Synthesis of *Bacillus cereus* Spore Coat Protein

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The major structural protein of *Bacillus cereus* spore coats was synthesized, commencing 1 to 2 h after the end of exponential growth, as a precursor with a mass of ca. 65,000 daltons. About 40% of this precursor, i.e. 26,000 daltons, was converted to spore coat monomers of 13,000 daltons each, perhaps as disulfide-linked dimers. The rate of conversion varied, being initially slow, most rapid at the time of morphogenesis of the coat layers, and then slow again late in sporulation, coincident with a decrease in intracellular protease activity. There was a second major spore coat polypeptide of about 26,000 daltons that was extractable from mature spores in variable amounts. This protein had a peptide profile and a reactivity with spore coat protein antibody that were very similar to those of the 13,000-dalton monomers. It is probably a disulfide-linked dimer that is not readily dissociated.

The spore coat of *Bacillus cereus* is composed almost exclusively of protein arranged into three identifiable morphological layers (2). The proteins extractable from isolated spore coats or intact spores are primarily 13,000-dalton species containing about 3 mol% of half-cystine residues and variable amounts of a 26,000-dalton component (2). These two classes are the principal structural components of all the morphological layers, undergoing secondary alterations as a result of disulfide interchange reactions (2).

Evidence for the synthesis of a precursor to the spore coat proteins was first obtained from the study of a mutant with an altered intracellular protease that made spores defective in spore coat. This mutant also produced a spore coat antigen with a mass of ca. 65,000 daltons that appeared to be improperly processed in the mutant (6). Subsequently, another mutant conditionally altered in spore coat formation was isolated and found to accumulate a 65,000-dalton coat antigen both in sporulating cells and on spores (11).

The relationships between the two major structural proteins with masses of 26,000 and 13,000 daltons and of these to the precursor with a mass of 65,000 daltons have been further studied. The time of synthesis and the extent of conservation of this precursor during sporulation have been determined.

MATERIALS AND METHODS

B. cereus T was grown at 30°C in G-Tris medium as previously described (11). For labeling with [^{35}S]-methionine, cells were grown in a low-sulfur G-Tris medium (1).

Spores were washed and extracted, and the soluble proteins were fractionated on 12 to 15% acrylamide gels containing sodium dodecyl sulfate (SDS) as previously described (12). Slab gels were used for autoradiography or staining, and tube gels were used for freezing and for preparation of 1-mm slices for scintillation counting (11).

For the determination of spore coat antigen, cell cultures at the following three stages of sporulation were used: (i) 1.5 to 2 h after the end of exponential growth, before the appearance of any easily recognizable change by phase microscopy (but when forespores could be seen in electron microscopic sections stage II); (ii) when at least 70 to 80% of the cells contained a dull phase white structure (this morphological change correlates with the morphogenesis of the spore coat layers [stage IV (2)] and (iii) when 70 to 80% of the cells contained a phase bright structure (stage V), usually 90 min to 2 h after stage IV (at this time, there is deposition of dipicolinic acid in the spore).

Cells at these various stages were incubated with radioactive amino acids (usually [^{35}S]methionine [20 to 100 μCi/ml] or [^{3}H]leucine [5 to 10 μCi/ml]) for the times indicated with each experiment. For pulse-chase experiments, 100 μg of unlabeled amino acids per ml was added at the specified times. Samples were pipetted into chilled centrifuge tubes containing frozen crushed antibody buffer (0.5 M sodium phosphate–0.05% sodium deoxycholate–0.1 M NaCl–2 × 10^{-3} M phenylmethylsulfonyl fluoride–10^{-3} M Na$_2$-EDTA [pH 8.0]) plus 300 μg of chloramphenicol and 300 μg of unlabeled L-methionine or L-leucine. Five milliliters of unlabeled carrier cells was added, and the samples were kept on ice until the cells were pelleted by centrifugation in an SS34 rotor in a Sorvall RC2B refrigerated (Ivan Sorvall, Inc.) centrifuge at 8,000 × *g* for 3 min. The cell pellets were rapidly suspended in 8 ml of antibody buffer plus 50 μg of unlabeled amino acid per ml and immediately centrifuged as above. The washing was repeated twice, and the cell pellets were finally transferred to 1.5-ml conical tubes for a final wash.

The pellets were suspended in 0.2 ml of antibody buffer and sonicated for a total of 4 to 5 min, with 90-

s treatments (and cooling) with a Branson 200 sonifier and microtip (Branson Instruments Co.) with a 50% pulse time. The extracts were centrifuged at 15,000 × g for 15 min in a Sorvall RC2B refrigerated centrifuge, and the pellets were extracted with 0.1 ml of UDS (8 M urea–0.05 M dithioerythritol–1% SDS–0.005 M cyclohexylaminoethane sulfonic acid [pH 9.8]) at 37°C for 1 h, conditions known to solubilize spore coat (2). After centrifugation at 15,000 × g for 15 min, the supernatants were pooled with the initial supernatants, and the total was dialyzed against 1,500 ml of antibody buffer at 4°C for 12 to 16 h.

A constant amount of extract protein was incubated with varying concentrations (usually three) of spore coat antibody or preimmune serum at 37°C for 1 h and then at 4°C for 12 h. Either goat anti-rabbit antiserum or goat anti-rabbit antiserum linked to Sepharose was then added, employing one-half the quantity of antiserum protein used for the initial antibody reaction. The tubes were incubated at 4°C for 3 to 4 h and then centrifuged at 4°C at 15,000 × g for 20 min. The supernatants were carefully withdrawn and discarded. The pellets were suspended in 0.5 ml of antibody buffer and centrifuged as above. After three washings, the pellets were suspended in 20 to 50 μl of UDS and heated at 100°C for 2 min, and an aliquot was removed into 15% trichloroacetic acid. The precipitates were collected on glass fiber filters (no. 35, Schleicher & Schuell Co.) and counted in an Omnifluor scintillation cocktail (New England Nuclear Corp.).

The tubes containing the most counts per minute (and the corresponding extracts incubated with preimmune serum) were analyzed by gel electrophoresis. Tube gels were then frozen and sliced with a Mickle gel slicer as previously described (3, 12). After electrophoresis, slab gels were incubated in 15% trichloroacetic acid for 20 min and then in deionized water for 2 h before drying. Gels were exposed to Kodak X-omat RP film for varying periods of time. One slot of the slab gels and one tube gel of each series contained molecular weight standards as indicated on the figures, and these were stained with Coomassie blue and served as molecular weight references after corrections for changes in gel size due to freezing or drying.

[^{35}S]methionine (>200 Ci/mmol) was purchased from Schwarz/Mann, and DL-4,5 [^{3}H]leucine (15 Ci/mmol) was purchased from Amersham-Searle. Chymotrypsin was purchased from Worthington Biochemicals Corp.; urea, SDS, and ammonium sulfate were purchased from E-M Labs, and dithioerythritol was purchased from Pierce Chemical Co.

For antibody preparation, spore coat extracts were fractionated on Sepharose 6B-CL columns as previously described (3) to prepare a 13,000-dalton fraction purified to >98%. After removal of SDS by adding saturated KCl (0.05 ml per milliliter of column eluate) at 0°C, solid ammonium sulfate was added to 25% saturation (at 0°C). After 1 to 2 h at 0°C, the suspension was centrifuged at 12,000 × g for 10 min. The pellet was washed once with deionized water and suspended in 0.1 M NaCl–0.05 M potassium phosphate (pH 7.5) for cross-linking with glutaraldehyde (4). This suspension was used as antigen as previously described (8). In addition, a high-titer antibody was obtained by employing as antigen a coat fraction purified on Ren-

ografin (76% stock of 66% meglumine diatrizoate plus 10% sodium diatrizoate) gradients (12). The coat fraction was obtained from a mutant that produced coatless spores plus cytoplasmic deposits of coat material (12). All experiments reported were done with both antibody preparations, with essentially similar results; only data obtained with antibody to the cross-linked protein are reported.

RESULTS AND DISCUSSION

As reported previously, most of the protein extracted from *B. cereus* spore coats has a mass of ca. 13,000 daltons and consists of a few closely related species (2). There is also a 26,000-dalton component present in varying amounts (2) (Fig. 1). Other minor species may or may not be contaminants. In addition, a 65,000-dalton polypeptide was present on spores and was detected by labeling sporulating cells during the late
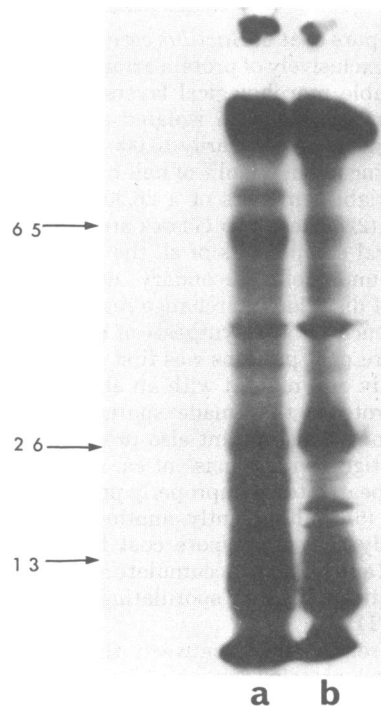

FIG. 1. *Autoradiograph of UDS extracts of spores fractionated on 12% polyacrylamide gels. [^{35}S]methionine (5 μCi/ml) was added to sporulating cells in low-sulfur G-Tris at 15 h (a) or 13 h (b). The former corresponds to phase white to bright forespores in at least 95% of the cells, and the latter corresponds to the forespore stage. After completion of sporulation (24 to 30 h), the spores were washed and extracted as previously described (11). The arrows indicate the positions (top to bottom) of 65,000-, 26,000-, and 13,000-dalton polypeptides. These gels were intentionally overloaded to provide labeled polypeptides for enzymatic digestion.*

stages of sporulation (Fig. 1). This component was precipitated by antibody to the 13,000-dalton spore coat protein and may be a spore coat precursor. Apparently, this precursor was not processed late (perhaps due to a decrease in the amount of intracellular protease [5, 6]), and at least some of it was deposited on the spores. Other labeled bands present in the overloaded profiles shown in Fig. 1 (i.e., about 40,000 and 20,000 daltons) were not always present.

The general pattern of synthesis of these major spore coat polypeptides during sporulation was examined by antibody precipitation of pulse-labeled extracts, fractionation on polyacrylamide gels, and autoradiography (Fig. 2–4). At the three stages examined, a 65,000-dalton spore coat antigen was seen, but its rate of turnover appeared to differ. At early stages (Fig. 2), there was a slow accumulation of this component that appeared not to have reached steady state by 8 min and very little synthesis of the 13,000-dalton monomers within 8 min. During the time of active deposition of spore coat, the turnover of the 65,000-dalton polypeptide seemed to be more rapid (Fig. 3). The accumulation of lower-molecular-weight polypeptides was more difficult to measure because of nonspecific precipitation of these monomers (or other polypeptides of the same size). At later

stages (Fig. 4), there appeared to be a turnover of the 65,000-dalton component, but it seemed not to be as rapid or as complete as at earlier times (Fig. 3). As already mentioned, there is deposition on the spores of this large polypeptide that is made late in sporulation. This accumulation correlates with the decrease of a proteolytic activity thought to have a role in the processing of spore coat precursors (5, 6).

The 65,000- and 13,000-dalton fractions were the major spore coat antigens found in extracts (Fig. 2–6), and the qualitative data presented here (Fig. 2–4), as well as more quantitative results (6), implied a precursor-product relationship.

The rates of turnover of the 65,000-dalton fraction and of accumulation of the 13,000-dalton polypeptides were determined by incubating cells in G-Tris medium until the desired stage of sporulation, as indicated in the legends to Fig. 5 and 6. After the pulse-chase period, extracts were prepared, and a constant amount of protein was used for precipitation with varying amounts of antibody or preimmune serum. The efficiency of antibody precipitation was determined by adding [^{35}S]-spore coat protein (primarily 13,000-dalton species prepared by fractionating the spore extract on a Sepharose 6B-CL column [3]) to a crude extract before the addition of
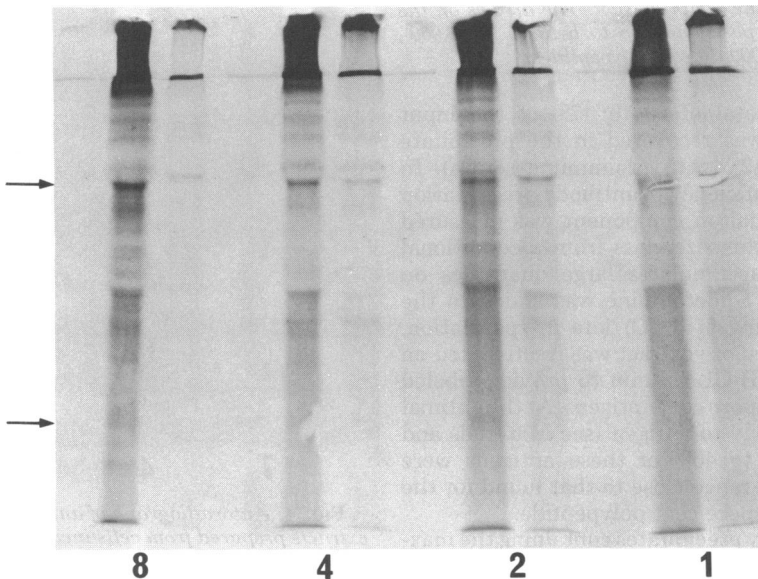

FIG. 2. *Autoradiograph of antibody precipitates of extracts prepared from cells after 12 h of growth in low-sulfur G-Tris (probably early forespore, stage II). Cells were incubated with [^{35}S]methionine for 1, 2, 4, or 8 min and then processed as described in the text. The numbers indicate the minutes of incubation with [^{35}S]methionine (all incorporated in 4 to 8 min), and the channels on the right of each pair are preimmune serum precipitates. The arrows denote the positions (top to bottom) of 65,000- and 13,000-dalton polypeptides. (There was no internal control for the efficiency of antibody precipitation nor for the amount of protein in extracts.)*
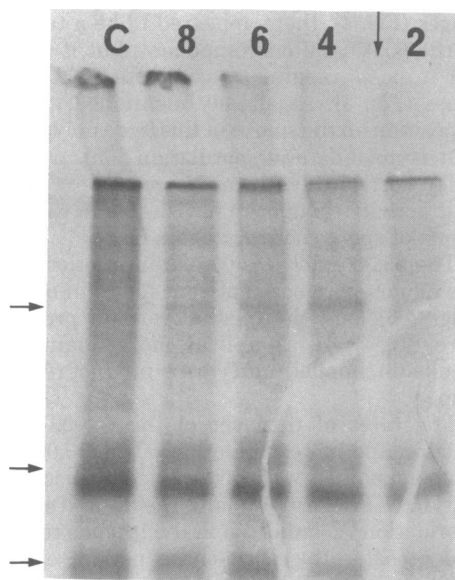
FIG. 3. *Autoradiograph of antibody precipitates of extracts prepared from cells after 15 h of growth in low-sulfur G-Tris (greater than 80% phase white, stage IV). Cells were incubated with [^{35}S]methionine for 2 min, and then unlabeled L-methionine was added to 100 µg/ml. Additional samples were taken at 4, 6, and 8 min. The numbers indicate the minutes of incubation with the addition of 100 µg of L-methionine per ml at 2 min (arrow at top). C, Control serum precipitate for the 6-min extract. The arrows on the left denote the positions (top to bottom) of 65,000-, 26,000-, and 13,000-dalton polypeptides.*

were subtracted. It was difficult to assess the total counts per minute in the 13,000-dalton region due to more extensive precipitation of polypeptides in this size range than in the 65,000-dalton size range in preimmune serum controls and to the accumulation of radioactivity near the dye front, as indicated in Fig. 5 and 6. The method of Girrard et al. (7) was used to estimate the peaks.

The data from Fig. 5 and 6 are summarized in Table 1. At the forespore stage, about 40% of the loss of the counts per minute from the 65,000-dalton region may be accounted for as an increase in the counts per minute in the 13,000-dalton region. These two classes are antigenically related, but further characterization of the polypeptides is needed to conclusively demonstrate a precursor-product relationship. It is also possible that the 65,000-dalton antigen is con-
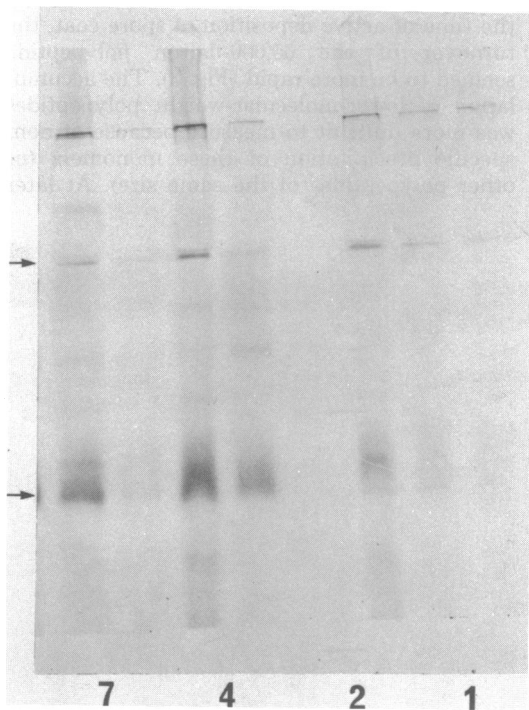
antibody. A total of 38 to 47% of the input radioactivity was recovered in the precipitate (versus 5 to 12% with preimmune serum). In addition, the efficiency of antibody precipitation of the 65,000-dalton component was measured by preparing this size class from a conditional mutant that accumulates large quantities on spores (11). [^{35}S]methionine was added to the mutant (growing at 36°C) late in sporulation, and the crude spore extract was fractionated on a Sepharose 6B-CL column to provide labeled 65,000-dalton spore coat antigens. At the optimal ratio of antibody to antigen (see Materials and Methods), 35 to 40% of these antigens were precipitated, a range close to that found for the 13,000-dalton spore coat polypeptide.

The antibody precipitates containing the maximum amounts of radioactivity (values agreed to within ±10% for any given experiment) were electrophoresed and processed as described in Materials and Methods. Recoveries from the gels were 95 to 105% of the input radioactivity. The total counts per minute in the 65,000- and 13,000-dalton peaks were determined, and the values for preimmune serum control profiles

FIG. 4. *Autoradiograph of antibody precipitates of extracts prepared from cells incubated with [^{35}S]methionine after 16.5 h of growth in low-sulfur G Tris (all cells phase white to bright; 60% of final dipicolinic acid content, stage V). Cells were incubated with [^{35}S]methionine for 1 min, 100 µg of L-methionine per ml was added, and samples were removed at 2, 4, and 7 min. The Right-hand lanes of the 1-, 4-, and 7-min pairs are preimmune serum precipitates. The arrows on the left denote the positions (top to bottom) of 65,000- and 13,000-dalton polypeptides.*
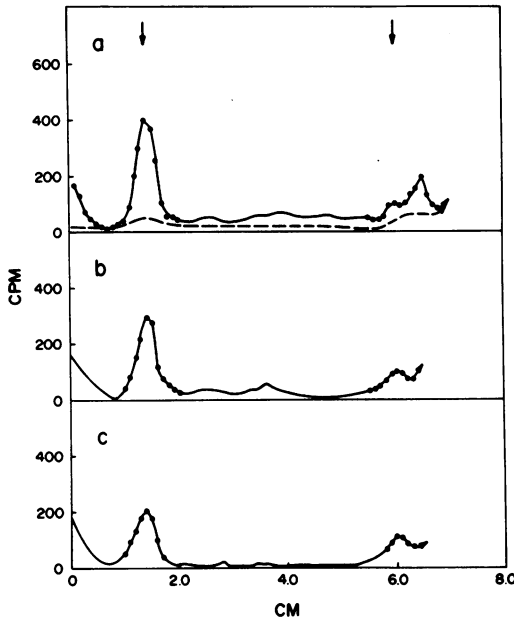
FIG. 5. *Distribution of labeled coat antigens after electrophoresis in 12.5% acrylamide gels. A total of 60 ml of cells grown in G-Tris for 13 h (primarily forespores, stage II) was incubated with [³H]leucine (5 µCi/ml) for 4 min (a), and then a 100-fold excess of unlabeled L-leucine was added for 1 min (b) or 4 min (c). Samples (20 ml) were used to prepare extracts, and antibody precipitates were electrophoresed as described in the text. B. cereus [³⁵S]-spore coat protein was added to the extracts to monitor the efficiency of antibody precipitation (35 to 47% of input S³⁵ counts per minute) as described in the text. The arrows indicate the positions (left to right) of polypeptides with masses of 65,000 daltons (relative to bovine serum albumin) and ca. 13,000 daltons (slightly faster than RNAase). The dashed line in (a) refers to values obtained with preimmune serum. Similar control values were found for (b) and (c).*

verted in part to nonantigenic components. Because the same results were obtained with antibodies to purified 13,000-dalton protein and to total spore coats purified on Renografin gradients (12), any such product is not likely to be part of the spore coat.

The data for the late stage of sporulation (16.5 h in Table 1) was similar but the extent of loss of the counts per minute from the 65,000-dalton antigen rapidly decreased. Some of this large antigen was deposited on the spores late in sporulation (Fig. 1) and may have become inaccessible for cleavage (and possible prior modification). In four independent experiments, as in Fig. 5 and 6 but with somewhat different stages of sporulation, a range of values for Δ13,000/Δ65,000 of 0.32 to 0.52 was obtained, with a mean of 0.41. Within the limits of accuracy of the

methods and molecular weight determinations, there could be two coat monomers processed from each precursor, perhaps as disulfide-linked dimers.

The 26,000-dalton species found in variable amounts in extracts of spores or spore coats has never been seen in antibody precipitates of extracts of sprculating cells. It has been established, however, that the 26,000-dalton species is precipitable by the antibodies used in the present experiments (unpublished results). In addition, the amino acid composition and amino terminal residues (glycine and serine) are identical. Peptide maps were difficult to obtain as both species are rather resistant to an array of proteolytic enzymes, and in cases where digestion was obtained, there were few peptide products. The 26,000-dalton component could be a dimer formed during the maturation of the spore coat, and the variable quantities extracted could be due to slight variations in the extraction conditions (Fig. 7). An increase in the pH of the extraction buffer and the addition of more mercaptan reduced the relative amount of 26,000-dalton protein found in coat extracts under conditions where virtually all of the coat protein
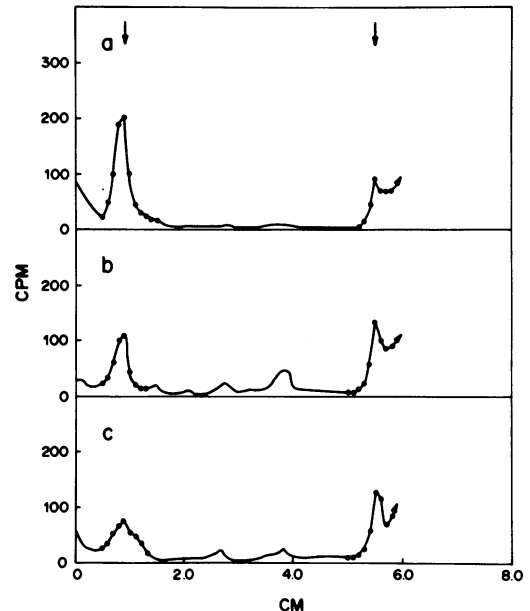

FIG. 6. *Distribution of labeled coat antigens for cells grown for 16.5 h in G-Tris (20% final content of dipicolinic acid, early stage V). Cells were processed, and antibody precipitates were electrophoresed as described in the legend to Fig. 5. 4-Min incubation with [³H]leucine (a); additional 1-min (b) and 4-min (c) incubations with a 100-fold excess of unlabeled L-leucine. The arrows indicate the positions (left to right) of 65,000- and 13,000-dalton polypeptides.*

was solubilized. Attempts to find cross-links via dityrosine (3, 9) or γ-glutamyl-ε-amino lysine were unsuccessful. A rather stable disulfide linkage is proposed.

The primary *B. cereus* spore coat polypeptide is one species or a few closely related species with masses of 13,000 daltons each. The only other coat antigen detectable in extracts when either antibody to the purified 13,000-dalton species or to total spore coat is used has a mass of ca. 65,000 daltons. This class behaves as a precursor because it turns over fairly rapidly at the time of maximum coat deposition (Fig. 3 and 5), and mutants defective in their ability to process this precursor produce spores with altered coats (6, 11). If it were assumed that there is only one species of large-molecular-weight precursor, then the 40% conservation of antigen found in pulse-chase experiments (Fig. 5 and 6) implies conversion to two monomers of 13,000 daltons each. Because disulfide bonds are very important in spore coat structure (1, 2), perhaps the formation of intramolecular disulfide bonds in the precursor ensures the proper alignment of the two monomers after processing. As has been demonstrated for the processing of insulin, the orientation of correct intramolecular disulfide bonds is thermodynamically more favorable than attempting to form such bonds between molecules (10). A relatively more stable dimer, i.e., the 26,000-dalton species found only in spore coat extracts, may be a consequence of subsequent packing of dimers and multimers in the spore coat layers.

FIG. 7. *Profiles of spore extracts fractionated on a 15% acrylamide gel and stained for protein. (A) Extracted with UDS; (B) extracted with UDS adjusted to pH 10.5; (C) extracted with 0.05 M dithioerythritol (pH 9.8); (D) extracted with UDS (pH 10.5) supplemented with 0.15 M dithioerythritol. The markers on the left indicate the positions (top to bottom) of polypeptides with masses of 65,000, 26,000, and 13,000 daltons as deduced from molecular weight standards.*

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TABLE 1. *Conversion of spore coat precursors to monomers in a pulse-chase experiment*[a]

Culture age (h)	Min of pulse-chase	Total cpm[b]				
		65K	Δ	13K	Δ	Δ13K/Δ65K
	1	1,690		290		
			340		140	0.41
13	2	1,350		430		
			450		160	0.36
	4	900		590		
	1	650		200		
			290		120	0.41
16.5	2	360		320		
			40		20	0.50
	4	320		340		

[a] Data from experiments illustrated in Fig. 5 and 6. See figure legends for details.

[b] Values from preimmune serum (5 to 12% of antibody precipitate counts per minute) subtracted after summation of the counts per minute over the peak as described by Girard et al. (7). 65K, 65,000 daltons; 13K, 13,000 daltons.
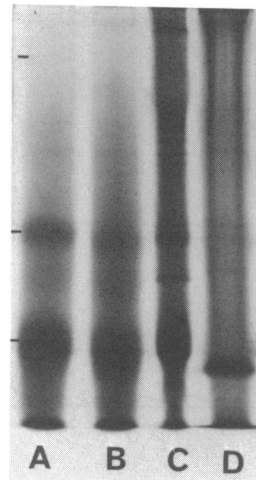
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