

Commitment to Sporulation in *Bacillus subtilis* and its Relationship to Development of Actinomycin Resistance

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1. Experiments to determine the point of commitment to sporulation were carried out by restoring nutrients at different times to suspensions of sporulating *Bacillus subtilis*. 2. No single point of commitment to the process as a whole was found. Instead, the cells became committed in turn to the following successive events connected with sporulation: formation of alkaline phosphatase, development of refractility, synthesis of dipicolinic acid and development of heat-resistance. 3. Each point of commitment was followed within about 30 min. by a period in which the event concerned ceased to be inhibited by actinomycin D. 4. The implication of these results is that each point of commitment is probably due to the formation of a species of long-lived messenger RNA and that, in any case, sporulation is regulated at the level of both transcription and translation. 5. It is also shown that sporulation and growth are perhaps not mutually exclusive functions and that histidase, an enzyme typical of the vegetative state, can be induced in sporulating suspensions.

Commitment is a biological term that has been used to describe a 'point of no return' in differentiating systems such as slime moulds and sporulating bacteria (Foster, 1956), but it has never been biochemically defined. The time at which it occurs in *Bacillus cereus* was measured by Hardwick & Foster (1952) as follows. The bacteria were suspended in a solution lacking glucose, to induce sporulation. Then, at various times, glucose was restored to the medium. If the addition was made at any time up to 5 hr. sporulation was inhibited, whereas later additions had progressively less effect. At 7 hr., when the population was completely committed, the cells were incapable of forming the inducible 'vegetative enzyme' maltase.

The present paper describes experiments done to determine whether a specific point of commitment could be found in *Bacillus subtilis*. When this organism is transferred to a deficient medium a sequence of biochemical events is initiated leading to sporulation (Schaeffer, Ionesco, Ryter & Balassa, 1965; Szulmajster, 1964; Warren, 1968). Of these we have examined the appearance of alkaline phosphatase, refractility, the formation of DPA,* and the development of heat-resistance. There is no single point of commitment for all these events. Instead, the cells become committed to each of them in turn and each point of commitment is

associated with the development of resistance to actinomycin D of the event concerned.

These results have been reported in a preliminary form (Mandelstam, Waites, Warren & Sterlini, 1968; Mandelstam & Sterlini, 1969).

METHODS

Organism and culture. *B. subtilis* 168 (Marburg) is an auxotrophic mutant blocked in the formation of indole. It grew well in a medium supplemented with either indole or tryptophan. It was grown with shaking at 37° in a medium containing (per l.): hydrolysed casein (Difco Casamino [Acids, vitamin-free], 8.6 g.; L-glutamic acid, 3.16 g.; DL-alanine, 2.14 g.; L-asparagine monohydrate, 1.2 g.; KH₂PO₄, 1.36 g.; Na₂SO₄, 0.107 g.; FeCl₃.6H₂O, 0.6 mg.; NH₄Cl, 0.535 g.; NH₄NO₃, 0.096 g.; MgSO₄.7H₂O, 0.0986 g.; CaCl₂.6H₂O, 0.02 g.; MnSO₄.4H₂O, 0.022 g.; L-tryptophan, 0.02 g. The pH was adjusted to 7.1. When the density of organisms had reached 0.25 mg. dry wt./ml., the culture was centrifuged and the cells were transferred to the same volume of a medium based on that of Donnellan, Nags & Levinson (1964) but having a higher concentration of Mg²⁺ and no glucose. Its composition (per l.) was as follows: FeCl₂, 0.046 mg.; MgSO₄, 4.8 g.; MnCl₂, 12.6 mg.; NH₄Cl, 535 mg.; Na₂SO₄, 106 mg.; KH₂PO₄, 68 mg.; NH₄NO₃, 96.5 mg.; CaCl₂, 219 mg.; L-glutamic acid, 2 g.; L-tryptophan, 20 mg. The pH was adjusted to 7.1 with KOH. This is referred to below as 'resuspension medium' and, unless otherwise stated, all experiments were done with bacteria in this medium in which shaking at 37° generally gave a yield of about 80% spores in 8 hr.,

* Abbreviations: DPA, dipicolinic acid; m-RNA, messenger RNA.

though in some experiments the time taken to reach this yield was 1-2 hr. longer.

Estimation of incidence of spores. Refractile spores were counted in the phase-contrast microscope and heat-resistant spores were counted on agar plates after the suspension had been heated at 80° for 10 min. (see Mandelstam & Waites, 1968).

Alkaline phosphatase assay. Samples (15 ml.) were centrifuged and the cells resuspended in 5 ml. of 1.0 M-tris-HCl buffer, pH 8.0. Toluene (2 drops) was added, and the tubes were stoppered, shaken for 30 sec. and kept at 37° for about 30 min. Then 1.0 ml. of the suspension (or a suitable dilution of it) was added to 1.0 ml. of tris buffer and 1.0 ml. of 0.1% *p*-nitrophenyl phosphate (Torriani, 1960). The reaction mixture was incubated at 37° until enough colour had developed. The time was noted and the reaction stopped with 1.5 ml. of 2 M-NaOH. A pinch of BaCO₃ was added to the samples, which were centrifuged and the *E*₄₁₀ of the supernatant was read within 1 hr., as the colour continued to develop slowly even after the addition of alkali. Readings were corrected for blanks set up with the omission of the cell suspension. One enzyme unit liberates 1 nmole of *p*-nitrophenol/min.

Histidase assay. This was assayed by a modification of the method of Hartwell & Magasanik (1963). Samples (2.5 ml.) were centrifuged and the pellet was cooled immediately and resuspended in 5 ml. of cold 0.1 M-sodium phosphate buffer, pH 7.0. Toluene (2 drops) was added, and the tubes were stoppered and shaken hard. After 5 min. the toluene was blown off by bubbling air through the suspension, 1.0 ml. of which was then added to a reaction mixture containing 0.2 ml. of 0.1 M-histidine, 0.2 ml. of 1.0 M-diethanolamine (adjusted to pH 9.4 with HCl) and 0.6 ml. of water. The incubation was carried out at 30° for a suitable time (5-180 min., depending on the activity) and the reaction stopped by the addition of 0.6 ml. of 12% (w/v) HClO₄. The suspension was centrifuged and the urocanic acid that had been formed was determined from the *E*₂₆₈ value. Readings were corrected for blanks in which HClO₄ had been added at the beginning of the incubation before the cell suspension. One enzyme unit converts 1 nmole of histidine into urocanic acid/min.

Determination of DPA. Samples (65 ml.) were centrifuged and the bacterial pellet was suspended in water (1.5 ml.). The tube was covered with tinfoil and autoclaved for 60 min. at 10 lb./in.² pressure. Then 0.15 ml. of 0.2 M-acetic acid was added and the suspension centrifuged. A portion of the supernatant (0.5 ml.) was then treated with 0.1 ml. of a solution containing Fe(NH₄)₂(SO₄)₂ and ascorbic acid (Janssen, Lund & Anderson, 1958). The *E*₄₄₀ value was measured. A standard curve was prepared with commercial DPA.

Measurement of bacterial growth. This was measured spectrophotometrically by using a calibration curve relating *E*₆₀₀ to bacterial dry weight.

Incorporation of [³⁵S]methionine and of [¹⁴C]adenine by sporulating cells. Resuspended cells were incubated with [³⁵S]methionine (0.1 μC/ml.; final concn. 100 μg./ml.). Samples (10 ml.) were taken at intervals, treated with trichloroacetic acid and lipid solvent and prepared for counting as described by Mandelstam & Waites (1968).

For adenine incorporation, the incubation was carried out in the presence of [¹⁴C]adenine (0.02 μC/ml.; final concn. 25 μg./ml.). Samples (10 ml.) were taken and added to

1.2 ml. of 50% (w/v) trichloroacetic acid. The precipitates were washed twice with 5% trichloroacetic acid (containing carrier adenine at 40 μg./ml.) and then with ethanol. The residue was quantitatively transferred with small washings of aq. 2 M-NH₃ to aluminium planchets and dried at 100° for counting. It was assumed that all incorporation was into RNA since it was known that at the stage of sporulation at which these experiments were done synthesis of DNA had ceased (J. Mandelstam & J. M. Sterlini, unpublished work).

RESULTS

Commitment time for refractility: measurement by enrichment of culture. Commitment was measured by determining the point at which development of refractility could no longer be prevented by adding hydrolysed casein to the sporulating culture. The procedure was as follows. Resuspended cells were divided into portions (50 ml.), which were shaken in flasks at 35°. To the first flask, at zero time, hydrolysed casein (5.5 ml. of a 10% solution, w/v) was added, and the succeeding flasks were treated in the same way at hourly intervals. Small samples

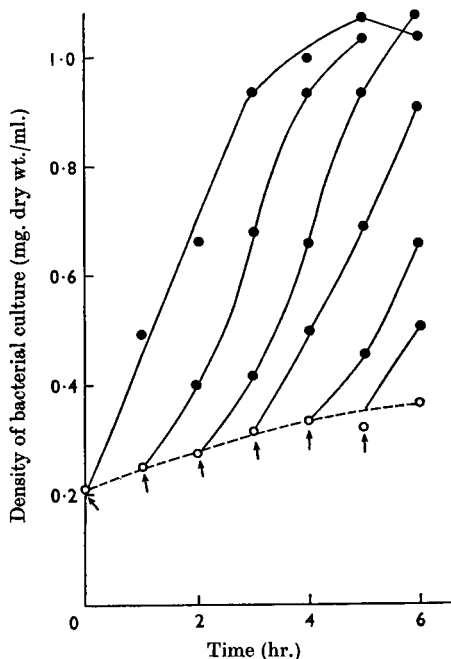


Fig. 1. Ability of cells initiated to sporulation to grow. Bacteria were transferred to resuspension medium to initiate sporulation. At hourly intervals (↑) a portion of the culture was treated with hydrolysed casein to restore growth conditions, and subsequent growth of the cultures was then followed. This is the same experiment as that described in the legend to Fig. 2. ○, Culture in resuspension medium; ●, cultures under growth conditions.

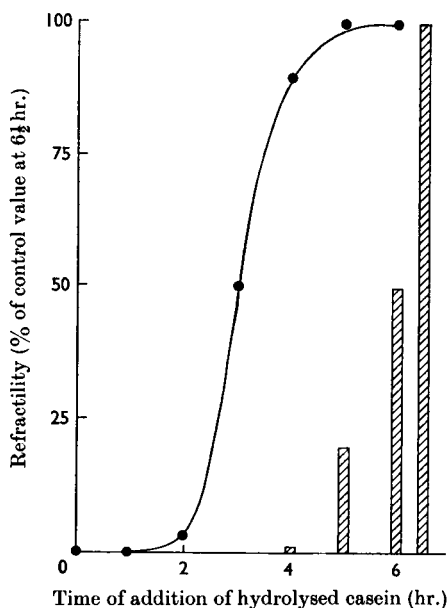


Fig. 2. Determination of commitment time for refractility. Bacteria were transferred to resuspension medium to initiate sporulation. At hourly intervals a portion of the culture was supplemented with hydrolysed casein. At the end of 6½ hr., when 50% of the cells in the control portion of the culture contained refractile spores, the experiment was stopped and spores were counted in all cultures. The continuous curve (●) represents spore counts in the treated cultures at 6½ hr.; hatched rectangles (▨) show the time-course of development of refractile spores in the control culture. Growth of each culture is shown in Fig. 1, and comparison of the two figures indicates that commitment to refractility occurred after 2 hr. but that the cells retained the ability to grow for at least another 3 hr.

(0.5 ml.) were taken periodically for measurement of bacterial growth and for spore counts. At 6½ hr., when 50% of the cells in the control flask had refractile spores, the experiment was stopped and the spores were counted in all cultures. The effect on growth of addition of hydrolysed casein is shown in Fig. 1. The cells retained the capacity to resume growth with little or no lag even after 5 hr. in the resuspension medium. At this point, in most experiments, the refractile-spore count had reached 20% or more.

The effect of addition of hydrolysed casein on the final spore yield in the same experiment gave a fairly well-defined commitment time. Thus (see Fig. 2) the addition of hydrolysed casein at any time before 2 hr. prevented almost completely the appearance of spores. After this time hydrolysed casein had progressively less of an inhibitory effect and, after 4 hr., the spore count reached a value

about as high as that of the control. The commitment time measured in a number of experiments occurred usually at 2–3 hr., but varied somewhat from day to day. Fig. 2, which shows the rate of appearance of spores in the untreated control organisms, also indicates that at 4 hr., when the cells were almost completely committed, almost no refractile spores were yet visible.

Relationship of commitment time for refractility to actinomycin D resistance. Aronson & del Valle (1964) found that once sporulation was established in *B. cereus*, the rest of the process was resistant to actinomycin D, and this implied that a stable m-RNA was involved.

It seemed possible that the commitment measured in our experiments might coincide with the formation of stable m-RNA. However, Szulmajster, Canfield & Blicharska (1963), working with the same strain of *B. subtilis* as we were using, found that actinomycin D inhibited sporulation whenever it was added. They concluded that the m-RNA molecules in sporulation had the same short half-life (a few minutes) as the m-RNA of vegetative cells. The apparent discrepancy between these results could have been due to the fact that the concentration of actinomycin D used (10 µg./ml.) might have been more toxic to *B. subtilis* than to *B. cereus*.

Experiments were therefore done to determine the lowest concentration of actinomycin that would inhibit sporulation without causing general cell damage. Resuspended cells, in the presence of actinomycin at concentrations up to 5 µg./ml., were placed in 2 ml. amounts in test tubes fixed at an angle of 45° and shaken to obtain good aeration. Concentrations of actinomycin below 0.5 µg./ml. did not completely inhibit sporulation, whereas those above 1.0 µg./ml. caused lysis, as measured by a fall in extinction of the culture. Further, before there was any measurable effect on extinction, microscopic observation showed that many of the cells were pale when viewed by phase-contrast microscopy and were obviously damaged. Higher concentrations of actinomycin (10–15 µg./ml.) caused more rapid and more extensive lysis. The experiments described below cannot be compared with those of Balassa (1963), who used even larger amounts of actinomycin (40 µg./ml.).

As a result of these experiments a final actinomycin concentration of 1 µg./ml. was used in experiments of the following type. Two parallel sets of resuspended cell cultures (2 ml. each) were set up. The first was used for determination of the commitment time with hydrolysed casein as described above. To the tubes in the second series, additions of actinomycin were made at the same time-intervals. The experiments were stopped at 7½ hr. and refractile spores were counted in all

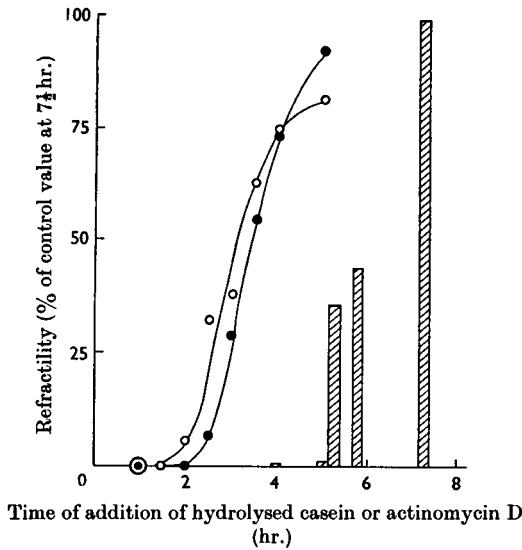


Fig. 3. Relationship between commitment time for refractility and development of resistance to actinomycin D. Cells were transferred to resuspension medium to initiate sporulation. At intervals portions of the culture were treated with hydrolysed casein (cf. Figs. 1 and 2). A parallel set of samples was treated with actinomycin D. At $7\frac{1}{2}$ hr. when 80% of the cells in the control culture contained refractile spores the experiment was stopped and the spores were counted in all cultures. The continuous curves represent spore counts in the two sets of treated cultures at $7\frac{1}{2}$ hr.; hatched rectangles (■) show the time-course of development of refractile spores in the control culture. ○, Hydrolysed casein; ●, actinomycin D.

cultures. Actinomycin D, added in the initial stages of incubation, prevented the emergence of spores. However, a definite point of 'escape' occurred after 2 hr. and the actinomycin then ceased to be inhibitory (Fig. 3). This point was about 20 min. after the commitment time obtained with hydrolysed casein in the same experiment. A number of experiments were done, which all resulted in a pair of curves that were roughly parallel. The commitment time measured with hydrolysed casein always preceded the time of onset of resistance to actinomycin D. The two curves were integrally related, as shown by the fact that in the day-to-day variation obtained in these experiments they were both displaced in the same way and were always separated by an interval of 20–30 min.

Also, as before, commitment to refractility preceded the actual appearance of refractile spores by about 2 hr. The implications of this observation are considered in the Discussion section.

Commitment times for formation of alkaline phosphatase and DPA, and development of heat-resistance.

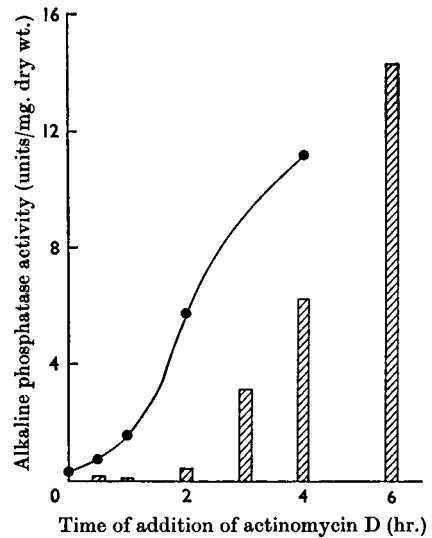


Fig. 4. Effect of actinomycin D on ability of initiated cells to form alkaline phosphatase. Cells were transferred to resuspension medium to initiate sporulation and portions of the culture were treated at intervals with actinomycin D. At 5 hr. the experiment was stopped and alkaline phosphatase activity was measured in all cultures. The continuous curve (●) represents the enzymic activity in treated cultures at the end of the experiment; hatched rectangles (■) show the time-course of enzyme synthesis in the control culture.

Whether there was a single point of commitment for the whole process of sporulation remained to be determined. In *B. subtilis* this process involves a definite sequence of events among which the formation of alkaline phosphatase precedes the appearance of refractility, whereas the formation of DPA and the development of heat-resistance occur later (see Warren, 1968; Kay & Warren, 1968; Mandelstam, 1969). The commitment point for each of these additional events was therefore determined with actinomycin D. It was not possible to determine commitment for all these events in any one experiment. It was therefore decided to use refractility as a standard event that was measured in all experiments and to relate to it the commitment time for the other events.

A typical result for alkaline phosphatase is shown in Fig. 4, in which the commitment time for the enzyme occurred soon after the beginning of the incubation whereas that for refractility occurred, as described above, at about $2\frac{1}{2}$ hr. When further comparative experiments were done to determine the effects of hydrolysed casein and actinomycin D on the formation of alkaline phosphatase the results confirmed those already obtained for refractility,

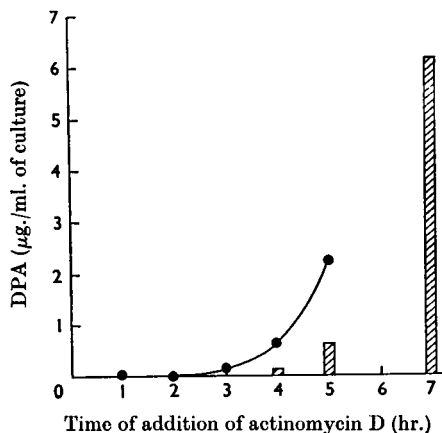


Fig. 5. Effect of actinomycin D on ability of initiated cells to form DPA. The procedure was as described in the legend to Fig. 4, except that the experiment was continued for 7 hr. The continuous curve (●) represents DPA in actinomycin-treated cultures at the end of the experiment; hatched rectangles (▨) show the time-course of DPA synthesis in the control culture.

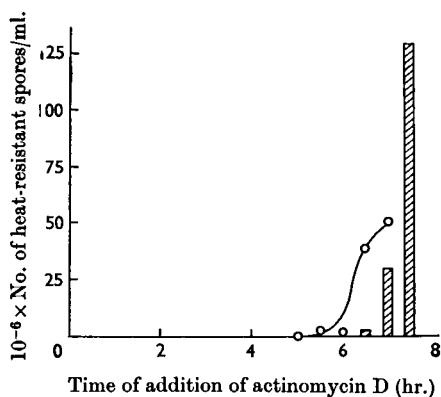


Fig. 6. Effect of actinomycin D on ability of initiated cells to form heat-resistant spores. The procedure was as described in the legend to Fig. 4, except that the experiment was continued for 8 hr. The continuous curve (○) represents the incidence of heat-resistant spores in actinomycin-treated cultures at the end of the experiment; hatched rectangles (▨) show the time-course of development of heat resistance in the control culture.

i.e. the point of commitment obtained by adding hydrolysed casein preceded the development of resistance to actinomycin D by about 30 min. and both of these events were largely completed before the enzyme began to be synthesized.

The commitment times for DPA formation and

for heat-resistance relative to that for refractility were found from similar experiments and they occurred at about 4 and 5½ hr. (Figs. 5 and 6). Again there was some day-to-day variation.

Effect of actinomycin D on incorporation of [¹⁴C]-adenine and of [³⁵S]methionine by sporulating cells. From these results it appeared that sporulation might involve the formation of a stable species of m-RNA, but it was necessary to rule out the possibility that the effect was non-specific. A non-specific effect could have been due to inactivation or destruction of the ribonuclease in the sporulating cells. This would have conferred stability on all types of m-RNA and made protein synthesis in general resistant to actinomycin. To check this possibility the effect of actinomycin D on the incorporation of methionine into protein was determined. It was also necessary to know that the amount of actinomycin added was sufficient to cause a substantial decrease in the rate of RNA synthesis.

Bacteria were transferred to resuspension medium and incubated for 1½–2½ hr. to get the culture to the point where commitment to refractility might be expected to have taken place. At this stage the radioactive material was added and samples were taken immediately and at short intervals thereafter (see Figs. 7 and 8). At the point indicated by the arrow the culture was divided into two portions, to one of which actinomycin D was added. Incubation was continued and further samples were taken. The results were very similar with cells that had been in resuspension medium for 1½ or 2½ hr. The drug had a very rapid effect on the incorporation of adenine, which stopped immediately and then recovered to about 20% of the control value (Fig. 7). When similar experiments were done with [¹⁴C]uracil, actinomycin produced the same immediate effect, but the subsequent recovery was more variable and the rates were 20–40% of the control values.

The effect of actinomycin on protein synthesis was more lasting as well as more reproducible. A small amount of methionine was incorporated during the 10 min. after addition of the drug (Fig. 8). After that the rate of incorporation was inhibited by 95% and sometimes more.

A similar experiment was carried out to determine the effect of actinomycin D on protein synthesis in an actively growing culture. Cells growing in hydrolysed-casein medium were treated with radioactive methionine (0.1 µC/ml.). Since the medium already contained carrier methionine no more was added. After a short time the culture was divided into two portions, one of which was treated with actinomycin as before. The effect on methionine incorporation was very similar to that obtained in sporulating cells (i.e. almost complete

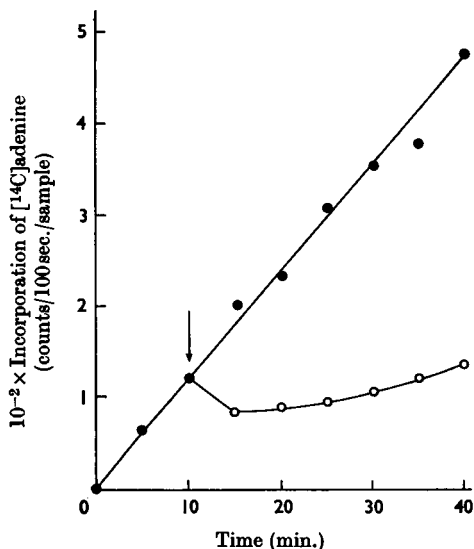


Fig. 7. Effect of actinomycin D on incorporation of $[^{14}\text{C}]$ adenine into acid-insoluble material of initiated cells. To bacteria that had been in resuspension medium for $2\frac{1}{2}$ hr. $[^{14}\text{C}]$ adenine was added and its incorporation was measured for 10 min. The culture was divided and one portion treated with actinomycin. \circ , Actinomycin D; \bullet , control.

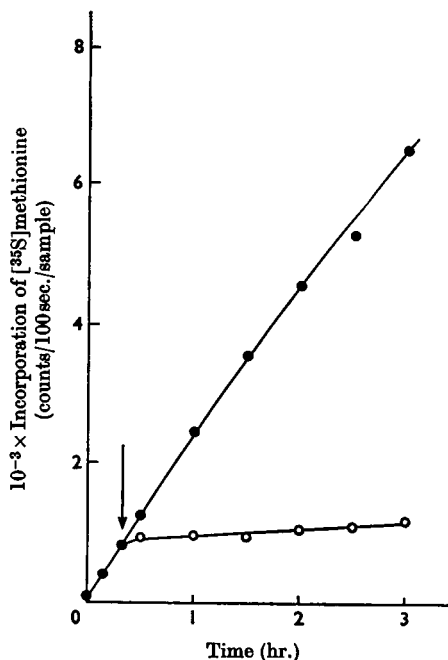


Fig. 8. Effect of actinomycin D on incorporation of $[^{35}\text{S}]$ methionine into protein of initiated cells. The procedure was as described in the legend to Fig. 7, with adenine replaced by $[^{35}\text{S}]$ methionine. \circ , Actinomycin D; \bullet , control.

inhibition), and the results did not differ significantly from those reported by Levinthal, Keynan & Higa (1962) with another strain of *B. subtilis*.

Effect of actinomycin D on the induction of histidase. The experiments with methionine showed that the major part of protein synthesis was inhibited by actinomycin D. It was desirable, however, to extend this observation to include a characterized protein. For this, the inducible enzyme histidase was chosen as representing a normally vegetative function of the cells.

Bacteria that had been in resuspension medium for $2\frac{1}{2}$ hr., and in which therefore sporulation had been initiated, were treated with histidine ($500\ \mu\text{g./ml.}$). Then 30 min. later, when induced formation of the enzyme was well established, the culture was divided and actinomycin ($1\ \mu\text{g./ml.}$) was added to one portion. The drug allowed a small increase in enzyme during the first 8 min., but after this inhibition was complete (Fig. 9). The result closely resembled that obtained for methionine incorporation. The slow fall in activity after 80 min. is attributable to protein degradation, which is known to proceed at an appreciable rate under these conditions (Mandelstam & Waites, 1968).

Comparison of effects of actinomycin D and of chloramphenicol on the rate of formation of alkaline phosphatase and of refractile spores. The results of the experiments so far described could be explained in either of two ways. (a) The proteins required for sporulation were exceptional in their synthesis,

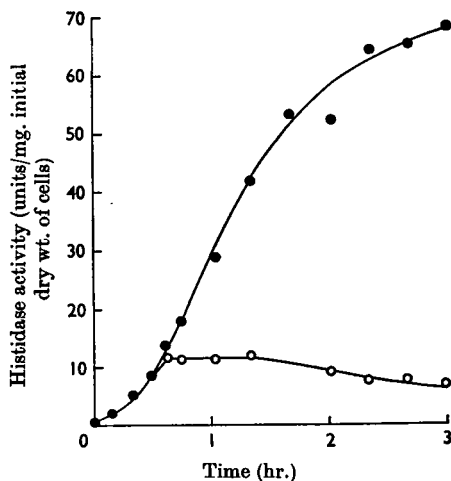


Fig. 9. Effect of actinomycin D on induction of histidase in a sporulating culture. To bacteria that had been in resuspension medium for $2\frac{1}{2}$ hr., histidine was added, and induced formation of histidase was allowed to proceed for 30 min. The culture was split and actinomycin added to one portion. \circ , Actinomycin D; \bullet , control.

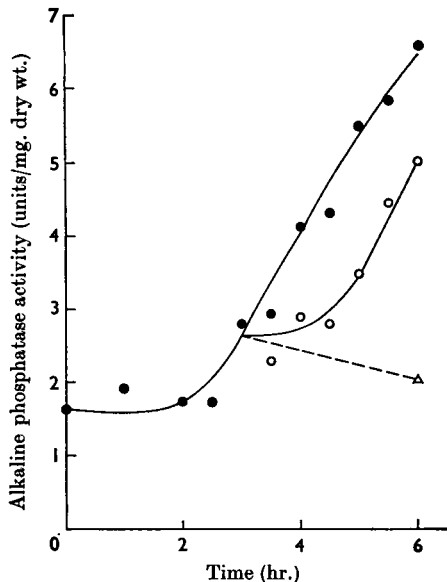


Fig. 10. Effect of actinomycin D and of chloramphenicol on the formation of alkaline phosphatase in a sporulating culture. After the bacteria had been in a resuspension medium for 3 hr. and the characteristic increase in amount of alkaline phosphatase had begun, the culture was divided into three portions, which were treated as follows: ○, actinomycin D; △, chloramphenicol; ●, control.

being resistant to inhibition by actinomycin though protein synthesis in general was as susceptible to actinomycin in sporulating cells as in growing cells. (b) The spore proteins were not exceptional and their synthesis had been just as much affected as that of all other proteins, and what had been observed was the assembly of protein subunits that had already been formed by the time the actinomycin was added.

To examine the latter possibility the following experiment was done. Cells were incubated in resuspension medium for 3 hr. so that they had reached the point when the production of alkaline phosphatase at a steady rate had been established. The culture was then divided into three portions. To the first actinomycin was added, to the second chloramphenicol (final concn. 50 $\mu\text{g./ml.}$) was added, and the third was the control. Incubation was continued and further samples were taken for assay of alkaline phosphatase. Actinomycin caused transient inhibition of formation of alkaline phosphatase. Then the cells recovered and enzyme synthesis proceeded at a rate not far removed from that of the control (Fig. 10). Even after a further 2 hr. of incubation, synthesis of alkaline phosphatase was still proceeding at a linear rate. This contrasts markedly with the behaviour of the

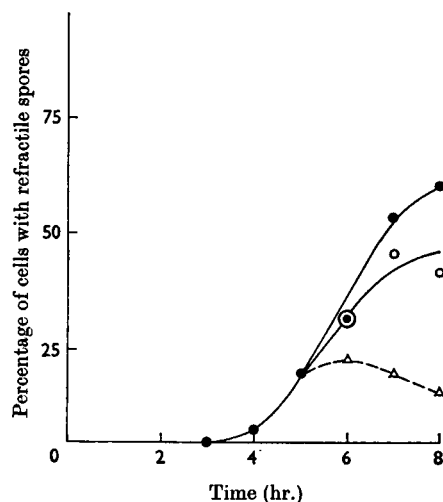


Fig. 11. Effect of actinomycin D and of chloramphenicol on the formation of refractile spores. The procedure was as described in the legend to Fig. 10 except that the culture was divided and treated with the drugs at 5 hr.: ○, actinomycin D; △, chloramphenicol; ●, control.

proteins generally and of induced histidase in particular (cf. Figs. 8 and 9). That the actinomycin in this experiment was active was shown by the fact that when it was added to another portion of the culture at the beginning of the experiment it completely prevented appearance of the enzyme. Fig. 10 also shows that addition of chloramphenicol at 3 hr. prevented further formation of alkaline phosphatase, making it clear that the appearance of enzyme in the actinomycin-treated culture required protein synthesis and was not due to the assembly of preformed subunits.

A similar experiment with actinomycin and chloramphenicol was then done by using refractility as the indicator, although this is, biochemically, an ill-defined event. Nevertheless the two drugs had the same qualitative effects as before (Fig. 11): after addition of actinomycin D refractile spores continued to be made at a rate comparable with that of the control culture, whereas chloramphenicol added at the same time was almost totally inhibitory. It also seems that the incomplete spores present at the time were unstable because the number of refractile bodies fell by about 50% in the last 2 hr. of the experiment.

DISCUSSION

The term 'commitment' has never been precisely defined, but is generally assumed to mean a state in which the metabolism of the cells is so

definitely channelled in the direction of sporulation that it cannot be reversed by enrichment of the medium. The experiment illustrated in Fig. 2 shows that there is indeed such a point and that, once it has been reached, addition of hydrolysed casein will no longer prevent development of spores, at least to the stage of refractility.

The same experiment shows that commitment is not an 'either/or' event. In some of the experiments, in the later stages of incubation, about 25% of the cells contained refractile spores by the time the medium was enriched and at least as many other cells had become committed and developed spores shortly afterwards. Nevertheless growth was resumed at a rate almost as high as that exhibited at the beginning of the experiment. This suggests that vegetative growth and sporulation may perhaps be able to occur in the same bacterial cell at the same time (see also Mandelstam, 1969).

The remaining results are best discussed in the context of what may reasonably be supposed to be happening in a sporulating bacterium (see Halvorson, 1965; Mandelstam, 1969). Since the information in the genes responsible for spore formation is not expressed in a rich medium one can assume that in such a medium the cells make an inhibitor or repressor that has this effect. One can go further and argue that, because there is a definite sequence of events expressed in a definite order (Warren, 1968), there may be several such inhibitors.

The individual inhibitions caused by this pool of substances are released in turn when the cells are placed in a deficient medium. The fact that if actinomycin D is added at any time before commitment it completely prevents all later development suggests that commitment consists of the transcription of the relevant m-RNA. The 20 min. gap between commitment and development of resistance to actinomycin might represent the time taken to build up a sufficient store of m-RNA, and the gap after addition of hydrolysed casein the time taken to restore an effective concentration of inhibitors. Now, if the m-RNA molecules are stable (see below) and restoration of a rich medium results in replenishment of the pool of inhibitors there will be no effect on those events for which m-RNA molecules have already been formed, but there will be inhibition of transcription of all later m-RNA molecules.

The experiments that have been described show that there is no commitment to sporulation as a whole and that, instead, the cells become committed in turn to successive parts of the process. The results are consistent with the model we have proposed in which there is a series of inhibitors that are removed one by one so that the genes on the chromosome can be transcribed.

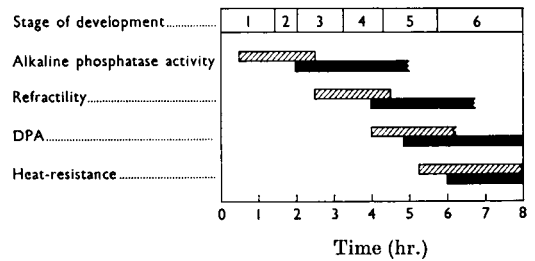


Fig. 12. Composite diagram illustrating the times of appearance of events connected with sporulation. Hatched rectangles (▨) indicate intervals during which cells acquire the potential ability to carry out the process concerned. This is the period during which that process becomes resistant to actinomycin D. The black rectangles indicate the period of expression of the potential ability. The stages of spore development are those defined by Schaeffer *et al.* (1965) and the times are approximate only. The results illustrated here are based on a large number of experiments, and there is considerable day-to-day variation in the time taken for cells to form complete spores.

An alternative to this idea of sequential transcription is to assume that all the m-RNA molecules are stable and are all produced at the beginning of the resuspension period. The observed facts could then be explained by saying that actinomycin has some secondary effect that takes an hour or two to manifest itself. Sporulation probably consists of a sequence of events in which the occurrence of any event depends on the successful completion of all earlier events (see Mandelstam, 1969). It follows that, if actinomycin is added, sporulation events will continue to be expressed for a while and the whole process will then stop. The assumption would also have to be made that addition of hydrolysed casein produces a qualitatively similar result. The net effect would be to produce an appearance of sequential transcription, although, in fact, the regulation would be exerted at the level of translation. It is shown below that, even if we do not accept this somewhat unlikely explanation, it still appears that there must be some element of control at the level of translation.

It has been noted above that the point of commitment for each event precedes the expression of that event by over an hour. The relationship of potentiality to expression for the four sporulation events we have examined is shown in the composite diagram (Fig. 12). Before this time gap is discussed it is necessary to consider the implications of the experiments with actinomycin. The point has been made above that, if the drug is added at any time to a sporulating culture, it will allow the expression of those events that have already passed the point of commitment but will inhibit the rest.

The most likely explanation is that the DNA is continuously accessible to actinomycin and that the concentration used is effective in preventing synthesis of m-RNA. If this argument is correct and no more m-RNA molecules can be synthesized it follows that the residual maturation observed must be governed by m-RNA that has been formed before addition of the drug and is more stable than bacterial m-RNA molecules are usually assumed to be. Indeed, a comparison of Figs. 4, 8, 9 and 10 shows that synthesis of alkaline phosphatase is far more resistant to the action of actinomycin than is protein synthesis generally or the induced formation of histidase in particular. The same appears to be true for other events of sporulation, such as refractility and the development of heat-resistance.

The assumption that the m-RNA concerned in sporulation is stable is supported by work with other developing systems. In particular, the results obtained with actinomycin are similar to those reported by Aronson & del Valle (1964) in *B. cereus* and by Sussman (1966) in experiments with developing slime moulds. There are many other experiments to show that m-RNA, particularly in higher organisms, is stable for long periods. For a detailed discussion see Harris (1968).

However, when the effect of actinomycin on the incorporation of adenine or uracil is examined it is found that, although there is initially a period of complete inhibition, incorporation begins again after 20 min., albeit at a decreased rate. There is no easy way to tell whether this incorporation represents synthesis of m-RNA. Certainly the renewed incorporation is not followed by a resumption of general protein synthesis, nor does it allow the expression of sporulation events to which the cells are not already committed. It therefore seems unlikely that the observed incorporation indicates the synthesis of meaningful m-RNA molecules. However, it could be argued that the particular segment of the sporulation genome that is actually being transcribed at the time the drug is added is in some way protected and continues to act as a template for meaningful short-lived m-RNA molecules, although all the later sporulation genes are accessible to actinomycin and are therefore not transcribed.

Whether long-lived m-RNA molecules are produced at the time of commitment or whether there is continuous synthesis of unstable m-RNA, we still have to explain the time gap of 1–2 hr. before these molecules are functionally expressed as protein. The most reasonable explanation is that there is a point of control affecting translation. It also follows that this control of the functioning of

m-RNA must be highly specific. If it were not, there would be simultaneous expression of all those events for which m-RNA molecules had already been formed. For example, at 3 hr. the points of commitment for alkaline phosphatase and for refractility have both been passed, but only one of these is being expressed while the other is dormant for a further 1 hr. (Fig. 12).

To sum up, it seems that sporulation involves a sequence of points of commitment, each of which allows the transcription of a species of m-RNA molecule. This control is exerted, possibly but not necessarily, by the removal in turn of specific repressors. Apart from this type of regulation there appears to be a second type of control that is highly specific and that ensures that the m-RNA molecules are expressed in the proper sequence. We have at present no way of knowing whether this regulation is positive or negative, nor do we know what type of molecule might be involved.

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