SEROLOGICAL STUDIES ON THE FORMATION OF PROTEIN PARASPORAL INCLUSIONS IN *BACILLUS THURINGIENSIS*

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ABSTRACT

A strain of *Bacillus thuringiensis* has been isolated, and methods have been developed for separation of the crystalline, parasporal inclusions in a pure form. Normal sporulation with concomitant crystal formation takes place when cells are incubated under suitable conditions in a nutrient free medium. Serological techniques have been used to study the origin and development of the crystals. Rabbit antisera have been prepared to a vegetative cell extract, suspensions of crystals, and a solution of crystal protein (obtained by alkali treatment of crystals). Tests have been carried out mainly by the Ouchterlony gel plate technique. Crystal protein solutions were found to be more active than suspensions of intact crystals both in reaction with, and in neutralization of, the crystal antibodies. Antisera to the vegetative cell extract gave no reaction with crystal protein. Ultrasonic extracts of cells taken before or during crystal formation gave no reaction with the crystal antibodies. Tests with alkali extracts of disrupted cells showed that the crystal antigen is absent in vegetative cells but arises during sporulation. The appearance of the antigen can be correlated with the formation and growth of the crystals as followed by examination of disrupted cell preparations under the electron microscope. It can be concluded that the crystalline protein inclusions do not arise from precursors in the same antigenic state.

INTRODUCTION

During sporulation of the insect pathogen *Bacillus thuringiensis*, a spore and a crystalline inclusion are formed within each cell. The inclusions are of approximately the same size as the spores and are composed of protein (5, 6). The isolated inclusions or protein therefrom are toxic to insect larvae (2, 4). The inclusions have a regular shape and surface structure and they are referred to as "crystals" for convenience. Some properties of the isolated inclusions are presented in another paper (10). Similar inclusions have been found in other *Bacilli* (5). These strains are closely related to *Bacillus cereus*, but their nomenclature has not yet been fixed (4, 7, 13). The general field of "bacterial insecticides" has recently been reviewed by Heimpel and Angus (7).

The present studies were concerned with the way in which the crystal protein is formed and laid down. Investigation was stimulated by the observation that normal sporulation takes place when cells are incubated under suitable conditions in a nutrient free medium. This observation led to a search for precursors of the crystals. In the present paper the results of a serological study are described; chemical and tracer studies are recorded elsewhere (9). The problem of crystal

origin is considered to be of interest in connection with the way in which insoluble protein structures are laid down in cells in general. The results are also of interest from the viewpoint of protein turnover.

Young and Fitz-James (15) have recently reported elegant studies on spore and crystal formation in *Bacillus cereus* var. *alesti*, a strain related to *Bacillus thuringiensis*. By use of chemical and cytological techniques they showed that the inclusions form and grow in a position adjacent to the developing forespore. An alkali fractionation method for separation of the crystal protein was developed and used in conjunction with S^{35}-methionine incorporation to study the synthesis of the inclusion protein. Evidence was presented that most if not all of the crystal protein is synthesized during the same period that the crystals are formed. In the present paper this conclusion is supported by correlation of the formation of crystal protein antigen with the appearance of the crystals. It is also shown that appreciable quantities of water-soluble, antigenically related precursors are absent from the cells even during the period of crystal formation. A preliminary report of this work has already been published (8).

Young and Fitz-James (15) also found that the crystal protein is derived in part from vegetative cell constituents and in part from the medium. In the present paper it is shown that, under suitable conditions, the crystal protein is derived entirely from vegetative constituents. Tracer work, reported elsewhere (9), shows that, under such conditions, vegetative cell proteins break down to free amino acids, which are then incorporated into the crystal and other new proteins, synthesized during sporulation. If a labelled amino acid is added to a culture of sporulating cells, it equilibrates with free amino acid in the cell and is partly incorporated into the new protein, even though net utilization of amino acid from the medium does not necessarily take place.

These results show that once commitment has taken place, sporulation can go to completion in the absence of added sources of carbon or nitrogen. Although the evidence suggests that the process can be wholly endogenous, supported by intracellular turnover, there remains some possibility that small amounts of nutrients are essential, and that in the system used, these were supplied by lysis of a small percentage of the population.

MATERIALS AND METHODS

Organism

A newly isolated strain of *Bacillus thuringiensis* has been used throughout the present studies. The organism was obtained from diseased larvae of the flour moth and is an aerobic, spore-forming rod. Examination of sporulated cultures by electron microscopy (10) revealed the presence of parasporal inclusions similar to those reported for *Bacillus thuringiensis* and related strains. The organism has been maintained both in a freeze-dried form and as washed spore suspensions in distilled water, kept at 5°C.

Growth and Sporulation

These processes were effected in liquid media aerated at 31°C. The growth medium consisted of 3 per cent casein digest supplemented with $MnSO_4$ (4.5 μM). Cultures were taken in the early stationary phase about one hour after aggregation set in (see Results), and either transferred directly for sporulation or stored at 5°C for up to 5 days before use. Such cultures were centrifuged and the cells washed with distilled water and resuspended in a nitrogen-free buffered salts medium (KH_2PO_4, 0.6 gm; Na_2HPO_4, 2 gm; $MgSO_4 \cdot 7H_2O$, 0.4 gm; NaCl, 0.6 gm; distilled water, 1000 ml; pH 6.7). Sporulation with concomitant crystal formation was complete after about 10 hours incubation in the salts medium.

Light Microscopy and Cell Counts

Liquid mounts were viewed under the microscope using contrast or the Reichert anoptral phase contrast illumination. In cases where cells had aggregated, samples were sucked up and down several times through a fine hypodermic syringe to disperse the aggregates. Cell counts were carried out using a calibrated counting chamber. Cells occur in chains, and each unit which was seen to be separated from other like units by a transverse wall was counted as one cell.

Ultrasonic Treatment

Suspensions of cells or spores and crystals in distilled water at concentrations up to 150 mg dry weight per ml were subjected to ultrasonic vibration in the type B apparatus described by Davies (3). The power, adjusted to 500 watts, was supplied by the Mullard type E7590B unit. The volume of the suspensions ranged from 1 to 10 ml, and treatment was continued until more than 95 per cent of the rods were broken (2 to 30 minutes).

Preparation of Parasporal Inclusions

Crystals were obtained free from spores and cell wall material by (*a*) germination followed by autolysis of the spores, (*b*) isopycnic centrifugation, or (*c*) ultrasonic disruption of immature cultures. The final step in each of these methods was removal of slow-sedimenting debris by differential centrifugation. Suspensions of crystals and debris in water were subjected to ultrasonic vibration (*ca.* 1 minute) to disperse aggregates, and then centrifuged in a swinging bucket rotor, under conditions such that *ca.* 95 per cent of the crystals were sedimented. The sediment was resuspended, and the whole procedure repeated 3 to 5 times. The centrifuge used varied with the size of the preparation, but typical conditions were as follows: distances from axis of rotation to meniscus and bottom of tube, 9 cm and 14 cm, respectively; speed, 1800 RPM (average RCF, 420 *g*); time, 20 minutes.

a) *Germination and Autolysis of Spores:* A thick suspension or "paste" of sporulated cells was stored at 5°C for 6 weeks. Under such conditions nearly 100 per cent germination and autolysis of the spores took place. The released crystals were freed from debris by differential centrifugation, as described above.

b) *Isopycnic Centrifugation* (1): This method utilizes a small difference of density between spores and crystals. Centrifugation is carried out in sucrose of such a concentration that spores sediment whilst crystals remain stationary or rise. It is important to avoid germination of spores, since germinating spores also rise.

Spores and crystals were released from sporulated cells by ultrasonic treatment. A washed sediment of spores, crystals, and vegetative cell remnants was suspended in 81.5 per cent sucrose and centrifuged in a high speed, angle head centrifuge for a time depending upon tube size, centrifugal force, and temperature (using 1.5 × 7.5 cm tubes at 5°C, centrifugation was for 1.5 hours at 90,000 *g*). The supernatant was combined with material which had formed a pellet at the top of the tube, diluted with an equal volume of water, and centrifuged at 10,000 *g* for 1 hour. The resulting sediment consisted of crystals and small debris, and the latter was removed by differential centrifugation, as described above.

c) *Ultrasonic Disruption of Immature Cultures:* Mature spores are resistant to ultrasonic vibration. It was found, however (see Results), that crystals attained full size before spores developed resistance to ultrasonic vibration. Cultures were harvested at a stage when crystals were nearly grown to full size as viewed under anoptral phase contrast (*ca.* 6 hours after transfer), and treated by ultrasonic vibration, as described above, until the rods and immature spores were disrupted. The crystals were then separated from small debris by differential centrifugation, as described above.

Electron microscopic examination of crystal preparations showed that they were nearly pure, and no anatomical or chemical differences were observed between crystals prepared by the different methods. X-ray diffraction patterns were the same for crystals prepared by the method of isopycnic centrifugation and for those prepared by the method of ultrasonic disruption of immature cultures.[1]

Miscellaneous Methods

Vegetative Cell Extracts: Washed cell suspensions were disrupted in the ultrasonic disintegrator, then centrifuged at 2000 *g* for 20 minutes, and the supernatant liquid was used as extract.

Crystal Protein Solutions: Suspensions of crystals were dialysed against 0.1 N NaOH for 2 hours at 20°C and then dialysed against 0.1 M phosphate buffer, pH 7.5. Crystals were dispersed completely by this treatment. Any insoluble residue arose from spore or cell wall contamination and was removed by centrifugation at 2000 *g* for 20 minutes.

Alkali Extracts of Cells or Disrupted Cells: These were prepared in a manner identical with that used for the preparation of crystal protein solutions.

Serology

Antisera: Preparations used for injection were as follows: (i) vegetative cell extract prepared, as described above, from a transition phase culture; the final concentration corresponded to material obtained from 20 mg dry weight of cells per ml; (ii) crystal suspensions (5 mg dry weight per ml) prepared by the method of isopycnic centrifugation (for first course) and ultrasonic treatment (for second course); (iii) solutions of the crystal protein, prepared as described above; concentration 5 mg dry weight per ml.

Rabbits were injected in duplicate with each preparation. Each rabbit received 3 iv. injections of 0.25 ml followed by 4 injections of 0.5 ml at 2 to 3 day intervals. The animals were bled 10 days after the last injection. A second course of injections was given, starting 4 weeks after bleeding. In this series, the first iniection of 0.5 ml was given intraperitoneally and was followed by 5 iv. injections of 0.5 ml given at 2 to 3 day intervals. Animals were bled 8 days after the last injection. The sera were

[1] A. Klug and K. C. Holmes, personal communication.

stored separately at −20°C without the addition of preservative.

Agglutination Tests: (*a*) Slide tests: One drop of crystal suspension was added to one drop of serum on a slide. The slide was rocked in a moist atmosphere and the degree of aggregation determined by examination under a hand lens. (*b*) Tube tests: Two drops of spore or crystal suspension were added to two drops of serum in a 3 ml tube and incubated at 37°C. The degree of aggregation was assayed at 15 minute intervals by visual examination.

Interfacial Precipitin Test: Two drops of serum (clarified by centrifugation if necessary) were layered below two drops of the preparation to be tested in Dreyer's agglutination tubes. The extent of flocculation at the interface was noted at 5 minute intervals.

Precipitation in Agar Gel: The method of Ouchterlony (11) was used. The medium consisted of 1 per cent Noble's agar and 0.8 per cent NaCl with 0.1 per cent NaN_3 added as bactericide. After a thin layer (about 1 mm) of this medium had been poured into a Petri dish and allowed to set, a paraffin-coated matrix was put into place and a second layer of medium poured (2 to 4 mm). After this layer had set, the matrix was removed, leaving holes in the required arrangement. Sera and antigen preparations were placed in the holes and in some cases mixtures of sera and antigens were used. Plates were examined daily, and photographs taken, using indirect illumination from below, when a suitable stage of development was reached (6 to 10 days).

Notation

VC, vegetative cell extract
C, crystal suspension
CP, crystal protein solution
Anti implies "antiserum to." Thus:
Anti-VC, antiserum to vegetative cell extract
Sera obtained from duplicate rabbits are designated *a* and *b*. Numbers refer to the course of injections. Thus:
Anti-Ca_2, serum obtained after the second course of injections of crystals into the first of a pair of rabbits

RESULTS

The conditions employed for sporulation were worked out with a view to obtaining a high percentage of synchronous sporulation in a nutrient free medium. If incubation is continued in the growth medium which was employed, without transfer, sporulation does take place but there is little synchrony, and recycling (germination and growth of spores) occurs before many of the cells have sporulated.

Aggregation of cells, accompanied by a fall in turbidity, takes place in the early stationary phase and may be considered to be a sign that sporulation has begun (15). The process of sporulation is not much accelerated by transferring cells from the late exponential or transition phase of growth to the nutrient free medium; aggregation followed by sporulation takes place at about the same time as in the untransferred culture.

There is a lower percentage of sporulation when cells are transferred before aggregation. However, when cells are taken 1 hour after aggregation, *i.e.* under the conditions employed, sporulation is synchronous and takes place in a high proportion of the cells. Cell counts taken at the time of transfer and after 10 hours incubation show that there is no change in the total number of cells over the course of sporulation. At 10 hours, over 75 per cent of the cells contain a spore and a crystal; the remainder appear either empty (as "ghosts") or undifferentiated (dark under phase contrast). The first differentiation of the forespore is observed in the majority of the cells after about 2 hours, and crystals form from about 3 to 6 hours. The spores which are formed after 10 hours show the characteristic refractile appearance of spores, under phase contrast, and are resistant to incubation at 70°C for 20 minutes. If spores are stored at 5°C in water, some remain viable for months whilst others germinate within a day or two.

Experiments have been carried out to investigate whether sporulation can in fact take place in the absence of external nutrients or whether substances released into the medium through lysis of a small percentage of the cells are necessary for sporulation of the remaining cells. When suspensions of one-tenth the normal cell density were incubated, excellent sporulation was obtained, showing that, if the utilization of lytic products is necessary for sporulation, they are readily taken up at very low concentrations. Furthermore, cells can be repeatedly resuspended

in fresh medium during sporulation without any noticeable effect on their development. This shows that if lytic products are needed, they are taken up by the healthy cells from the medium immediately after they are released.

Serology

The observation that normal crystals are formed during sporulation in the absence of external nutrients raised the question of the origin of the crystals. Clearly, under such conditions, the crystal protein must be derived from material present in the cells at the time of transfer to the nutrient free medium. Two hypotheses were formulated to account for crystal formation: (i) crystals arise through crystallization or modification of protein or polypeptide precursors, present at the time of transfer; (ii) the crystal protein is synthesized *de novo* from amino acids during sporulation. The first hypothesis has been studied by serological methods and the results are described below.

Experiments have been designed to determine whether vegetative cells contain any material which is immunologically related to the crystals. Such a possibility has been tested by investigating (*a*) whether vegetative cell extracts, when injected into rabbits, give rise to "crystal antibodies" (*i.e.*, antibodies which react with the crystal protein), and (*b*) whether vegetative cells contain a component which reacts with crystal antibodies. Rabbits have been injected with preparations of vegetative cell extract, crystals, or dissolved crystal protein, and reactions of the resulting antisera have been studied. Details of the preparations are given under Methods.

Flocculation and Interfacial Precipitin Tests: Isolated crystals reacted only weakly with crystal antiserum (flocculation test) but strong reactions were given when solutions of the crystal protein were used (interfacial precipitin test). Such solutions gave precipitates with antisera to crystals and crystal protein solution but not to the vegetative cell extract. It follows that the vegetative cell preparation does not give rise to crystal antibodies when injected into rabbits.

Vegetative cell extracts were also tested by the interfacial precipitin method. Strong reactions were given with antisera to vegetative cell extract and to crystals, and a weak reaction was given with crystal protein antiserum. There was no reaction with the control preinoculation sera in any case. It may be concluded that the crystal antisera contain antibodies reacting with the vegetative cells, but that the vegetative cell antisera contain no antibodies to the crystal protein. In experiments with gel plates, described below, the reaction between vegetative cell extract and crystal antiserum is shown to be due to antibodies unrelated to the crystals.

Reactions of Crystal Protein on Gel Plates: Crystals gave no reaction with antisera when tested on gel plates, but crystal protein solutions reacted strongly, forming precipitation bands with antisera to crystals and crystal protein solutions within 24 hours. Effects of varying extraction conditions on the reactions are shown in Fig. 1. Treatment of crystals at pH 11.8 for 2 hours extracted some of the antigen, but maximum activity was obtained after 0.5 to 2 hours incubation with 0.1 N NaOH. The precipitation patterns varied with the antiserum used, but in all cases more than one band was given. The 2 hour extraction with 0.1 N NaOH has been selected for general use. Fig. 2 shows the patterns formed by crystal antiserum with serial dilutions of a crystal protein solution.

Relation between Crystals and Vegetative Cells: Fig. 3 shows that the vegetative cell antiserum (upper left hand pool) gave a strong multiple reaction with vegetative cell extracts (upper right hand pool) but no reaction with the crystal protein solution (lower left hand pool). The crystal antiserum (lower right hand pool) gave a strong reaction with the crystal protein solution and a reaction with the vegetative cell extract. The extension of the main *CP* × *anti-C* band into the region of reaction between *VC* and *anti-VC* is fortuitous. Non-identity of the *VC* antigens with the main crystal antigens is suggested by this plate, and proof of the non-identity is shown in Fig. 4. Vegetative cell extract was placed in the upper left hand triangle and crystal antiserum in the upper right hand triangle. In the lower triangle crystal antiserum was placed along with excess crystal protein solution. The formation of typical crystal bands between the upper right hand and lower triangles confirms that in the lower triangle, crystal protein antigens were in excess of crystal antibodies. The reaction of *VC* with *anti-C* was the same with both the upper right hand and the lower triangles, showing that the reaction was not affected by the neutralization of crystal antibodies. The absence of a relation between the main *VC*

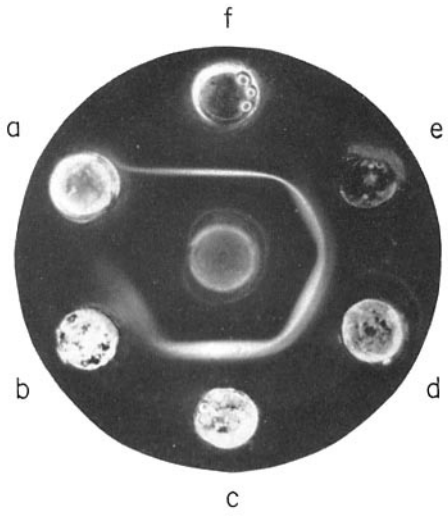


FIGURE 1

Extraction of crystal antigen. Crystal suspensions were dialyzed against extraction agents for varying lengths of time and then dialyzed against 0.1 M phosphate buffer, pH 7.5. The final concentration of protein was about 1.3 mg dry weight per ml. Extraction agents were: *a*, water; *b*, 0.01 M Na_3PO_4 (pH *ca.* 11.8), 2 hours; *c*, 0.1 M Na_3PO_4, 2 hours; *d*, 0.1 N NaOH (pH *ca.* 12.8), 0.5 hour; *e*, 0.1 N NaOH, 2 hours; and *f*, 1 N NaOH, 2 hours. Serum, *anti-*Ca_2, was placed in the center hole.

and crystal reactions is confirmed by crossing over of the respective bands. The reaction of *VC* with the lower triangle was slightly weaker than that with the upper, owing to dilution of the serum. The relations of the weaker reactions are less clear, but careful examination of the minor bands formed by *VC* with both upper and lower triangles reveals that in all probability there is no relation between the minor *VC* and *CP* bands. Similar results were obtained with the other sera, and it can be concluded that the vegetative cell extract contains no soluble material related antigenically to the crystals. The weak reactions of vegetative cell extract with crystal antisera were due to the presence of antibodies unrelated to the crystals, and it is shown below that these antibodies arose through the presence of small amounts of contamination in the crystal preparations used for injection of rabbits.

The Formation of Crystals: Experiments have been carried out to investigate whether crystal antigens are present in a soluble form at any stage during sporulation. Ultrasonic extracts were taken of cells sampled at intervals over the course of sporulation. Fig. 5 shows the pattern obtained when such extracts were tested against crystal antiserum. Bands other than those given by vegetative cell extracts appeared with later

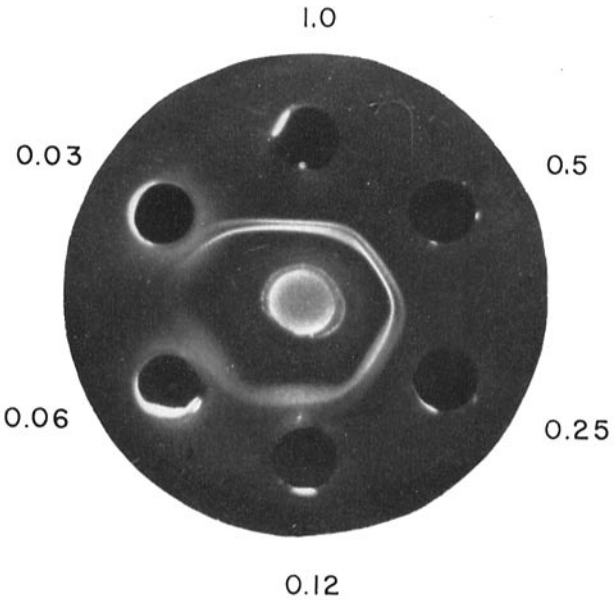


FIGURE 2

Serial dilutions of crystal protein solution. Initial concentration of the crystal protein solution was 5 mg dry weight per ml. Dilutions were made with saline. Numbers around the photograph refer to dilutions. The center hole contained serum, *anti-*Ca_2.

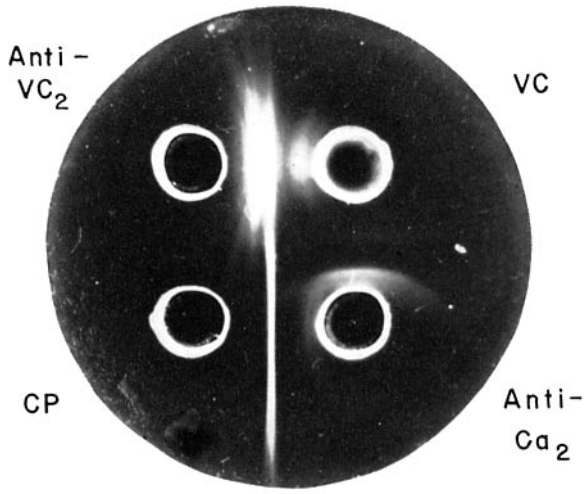


FIGURE 3

The crystal protein solution (*CP*) was at a concentration of 5 mg dry weight per ml. The vegetative cell extract (*VC*) was obtained from a transition phase culture, and each milliliter of extract was equivalent to 20 mg dry weight of cells.

samples, and it can be seen that new antigens are formed during sporulation. As with the reaction between vegetative cell extract and crystal antiserum (Fig. 4), it was found that such antigens are not related to the crystals (Fig. 6). Similar results were given with the other crystal antisera. It is estimated that less than 2 per cent of the final crystal protein could be present in soluble form. By use of extracts obtained from a pure spore preparation, the reaction given by ultrasonic extracts of sporulating cells with crystal antisera has been shown to be due to antigens identical with those present in spores (Fig. 7). It seems probable that the antibodies reacting with the spore extract arose as a result of small amounts of spore contamination in the crystal preparation used for injections, and that the weak reaction of vegetative cell extracts with crystal antisera likewise arose from contamination of crystal preparations used in preparation of the antisera. Spore extracts were also found to be free from soluble crystal antigen. It may be concluded that to a first approximation, soluble antigens related to the crystals are absent from vegetative cells, sporulating cells, and spores.

In other experiments, alkali extracts of cells have been tested. Little crystal antigen was extracted from intact sporulating cells by alkali treatment even though the crystals were seen by microscopic examination to be present in the cells before but not after treatment. The antigen can be extracted, however, if the cells are first disrupted by ultrasonic vibration, and then treated with alkali. In order to follow the course of crystal development, cells have been sampled, broken, and extracted with alkali at intervals over the

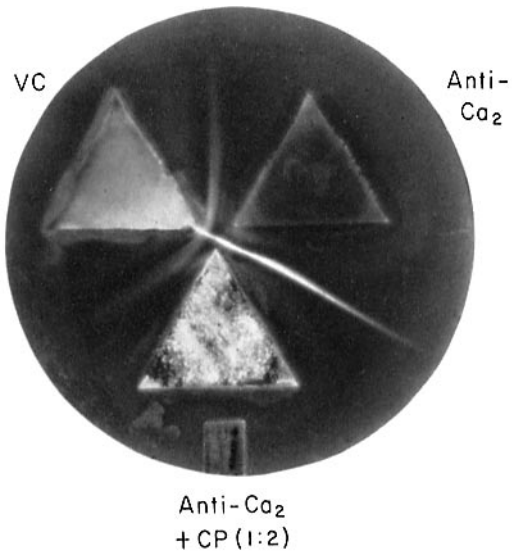


FIGURE 4

CP was at a concentration of 5 mg dry weight per ml; *VC* was derived from a culture which had just aggregated. The extract was concentrated by placing it in a dialysis bag and subjecting it to a stream of air. The final concentration corresponded to extract from *ca.* 300 mg dry weight of cells in 1 ml.

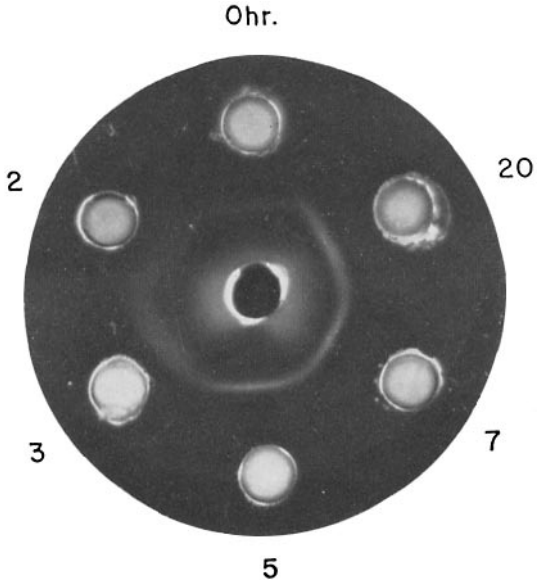


FIGURE 5

Samples were taken at intervals from a sporulating culture and extracted the same way as for preparation of *VC*. Each milliliter of extract was derived from 100 mg dry weight of cells. Numbers around the photograph refer to sample time (hour after transfer). The center hole contained serum, *anti-*Ca_2.

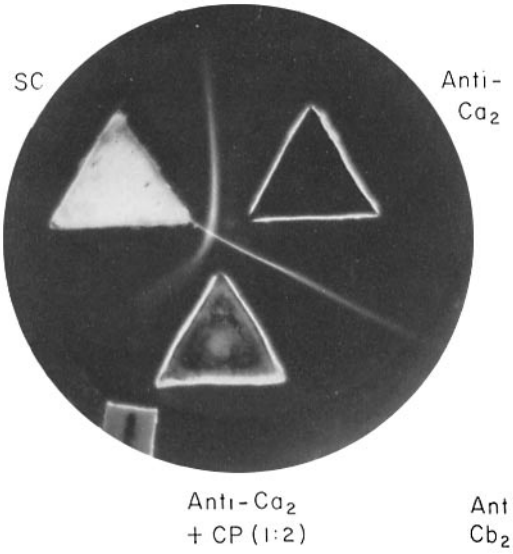
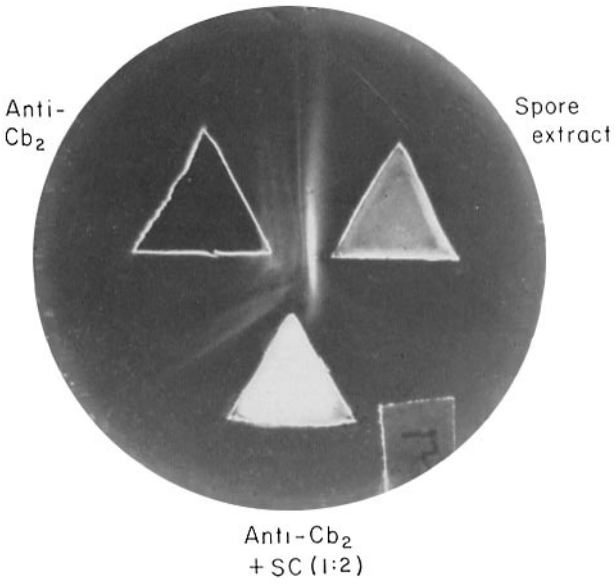


FIGURE 6

SC is the same as 7 hour sample in Fig. 5. *CP* was at a concentration of *ca.* 4.3 mg dry weight per ml.

FIGURE 7

A spore extract was prepared by ultrasonic disruption of a crystal free spore preparation (from isopycnic centrifugation). *SC* is the same as the 7 hour sample in Figs. 5 and 6.



course of sporulation. Fig. 8 shows the reaction of such extracts with crystal antisera. The two characteristic crystal bands are not given by samples taken at the beginning of sporulation, but are given by samples taken after 3 to 4 hours incubation. The bands do not increase in intensity after 6 hours. Antigens present in initial extracts and two others in later extracts were shown to be unrelated to the crystal antigens (Fig. 9). One of the two crystal bands is formed by earlier extracts than the other; this effect takes place with each of the three sera tested. It might be deduced that there are two main crystal antigens and that one of them is synthesized before the other. Fig. 2 shows, however, that the effects can also be explained in terms of the concentration of crystal protein in the extracts.

DISCUSSION

Endotrophic Sporulation

The purpose of the present work was to study the formation of the parasporal inclusions and the possibility of protein turnover in sporulating cells. Research was not expressly directed at the question whether or not sporulation is an endogenous process. The endotrophic system (*i.e.* sporulation in a nutrient free medium) was, however, chosen (*a*) for the theoretical reason that turnover would be expected to be accentuated under such conditions, and (*b*) for the technical purpose of simplifying the design and interpretation of isotope incorporation experiments. In view of the controversy over endotrophic sporulation (15), special attention has been directed to the matter in order to ensure the validity of the conclusions which have been drawn.

The present work shows that good sporulation can be obtained when cells from the early, stationary phase of growth (after commitment to sporulation) are transferred to a nutrient free medium. Thus, cell numbers are conserved, over 75 per cent of the cells form spores and crystals, and the possibility that there is a turnover of the cell population is eliminated by the synchrony observed. Criticisms applied to the assessment of

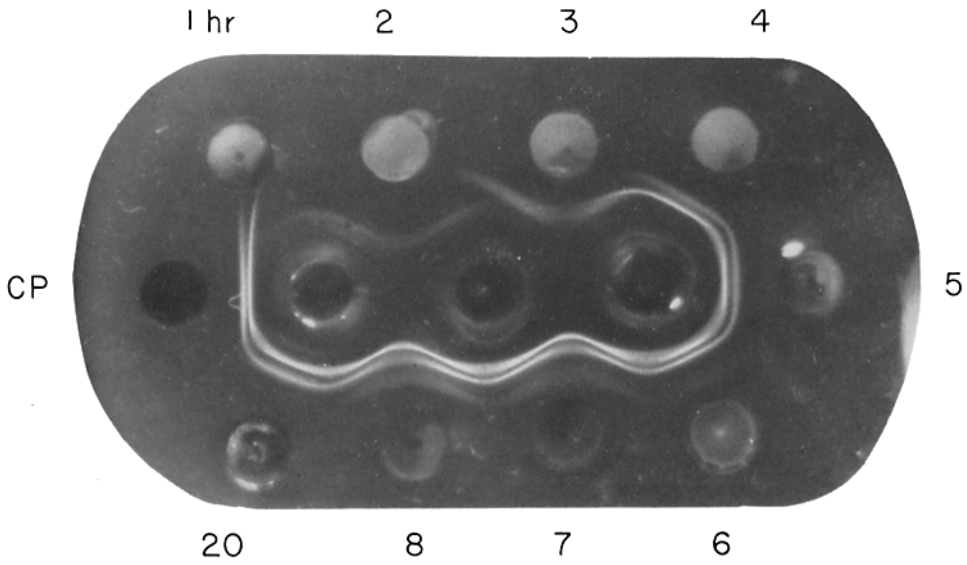


FIGURE 8

Samples were taken at intervals from a sporulating culture and, after resuspension in distilled water, cells were disrupted by ultrasonic vibration. Suspensions were then centrifuged for 10 minutes at 15,000 *g* and the sediments were extracted with alkali (as in the preparation of crystal protein solution). Each milliliter of extract was derived from *ca.* 130 mg dry weight of cells. Numbers around the photograph refer to sample time (hour after transfer). *CP* was at a concentration of 3 mg dry weight per ml. Serum, *anti-*Ca_2, was placed in the center holes.

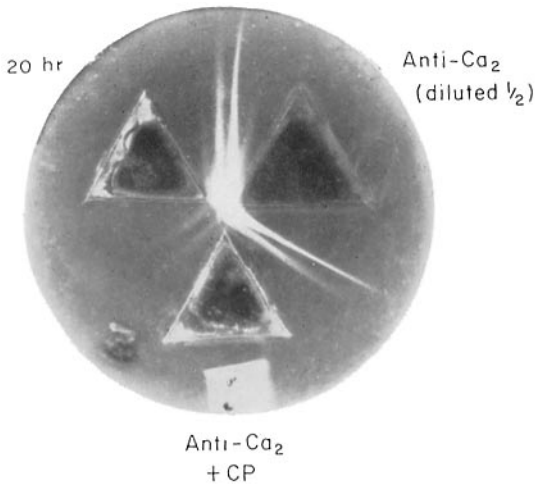


FIGURE 9

Preparations as in Fig. 8. A similar plate was also made using the 0 time sample instead of the 20 hour sample.

percentage of sporulation by means of viable counts do not apply to the method of counting used, in which each unit, separated from other like units by a wall, was counted as one cell. It is also improbable that nutrients, released by the small proportion of cells which partially lyse, are necessary for sporulation of the remaining cells. Thus, neither dilution nor repeated washing of cells affects the course of sporulation.

It is known that cells increase in dry weight during sporulation in suitable media (14) and that carbon and nitrogen compounds may be depleted from the medium (12). It is also probable that crystals are smaller and spores less resistant when cells are sporulated endotrophically. Such differences are to be expected, however, and the fact that sporulating cells can utilize external nutrients is no reason to believe that they have to. It is not surprising that bacilli can sporulate under a variety of conditions, since a selective advantage would be gained by such versatility.

Finally, it should be noted that other workers have designed media which support excellent growth and sporulation without transfer. Using such a medium, Young and Fitz-James (15) report 100 per cent sporulation with synchrony. It is thought that the degree of sporulation (*ca.* 75 per cent) obtained in the present system could be increased by refinement of conditions.

Serology

It is interesting that crystal protein solutions react with antisera to the crystals as well as with the crystal protein antiserum. The reaction shows that at least part of the configuration of the crystal protein remains intact after alkali treatment.

In reactions on gel plates the number of distinct antigens may be greater or smaller than the number of bands formed (11). It is therefore not possible to conclude from the data presented how many distinct antigens are present in the crystal protein solution, especially as the number of bands is not the same with different sera. However, the finding that the number of bands varies according to extraction conditions shows that at least some of the bands are formed from distinct components in the extracts; such components are heterogeneous as regards conditions of formation and stability. Chemical and physical data bearing on the number of components of the crystal protein are presented elsewhere (10).

The possibility that there are small amounts of antigens common to crystal protein solutions and vegetative cells has not been completely excluded. It is clear, however, that the main crystal antigens are absent from vegetative cells and are formed during sporulation. The period over which crystal antigens are formed (2 to 6 hours) correlates with the period of crystal formation, as observed by light microscopy. Confirmation that this is the period over which the crystals are formed has been obtained by electron microscopy (10).

It can be concluded that inclusions are not formed by simple crystallization of a protein which exists in vegetative cells prior to sporulation. This conclusion is supported by the finding of Fitz-James, Toumanoff, and Young (4) that the protein inclusions of *Bacillus cereus* var. *alesti* are toxic to insect larvae and that the toxin is formed over the course of sporulation. However, the possibility is not eliminated that crystals might arise through modification of protein or polypeptide components, which are non-toxic to larvae and of different serological properties from the crystal protein. The origin of the crystals and the possibility of protein turnover have been further investigated by use of isotopic tracer techniques in work reported elsewhere (9). The related work of Young and Fitz-James (15) has already been discussed in the Introduction.

It is interesting that no soluble, antigenically related precursor of the crystals has been detected even during the period over which the crystals are formed. This finding might be explained in either of two ways: (i) the protein does not take on the antigenic properties of the crystals until the stage at which it is built into the crystals, or (ii) there are soluble, antigenically related precursors present during crystal formation, but the concentration of such precursors in the cytoplasm is low and their rate of turnover high.

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REFERENCES

1. ANDERSON, N. G., *Canad. J. Microbiol.*, 1955, **2,** 111.
2. ANGUS, T. A., *Canad. J. Microbiol.*, 1956, **2,** 416.
3. DAVIES, R., *Biochim. et Biophysica Acta*, 1959, **33,** 482.
4. FITZ-JAMES, P. C., TOUMANOFF, C., and YOUNG, I. E., *Canad. J. Microbiol.*, 1958, **4,** 384.
5. HANNAY, C. L., *in* Bacterial Anatomy, (E. T. C. Spooner and B. A. D. Stocker, editors). *6th Symp. Soc. Gen. Microbiol.*, Cambridge University Press,1956, 318.
6. HANNAY, C. L., and FITZ-JAMES, P. C., *Canad. J. Microbiol.*, 1955, **1,** 694.
7. HEIMPEL, A. M., and ANGUS, T. A., *Bact. Revs.*, 1960, **24,** 266.
8. MONRO, R. E., *J. Gen. Microbiol.*, 1959, **21,** iv.
9. MONRO, R. E., *Biochem. J.*, in press.
10. MONRO, R. E., and HORNE, R. W., to be published.
11. OUCHTERLONY, O., *Progr. Allergy*, 1958, **5,** 1.
12. POWELL, J. F., and Strange, R. E., *Biochem. J,.* 1956, **63,** 661.
13. TOUMANOFF, C., and LECORROLLER, Y., *Ann. Inst. Pasteur*, 1959, **96,** 680.
14. YOUNG, I. E., and FITZ-JAMES, P. C., *J. Biophysic. and Biochem. Cytol.*, 1959, **6,** 467.
15. YOUNG, I. E. and FITZ-JAMES, P. C., *J. Biophysic. and Biochem. Cytol.*, 1959, **6,** 483.