JOURNAL OF BACTERIOLOGY, Sept. 1968, p. 713-720
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Vol. 96, No. 3
Printed in U.S.A.

Immunological Homology Between Crystal and Spore Protein of *Bacillus thuringiensis*

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Received for publication 12 June 1968

Spore suspensions containing about 0.3% crystals and crystal suspensions containing about 0.1% spores were obtained from cultures of *Bacillus thuringiensis* by extraction with a two-phase system. Both preparations were tested for the presence of contaminating material from vegetative cells and were judged to be clean. Solutions of spore protein were obtained by extracting broken spores with phosphate buffer followed by extraction with either alkali- or urea-mercaptoethanol. The alkali spore or urea spore extracts had the same isoelectric point as crystal protein solubilized with these reagents. An antiserum prepared against alkali crystal solution precipitated alkali or urea spore extracts and crystal solutions but not phosphate spore extracts or extracts of whole cells. Lines of identity between spore and crystal precipitates were observed by using the Ouchterlony double-diffusion technique. Absorption of the antiserum with an excess of urea spore extract caused a disappearance of the precipitin bands originating from the spore protein and the homologous bands from the crystal protein. The results suggest that the crystal and endospore contain one or more common proteins.

The species *Bacillus thuringiensis* denotes a group of aerobic sporeformers that characteristically develop a massive intracellular crystal when they sporulate. It has been known since 1915 (4) that these bacteria cause the death of lepidopteran larvae, but not until 1955 were the crystals isolated and shown to be the toxic factor (2, 9). Since then, many studies have been made on the insecticidal properties of *B. thuringiensis* (10), but relatively few investigations have been concerned with the possible physiological significance of the crystals to the bacteria which form them. This report presents preliminary observations bearing on that question.

The crystal is an octahedron composed of spherical subunits in cubic close packing (11, 12). It is insoluble in water but dissolves readily in alkali and remains in solution after neutralization (9). The resulting solution contains mainly protein, with only traces of phosphorous and carbohydrate. All of the common amino acids are present and may be distributed among several polypeptide chains (14, 15). No enzymatic activity has yet been attributed to the dissolved crystal.

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The crystal first appears as a minute granule near the forespore, enlarges as the spore develops, and reaches its full size as the spore becomes mature (22). The crystal is synthesized from amino acids formed by protein turnover within the sporangium (17), a conclusion supported by the observation that crystal antigen is not found in the cell prior to sporulation (18). Furthermore, it has been asserted that crystal antigen is absent from the spore (18).

The foregoing observations imply that the crystal is composed of unique protein whose synthesis is fortuitously coincident with sporulation. An alternate possibility is that the crystal accumulates as a result of unregulated synthesis of spore protein. We have therefore reinvestigated the relationship of the crystal to the spore, to determine whether or not they contain common protein components.

MATERIALS AND METHODS

Organisms. Most of the reported experiments were done with a strain of *B. thuringiensis* var. *alesti anduze* doubly resistant to penicillin (1,000 units/ml) and streptomycin (250 μg/ml). The strain was derived by standard procedures from *B. thuringiensis* var. *alesti anduze* (EAS 0-24-3) obtained from E. A. Steinhaus.

Cultivation. Bacteria were spread uniformly on plates of the following medium: 8.0 g of Nutrient Broth (Difco), 20.0 g of agar, 0.08 g of $CaCl_2$, 0.05 g of $MnCl_2 \cdot 4H_2O$, 0.005 g of $ZnSO_4 \cdot 7H_2O$, and 0.005 g

of $CuSO_4 \cdot 5H_2O$, in 1 liter of distilled water. After incubation for 72 hr at 30 C, growth and sporulation were virtually complete, and nearly all of the sporangia had lysed.

Separation of spores and crystals. Autolyzed cultures were scraped from the surface of 250 petri dishes and were suspended in 1 M NaCl-0.02 M potassium phosphate buffer (*p*H 7.0) containing 0.01% Triton-X-100. The suspension was filtered through cheesecloth to remove small pieces of agar and was centrifuged. The sediment was washed repeatedly with fresh portions of the same solution until only traces of material absorbing at 260 nm remained in the supernatant liquid. The particles were then washed once in 0.2 M NaCl-0.004 M phosphate buffer (*p*H 7.0)-0.01% Triton-X-100 and once in 0.01% Triton-X-100, and were suspended in water. Residual cells were then removed from the suspension by extracting five times in 1.5 liters of Phase Mixture I of Sacks and Alderton (19).

The remaining crystals and spores were centrifuged and washed three times in 0.02 M phosphate buffer (*p*H 7.0)-0.01% Triton-X-100. The suspension, in 182 ml of the same buffer, was added to a cylindrical separatory funnel containing 105 g of a 20% (w/w) aqueous solution of sodium dextran sulfate 500 (Pharmacia Inc., New Market, N.J.), 13.2 g of solid polyethylene glycol 6000 (Union Carbide), 3.3 ml of 3 M phosphate buffer (*p*H 7.0), and 7.5 g of NaCl. After shaking to dissolve the solids, the volume was adjusted to 600 ml by adding a well-shaken solution of the same composition, but without bacterial particles. The complete mixture was shaken vigorously and placed at 5 C for 30 min. The mixture separated into two phases, the upper phase rich in polyethylene glycol and the lower phase rich in sodium dextran sulfate. This two-phase system was empirically developed from methods described by Albertsson (1); a similar one was independently described by Goodman et al. (7).

The upper phase contained most of the spores initially present, but very few crystals. The lower phase contained a mixture of crystals and spores. The upper phase was drawn off and centrifuged. The supernatant solution was poured back into the separatory funnel and the extraction was repeated. Spores in the first two upper phases constituted the bulk of those initially present and were saved; spores subsequently extracted from the lower phase were discarded. After the tenth extraction, the crystals in the lower phase were virtually free of spores and were collected by centrifugation. The spores and crystals were both washed five times in cold distilled water. The spores were stored at −20 C as freeze-dried powders. The crystals were stored at −5 C as suspensions in water.

Enumeration of crystals and spores. Crystals and spores are both refractile and of roughly equal size. Since the crystals have pointed apices, they may easily be differentiated from the oval spores by phase contrast microscopy. Differential counts were made in a Petroff-Hauser counting chamber, with suspensions containing not less than 10^8 particles per ml.

Fractionation of crystals and spores. Freeze-dried spores were ballistically disintegrated (20). A ball bearing weighing 180 mg, 50 mg of spore powder, and 50 mg of 50-mesh NaCl were shaken for 10 min in the cup of a Toothmaster Hi Speed Amalgamator (Toothmaster Co., Racine, Wisc.). The spores, by microscopy judged to be nearly all broken, were extracted twice with 0.02 M potassium phosphate buffer (*p*H 6.8) for 1 hr at 0 C. The supernatant solutions were pooled; they are referred to as "phosphate spore" extract.

The residue remaining after phosphate extraction was reextracted twice for 1 hr at 23 C and centrifuged for 15 min at 15,000 × *g* with either 0.1 N NaOH or 8 M urea-10% β-mercaptoethanol (*p*H 8.5) as the solvent. The pooled solutions, referred to as "alkali spore" or "urea spore" extracts, respectively, and the phosphate spore extracts were thoroughly dialyzed at 5 C against 0.01 M tris(hydroxymethyl)aminomethane (Tris)-chloride buffer (*p*H 8.2). The solutions were clarified, if necessary, by centrifugation and frozen until needed.

The crystals were solubilized directly with 0.1 N NaOH or with the urea-mercaptoethanol reagent. The solutions were dialyzed and clarified as described above to give "alkali crystal" or "urea crystal" solutions, respectively.

Vegetative cells were harvested during exponential growth in Nutrient Broth. They contained no visible crystals or spores. The procedures for extracting the cells were the same as those used for the spores.

Serology. Rabbits were immunized with vegetative cells, purified whole crystals, or with alkali crystal solution centrifuged at 60,000 × *g* for 1 hr to remove residual spores. Vegetative cells and whole crystals were injected intravenously at 1.0 mg (dry weight) daily for 5 days. The alkali crystal solution was mixed with an equal volume of Freund's complete adjuvant (Hyland Laboratories, Inc., Los Angeles, Calif.), and a total of 1.0 ml, containing 5.0 mg of protein, was injected subcutaneously into the footpads and dorsal skin of rabbits. Three weeks after the initial course of injections, each rabbit received 10.0 mg of the appropriate antigen intravenously. One week later, each rabbit was bled from the heart.

Precipitation reactions in agar-gel were studied by the double-diffusion technique of Ouchterlony (6). The gel consisted of 1.0% Difco Agar in 0.077 M barbital buffer (*p*H 8.2) containing 0.025% Merthiolate. The agar was poured into plastic boxes to a depth of 2 mm, and after solidification was drilled with 20-μliter wells spaced 8 mm apart. The wells were filled with solutions of antigen or antiserum, and the boxes were incubated in a moist atmosphere at 5 C for 4 days before examination.

Enzyme determinations. Catalase activity was estimated in the Warburg apparatus at 30 C. The side arm contained 0.2 ml of a suspension of intact spores which, after thermal equilibration, was mixed with 1.9 ml of 0.066 M phosphate buffer (*p*H 7.0) containing 14 μmoles of H_2O_2 per ml. Activity, measured as the initial linear rate of O_2 evolution in microliters of gas per hr per mg (dry weight) of spores, was proportional to the amount of spores tested.

Alanine racemase activity was assayed by measuring the uptake of oxygen with a Clark electrode at 30 C

with L-(+)-alanine as substrate and an excess of D-amino acid oxidase (Boehringer). The assay mixture contained 0.9 ml of 0.01 M Tris-chloride buffer (*p*H 8.2), 0.1 ml of 0.1 M L-alanine, and 0.1 ml of the oxidase solution. After thermal equilibration, 0.1 ml of diluted intact spore suspension was added to start the reaction, and oxygen uptake was recorded. Under the conditions of the assay, the rate of oxygen uptake, which was proportional to the number of spores used, was a direct measure of D-alanine formation.

Protein determinations. Protein was usually estimated by the method of Lowry et al. (16). Since Tris-chloride buffer interferes with protein determinations by this method, protein in Tris buffer was estimated spectrophotometrically by the method of Warburg and Christian (23). Determination of crystal protein by this method agreed closely with that obtained by the Lowry method in the absence of Tris. By either method, with bovine serum albumin as standard, the ratio of crystal protein to crystal dry weight was close to 1.4.

Miscellaneous methods. Phosphorous was estimated by the method of Chen et al. (5), and carbohydrate by the anthrone procedure with glucose as a standard. Calcium and magnesium were determined by flame photometry.

RESULTS

Separation of crystals and spores. By using the procedures described, overall recovery of both spores and crystals, based on their initial contents in the harvested autolysate, approximated 70% (Table 1). The final spore preparation (step 4A) contained about 0.3% crystal and the crystal preparation about 0.1% spores (step 4B).

Cleanliness of crystals and spores. Although the purified suspensions were microscopically homogeneous, their possible contamination with cellular debris was examined, since such debris might give the false impression that spores and crystals share common components. Purified crystal suspensions contained, by weight, less than 0.001% phosphorous, less than 0.05% carbohydrate, and less than 0.02% calcium and magnesium. Dissolved crystal protein had an absorption ratio ($OD_{280\ nm}$: $OD_{260\ nm}$) of 1.5. This value is consistent with a pure protein preparation and is, in fact, the highest yet reported for soluble crystal preparations.

The spores of *B. thuringiensis*, washed 15 to 20 times in the course of purification, evolved gas from H_2O_2 at a rate equivalent to 153 μliters of O_2 per hr per mg (dry weight). This catalase activity was largely thermolabile, being rapidly destroyed at 70 C. However, a fraction of the activity was stable to heating for at least 15 min, so that a bimodal curve of thermal inactivation was observed. As estimated from this curve, 30% of the total activity is heat-stable (Table 2), and, accepting the interpretation of other workers, would be an integral part of the spore. The rest of the activity, or 70%, is heat-labile and could be cellular enzyme. To discover whether or not any of the catalase activity could be solubilized, as should be the case were it simply adsorbed enzyme, the spores were subjected to ultrasonic vibration under conditions described by Berger and Marr (3). The treatment disintegrated few if any of the spores, but removed the exosporia and associated alanine racemase from more than 85% of them. However, even after washing the stripped spores four more times in distilled water, the amounts of heat-labile and heat-stable catalase were unchanged (Table 2). Accordingly the heat-labile component appears to be part of the spore rather than an adsorbed cellular enzyme.

Heat-shocked (at 75 C for 10 min) spores were incubated at 2 mg (dry weight) per ml of 0.02 M phosphate buffer (*p*H 7.0) containing 1.2 mM glucose, 6 mM L-(+)-alanine, and 0.012 mM

TABLE 1. *Separation of crystals and spores*

Separation step	Total particles remaining after each step		
	Spores	Crystals	Cells
1. Harvest and filtration	4.25×10^{12}	4.20×10^{12}	0.08×10^{12}
2. Preliminary washings	3.10×10^{12}	3.60×10^{12}	0.08×10^{12}
3. Removal of cells			
(A) Polyethylene glycol-rich phase	2.79×10^{12}	3.25×10^{12}	Undetectable
4. Separation of crystals and spores			
(A) Polyethylene glycol-rich phase[a]	2.93×10^{12}	0.01×10^{12}	Undetectable
(B) Sodium dextran sulfate-rich phase[b]	0.003×10^{12}	2.90×10^{12}	Undetectable

[a] Combined harvest from first two extractions.
[b] Harvest after 10th extraction.

TABLE 2. *Removal of exosporia and enzymes from intact spores*[a]

Length of oscilla- tion (min)	Spores (percentage of initial)			Alanine racemase (micromoles of D-alanine per min per 3X 10^{10} spores)		Catalase activity (microliters of O_2 per min per 3 X 10^{10} spores)	
	Refrac- tile	Phase- dark	With exos- poria	Total	Sedimen- table	Labile	Stable
0	99	1	85	12.7		3,210	1,380
80	89	11	15	12.0	1.4	2,970	1,290

[a] Spores were treated in a 10-kc Raytheon sonic oscillator containing 3 × 10^{10} spores (30 mg, dry weight) in 50 ml of 0.02 M phosphate buffer (*p*H 6.8) at 5 C under an atmosphere of N_2. Samples were withdrawn at intervals, and the fraction of spores with exosporia was determined by phase contrast microscopy. The first-order rate constant for removal of exosporia was 0.01 min^{-1}. Alanine racemase and catalase activity of the intact spores was determined before and after the ultrasonic treatment. The treated spores were then washed four times in cold distilled water, and their alanine racemase and catalase activities were again determined. By direct count, less than 2% of the initial spores were lost during the experiment; this number is not noted in the table, as its significance is questionable.

adenosine. After shaking for 4 hr at 30 C, the suspension was stained with 0.5% aqueous methylene blue and examined microscopically. Judging from the absence of swollen, blue spores, there was no germination under these conditions. In a parallel experiment, in which five times as much glucose, alanine, and adenosine was used, 62% of the spores germinated. Omission of glucose from the concentrated mixture reduced the numbers germinating to 43%. Since germination under the latter condition is clearly far from optimal, 30 mM L-alanine and 0.06 mM adenosine approximate the *minimal* germination requirements for these spores. Negligible germination (less than 10%) occurred when either alanine or adenosine was omitted from the concentrated incubation mixture.

Fractionation of the spores. The course of fractionation of ballistically disintegrated, freeze-dried spores is shown in Table 3. The total protein solubilized in this experiment is somewhat higher than usual. More typically, of the total protein, about 25% is solubilized by phosphate buffer and an additional 25% by either alkali or urea-mercaptoethanol. These solutions probably contain nucleic acid as well as protein, because the ratio of their optical density at 280 nm to that at 260 nm is low.

Solubility of extract protein. The solubility of protein in urea crystal and urea spore extracts was examined as a function of *p*H (Fig. 1). Both extracts have a fairly low isoelectric point

TABLE 3. *Fractionation of ballistically disintegrated spores*[a]

Course of fractionation	Batch 1	Batch 2
	mg	*mg*
Initial dry weight	200.0	200.0
Total protein initially present	108.0	106.5
Total protein dissolved by phosphate (OD 280/260 nm)	27.0 (0.59)	24.3 (0.60)
Phosphate-insoluble protein dissolved by urea-mercapto-ethanol (OD 280/260 nm)	52.0 (0.70)	
Phosphate-insoluble protein dissolved by NaOH		42.3
Residual insoluble protein	43.6	39.2
Total protein recovered	122.6	105.8

[a] See Methods for composition of solvents.

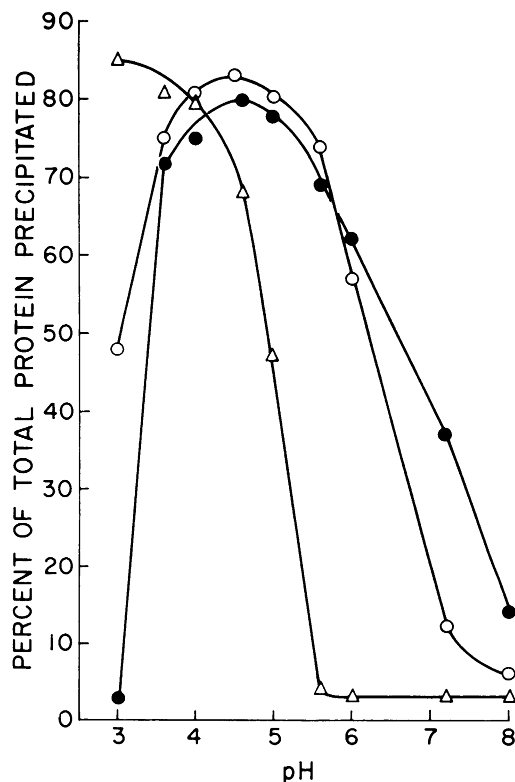

FIG. 1. *Solubility of protein in urea crystal (●), urea spore (○), and phosphate spore (△) extracts as a function of pH.*

at *p*H 4.6, a value in good agreement with previous observations for solubilized crystal protein (9). The shapes of the curves are very similar in the vicinity of the isoelectric point but less so at the extremes of *p*H studied. The solubility curve for phosphate spore extract is distinctly different.

Immunological comparison of spore and crystal extracts. Antisera were taken from rabbits immunized with (i) whole cells harvested during exponential growth and containing, as judged by microscopy, no spores or crystals; (ii) purified whole crystals; (iii) alkali-soluble crystal protein from which residual contaminating spores were removed by centrifugation at 60,000 $\times$ *g* for 60 min. Alkali was used to prepare this immunogen, since it would extract considerably less protein than urea-mercaptoethanol from whole spores if they were present. This immunogen represents the purest solution of crystal protein that we have achieved. Seven antigen solutions were tested with these antisera: phosphate spore, urea spore, alkali spore, phosphate cell, urea cell, alkali crystal, and urea crystal.

The specificity of each antiserum was examined by double immunodiffusion in agar-gel with each of the seven soluble antigens; the presence or absence of precipitin band formation is shown in Table 4. Antiserum to whole crystals was nonspecific and precipitated all of the antigens tested. In contrast, antiserum to soluble crystal protein was specific in the sense that it precipitated spore and crystal protein extracted with urea-mercaptoethanol or alkali but did not precipitate either of the vegetative cell extracts or the phosphate spore extract. This immunological pattern confirms a previous report that crystal antigen is not found in the vegetative cell (18). It also suggests that spore and crystal may share one or more similar antigens. Finally, antiserum

TABLE 4. *Precipitin patterns in double diffusion plate tests*

Rabbit sera	Antigens				
	Cells		Spores		Crystals (Alkali or urea extract)
	Urea extract	Phosphate extract	Alkali or urea extract	Phosphate extract	
Anti-whole crystal	±	+[a]	+	+	+
Anti-soluble crystal	−[b]	−	+	−	+
Anti-whole cell	+	+	+	+	−

[a] One or more precipitin lines, +.
[b] No precipitin lines observed, −.

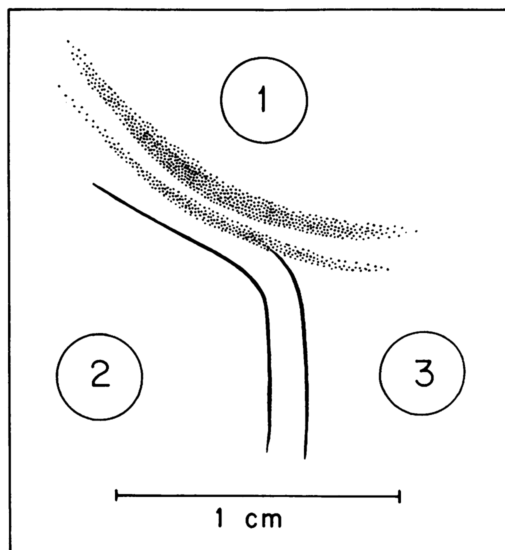

FIG. 2. *Precipitin patterns formed by urea-mercaptoethanol extracts of crystal (1) and spore (3) with antiserum to soluble crystal protein (2). Antiserum was used without dilution; 200 μg of protein was added to each antigen well.*

to vegetative cells precipitates cell and spore extracts but not crystal extracts. This observation supports the conclusion that the crystals are relatively free of cellular components. The precipitation of the spore extracts by this serum can be attributed to the known fact that spores are partially composed of vegetative cell proteins.

A detailed comparison of the precipitin patterns formed by the urea-mercaptoethanol extracts of spore and crystal with antiserum to soluble crystal protein is shown in Fig. 2. The most rapidly moving component from each extract forms a continuous precipitate without spur formation and, therefore, appears to be common to both spore and crystal. The more slowly moving spore antigen forms a precipitate that may be continuous with a component of the intermediate crystal band, but the precipitate is so faint that it is difficult to be certain. The unique band under the crystal well contains, to judge from its density, most of the crystal protein.

The precipitin bands shown in Fig. 2 are not formed if normal rabbit serum is substituted for the immune serum or if the immune serum is absorbed with an excess of soluble crystal protein. Absorption of the antiserum with an excess of urea spore extract causes a disappearance of the precipitin bands originating from the spore well and of their homologues from the crystal well.

One possible source of the common precipitin bands originating from the spore extract might be the traces of contaminating crystal protein that must be solubilized when extracting the spores with urea-mercaptoethanol. This idea, however, is not correct, for spores of a mutant strain that does *not* form crystals prove to have urea-mercaptoethanol-soluble antigens precipitable in a pattern indistinguishable from that already described.

DISCUSSION

To determine whether or not crystals and spores of *B. thuringiensis* share common components, it was essential to this investigation that these particles be effectively separated from each other and be free of common contaminating protein from the vegetative cells. The method devised for their separation, although unwieldy and monotonous to use, did yield effective separation with recovery of nearly three-fourths of the desired particles initially present in the cultures. The method had the additional virtue of subjecting spores and crystals to more than 20 washings, which should have rendered them free of all cellular components that are not part of their intrinsic structure. Analysis of the purified suspensions supported this prediction. The crystal suspension contained a negligible amount of phosphorous and anthrone-reactive material, and it was therefore judged to be free of nucleic acid, carbohydrate, and phospholipid. The ultraviolet absorption of dissolved crystals was characteristic of pure protein, and approximately 100% of the mass of the crystal could be accounted for as amino acids (21). The crystals did contain traces of calcium and magnesium, but these metals were not present in greater quantity than expected from the level of spore contamination. Finally, antiserum to vegetative cells did not give detectable precipitin reactions with crystal solutions.

The cleanliness of the spores could not be evaluated by direct chemical analysis. However, the spores failed to germinate unless both L-alanine and adenosine were provided. In this respect, they were similar to well-cleaned spores of the closely related *B. cereus* (8). The spore suspensions had considerable catalase activity, of which more than half was heat-labile. Lawrence and Halvorson (13), studying intact spores of *B. cereus*, concluded that heat-stable catalase was an integral part of the spore, whereas heat-labile catalase was probably a cellular enzyme on the spore surface, from which it could be removed by exhaustive washing. Considering the large number of washings involved in purifying the spores of *B. thuringiensis* and, more important, that removal of their exosporia and associated alanine racemase did not diminish their total catalase activity or alter the proportion of the heat-labile and stable components, it appears that the heat-labile enzyme is a spore component. Its presence is therefore not a criterion of cellular contamination in spores of *B. thuringiensis*. In fact, since the total catalase activity was not decreased by removal of the exosporia, it is probable that no cellular catalase was adsorbed on the spore surface, and by inference this would be true of other cellular protein as well. Considering the results of Lawrence and Halvorson (13), it appears that species or strain differences in the heat-lability of spore catalase activity exist, and a comparative study of this question is desirable. Although the foregoing observations do not prove that the spores and crystals are completely free of occluded components from the vegetative cell, they provide compelling reasons to believe that the level of contamination is low.

Crystals are completely dissolved by alkali or a urea-mercaptoethanol solution. The same solvents solubilize protein from broken spores that is not soluble in phosphate buffer, and the extracts obtained are closely similar in isoelectric points to the crystal solutions. These data suggest a common class of protein in crystal and spore. Serum from a rabbit immunized with soluble crystal protein, from which residual spores were removed by centrifugation, precipitated the urea-mercaptoethanol or alkali extracts of crystals and spores but did not precipitate the phosphate extract of spores. Spore protein soluble in phosphate buffer thus appears to be both chemically and immunologically different from crystal protein.

This observation suggests an explanation for Monro's (18) earlier failure to detect antigenic similarity of spores and crystals. He compared the antigenicity of alkali-soluble crystal protein and water-soluble spore protein obtained by ultrasonic disruption of whole spores. Both extracts were precipitated by rabbit antiserum to whole crystals. After absorption with a cell-free extract of sporulating cells, the serum still precipitated the crystal protein but no longer precipitated the spore protein. Since our experiments suggest that phosphate buffer or water does not dissolve crystal-like protein in broken spores, Monro's negative results could be attributed to the use of an inappropriate spore extract. If so, there are no experimental grounds for believing that the crystal is composed of unique protein.

Comparison of urea crystal and urea spore protein by double immunodiffusion showed partial antigenic homology. The homologous bands are not formed if the anticrystal serum is absorbed with spore extract, and no bands appear if it is absorbed with spore extract. Despite this evidence for homology, the bulk of the crystal protein, judging from the density of the various precipitin bands, appears to be antigenically unique. The significance of the homology is therefore not perfectly clear.

The partial homology can be explained in at least four different ways. Three of these would attribute it to contamination. That is, the unique precipitin band could represent native crystal antigen, and the homologous bands could originate from (i) extrinsic cellular antigens common to both suspensions; (ii) crystals in the spore suspension; or (iii) spores in the crystal suspension.

The first possibility is rendered unlikely by the apparent purity of the spore and crystal preparations as well as by the failure of crystal antiserum to precipitate cellular antigens. Possibilities (ii) and (iii) are unlikely because of the insensitivity of our immunodiffusion technique, which did not detect antigens at less than 10 μg of protein per well. Since 200 μg of protein was added to the antigen wells, a minimum of 5% contamination of spore protein in the crystal extract, or vice versa, would have been required for formation of detectable bands due to this cause. Calculations based on data in Tables 1 and 2 indicate that the antigen solutions could contain, at maximum, no more than 1% of such contaminating protein.

Alternatively, the homology could be the consequence of common protein(s) intrinsic to the spore and crystal. To test the validity of this fourth possibility, a more definitive biochemical study of the spore and crystal solutions was undertaken and is reported in the following paper (21).

ACKNOWLEDGMENTS

We thank Gareth Wyn Jones, who did the calcium and magnesium analyses, and H. Downs for general technical assistance.

This investigation was supported by grants GB 1225 and GB 6223 from the National Science Foundation. F. Delafield held a Public Health Service Postdoctoral Fellowship, 5-F2-AI 31,369, from the National Institutes of Allergy and Infectious Diseases.

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