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Sporulation in *Bacillus subtilis*

THE ROLE OF EXOPROTEASE

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1. Intracellular turnover of protein was measured in wild-type *Bacillus subtilis*, which produces exoprotease at stage I in the sporulation process. Protein is degraded at a rate of 8–10%/hr. 2. As a result of this turnover, the proteins of the mother cell are extensively degraded and resynthesized by about 6hr., so that the later stages of spore formation occur in a cytoplasm containing mainly 'new' protein. 3. The same protease appears to be responsible both for the intracellular turnover of protein and for extracellular proteolytic activity. In mutants that have lost the exoenzyme the intracellular protein is stable for many hours. In addition, these mutants fail to produce antibiotic and are asporogenous. When the exoprotease is regained as a result of back-mutation all the lost capacities of the cell are restored together. 4. Protease activity also accounts for the change in antigenic pattern of extracts of cells sampled during sporulation. Immunoelectrophoresis shows that, in the wild-type, the antigens characteristic of the vegetative cell have largely disappeared after a few hours; in the proteaseless mutants the vegetative-cell pattern is conserved. Apart from changing the protein pattern of the cell the protease could also have the function of removing protein inhibitors of sporulation. Other possible interpretations of the results are discussed.

Sporulation in *Bacillus subtilis* involves a definite sequence of biochemical and morphological events. These have been outlined by Ryter, Schaeffer & Ionesco (1966) and by Ryter & Szulmajster (1965) and described in greater detail by Warren (1968) and Kay & Warren (1968). One of the earliest of these events is the appearance of an exoprotease, which begins to be produced at stage I in sporulation (see Kay & Warren, 1968). The importance of this enzyme in sporulation is shown by the fact that mutants that have lost the ability to make it lack the ability to form spores. They also tend to have a number of associated defects. For instance, they do not produce any antibiotic, a product characteristically formed by sporulating cells, and their competence in genetic transformation is greatly decreased (see, e.g., Schaeffer, 1967; Balassa, Ionesco & Schaeffer, 1963). The almost invariable association of the antibiotic, the protease, genetic competence and the ability to form spores led to the suggestion that all these characteristics might be controlled by a group of closely linked genes (Spizizen, 1965).

The present work was undertaken in the hope that a study of the exoprotease, by using suitable mutants, would provide information on the following points: the role of the exoprotease in the intracellular turnover of protein, the extent to which this intracellular turnover accounts for the fact that the proteins of the spore are apparently 'new' proteins and, finally, the apparent necessity of the exoprotease for sporulation.

Some of the results have been briefly described elsewhere (Mandelstam, Waites & Warren, 1967; Mandelstam, Waites, Warren & Sterlini, 1968).

MATERIALS AND METHODS

Incubation procedure for sporulating organisms. B. subtilis (Marburg strain 168) was grown in a hydrolysed-casein medium (Donnellan, Nags & Levinson, 1964) at 35° with shaking to 0·25mg. dry wt./ml. The culture was centrifuged and the cells were resuspended in the same volume of resuspension medium pre-warmed to 35°. Resuspension medium consisted of the defined sporulation medium, containing glutamate and inorganic ions, of Donnellan *et al.* (1964), with glucose omitted. This medium caused sporulation with high incidence in about 8hr. Under these conditions the proteins of the cells usually increased by about 50% and the DNA by 10–20%, whereas the total RNA remained constant. However, it was found useful to do the experiments with extra $MgSO_4$ added at the beginning of the experiment to a final concentration of 0·04M. The reason for this high concentration of Mg^{2+} was that many of the asporogenous mutants that were isolated had a tendency to lyse in the resuspension medium but were stabilized by Mg^{2+}. Rates of intracellular proteolysis and spore formation in the wild-type were unaffected by the $MgSO_4$.

Determination of spore incidence. A qualitative estimate of spore incidence could be obtained by using the phase-contrast microscope and examining a drop of the suspension.

For more quantitative results a Thoma counting chamber was used. In addition, in many experiments, counts of viable spores were carried out as follows: the suspension was treated with lysozyme (50 μg./ml.) for 20 min. This was necessary because a significant proportion of the organisms formed long filaments, each of which was likely to have several refractile spores. The suspension was centrifuged to remove most of the lysozyme and washed once with potassium phosphate buffer, pH 7·0 (0·1 M). Vegetative cells that had survived the lysozyme treatment were killed by heating at 80° for 10 min. This did not affect the viability of the spores, which were then serially diluted and plated on germination medium (Donnellan *et al.* 1964) solidified with agar.

Determination of bacterial cell density. This was carried out by drawing a curve relating extinction at 600 mμ to dry weight. The curve was prepared with cells from an actively growing culture. It is known that the extinction of sporulating cultures is likely to increase for reasons that have nothing to do with an increase in dry weight. However, it was found that, even with samples taken late in sporulation (stage V; see Kay & Warren, 1968), the actual dry weight determined by drying a sample at 100° still corresponded, within 10–15%, to the value expected from the extinction reading. Later samples might well have shown a greater discrepancy, but no correction on this account was made because neither in this paper nor in any of the ensuing three do the results beyond stage V depend on extinction measurements for their significance.

Isolation of proteaseless (Pr$^-$) and asporogenous (Sp$^-$) mutants. A spore suspension (1 ml.) containing 5×10^8 spores was made in potassium phosphate buffer, pH 7·0 (0·1 M), and 0·1 ml. of ethyl methanesulphonate was added. The suspension was incubated in a water bath at 35° for 20 min. The spores were washed once, suspended in 5 ml. of hydrolysed-casein medium and 0·5 ml. amounts inoculated into each of ten flasks containing 25 ml. of the same medium. The flasks were shaken overnight at 35° and suitable dilutions of the culture obtained were plated on synthetic solid medium for the detection of asporogenous mutants (Sp$^-$). These produced white colonies after several days at room temperature whereas the wild-type colonies were brown.

Proteaseless mutants (Pr$^-$) were detected as follows: suitable dilutions of culture were plated on sporulation medium, solidified with 1·5% agar and containing serum albumin (1%). The albumin was included while the medium was still hot and it gave the final material a milky appearance. Wild-type colonies, which produced exoprotease, exhibited a distinct zone of clearing about 4 mm. wide round each colony after 36–48 hr. Proteaseless mutants showed no zones of clearing after this period, but usually developed a narrower zone on more prolonged incubation. This was probably due to the presence of a second (alkaline) protease, which some strains of bacilli are known to possess.

Immunoelectrophoresis. Samples (300 ml.) in a resuspension medium were taken at intervals, the cells broken in a Braun homogenizer and antigens in the high-speed supernatant (15000*g* for 20 min.) examined by immunoelectrophoresis by using antiserum to either vegetative-cell or to spore proteins. The details are as described by Waites (1968).

Assay of protease. Assays were carried out on cell extracts that had been prepared for immunoelectrophoresis as above. Samples were diluted to about 500 μg. of protein/ml. in tris–HCl buffer, pH 7·4 (0·1 M), containing calcium acetate (0·01 M). The diluted extract (0·2 ml.) was added to casein (1·0 ml. of 0·1% fat-free casein from British Drug Houses Ltd., Poole, Dorset) in tris–HCl buffer. The mixture was incubated at 37° for 1 hr. before addition of trichloroacetic acid (1 ml. of 12%, w/v). The sample was cooled in ice and centrifuged, and the protein in the precipitate was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). The protein precipitated in controls without casein and without cell extract was also determined. Activity is expressed as μg. of protein solubilized/mg. of protein of extract/min.

Qualitative assay of protease in supernatants from suspensions of sporulating cells. A solution of 1% (w/v) albumin in potassium phosphate buffer, pH 7 (0·1 M), with agar (1·5%) was prepared and poured into Petri dishes. When the solution had set, holes were punched in the agar with a cork borer. These were filled with samples of supernatant from sporulating suspensions. Before addition, the supernatants were shaken with a drop of toluene to kill any cells that might not have been removed by centrifuging. Residual toluene was then blown off with a stream of air. The plates were incubated at 35° and read after 36–48 hr. Zones of clearing (2–6 mm.) were found with supernatant from normal cells, but none with supernatant from Pr$^-$ strains.

Assay of antibiotic. Antibiotic activity was measured by plating samples of supernatant on nutrient agar plates inoculated with staphylococci as described by Brownlee *et al.* (1948). The test organism was *Staphylococcus aureus* 524.

Measurement of intracellular protein degradation during sporulation. Cells were grown from a small inoculum in hydrolysed-casein medium containing 10 μC of L-[^{35}S]-methionine/100 ml. at 35° with shaking. The cultures were harvested when the density reached 0·25 mg./ml. After centrifuging the tubes were drained carefully to remove as much as possible of the radioactive supernatant. Washing of cells was avoided because it tended to decrease the spore yield. Cells were suspended to the same density in resuspension medium. Unlabelled L-methionine (100 μg./ml.) was present to trap any labelled amino acid released and prevent its reincorporation into protein. The suspension was shaken at 35° and samples (20 ml.) were taken at the beginning of the experiments and at hourly intervals until refractile spores were observable. In some experiments shaking was continued until spore formation was complete.

Each sample was centrifuged and the pellet extracted with trichloroacetic acid and with lipid solvents as described by Mandelstam (1958). The final residue was dissolved in aq. NH_3 (1·5 N) and sufficient transferred to a tared aluminium disk (7·25 cm.^{2}) to give a weight of 1·5–2 mg. after drying.

For the measurement of radioactivity in spores a larger sample (100 ml.) was taken and centrifuged. The pellet was resuspended in 5 ml. of potassium phosphate buffer, pH 7 (0·15 M), containing lysozyme (50 μg./ml.) and trypsin (1 mg./ml.) and incubated at 35° until whole cells and debris had been destroyed (about 20 min.). The spores were washed three times with water (5 ml.) containing carrier L-methionine (100 μg./ml.), and about 2 mg. was plated on an aluminium disk and then weighed and counted in the same way as the protein precipitates.

Radioactivity measurements were corrected for back-

ground. Since the weights of all samples were very similar the small correction for self-absorption of the sample was disregarded. Sufficient counts were collected to ensure that the error was less than 3%.

RESULTS

Transfer of protein from vegetative cells to spores. Experiments carried out by previous workers indicate that the protein in the spore is newly formed and is not carried over from the cytoplasm of the vegetative cells. These experiments (see Young & Fitz-James, 1959; Canfield & Szulmajster, 1964) were done either with different species of bacteria or in complex media. As a preliminary to further work it was important to know whether the same was true for spores formed in the resuspension medium we have described.

Cells were grown up from a small inoculum in hydrolysed casein (200ml.) containing 20μc of [^{35}S]methionine to a density of 0·25mg. dry wt./ml. The cells were suspended in 200ml. of resuspension medium and two samples (20ml.) were removed for determination in duplicate of the specific radioactivity of vegetative-cell protein. The remainder of the suspension (160ml.) was divided into two equal portions. To the first was added carrier L-methionine (100mg./ml.). To the other flask was added 20ml. of hydrolysed casein with radioactive methionine as in the original growth medium. This was to allow spores to be formed in the presence of labelled methionine and hence to get a value for 'fully labelled spores'.

Both flasks were shaken at 35° for 15hr. to allow formation of complete spores, which were then isolated for radioactivity measurement.

The protein from the cells at the time of resuspension had a specific radioactivity of about 1000counts/min./mg. and the spores formed in the presence of radioactive methionine of the same specific radioactivity had a comparable count (Table 1). However, spores that had been formed in the presence of carrier methionine were almost completely unlabelled. From the values it was apparent that only about 5% of the protein in the spore could have been derived from the vegetative cell. However, in other experiments higher values (up to 20%) were obtained.

Degradation of protein in cytoplasm of cells sporulating in resuspension medium. These results could have been due to the formation of new protein that was specific to the spore. Alternatively it was possible that the proteins of the cytoplasm in the cell had gone through a process of turnover and were therefore unlabelled by the time that the later stages of spore formation occurred. The specific radioactivity of the proteins in the spore would then simply reflect the specific radioactivity of the cytoplasm in which it was being formed. It was therefore decided to follow the changes in the specific radioactivity of cytoplasmic proteins during spore formation.

Cells were grown in hydrolysed casein with labelled methionine and then transferred to replacement medium with carrier methionine (see the Materials and Methods section). Samples were taken at zero time and at hourly intervals, usually up to 6 or 7hr., by which time a substantial number of cells contained refractile spores (about 25%). The specific radioactivity of the protein was measured and typical results obtained with the wild-type are shown in Figs. 1 and 2. Loss of radioactive label sometimes occurred linearly and sometimes at a falling rate. The reason for this difference is not known.

The labelled protein was broken down, usually without lag, at a rate of about 8–10%/hr., which is similar to the value obtained by Urbá (1959) with

Table 1. *Transfer of intact protein from vegetative cells to spores*

Proteins of cells were 'fully labelled' by growth from a small inoculum in the presence of [^{35}S]methionine. Duplicate samples were taken for determination of specific radioactivity and the culture was split. One portion was transferred to resuspension medium and allowed to form spores in the presence of [^{35}S]methionine with the same specific radioactivity as that in the growth medium. These spores were thus also 'fully labelled'. The other portion of cells was allowed to sporulate in the presence of excess of unlabelled methionine. Specific radioactivities were determined on both batches of spores.

	Wt. of sample (mg.)	Radioactivity (counts/min.)	Specific radioactivity (counts/min./mg.)	Protein derived from cells (%)
Vegetative-cell protein ('fully labelled')	1·70	1621	953	—
	2·30	2284	993	—
Spores from ^{35}S medium ('fully labelled')	1·99	2725	1368	—
	1·84	2596	1410	—
Spores from ^{32}S medium	1·59	94	59	6
	1·50	85	57	6

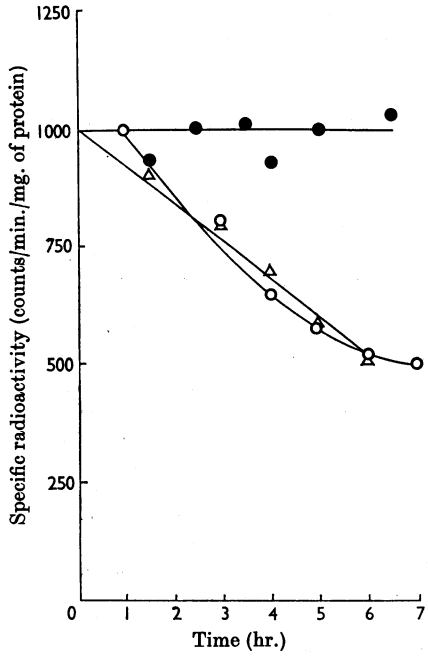

Fig. 1. Degradation of protein in sporulating cells and in two asporogenous mutants. Cells were labelled by growth in the presence of [^{35}S]methionine and sporulation was induced by transferring them to resuspension medium containing excess of unlabelled methionine. The specific radioactivity of protein was measured at intervals. There was no net loss of protein during the experiment. ○, Wild-type, has protease and forms spores; ●, mutant E22, proteaseless and asporogenous; △, E21, has protease, but asporogenous for other reasons.

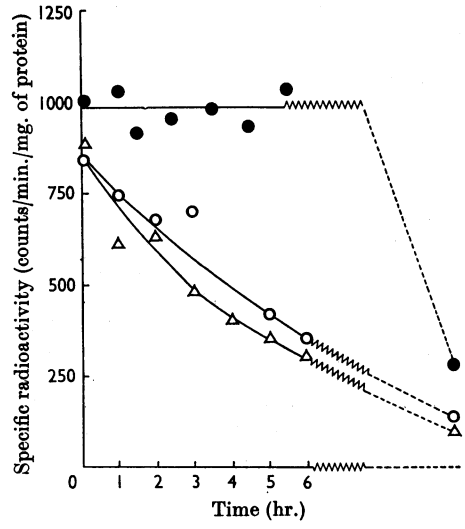

Fig. 2. Effect on protein degradation of reversion from the proteaseless state to wild-type. The procedure is as described in the legend to Fig. 1. ○, Wild-type; ●, mutant 3F1, proteasless and asporogenous; the late breakdown of protein is probably due to another enzyme (see the text); △, 3F1 (rev), a revertant that has regained protease and ability to form spores.

Bacillus cereus. By the time the counts of refractile spores had reached 20–30%, the specific radioactivity of the vegetative-cell protein had fallen by at least 50% and often much more. This represented intracellular turnover of protein, since there was no lysis (at least as measured by turbidity of the culture), nor was there any fall in the total cell protein. Indeed, there was invariably some increase in both cell mass and protein.

Effect of loss of exoprotease activity on intracellular turnover and on other events preceding sporulation. Two proteaseless (*Pr*$^-$) mutants E22 and 3F1 were examined in some detail.

Mutant E22 was very stable and reversion to the wild-type was never observed. Its properties may have been due to a deletion. It grew somewhat more slowly in hydrolysed-casein medium than the wild-type and had a tendency to lyse during prolonged incubation in resuspension medium. It was, however, completely stabilized in the presence of magnesium sulphate (0·04 M). When the stability of cytoplasmic protein was measured as described in the previous experiment no turnover of protein was observable over a period of up to 6 hr. (Fig. 1). In the same period, the wild-type had lost about half of the labelled sulphur from its protein and refractile spores had appeared. Fig. 1 also shows, for comparison, the results obtained with another mutant, E21, which possessed the exoprotease but which was asporogenous for some other reason.

Further, when the supernatant from the control culture was tested on albumin plates for exoprotease activity distinct zones of clearing were produced with all samples taken after 2 hr., and sometimes earlier, whereas corresponding samples of supernatant from mutant E22 produced no sign of protease action even when the plates were incubated for long periods (48 hr.).

There were parallel effects on the production of antibiotic. Samples of the supernatant taken from the wild-type suspension at 2 hr. or later all showed zones of inhibition on plates with *S. aureus* as the test organism, whereas samples from the suspension of mutant E22 had no detectable antibiotic activity.

Finally it was found, as reported by other workers (Schaeffer, 1967; Spizizen, 1965), that loss of exoprotease was associated with an inability to form spores. In the wild-type, refractile spores were usually visible from about 5 hr. onwards and

high yields (50–80%) were obtained after 8–10hr. incubation. Under the same conditions mutant E22 produced no refractile spores even after incubation for 24hr.

Mutant 3F1 was examined in the same way as mutant E22 with the following results. It exhibited no protein turnover during at least the first 6hr. (Fig. 2), though continued incubation did produce protein turnover. This was attributable to the activity of an alkaline protease (see below). Like mutant E22 this mutant produced no measurable exoprotease and no antibiotic and it failed to produce spores.

Consequences of reversion from Pr⁻ mutants to Pr⁺. Mutant 3F1 had a tendency to throw off revertants that had normal exoprotease activity. These were readily detected on albumin plates by the zones of clearing that surrounded them. A number of these revertants were isolated and were all found to have the following properties: degradation of protein in resuspension medium was restored to the normal rate (Fig. 2); antibiotic activity was detectable after 2hr. of incubation; the formation of heat-resistant spores occurred at the same rate and to the same extent as in the wild-type.

Intracellular protease activity in the wild-type and in exoprotease mutants. The experiments at this point suggested that the same protease was responsible for intracellular turnover and for extracellular proteolytic activity. If this were so, cell extracts prepared from the wild-type and from *Pr⁻* mutants should reflect the difference in proteolytic activity shown by the supernatant fluids. Now *B. subtilis* contains at least two proteolytic enzymes, one the neutral protease having maximum activity at about pH7, and the other having maximum activity at pH10 (Hagihara, 1960). The neutral protease, which has been partially purified by McConn, Tsuru & Yasunobu (1964), appears to be the enzyme that is responsible for intracellular protein turnover, and this is the enzyme that is lacking in the *Pr⁻* mutants. Thus, in a standard experiment with resuspension medium, the cell-free extracts of wild-type had a specific activity of 21·5 units of protease/mg. with samples taken at 6hr., whereas mutant E22, sampled at the same time, had a specific activity of 1·4 units/mg. Even this may have been an overestimate, because incomplete precipitation of protein at the end of the test period would have given a falsely high value. The intracellular protease values are thus in keeping with those measured as exoprotease.

Rates of protein synthesis in wild-type and in Pr⁻ mutants in resuspension medium. The experiments described in Table 1 show that, under our conditions, spore formation involves the synthesis of new proteins. The apparent requirement of protease for sporulation could have a number of explanations,

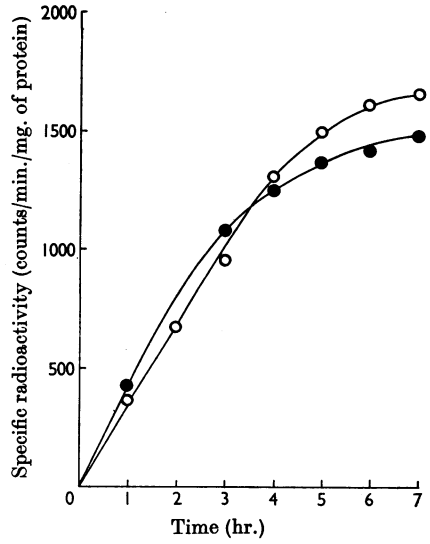

Fig. 3. Synthesis of protein in sporulating cells and in an asporogenous mutant. Unlabelled cells were induced to sporulate by transferring them to resuspension medium containing [^{35}S]methionine. The specific radioactivity of protein was measured at intervals. ○, Wild-type; ●, mutant E22, proteaseless and asporogenous. There were always increases in the total protein; in typical experiments these were 40–80% in the wild-type and 40–60% in mutant E22.

of which the most obvious is the following. In resuspension medium the possibilities for protein synthesis are greatly restricted and, in fact, the total protein increases only about 50% in 6hr., i.e. in six generation times. It was therefore conceivable that the new proteins required for making the spore could only by synthesized at the expense of the existing proteins of the vegetative cells. This would require the intervention of the protease and so be precluded in *Pr⁻* mutants.

This possibility was tested by measuring the protein-synthetic capacities of the two *Pr⁻* mutants. The organisms were grown in hydrolysed-casein medium without radioactive supplement and then transferred to resuspension medium containing 10 μc of [^{35}S]methionine/100ml. (final concentration 100 μg./ml.). The flasks were shaken and samples taken at hourly intervals for measurement of specific radioactivity of the protein.

In mutant E22 the rate of protein synthesis, as measured by incorporation, was consistently as high as in the wild-type (Fig. 3). Further, in this mutant net synthesis of protein occurred in much the same way as in the wild-type, and the values for two typical experiments are given in the legend to Fig. 3.

In mutant 3F1 the capacity for protein synthesis

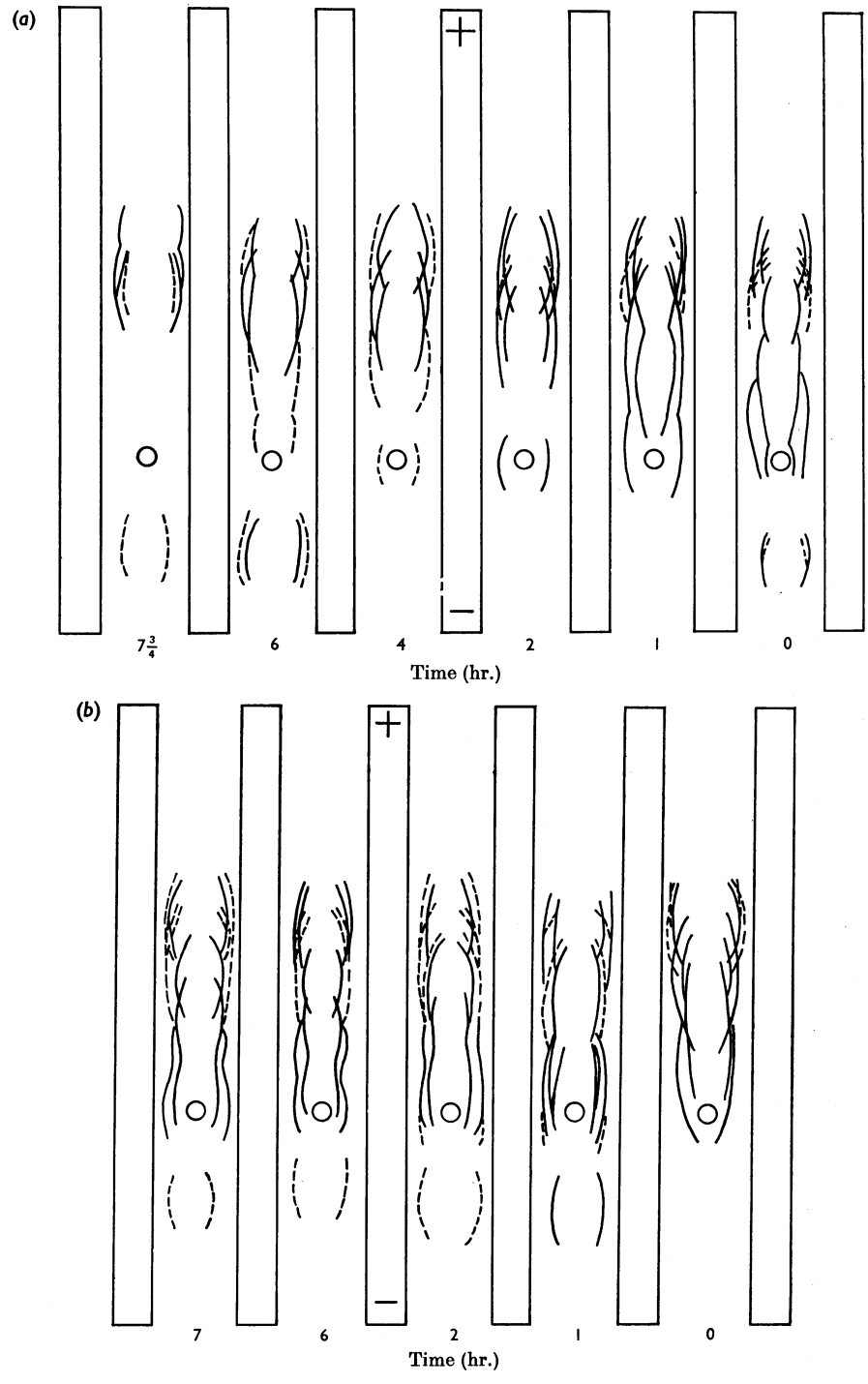

Fig. 4. Effect of loss of protease activity on the disappearance of vegetative-cell antigens during sporulation. (*a*) Wild-type cells grown in a hydrolysed-casein medium were transferred to resuspension medium to induce sporulation. Samples were removed at 0, 1, 2, 4 and 6 and 7¾ hr., and extracts from them examined by immuno-electrophoresis by using rabbit antiserum prepared against soluble proteins of the vegetative cells. (*b*) A similar experiment was done with the proteaseless mutant E22 but the last sample was taken at 7 hr. For details see Waites (1968).

in resuspension medium was usually 30–50% less than in the wild-type, and very variable from day to day. Both the low values and the variability could be attributed to the fact that this mutant had a greater tendency to lyse than mutant E 22. It was not investigated further.

Antigenic pattern of protein in the sporulating cell. The results described so far indicate that, during sporulation, the wild-type organism was degrading its protein extensively. However, this must have been more than balanced by protein synthesis. This is evident from the fact that the total protein increased appreciably during the incubation. It was desirable to know whether the cells were using their synthetic capacity to replace the vegetative-cell proteins they were degrading with proteins of the same type. Since the total amount of protein usually increased by at least 50%, they would have been able to do this and, in addition, to manufacture such new types of protein as were required for the spores. This problem could best be answered by the use of immunological methods and experiments of the following type were done.

Cells grown in germination medium were transferred to resuspension medium in the usual way and samples (300ml.) were taken at intervals. After centrifuging, the bacteria were disrupted and the supernatant obtained after high-speed centrifuging was examined by immunoelectrophoresis with an antiserum made against vegetative-cell extract (Waites, 1968). It was found that the precipitin lines produced by vegetative-cell proteins became progressively fainter during the incubation until at 7¾hr. they had virtually disappeared (Fig. 4). As a check, the experiment was also done with one of the proteaseless mutants, E 22, and as expected there was no comparable destruction of vegetative-cell protein. It is shown elsewhere (Waites, 1968) that at the same time the wild-type cells were synthesizing antigens of the spore types whereas the proteaseless mutant was not.

DISCUSSION

The control of protein stability has been studied extensively in other types of micro-organisms (see review by Mandelstam, 1960). In most of these, the imposition of conditions that stop or restrict growth will cause the onset of intracellular proteolysis, which, in *Escherichia coli*, proceeds at about 5%/hr. (Mandelstam, 1958; Borek, Ponticorvo & Rittenberg, 1958; Willetts, 1967). In Gram-positive bacilli the rate may be even higher. For example, Urbá (1959) reported a value of 8%/hr. for *B. cereus* and Monro (1961) an initial value of 10%/hr. falling after a while to 3%/hr. for *Bacillus thuringiensis*. Our results (Figs. 1 and 2) show that *B. subtilis* in resuspension medium degrades protein at a similar rate. The consequence of this is that by the time the protein layers of the spore coat begin to be formed, i.e. at about 6hr., the cytoplasm has lost at least half of its labelled methionine and the later stages of spore synthesis, 7–10hr., occur inside a cell containing even less. A similar rate of protein turnover during sporulation in *Bacillus mycoides* was found by Foster & Perry (1954).

The fact that most of the protein of the vegetative cell has been degraded by the time the refractile spore begins to be formed accounts for the general observation (Canfield & Szulmajster, 1964; Monro, 1961; Young & Fitz-James, 1959) that the proteins of the spore are 'new' in the sense of newly synthesized. Since, at this stage, the same is true of all the proteins, whether in the spore or in the surrounding cytoplasm, we are still left with the question whether the proteins are 'new' in the sense of different.

There are two obvious ways in which this question can be answered. The first is to choose a number of specific enzymes and to compare the properties of those extracted from the vegetative cell with those extracted from the spore or from the sporangium, i.e. the cytoplasm of the sporulating cell. Careful comparisons of this kind have failed to reveal any differences for a number of enzymes. Examples are the purine nucleoside phosphorylase of *B. cereus* (Gardner & Kornberg, 1967), the DNA polymerase of *B. subtilis* (Falaschi & Kornberg, 1966) and the inorganic pyrophosphatase of *Bacillus megaterium* (Tono & Kornberg, 1967). In the light of these studies it is reasonable to assume that at least some of the spore enzymes, though newly synthesized, are nevertheless the same protein types as the corresponding enzymes of the vegetative cells. Others, however, are almost certainly different, and this is based on results obtained with the other method of looking for differences in proteins, i.e. by immunological methods. Baillie & Norris (1964) showed that the spores of *B. cereus* contain a number of antigens that are immunologically distinct from those of the vegetative cell, and in a number of these, including catalase, this difference is associated with a different degree of heat-stability (Norris & Baillie, 1964). Cavallo, Falcone & Imperato (1963) have shown, and Waites (1968) has confirmed, that the spores of *B. subtilis* are characterized by antigens that differ from those of the vegetative cell and that these can begin to appear in the sporulating cell before refractile spores become visible in the microscope.

The combination of immunological and biochemical studies points to the conclusion that, during sporulation, some new, i.e. spore-specific, proteins are formed that are probably also present in the sporangial part of the cell, and that, though the vegetative-cell proteins are largely degraded,

some of them at least are resynthesized and incorporated into the spore. We have, however, no information about the rates at which these vegetative-cell-type proteins are being regenerated. The immunoelectrophoretic pattern (Fig. 4) showed a progressive disappearance of the precipitin lines of the vegetative-cell proteins. This could mean that the proteins concerned are not being regenerated at all or, if they are, that the rate is much lower than the rate of destruction. It is also possible that these proteins are atypical.

Finally we come to the possible role of the exoprotease. It seems fairly clear that, whatever other function it might have, the exoprotease is responsible for the intracellular turnover of protein and for the disappearance of some types of protein antigens. In mutants that have lost the ability to make the enzyme there is not only no turnover of protein but there is a concomitant inability to form spores. Further, back-mutations in mutant 3F 1 that restore the ability to make the exoprotease restore protein turnover and all the other activities associated with spore formation. These results are in keeping with the reports mentioned in the introduction.

However, it is still not certainly known why the protease is needed for sporulation. We first worked on the assumption, made by Mandelstam (1960), that protein turnover in sporulation was essential because the cells had to make new and different proteins and that they could do so only by degrading their existing proteins. This might be true under conditions of endotrophic sporulation, i.e. sporulation in water or in buffered solutions where net protein synthesis is impossible. It is not true for the conditions that exist in the resuspension medium because the glutamate and NH_4^+ ions supply sufficient carbon and nitrogen for a considerable net synthesis (50% or more) of protein. Since the spore is a much smaller object than the bacterial cell this should be ample to supply all the protein needed for incorporation into the spore. Further, the proteaseless mutant E 22 is in no way defective in its ability to make new protein in the resuspension medium (Fig. 3). It is therefore necessary to find some other reason for the fact that loss of protease entails an inability to make spores.

One possibility is that sporulation is inhibited in the vegetative cell by one or more protein inhibitors and that, in the resuspension medium, these are degraded by the protease in the same way as the other vegetative-cell proteins and are either not resynthesized at all or anyway not fast enough to maintain them at the necessary concentration. Their removal by the proteolytic enzyme would then allow the spore function to be expressed. The existence of such inhibitors would manifestly be difficult to demonstrate, but this explanation seems to be consistent with the facts that we know at present. An alternative possibility is that the protease has no essential function in sporulation and is merely one of an ordered sequence of biochemical events. We could then say that the proteaseless mutants have been damaged in some regulatory function so that the expression of the whole sequence of biochemical events (some essential and some not) leading to sporulation cannot occur. The fact that the protease is one of these events might then be regarded as more or less inadvertent.

Whatever the explanation it is clear that the protease enables the cells to change the immunoelectrophoretic pattern of their protein from that which characterizes the vegetative cells to that which characterizes the spores: loss of the proteolytic function results in retention of the vegetative-cell pattern and complete suppression of the events leading to sporulation.

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