.9	—	—
Aspartic acid	8.1	10.9	8.1	9.7
Threonine	3.4	5.5	3.1	5.0
Serine	4.3	4.6	3.6	5.0
Glutamic acid	8.1	10.2	7.3	11.4
Proline	8.2	6.9	10.9	5.1
Glycine	8.3	9.2	8.6	8.4
Alanine	4.4	6.0	3.7	8.8
Valine	3.4	7.0	3.8	9.4
Methionine	1.1	1.8	1.0	2.5
Isoleucine	3.2	4.9	3.1	7.4
Leucine	4.1	5.5	4.0	6.1
Tyrosine	12.9	4.9	13.3	5.3
Phenylalanine	2.8	5.4	2.7	4.7

that these extracts contain at least one common antigen, but the results do not preclude the possibility that procedure B extract contains additional non-cross-reacting antigens. Since protein from procedure C showed two additional precipitin bands, it was not completely equivalent immunologically to that prepared from procedures A and B. No precipitin bands were observed if serum from non-immunized animals was used, nor if immune serum was absorbed with an excess of protein from procedures A or B, and tested against extracts from procedures A, B or C. This suggests that the additional precipitin lines observed with extract from procedure C may also be due to very similar antigens to those present in extracts from procedures A and B. Extracts from whole or ultrasonically disrupted exponentially growing cells, prepared by procedures A or B gave no precipitin bands.

Immunological and electrophoretic properties of semi-crystalline protein from B. subtilis 12A⁻

Other workers (Somerville, 1971) have commented on the similarity of the semi-crystalline protein obtained by Rogolsky (1968) to spore-coat-protein fractions, in terms of amino acid composition and solubility properties. Preparations of the protein (see the Materials and Methods section) suspended in 0.1 M-diethanolamine-HCl buffer, pH 10.0, or dissolved in 0.1 M-NaOH, failed to give a precipitin line to alkali-extractable protein antibody. Since limited proteolysis of certain proteins is known to occur during sporulation (Sadoff, 1969) the possibility that *B. subtilis* 12A⁻ protein might be cleaved to an immunologically active form was tested. Samples of protein (2 mg) were incubated with soluble extracts of sporulating cultures (taken at t_0 , t_2 , t_4). These were known to contain several proteolytic enzymes (Mandelstam & Waites, 1968). Even after 36 h incubation at 37°C, samples of this protein (250 µg) failed to give precipitin lines. Further *B. subtilis* 12A⁻ protein did not remove antibody activity when mixed with immune serum. These observations suggest that it is immunologically distinct from alkali-extracted protein. Samples of *B. subtilis* 12A⁻ protein incubated with extracts of sporulating cells failed to liberate a protein fraction capable of entering 7.5% polyacrylamide gels.

Alkali extracts from other Bacillus species

It has been claimed that alkali extracts of spores of bacilli and clostridia show partial homology as judged by precipitin lines in immunological test plates (Somerville *et al.*, 1970). Accordingly, spores from *Bacillus* species were prepared (see the Materials and Methods section). These spores (500 mg dry wt.) and spores of *Clostridium roseum* were extracted by pro-

cedure A. Tests of protein extracts (200 µg) dissolved in 0.1 M-diethanolamine-HCl, pH 10.0, did not cross-react with the antiserum to the *B. subtilis* protein, although all the protein extracts showed a single band moving with tracker dye in 7.5% polyacrylamide gels. In a converse experiment antisera to *B. cereus* T spore coats or *B. thuringiensis* crystal protein also failed to give precipitin lines when tested against extracts (200 µg of protein) from *B. subtilis* prepared by procedures A, B or C, but precipitin lines were seen with extracts of *B. cereus* N.T.C.C. 7578.

Fine structure of alkali-treated spores

The change in fine structure of spores treated by all three extraction procedures seems to be confined to a loss in density in the outer darkly staining layers of the spore coats (see Plate 1). The inner portion of the spore shows very little detail of fine structure and no change was observable in these layers. Even if staining with lead citrate were omitted the outer coat layers were less electron dense in the treated spores.

Density-gradient centrifugation of spores

Since 6% of the dry weight of the spore was removed by alkali extraction it was decided to determine if any density changes had taken place, such as have been used to select mutants of bacilli with spores lacking 2,6-dipicolinic acid (Wise *et al.*, 1967). In fact, a small density difference was observed between treated and untreated spore suspensions. Untreated spores had an average density of 1.3340 g/cm³, whereas treated spores were slightly denser at 1.3480 g/cm³. A 1:1 mixture of the two spore types had an average density of 1.3406 g/cm³, indicating that the density difference was probably not an artifact.

Effect of extraction on other spore properties

Heat sensitivity, outgrowth and 2,6-dipicolinic acid content of treated spores remained completely unaltered after extraction by the procedures A, B or C. However, spores treated by any of the three procedures were sensitive to lysozyme (50 µg/ml in 0.01 M-sodium phosphate buffer, pH 7.2). The E_{600} value after the addition of lysozyme fell rapidly to a plateau value about 30% of the initial value by 5 min. After 20 min of incubation less than 0.1% of refractile spores were seen, compared with 99% in control suspensions of untreated spores.

Immunological assay for time of appearance of alkali-extractable protein during sporulation

The time of appearance of alkali-extractable protein was measured by using resuspension cultures of the wild-type strain and two asporogenous mutants

EXPLANATION OF PLATE I

Sections of spores of B. subtilis

See the Materials and Methods section for preparation, staining and sectioning procedure for electron microscopy. (a) Section of untreated wild-type spores. (b) Section of extracted spores (see procedure A, the Materials and Methods section), showing loss of staining in the outer electron-dense layers of the spore coat. The bars represent 1 μm.

derived from it, namely A23, a stage IV mutant, and E21, a stage II mutant (Waites *et al.*, 1970). Cross-reacting material could be detected in both the wild type and the mutants only 4 h after resuspension. In the wild-type strain this corresponds to a period when about 1% of the spores have attained refractility and probably represents the end of stage III of sporulation. Neither of the two mutants is known to make morphologically recognizable coat layers (Waites *et al.*, 1970).

To examine the possibility that a non-cross-reacting precursor form of this protein was present, samples of alkali extracts (100 ml of culture) taken at t_0 , t_2 , t_3 were incubated with small volumes of phosphate buffer-soluble extracts (see the Materials and Methods section) from cultures taken at t_5 and t_6 of sporulation. In no case did such incubation convert the proteins in the early samples into a cross-reacting form. Further, treatment of these earlier samples with proteolytic enzymes such as trypsin, chymotrypsin and ficin also failed to release cross-reacting material.

Inhibition of the appearance of immunologically active alkali-extractable protein by chloramphenicol, actinomycin and rifamycin

Chloramphenicol (50 $\mu\text{g/ml}$), actinomycin D (1 $\mu\text{g/ml}$) or rifamycin (1 $\mu\text{g/ml}$) inhibited appearance

of cross-reacting material if added to sporulating cultures before t_4 . These concentrations of chloramphenicol and actinomycin D are known to inhibit protein and RNA synthesis almost completely in this organism (Sterlini & Mandelstam, 1969). Rifamycin at this concentration is also known to inhibit RNA synthesis by more than 95%. Subsequent additions of drugs allowed cross-reacting material to be formed. Thus it seems that both protein and RNA synthesis before t_4 are necessary for the synthesis of this protein. Since this assay system was only qualitative it cannot be determined whether continuing protein and RNA synthesis is required after 4 h in sporulation.

Label 'chase' experiments with alkali-extractable protein

Label 'chase' experiments (see the Materials and Methods section) also indicated (see Fig. 1) that at least part of the alkali-extractable protein was synthesized early in sporulation. The bulk (>70%) of synthesis occurred after t_{2+} ; nevertheless over 60% of the total protein was synthesized by t_{4+} , a point when only 5% of the final amount of refractile spores were seen.

Gel electrophoresis in 7.5% polyacrylamide gels of alkali extracts of lysed cells (see the Materials and Methods section) showed the presence of a major

Fig. 1. Time-course of synthesis of alkali-extractable spore protein studied by using [^3H]leucine

A culture (1 litre) was set up in sporulation medium containing 75 μCi of L-[^3H]leucine and carrier leucine (final concn. 20 $\mu\text{g/ml}$). At hourly intervals from t_4 samples (90 ml) were withdrawn, washed and 'chased' with excess of unlabelled amino acid (see the Materials and Methods section). The cultures were incubated until t_{24} . The spores were purified and the alkali-extractable protein was removed and its specific radioactivity determined. Alkaline phosphatase activity and the development of refractility were determined in the control culture. Δ , Specific radioactivity (c.p.m./mg of protein) of alkali-extractable spore protein; $\square$, alkaline phosphatase (enzyme units/ml of culture); $\bullet$, % of refractile spores.

Fig. 2. Specific radioactivity of the 'fast band' in gel electrophoresis during sporulation

Samples (20 ml) from a continuously labelled ($[U-^3H]$ leucine) sporulating culture were removed, lysed with lysozyme and extracted with alkali (see the Materials and Methods section). Samples of the alkali extract were analysed on polyacrylamide gels. The band moving with tracker dye was removed and its specific radioactivity determined. Δ, Specific radioactivity of 'fast band' (c.p.m./mg of protein); ●, % of refractile spores.

protein band moving with the tracker dye, even in samples 30 min from the initiation of sporulation. Since it was known that the alkali-extractable spore protein exhibited a similar electrophoretic mobility experiments were set up to determine if this 'fast band' was labelled at the same time as the total alkali-extractable protein during sporulation. Samples were taken from a continuously labelled sporulating culture (see the Materials and Methods section), treated with lysozyme, and alkali-extracted, and then analysed on polyacrylamide gels. The 'fast band' was removed after staining and its specific radioactivity measured. The results (Fig. 2) show that it was labelled at the same rate as alkali-extractable protein and was of similar specific radioactivity.

Presence of cross-reacting material in asporogenous mutants

Resuspension cultures (200 ml) of a series of asporogenous mutants were set up and incubated for 7 h, i.e. a period at which wild-type cultures would have developed about 70% refractile spores and alkali-extractable protein. The cells were centrifuged, the pellet was extracted by procedure A and the proteins were tested for cross-reaction to antiserum. The results (Table 2) show that all stage O mutants fail to produce cross-reacting material, as did the stage II mutants N25 and N38, but all other stage II mutants

and all stage III and stage IV mutants gave visible cross-reaction.

Discussion

Although it has been suggested by Aronson & Fitz-James (1968) that the spore coat may contain only a few types of polypeptides, the number of these remains unknown. Sodium dodecyl sulphate gel electrophoresis of alkali extracts and the *N*-terminal analyses show that there are at least two distinct proteins. Immunological tests show that the extracted protein is spore-specific. All three extraction procedures yielded at least one similar protein on both this and other criteria. The nature and function of the semi-crystalline protein from *B. subtilis* 12A⁻ (Rogolsky, 1968) remain obscure, but it is immunologically quite distinct and unlikely to be a precursor form of the spore-coat protein.

In common with alkali extracts of spores or crystals of *B. thuringiensis* (Somerville *et al.*, 1968) the extracted protein seems capable of aggregation. This was shown by its behaviour on gel filtration, gel electrophoresis and sucrose-density-gradient analysis. Such spontaneous aggregation may be an indication of the properties of the protein *in vivo* during sporulation. Morphologically the spore coat of *B. subtilis* consists of two distinct layers (Ryter, 1965; Kay & Warren, 1968). The fine-structure changes seen in

Table 2. *Cross-reaction of extracts of asporogenous mutants to antiserum of alkali-extractable spore protein*

Resuspension cultures of asporogenous mutants and the wild-type were set up (200 ml) and after 7 h alkali-soluble protein was extracted by procedure A (see the Materials and Methods section). Samples of the protein were then tested against the antiserum. The morphological block and alkaline phosphatase activity of the mutants were known from previous experiments (Waites *et al.*, 1970; Coote, 1972).

Mutant	Morphological block	Alkaline phosphatase activity	Cross-reaction to alkali-extractable-protein antiserum
Y1	O	—	—
NG1.161	O	—	—
E22	O	—	—
U27	O	—	—
U42	O	—	—
N25	II	—	—
N38	II	—	—
NG82	II	—	+
E21	II	+	+
A24	II	+	+
E34	III	+	+
NG13	III	+	+
A19	IV	+	+
U28	IV	+	+
A8	IV	+	+
A23	IV	+	+
E31	IV	+	+
E33	IV	+	+
168		+	+

alkali-treated spores showed that the outer coat layer had lost electron density. Correspondingly, the only gross changes observed in spore properties were a transformation to lysozyme sensitivity, and a change in their surface properties shown by their aggregation.

Proteins of similar properties exist in the spores of other bacilli, a finding that confirms the results of earlier workers (Somerville *et al.*, 1970; Gould *et al.*, 1970). My results fail to show the immunological relationship of this group of proteins that has been claimed by Somerville *et al.* (1970).

The appearance of alkali-extractable protein was first detectable immunologically some 4 h after the initiation of sporulation in cultures of wild-type and two asporogenous mutants. This is at a point in sporulation that probably corresponds to the end of stage III (Dawes *et al.*, 1969). Addition of inhibitors of RNA or protein synthesis up to this time (4 h) prevents its appearance. However, examination of a series of asporogenous mutants showed that some event in stage II is critical for production of alkali-extractable protein, because mutants in this class fell into two groups: producers and non-producers. This very early point for commencement of synthesis seems more likely in view of the findings from

radioactive-labelling experiments. These show that formation of the protein begins early in sporulation and probably before the synthesis of alkaline phosphatase, a sporulation-specific enzyme associated with stage II (Waites *et al.*, 1970; Glenn & Mandelstam, 1971). This finding parallels that of Aronson & Fitz-James (1968), who showed incorporation of label into spore-coat polypeptides of *B. cereus* early in sporulation. Similarly, Somerville (1971), working with *B. thuringiensis* crystalline protein, was able to detect synthesis of the latter by both immunological and radioactive means very early in sporulation.

Since it is generally agreed that the morphological process of coat deposition takes place very late in sporulation, certainly subsequent to stage IV, these results raise two questions. First, where in the sporulating cell is this protein synthesized? Since it is a surface component of the completed spore it is most likely to be synthesized in the mother cell compartment, and in fact mutants exist that contain material very similar to coat layers in the mother cell compartment (Schaeffer, 1969; Coote, 1972). The second question concerns the role of assembly processes in the sporulating cell. The evidence suggests that this alkali-extractable protein, having been formed at

some early stage, remains (presumably free) in the cytoplasm until the spore has reached the appropriate state of development. At this stage the protein is deposited on the surface of the spore either by self-assembly or by some more active process.

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