JOURNAL OF BACTERIOLOGY, Jan. 1973, p. 313-321

Vol. 113, No. 1
Printed in U.S.A.

Development of a Quantitative Immunological Assay for the Study of Spore Coat Synthesis and Morphogenesis

DIANE HORN, A. I. ARONSON, AND E. S. GOLUB
Department of Biological Sciences, Purdue University, Lafayette, Indiana 47907

Received for publication 31 July 1972

A quantitative assay employing ^{125}I-labeled antibody has been developed for *Bacillus cereus* T spore coat protein. Populations of antibody molecules with various affinities for inner or outer coat can be prepared by selective adsorption to and elution from different coat preparations. Adsorption to and elution from intact spores results in an antibody preparation at least 15 times more reactive to outer coat. This antibody is useful for measuring the time and extent of spore coat maturation, i.e., outer spore coat formation. Rifampin inhibits the increase in content of this coat antigen.

The spore coat of *Bacillus cereus* T contains primarily protein (3, 16, 23) and consists of two morphological layers, an inner or under coat most readily seen in freeze-etched preparations (6, 11), comprising 80% of the total coat protein, and an outer coat discernible in thin sections (3, 4, 9, 11, 23, 24). We have evidence that there are only one or two kinds of polypeptides in the total coat and that the two layers are composed primarily of identical kinds of polypeptides (6). The morphogenesis of the outer coat involves a cystine interchange reaction (3, 5, 22) which presumably results in an alteration of the coat polypeptides and the resulting deposition of the outer layer.

Whereas coat protein synthesis in *B. cereus* T commences at the end of exponential growth and rapidly reaches a maximal rate (3), there is evidence for a greatly increased rate of coat synthesis very late in sporulation in *B. subtilis* (19). In both cases, coat synthesis was measured by a very short or continuous incubation with labeled amino acid and isolation of the radioactive coat from intact spores. A more specific quantitative assay, especially one applicable to a direct study of coat synthesis and maturation during the course of sporulation, would be most useful. Our previous attempts at developing a quantitative microcomplement fixation assay (5) were not satisfactory because of the extensive nonspecific complement fixation. We have therefore developed an assay employing ^{125}I-labeled coat antibody to measure total coat antigen. Because of the multiple forms of coat, we have been able to isolate, by adsorption to and elution from coat preparations, coat antibody preparations with different reactivities for inner or outer coat. One such species, more reactive with outer coat, has been used to measure the time and extent of coat maturation. In addition, we have used this antibody species to show that rifampin added either early or late in sporulation inhibits outer coat formation.

MATERIALS AND METHODS

Preparation of spores. *B. cereus* T was grown at 30 C for 24 hr in G-medium (10) modified by the addition of tris(hydroxymethyl)aminomethane (Tris), *p*H 7.8, to a concentration of 0.01 M. Spores were harvested and washed as described previously (3). The spores were further purified by centrifugation through 55% Renografin at 27,000 × *g* for 15 min in an SS-34 rotor of the Sorvall RC-2 refrigerated centrifuge and then washed at least three times with deionized water.

Extraction of coat protein. Total coat protein was extracted by incubation of 3×10^9 to 5×10^9 spores/ml with 50 μmoles of dithioerythritol (DTE) per ml plus 8 M urea or 1% (w/v) sodium dodecyl sulfate (SDS), *p*H 10.5, at 27 C for 3 hr. Extracted coat protein was separated from the spores by centrifugation at 8,900 × *g* for 10 min at 27 C in a Sorvall SS-1 centrifuge. Spores were extracted a second time for 1.5 hr and centrifuged as before to separate the stripped spores from coat protein, and the two supernatant fractions were pooled.

To solubilize the inner coat selectively, the same procedure was followed but with the 8 M urea or SDS omitted from the extraction mixture (3). Subsequently, the outer coat was extracted as described

above for total coat. A 0.1-ml amount of saturated KCl per ml of coat protein was added, the solution was incubated at 0 C for 20 min, and the insoluble potassium salt of SDS removed by centrifugation at 12,000 × *g* for 10 min. This protein was then divided into soluble and insoluble fractions by either of two procedures: (i) dialysis of the protein for 16 hr at 4 C against 500 volumes of 0.1 M sodium phosphate (SPB), *p*H 7.0, and centrifugation at 12,000 × *g* for 10 min to provide a soluble and insoluble fraction, or (ii) addition of a saturated solution of $(NH_4)_2SO_4$ to 25% (v/v) saturation followed by incubation at 0 C for 1 hr and centrifugation at 12,000 × *g* for 10 min. Between 80 and 90% of the protein was precipitated by the addition of $(NH_4)_2SO_4$. The pellet was suspended in SPB, and the soluble and insoluble fractions were separated by centrifugation at 12,000 × *g* for 15 min. The soluble fraction was then dialyzed as above. With either procedure, 80 to 90% of the inner coat protein and 25 to 40% of outer coat protein are in the soluble fraction.

Preparation of antibody. Coat protein was extracted with SDS plus DTE from purified coats as described by Aronson and Fitz-James (3) and stored in lyophilized form at −20 C. After suspension in 8 M urea, the protein was dialyzed overnight against 500 volumes of 0.1 M sodium phosphate buffer, *p*H 7.2. The purified coats contain an exosporium (3), but this component remains largely insoluble after extraction.

Three 9-month-old Purdue Dutch rabbits were injected intramuscularly (once a week for 4 weeks) with 0.91 mg of coat protein emulsified in Freund complete adjuvant. On the fifth week, 0.1 mg of protein was injected intravenously via the marginal ear vein. Five and eleven days after the intravenous injection, the rabbits were bled by cardiac puncture, and the antiserum was stored at −20 C.

To prepare the antiserum for iodination, the antiserum was thawed and centrifuged in the Sorvall RC-2 refrigerated centrifuge at 10,000 × *g* for 20 min, and the supernatant fraction was filtered through a Millipore Swinnex-13 filter unit (0.45-μm pore size). The gamma globulin fraction was collected by adding saturated $(NH_4)_2SO_4$ slowly with constant stirring at 0 C to 50% saturation (v/v). After 10 to 15 min, the sample was centrifuged for 10 min at 7,710 × *g* in the Sorvall RC-2 refrigerated centrifuge, and the pellet was washed twice with 50% (v/v) saturated $(NH_4)_2SO_4$. The pellet was then suspended in distilled water to a volume equal to that of the original serum. Dialysis against distilled water and finally 0.05 M sodium phosphate buffer, *p*H 7.0, was carried out to remove the remaining $(NH_4)_2SO_4$. The globulin fraction was then either used directly for iodination or stored at −20 C.

A 5-mg amount of the partially purified coat protein antibody was diluted to 4.0 ml with 0.05 M phosphate buffer, *p*H 7.0, and filtered through a Swinnex-13 filter unit (0.45-μ pore size). Iodination with 300 μCi of ^{125}I/mg of protein was carried out by using the chloramine-T method of McConahey and Dixon (16). The labeled protein was then dialyzed for at least 24 hr against several changes of SPB to remove the unbound ^{125}I and finally was centrifuged at 12,000 × *g* for 10 min to remove any precipitated protein.

A modification of a procedure of Kabat and Mayer was used to purify antibody (13). ^{125}I-antibody was reacted with purified *B. cereus* T spores or purified spore coat protein for 1 hr at 37 C and then allowed to stand for 12 to 24 hr at 4 C or, in certain experiments with spore coat protein, at 0 C for 5 min. The resulting antigen-antibody complex was centrifuged at 12,000 × *g* for 10 min at 4 C, and the pellet was washed 3 to 5 times with several milliliters of SPB. The antibody was released from the insoluble coat or spores by incubation in 0.1 M sodium phosphate, *p*H 2.0, for 5 to 8 hr at 4 C. The suspension was then centrifuged at 10,000 × *g* for 15 min at 4 C, and the supernatant fluid was dialyzed against 500 volumes of SPB for 16 hr at 4 C. Purified crystalline bovine serum albumin (10 mg/ml) was added to the antibody as a protective protein, and the supernatant fraction was again centrifuged to remove any denatured, precipitated protein. In some cases, bovine serum albumin was added at the *p*H 2.0 extraction step. Purified ^{125}I-antibody was stored at 4 C.

Assay for coat protein. Insoluble and soluble coat protein fractions were prepared, as previously described, for analysis with ^{125}I-antibody. The reactivity of soluble coat protein with purified ^{125}I-antibody was determined by a modification of the procedure of Farr (8). Bovine serum albumin (10 mg/ml) in SPB was used as diluent, and antibody was diluted so that a 0.2-ml sample gave a convenient number of counts per minute. With the antibody concentration held constant, various amounts of spore coat protein were added to each assay tube, and the total volume was adjusted to 0.9 ml with phosphate buffer. The antigen-antibody mixture was then incubated for 12 to 18 hr at 4 C. Each sample was then diluted to 1.5 ml with SPB, and saturated $(NH_4)_2SO_4$ was added with constant stirring at 0 C to 25% (v/v) saturation. After 1 hr at 4 C, the samples were centrifuged at 12,100 × *g*. Supernatant and pellet fractions were assayed for the presence of ^{125}I-antibody by measuring gamma radiation in a Baird Atomic spectrometer. Since coat protein precipitates in 25% saturated $(NH_4)_2SO_4$, whereas the antibody does not, ^{125}I activity in the pellet is dependent upon the reaction of coat protein with the antibody. Where possible, tubes containing 50% of the plateau value of ^{125}I in the pellet were used for calculating coat content of the preparation.

Insoluble coat protein suspended in SPB was assayed as above but with the $(NH_4)_2SO_4$ step omitted. A standard curve was prepared for each preparation of ^{125}I-antibody, and these values were constant for at least 3 weeks.

Preparation of crude extracts. Samples of cells at various stages of growth or sporulation were centrifuged, washed twice with 10 ml each of SPB, suspended in 2.0 ml of SPB, and broken by two passages through a French pressure cell at 8,000 to 10,000 psi. The extracts were centrifuged in a Sorvall RC-2B refrigerated centrifuge at 12,000 × *g* for 10 min, and the supernatant fluids were decanted, again centrifuged as above, frozen in an acetone-dry ice mixture, and stored at −20 C or −80 C. Growth was

monitored in a Coleman 8 colorimeter employing a 660-nm filter. Stages of sporulation were determined by examination of wet mounts in a Zeiss phase microscope. Stages readily discernible were the appearance of a phase-dark region within the cell about 3 to 5 hr after the end of growth and a phase-white structure about 5 to 6 hr after the end of growth. The latter stage correlates with the time of appearance of the outer coat in electron micrograph sections (9, 27).

Rifampin addition to cultures. Rifampin (30 μg/ml) was added to cultures at different stages of sporulation. At various times, samples were removed, and crude extracts were prepared for immunological analysis as described above.

To determine the incorporation of labeled amino acids, 30 μg of rifampin/ml, 0.05 μCi of ^{14}C-isoleucine per ml, and 25 μg of unlabeled isoleucine per ml were added concomitantly to a separate 20-ml portion of sporulating cells. Duplicate 1-ml samples were taken as a function of time and pipetted into equal volumes of cold 20% trichloroacetic acid containing 50 μg of unlabeled isoleucine per ml. The radioactivity in each sample was determined as described under general chemical methods.

To determine the incorporation of ^{3}H-uridine, 10 ml of cells was treated as above but with 1 μCi of ^{3}H-uridine per ml and 5 μg of unlabeled uridine per ml. In one experiment, carrier-free ^{3}H-uridine (1 μCi/ml) was added to the cells followed by the addition of rifampin 10 min later. To determine incorporation of ^{3}H-uridine, samples of 0.2 ml were taken and treated as above.

General chemical methods. For protein determinations, samples were either pipetted directly into NaOH to a final concentration of 0.2 N or precipitated with 10 to 15% trichloroacetic acid and then suspended in 0.2 N NaOH after centrifugation at 12,000 × *g* in a Sorvall RC-2 refrigerated centrifuge. Protein was measured by the procedure of Lowry et al. (15) with bovine serum albumin as a standard.

Samples labeled with ^{3}H or ^{14}C were precipitated with 10 to 15% trichloroacetic acid (final concentration), incubated at 0 C for at least 20 min, and filtered onto 2.4-cm glass-fiber filters (Reeve-Angel 934 AH). The filters were then dried under high-intensity lamps for at least 15 min and counted in 5 ml of standard toluene scintillation fluid (Omnifluor) in a Packard Tri-Carb scintillation counter.

DTE was purchased from California Biochemical Corp. or Pierce Chemical Co.; SDS was from British Drug Houses, Ltd.; purified urea and guanidine-hydrochloride were from Schwarz-Mann; and bovine serum albumin was purchased from Pentex. [*U*-^{14}*C*]Isoleucine (180 μCi/μmole), [5-^{3}*H*]uridine (2 mCi/μmole), and ^{125}I were obtained from New England Nuclear; rifampin was a gift of CIBA Pharmaceutical Co.

RESULTS

^{125}I assay of coat protein. Initially, antibody isolated by adsorption to and elution from purified spores was used. The specificity was determined by comparing the reactivity of spores and vegetative cells of *B. cereus* T (Fig. 1). For a 30% precipitation of ^{125}I, at least 10 times as many cells as spores were required. It was not practical to assay higher cell concentrations due to the difficulty of washing the large pellets and removing nonspecifically bound antibody. ^{125}I-anti-bovine serum albumin antibody did not react with spores up to the highest spore concentration used.

Standard curves were prepared for the soluble and insoluble coat protein fractions (Fig. 2). A plateau of less than 100% ^{125}I precipitation (45–80% with various preparations) was reached when increasing amounts of coat were used, even to values as great as 1,000-fold above the lowest protein concentration giving the peak value. The observed leveling off may be due to the presence of either denatured or contaminating antibody.

On the basis of the data in Fig. 1 and 2, it has been calculated that the coat protein on the spore is about ten times less reactive than isolated insoluble coat protein. For a 34% precipitation of ^{125}I antibody, 5.5 × 10^{7} spores (Fig. 1) or 0.795 μg of isolated coat protein (Fig. 2b) are required. Since each spore contains about 1.45 × 10^{-13} g of coat protein (1), there is 7.95 μg of coat protein in the spore precipitate.

Use of the ^{125}I immunological assay for detection of coat protein in sporulating cells. Crude extracts were prepared from cells at various stages of sporulation as described in Materials and Methods and assayed for solu-

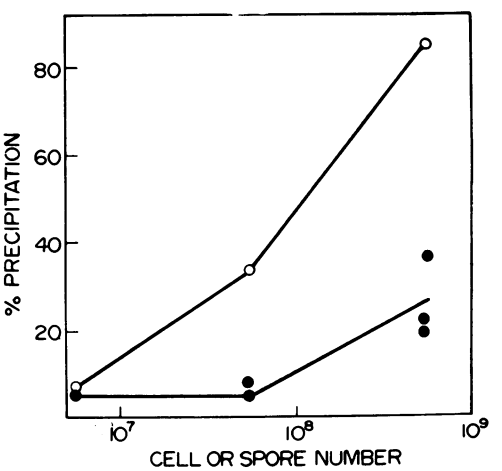

FIG. 1. *Reaction of ^{125}I-antibody with spores and vegetative cells. Various concentrations of spores and vegetative cells were added to a constant amount of antibody. The percent ^{125}I precipitated is plotted versus the cell or spore number by using semilog coordinates. Spores, ○; vegetative cells, ●.*

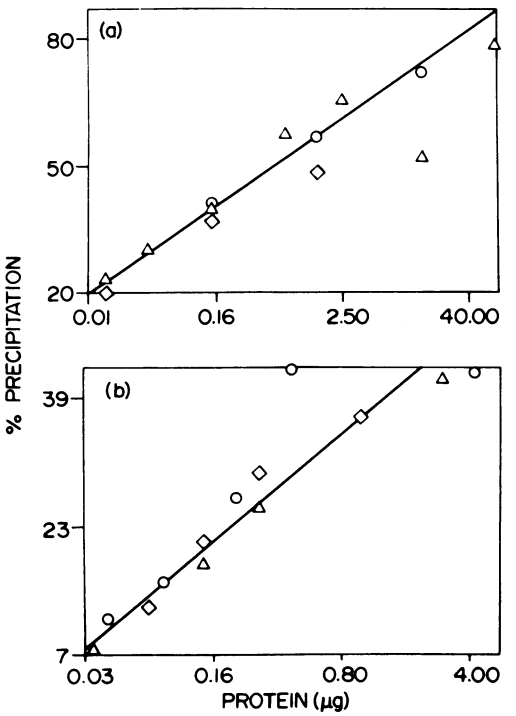

FIG. 2. *Standard curves for soluble coat protein (a) or insoluble coat protein (b). Total coat protein extracted with urea plus DTE was treated with $(NH_4)_2SO_4$ to prepare soluble and insoluble fractions (see Materials and Methods). Various amounts of coat protein were incubated with a constant amount of ^{125}I-antibody and treated as described in Materials and Methods for the soluble or insoluble coat protein assay. The percent ^{125}I-antibody precipitated of the total ^{125}I in each sample is plotted versus the coat protein concentration (on a log scale). The symbols represent different experiments run under the same conditions. Control (no coat protein added) = 16% precipitation for the soluble; 7% for the insoluble. The lines were determined by the method of least squares.*

ble coat protein content. At least two concentrations of crude extract were assayed each in duplicate, and only experiments where the values were within the range of those in Fig. 2 were averaged (Table 1).

Although the time of outer spore coat formation as judged by the appearance of phase-white structures varied somewhat, the values for coat protein production relative to the time before or after phase-whitening were quite comparable. When purified coat protein was assayed in the presence of crude extracts from sporulating cells, the values obtained were equal to the sum of the values of coat protein and the crude extract assayed separately, i.e., there is no inhibition by other components in the crude extract.

An attempt was made to measure coat protein which was in an insoluble form. The washed pellet from the original crude extract was treated under the conditions used to solubilize total coat protein and prepared for the ^{125}I antibody assay. A similar pattern of coat synthesis is observed for soluble crude extracts and soluble pellet extracts [Table 1, columns (a) and (b)]. The latter was not assayed as extensively as the crude extract, so the apparent difference in the time of increase of antigen content may not be real.

When pellet extracts were dialyzed against phosphate buffer to remove the solubilizing

TABLE 1. *Spore coat protein content of vegetative and sporulating cells*[a]

Time (hr)[b]	Total protein reacting with ^{125}I-Antibody (%)			
	(a) Crude extract	(b) Soluble pellet extract	(c) Insoluble pellet extract	(d) Total protein in the pellet[c] (%)
Exponential growth	0.006			
10	0.034	0.03	2.6	5.0
12	2.87	0.25	6.8	9.0
14	22.5	0.44	>100[d]	32.0
17	16.8[e]	2.32	>100[d]	47.0

[a] Crude extracts from cells in exponential growth and at several times during sporulation were analyzed by the ^{125}I assay for soluble coat protein (column a). The percent coat protein present at a given time was calculated by dividing the amount of coat protein as determined by the ^{125}I assay by the total protein in the extract. The pellet from the crude extract was extracted under conditions for solubilizing total coat protein (*see* Materials and Methods). The fraction which remained soluble after dialysis against SPB (90–95% of the total pellet extract) was analyzed by the ^{125}I assay for soluble coat (column b). After dialysis, the insoluble fraction was assayed by the insoluble coat assay (column c).

[b] The sample for exponential growing cells was taken at 8 hr. Exponential growth stopped at about 9 hr. At 12 hr, phase-white structures (correlates with appearance of outer coat in electron micrograph sections) appear. At 17 hr, the spores contain about 50% of the final concentration of dipicolinic acid and are becoming heat resistant.

[c] Protein in columns (b) + (c)/protein in columns (a) + (b) + (c).

[d] These values are very high because this fraction probably contains an antigenic form of coat protein which is much more reactive than the total coat protein used to prepare the standard curve (*see* Table 2).

[e] Three different determinations resulted in values of 4.2, 13.3, and 33%. A great fluctuation of values was often found for extracts prepared from cells very late in sporulation.

reagents, a small fraction (5–10%) of the protein precipitated. This fraction was assayed separately by the insoluble coat protein assay and is listed in Table 1, column c. Although the insoluble pellet extract was enriched in coat antigen content relative to the soluble pellet extract (Table 1, column b), the same pattern of increase of coat antigenicity was observed for both fractions. The apparent high content of coat antigen in this fraction was due to the presence of an antigenic form of the coat much more reactive than the soluble fraction of total coat used to prepare the standard curve (*see* Table 2).

That the increase in the percentage of spore coat protein in the crude extracts after 12 hr is due to a decrease in the extractibility of soluble protein rather than to an increase in coat protein per se must be considered. The forespore compartment becomes closed off from the mother cell about 2 to 3 hr prior to the time of phase whitening (27). Proteins which would normally become solubilized upon passage through the French pressure cell may now be retained in the forespore. Extracts made after this time may therefore represent primarily mother cell soluble protein. Consistent with this interpretation is the fact that the percentage of insoluble protein, i.e., that requiring treatment with DTE and urea for solubilization, increases from 5 to 47% during sporulation (Table 1, column d). Only a small part of this fraction appears to be coat protein being laid

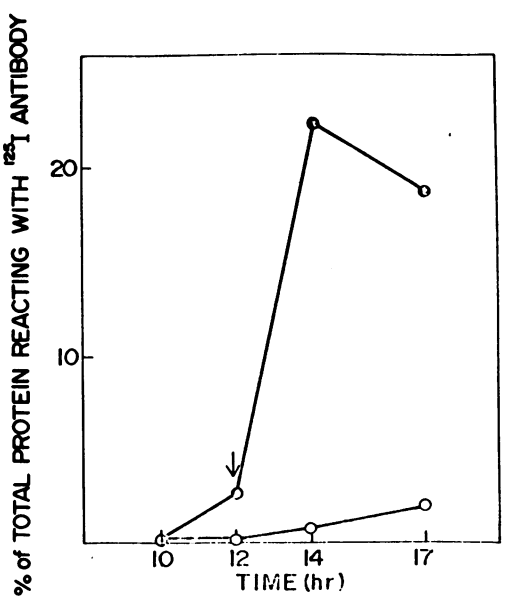

FIG. 3. *Synthesis of coat protein during sporulation. Plot of percent total protein reacting with ^{125}I-coat antibody versus time during sporulation for crude and soluble pellet extracts. Numbers and description are given in Table 1. Crude extract, ●; pellet extract, O. Arrow indicates the time of initiation of phase-whitening.*

TABLE 2. *Antigenic reactivity of various forms of coat protein*[a]

Coat form	Coat protein reacting with ^{125}I-antibody[b] (μg)		
	A Soluble fraction	B Insoluble fraction	Recovered protein in soluble fraction (%)
Inner coat			
1. Solubilized in DTE, dialyze vs SPB	0.12	2.7	83
2. Dialyze vs cystine in SPB	0.25	12.7	90[c]
3. Dissolve in 6 M guanidine - hydrochloride, dialyze vs cystine in SPB	0.40	6.7	81
Outer coat			
4. Solubilized in 8 M urea plus DTE (following extraction with DTE)	>15[d]	41	24
5. Dissolved in 6 M guanidine - hydro - chloride, precipitated with $(NH_4)_2SO_4$	0.25	13.5	21[e]

[a] Inner and outer coat were isolated as described in Materials and Methods, and the soluble and insoluble fractions were prepared by dialysis and centrifugation (lines 1 and 4). Freshly extracted inner coat was dialyzed at room temperature against 70 μg of cystine per ml dissolved in SPB (line 2). Another sample of inner coat was dissolved in 6 M guanidine-hydrochloride, *p*H 10.5, incubated for 3 hr at 37 C, and then dialyzed against cystine in SPB (line 3). A sample of outer coat was dissolved in 6 M guanidine-hydrochloride at *p*H 10.5, incubated as described above, and precipitated with a final concentration of 50% (v/v) $(NH_4)_2SO_4$. The precipitate was extracted twice with SPB, and the pooled supernatant fluids were dialyzed against SPB for 16 hr at 4 C (line 5).

[b] Values are based on those obtained with 1.44 μg of protein using standard curves as in Fig. 2. A higher value than 1.44 implies that the preparation is more antigenic than the standard (a total coat preparation) and the converse for a lower value.

[c] Value probably high since recovery of insoluble fraction was only 50% after dialysis.

[d] Values of 3.7, 6.4, 42, and >10 were obtained in separate experiments.

[e] Recovery of protein in this experiment was only 10% of the initial amount.

down in insoluble form (Table 1, column b). In addition, intact phase-white bodies (spores that have been prematurely released) can be seen under the microscope after passage through a French pressure cell. Thus, the percentage of coat protein calculated for 14 and 17 hr may reflect, in part, the removal from crude extracts of previously soluble protein as well as the synthesis of new coat protein. If the percentage of soluble protein is standardized to the 10-hr value, however, the qualitative pattern of coat protein synthesis is not changed, even though the quantitative values are less for the later times. The 12-hr point in Table 1 becomes 2.76%; the 14-hr point, 17.7%; and the 17-hr point, 11.8% of the crude extract protein. The values for the 17-hr point varied considerably, however, as indicated in the footnote to Table 1.

Effect of coat form on antigenic activity. It was noted that a large increase in antigenic reactivity of crude extracts occurred at the time of phase whitening when there is an increased cystine uptake into coat and when outer coat becomes morphologically apparent (Fig. 3) (3, 9, 22). This correlation suggested that outer coat may be more reactive with the antibody preparation used. To test this possibility, inner and outer coats were separated from spores as described in Materials and Methods. Soluble outer coat is much more reactive than inner coat (Table 2, columns 1A and 4A). Insoluble outer coat is approximately 15 times more reactive than an equivalent amount of inner coat (Table 2, columns 1B and 4B). If the insoluble fractions were prepared by $(NH_4)_2SO_4$ precipitation and dialysis (see Materials and Methods), then values of 3.1 and 0.08 μg of coat protein were obtained for outer and inner coat, respectively, as compared to 41 and 2.7 μg for insoluble fractions prepared directly by dialysis and centrifugation (Table 2).

Since secondary alterations of coat polypeptides, i.e., cystine interchange reactions, are believed to be responsible for outer coat formation (3, 5), attempts were made to convert inner coat to the more reactive form. Inner coat extracted at *p*H 10.5 was dialyzed for 16 hr at 27 C against cystine (70 μg/ml) in SPB. This procedure increased the reactivity of soluble inner coat twofold and insoluble inner coat approximately fivefold (Table 2, columns 2A and 2B), values that are still less than the values for outer coat. The reactivity of soluble and insoluble inner coat were increased 3.5- and 2.5-fold, respectively, by adding guanidine-hydrochloride to 6 M, incubating at *p*H 10.5 for 3 hr at 37 C, then dialyzing against cystine as before (Table 2, columns 3A and 3B). Both

TABLE 3. *Reactivity of coat preparations with antibody eluted from inner or outer coat*[a]

Coat form	Micrograms of coat protein per 10 μg of sample protein	
	Antibody eluted from inner coat	Antibody eluted from outer coat
Soluble[b]		
Inner	10	1
Outer	40	1
Insoluble[c]		
Inner	10	[d]
Outer	10	[d]

[a] Ten micrograms of soluble and insoluble inner and outer coat protein were reacted with antibody isolated by adsorption to and elution from inner or outer coat protein. For both soluble and insoluble coat protein, inner coat was used to construct a standard curve with the antibody eluted from inner coat. Equal quantities of inner and outer coat antibody were used, as judged by ^{125}I activity. The coat protein values are extrapolated from the standard curve.

[b] Prepared after $(NH_4)_2SO_4$ precipitation as described in Materials and Methods.

[c] Prepared from dialyzed coat preparations by centrifugation.

[d] Not tested.

Na_2SO_3 (5×10^{-3} M) and cystinyl-penicillamine (20 μg/ml) used in place of cystine gave comparable results. Treatment with 1% SDS or 8 M urea, *p*H 10.5, at 27 C followed by dialysis against cystine did not increase the reactivity of inner coat. Whereas inner coat can be converted to a more reactive form, none of the treatments attempted resulted in an elevation of the reactivity to the level of outer coat.

Attempts were also made to convert the more reactive outer coat to a less reactive form. Presumably, cystine removal from the outer coat should alter the antigenicity of the polypeptides. Outer coat suspended in 6 M guanidine-hydrochloride, *p*H 10.5, plus 50 μmoles of DTE/ml was incubated at 37 C for 3 hr and then precipitated with $(NH_4)_2SO_4$ to a final concentration of 50% (v/v). The pellet was then extracted with SPB and washed once with SPB, and the pooled supernatant fractions were dialyzed against SPB for 16 hr at 4 C. This treatment reduces the reactivity of the insoluble form threefold and the soluble form to a value similar to inner coat (Table 2, columns 5A and 5B).

Comparison of antibodies isolated with purified spores, inner coat, and outer coat. When ^{125}I-antibody was incubated for 12 hr with purified inner and outer coat, it was found that over 95% could not be extracted at *p*H 2.0

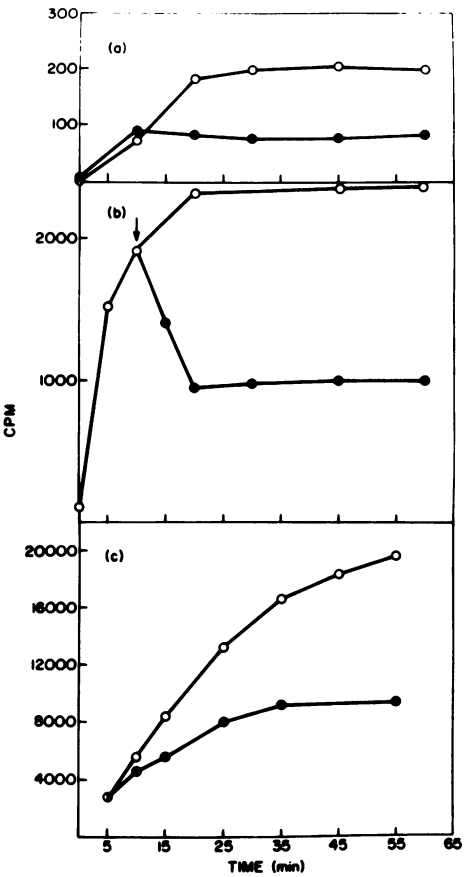

FIG. 4. *Effect of rifampin (30 μg/ml) on incorporation of ^{14}C-isoleucine or [5-^{3}H]uridine into sporulating cells of B. cereus T. Twelve-hour cultures (early forespore) grown in G medium-Tris. (a) Simultaneous addition of rifampin, [5-^{3}H]uridine (1 μCi/ml) and 5 μg of unlabeled uridine per ml. Samples (0.2 ml) were pipetted into 2 ml of cold 10% trichloroacetic acid and into 0.2 ml of 1 N KOH. O, Control; ●, rifampin. (b) Incubation of cells with [5-^{3}H]uridine (1 μCi/ml) for 10 min followed by addition of rifampin to 30 μg/ml (arrow) to one-half of the culture. Sampling into trichloroacetic acid and KOH as in (a). O, Control; ●, rifampin. (c) Simultaneous addition of rifampin, ^{14}C-isoleucine (0.05 μCi/ml) and 30 μg of unlabeled isoleucine per ml. Duplicate 1-ml samples were taken with time and pipetted into an equal volume of cold 20% trichloroacetic acid containing 500 μg of unlabeled isoleucine per ml. O, Control; ●, rifampin. All trichloroacetic acid-insoluble radioactivity in (a) and (b) was alkali labile (0.5 N NaOH, 37 C, for 16 hr).*

in contrast to a 40% recovery of antibody incubated with intact spores for 16 hr. Extraction of the antibody immediately after the reaction with antigen resulted in solubilization of approximately 30% of the antibody reacted with outer coat and 46% of that reacted with inner coat.

TABLE 4. *Effect of rifampin on coat protein production and sporulation*[a]

Time of sampling for assay with ^{125}I-antibody (hr)	Percent total extract protein as coat protein		
	−R	+R (12.5 hr)	+R (15.5 hr)
12.5	0.023	0.023	0.023
15.5	2.1	0.064	2.1
19.5	18.9	0.46	2.6
Final % sporulation	100	9	28

[a] A *B. cereus* T culture was grown for 12.5 hr. Thirty μg of rifampin per ml was added to a portion of the culture immediately and to another sample 3 hr later (+R). Crude extracts were assayed by the ^{125}I-antibody assay employing a standard curve as in Fig. 2a. Phase-whitening in the control (−R) began at 16 hr in this experiment. A sample from each culture was incubated until free spores had been released (at least 48 hr for the rifampin-treated cultures). The percent sporulation was determined by direct counts in a Petroff-Hauser chamber of the number of phase-white spores with the control (5.3×10^{8}/ml) as 100%.

Antibodies which had been adsorbed to and eluted from inner or outer coat were then tested for reactivity (Table 3). Antibody extracted from inner coat was only four times more reactive with soluble outer coat than soluble inner coat, rather than the 15- to 40-fold difference found with antibody extracted from spores (Table 2). For antibody eluted from outer coat, the reactivity of inner and outer coat were the same, although at least 10 times as much coat protein was required to precipitate the same percentage of this antibody.

Effect of inhibition of RNA synthesis on coat formation. With the use of the ^{125}I immunological assay, the effect of inhibition of ribonucleic acid (RNA) synthesis on a spore-specific product could be analyzed. Rifampin, a drug which inhibits RNA polymerase (25), was added to sporulating *B. cereus* T cultures as described in Materials and Methods to determine whether coat protein synthesis required continuous synthesis of RNA. Rifampin completely stops the incorporation of radioactive uridine into the trichloroacetic acid-insoluble fraction within 10 min (Fig. 4a), and addition of rifampin to prelabeled results in a loss of about 50% of the trichloroacetic acid-insoluble radioactivity (Fig. 4b). There is some incorporation of ^{14}C-isoleucine in the presence of rifampin for 20 to 30 min (Fig. 4c). A similar pattern of amino acid incorporation had been obtained previously in the presence of actinomycin D (2).

Rifampin initially blocks the increase in antigenic activity when added either several hours before (12.5 hr) or just prior to phase

whitening (15.5 hr) (Table 4). Upon longer incubation, however, some recovery does take place, as can be seen for cells in which rifampin was added at 12.5 hr. Seven hours later, the percentage of coat protein had increased 20 times over the earlier value but is still only 2.4% of the control value. Eventually, some spores are formed, but only 9% of the control value with most of the remaining cells lysing. When rifampin was added late, release of free spores was prolonged for over 48 hr, although phase-white endospores were produced within 24 hr. Cultures of these cells contained a large amount of cellular debris and phase-dark bodies which may be immature endospores. Mixing of crude extracts containing high concentrations of coat antigen with extracts in which coat production had been inhibited by rifampin gave an additive result, suggesting that there is no inhibition of the reaction by the latter extract.

DISCUSSION

A quantitative assay for spore coat protein has been developed which should be useful for measuring the rate of coat protein synthesis during sporulation, studying effects of inhibitors, and possibly for in vitro studies of coat synthesis. Since the state of aggregation of the coat protein, i.e., whether on a spore or "solubilized" or precipitated with $(NH_4)_2SO_4$, influences the capacity to bind antibody, it is important to compare only proteins prepared by identical procedures.

The antigen originally used to elicit antibody production was protein extracted from purified spore coats. There was no detectable carbohydrate present nor was the exosporium solubilized by the procedures used as judged by the appearance in the electron microscope (4). The solubilized coat protein appears to contain only one species of polypeptide on the basis of gel electrophoresis and number of NH_2-terminal polypeptides (6), but neither method of analysis is sufficiently sensitive to rule out 10 to 20% of other kinds of protein. It is quite likely, therefore, that a relatively homogenous protein preparation was used as antigen.

If we assume one species of polypeptide, then the heterogeneity in the antibodies produced must be due to secondary alterations (or states of aggregation) of the coat protein. By appropriate adsorption and elution procedures, various populations of antibody molecules have been selected with varying reactivities for the antigenic forms of the protein present in inner or outer coat preparations. It is not feasible to measure accurately the homogeneity of these antibody preparations by immunodiffusion or electrophoresis because of the insolubility of this protein, but a miminal estimate can be obtained. For example, only two major bands were formed in an immunoelectrophoretic analysis of total coat antibody and total solubilized coat (Fitz-James, *personal communication*) so it is unlikely that our purified antibody preparations are very heterogeneous.

The great difference in antigenic properties of inner and outer coat protein provides a basis for examining the morphogenesis of outer coat. Employing an antibody preparation selective for outer coat, a marked increase in reactivity was found at the time when outer coat is first seen in electron micrograph sections (9, 23). Since this is also the time of increased incorporation of half-cystine residues into the spore coat (3, 22), the morphogenesis could be due to synthesis of cystine-rich proteins unique to outer coat or to disulfide interchange reactions resulting in the altered conformation of coat polypeptides. As previously mentioned, there is evidence for the latter mechanism (3, 5, 22) which is supported by the ability to interconvert the antigenic forms in vitro by appropriate treatments (Table 2). These in vitro conversions were only partially successful probably due to alterations of proteins treated with alkali (21).

Rifampin does inhibit the incorporation of uridine into sporulating *B. cereus* and appears to prevent the reutilization of messenger RNA (mRNA) degradation products (Fig. 4) which could be selectively reincorporated from a nonexpanding pool (18). In *Escherichia coli*, about two-thirds of the pulse-labeled RNA is mRNA (26), whereas estimates from the base ratios of pulse-labeled RNA from sporulating *B. cereus* suggest 50 to 60% may be mRNA (2). A loss of 50% of the trichloroacetic acid-insoluble radioactivity after rifampin addition (Fig. 4b) is consistent with these estimates and suggests that the stable radioactive RNA is ribosomal and transfer species.

On the basis of results using an antibody selective for outer coat, it is clear that coat maturation is inhibited by rifampin and presumably dependent on continued mRNA synthesis. Because all of the reactions involved in the formation of outer coat are not yet understood, however, the exact significance of this result is not known. The inhibition may involve coat protein synthesis, some enzyme(s) required for maturation, or some combination of these.

Various workers have reported conflicting results as to the role of stable mRNA in spore formation (2, 7, 14, 20), but in no case was the effect of blocking RNA synthesis on a well-defined, spore-specific product analyzed. In

addition, inhibitors such as actinomycin D and rifampin with potential side effects, especially when examining a complex process such as sporulation, were used in most of the experiments, although Leighton and Doi (14) did employ a *B. subtilis* mutant with a temperature-sensitive RNA polymerase. Although the present results are consistent with the conclusions reached by others (7, 14), i.e., continued mRNA synthesis is necessary, none of the experiments is definitive. The question can only be answered when the synthesis of an absolutely essential spore-specific component is studied. Even then, different answers may be obtained depending on the spore component examined.

ACKNOWLEDGMENTS

Research supported by National Science Foundation grant GB 20765 and by Public Health Service grant AI-08800 from the National Institute of Allergy and Infectious Diseases. D. Horn was supported by a traineeship from a cell biology training grant from the National Institutes of Health; E. S. Golub is a recipient of a Public Health Service Career Development Award.

LITERATURE CITED

1. Aronson, A. I., N. Angelo, and S. C. Holt. 1971. Regulation of extracellular protease production in *Bacillus cereus* T: characterization of mutants producing altered amounts of protease. J. Bacteriol. **106**:1016-1025.
2. Aronson, A. I., and M. R. del Valle. 1964. RNA and protein synthesis required for bacterial spore formation. Biochim. Biophys. Acta **87**:267-276.
3. Aronson, A. I., and P. C. Fitz-James. 1968. Biosynthesis of bacterial spore coats. J. Mol. Biol. **33**:199-212.
4. Aronson, A. I., and P. C. Fitz-James. 1971. Reconstitution of bacterial spore coat layers in vivo. J. Bacteriol. **108**:571-578.
5. Aronson, A., and D. Horn. 1969. Synthesis and regulation of the bacterial spore coat, p. 72-81. *In* L. L. Campbell (ed.), Spores IV. American Society for Microbiology, Bethesda, Md.
6. Aronson, A. I., and D. Horn. 1972. Characterization of the spore coat protein of *Bacillus cereus* T, p. 19-27. *In* L. L. Campbell, H. O. Halvorsan, R. Hansen (ed.), Spores V. American Society for Microbiology, Washington, D.C.
7. Balassa, G. 1966. Synthèse et fonction des ARN messagers au cours de la sporulation de *Bacillus subtilis*. Ann. Inst. Pasteur **110**:60-76.
8. Farr, R. S. 1958. A quantitative immunochemical measure of the primary interaction between ^{125}I bovine serum albumin and antibody. J. Infect. Dis. **103**:239-262.
9. Fitz-James, P. C., and I. E. Young. 1969. Morphology of sporulation, p. 39-71. *In* G. W. Gould and A. Hurst (ed.), The bacterial spore. Academic Press Inc., London.
10. Gollakota, K. G., and H. O. Halvorson. 1960. Biochemical changes occuring during sporulation of *Bacillus cereus*. Inhibition of sporulation by α-picolinic acid. J. Bacteriol. **79**:1-8.
11. Hashimoto, T., and S. F. Conti. 1971. Ultrastructural changes associated with activation and germination of *Bacillus cereus* T spores. J. Bacteriol. **105**:361-368.
12. Holt, S. C., and E. R. Leadbetter. 1969. Comparative ultrastructure of selected aerobic spore forming bacteria: a freeze-etching study. Bacteriol. Rev. **33**:346-378.
13. Kabat, E. A., and M. M. Mayer. 1961. Purification of antibodies, p. 787-792. *In* Experimental immunochemistry. Charles C. Thorner, Springfield, Ill.
14. Leighton, T. J., and R. H. Doi. 1971. The stability of messenger ribonucleic acid during sporulation in *Bacillus subtilis*. J. Biol. Chem. **246**:3189-3195.
15. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
16. McConahey, P. J., and F. J. Dixon. 1966. A method of trace iodination of proteins for immunologic studies. Int. Arch. Allergy **29**:185-189.
17. Murrell, W. G. 1969. Chemical composition of spores and spore structure, p. 215-269. *In* G. W. Gould and A. Hurst (ed.), The bacterial spore. Academic Press, Inc., New York.
18. Nierlich, D. P. 1968. Amino acid control over RNA synthesis: a re-evaluation. Proc. Nat. Acad. Sci. U.S.A. **60**:1345-1352.
19. Spudich, J. A., and A. Kornberg. 1968. Biochemical studies of bacterial sporulation and germination. VI. Origin of spore core and coat proteins. J. Biol. Chem. **243**:4588-4599.
20. 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.
21. Tanford, C. 1968. Protein denaturation. Adv. Protein Chem. **23**:121-282.
22. Vinter, V. 1959. Sporulation of bacilli. VII. The participation of cysteine and cystine in spore formation of *Bacillus megaterium*. Folia Microbiol. **4**:216-221.
23. Walker, P. D. 1970. Cytology of spore formation and germination. J. Appl. Bacteriol. **33**:1-12.
24. Warth, A. D., O. F. Ohye, and W. G. Murrell. 1963. The composition and structure of bacterial spores. J. Cell Biol. **16**:579-592.
25. Wehsli, W., F. Knüsel, K. Schmid, and M. Staehlin. 1968. Interaction of rifamycin with bacterial RNA polymerase. Proc. Nat. Acad. Sci. U.S.A. **61**:667-673.
26. Winslow, R. M., and R. A. Lazzarini. 1969. The rates of synthesis and chain elongation of ribonucleic acid in *Escherichia coli* J. Biol. Chem. **244**:1128-1137.
27. Young, I. E., and P. C. Fitz-James. 1959. Chemical and morphological studies of bacterial spore formation. J. Biophys. Biochem. Cytol. **6**:467-482.