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Sporulation in *Bacillus subtilis*

CORRELATION OF BIOCHEMICAL EVENTS WITH MORPHOLOGICAL CHANGES IN ASPOROGENOUS MUTANTS

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A comparison was made of morphological changes and successive, mainly biochemical, marker events for sporulation in 14 asporogenous mutants. The morphological and biochemical sequences are linked so that arrested development in one is accompanied by corresponding effects in the other. Thus mutants that fail to produce both protease and antibiotic do not progress beyond stage 0, formation of alkaline phosphatase appears to be associated with the transition from stage II to stage III and glucose dehydrogenase with that from stage III to stage IV. Stage II mutants may produce 'pygmy' cells or other bizarre cell-division forms. The biochemical sequence is dependent in the sense that if the occurrence of any one event is blocked that of all the succeeding events is also blocked. This has implications for biochemical models that have been proposed to explain the temporal sequence observed in spore development.

A number of studies have been made of the biochemical events that accompany spore formation in aerobic bacilli, and these have been discussed in several reviews (Szulmajster, 1964; Halvorson, 1965; Murrell, 1967; Kornberg, Spudich, Nelson & Deutscher, 1968; Schaeffer, 1969; Mandelstam, 1969). Biochemical substances that appear during sporulation can be subdivided into at least two classes (see Mandelstam, 1969). (*a*) Enzymes, or simpler substances such as DPA,‡ that are characteristic of the spore, or that are associated with sporulating cells and are not normally found during vegetative growth. These can be presumed to serve some specific function in sporogenesis. (*b*) Enzymes that may have a vegetative function and that may also be used by sporulating cells. Some of these are likely to be repressed in a full growth medium but to appear inadvertently in the nutritionally defective media commonly used to initiate sporulation. This category would include enzymes of the glyoxylate cycle and citric acid cycle, and in this work we have excluded investigation of events that appear to be of this type.

We have chosen for study two early sporulation events, namely formation of exoprotease and of antibiotic, two intermediate events, namely formation of alkaline phosphatase and glucose dehydrogenase, and two late events, namely refractility and the formation of DPA. The morphological stages with which these marker events are associated in the wild-type are shown in Scheme 1. Refractility has been included because, although it is a property of the developing spore that has not been chemically defined, its occurrence depends on continued protein synthesis (Sterlini & Mandelstam, 1969). Whether the protease in its extracellular form has any function in sporulation is doubtful, but some of the enzyme is cell-bound and this appears to be responsible for the extensive intracellular turnover of protein that accompanies sporulation (Mandelstam & Waites, 1968). The part played by the other four markers is unknown, but their appearance is regularly associated with certain stages of sporogenesis and they occur in several different spore-forming types of bacilli. It seems reasonable to consider them provisionally as belonging to category (*a*).

The experiments described below were undertaken with two ends in view. The first was to determine whether the correlation of biochemical events with morphological changes that is found in the wild-type is maintained in asporogenous mutants blocked at different stages of development.

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‡ Abbreviation: DPA, dipicolinic acid.

The second was to determine whether the sequence of events is, or is not, a dependent sequence, i.e. one in which the occurrence of any event depends on the occurrence of earlier events. The assumption is often tacitly made that the sequence is dependent in this sense and it is, in fact, often found that a single mutation may cause the simultaneous loss of a number of characters successively connected with sporulation (Schaeffer, 1969; Spizizen, 1965). However, it seemed desirable to try to test the assumption directly because of its theoretical implications, which are considered in the Discussion section.

METHODS

Organisms. Bacillus subtilis 168 (Marburg) was used. Although it is an auxotroph requiring indole or tryptophan it forms spores normally and is therefore referred to as the wild-type. All asporogenous mutants described in this paper were derived from it by mutagenic treatment, indicated by the following prefixes: NG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; E, ethyl methanesulphonate; A, Acridine Orange; N, nitrous acid; U, u.v. irradiation. T20 was a transformant derived from a mutant rendered asporogenic by treatment with ethyl methanesulphonate. Asporogenous mutants were recognized because they formed white colonies on nutrient agar plates whereas the wild-type colonies were brown. For details see Mandelstam & Waites (1968).

Culture and procedure for obtaining sporulation. The organisms were grown with shaking at 37°C in a medium containing hydrolysed casein and inorganic ions (Sterlini & Mandelstam, 1969). When the bacterial concentration had reached 0.25 mg dry wt./ml and the cells were still in the exponential phase of growth, the culture was centrifuged and the cells were transferred to the same volume of a medium containing glutamate and inorganic ions with a high concentration of Mg^{2+} (40 mM). This is a modification (Sterlini & Mandelstam, 1969) of a medium described by Donnellan, Nags & Levinson (1964). It is referred to below as 'resuspension medium' and, except for the continuous cultures to be described, all experiments were done with bacteria in this medium in which shaking at 37°C generally gave a yield in the wild-type of about 80% refractile spores in 7–9 h.

Sporulation in continuous cultures. In addition to the studies in resuspension medium, the wild-type and three of the mutants were also grown in a synthetic medium in a chemostat. This, and the conditions of growth, were described by Dawes, Kay & Mandelstam (1969), who showed that the incidence of spores in the wild-type increases as the cells are made to grow more slowly. For example, at a doubling time of 5.2 h, about 40% of the cells are initiated to sporulation. If a sample is taken when the culture has reached a steady state and the cells are sectioned and examined in the electron microscope, all stages from the vegetative form to the complete spore are present and the relative incidence of the stages can be used to calculate the time taken for the completion of each stage (Dawes *et al.* 1969).

Estimation of spore incidence. Refractile spores were counted in the phase-contrast microscope and heat-resistant spores were determined by viable plate counts after heating at 80°C for 10 min.

Bacterial growth. This was measured spectrophotometrically by using a calibration curve relating E_{600} to bacterial dry wt.

Electron microscopy. Mutant strains from a growing culture were transferred to resuspension medium to initiate sporulation. They were sampled after 6 h, fixed, and sections were prepared as described by Kay & Warren (1968). Cells were assessed for their stage of development only if it appeared from their outline that they had been sectioned longitudinally and sufficiently near the equatorial level to give a representative view of the whole cell.

Qualitative detection of exoprotease and antibiotic in supernatants from resuspension experiments. Samples of supernatant were added to agar (1.5%) containing albumin and phosphate buffer as described by Mandelstam & Waites (1968). Supernatants with proteolytic activity produced zones of clearing of 2–6 mm after 48 h whereas those from proteaseless mutants did not. Antibiotic activity was assayed on plates with *Staphylococcus aureus* 524 as test organism (Brownlee *et al.* 1948).

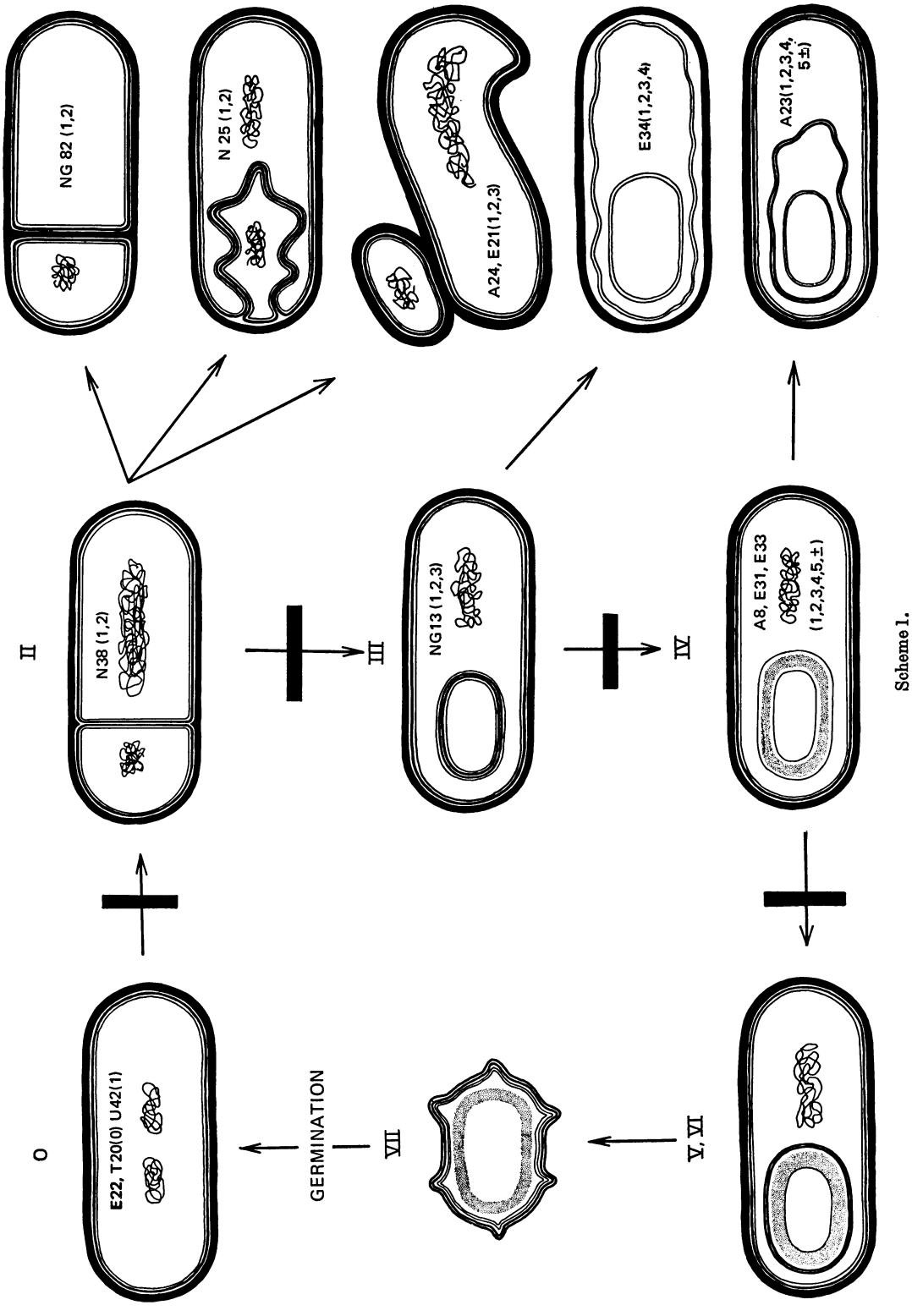
Enzyme assays. Alkaline phosphatase and glucose dehydrogenase were determined in bacterial samples taken from the resuspension medium by using the methods described by Warren (1968).

Enzyme units. One unit of enzyme is defined as that amount of enzyme catalysing the reaction of 1 nmol of substrate/min and specific activities are expressed either as enzyme units/mg of bacterial protein or as units/ml of culture.

Determination of protein, DNA and RNA. Samples (6 ml) from resuspension medium were taken at intervals and treated with trichloroacetic acid (final concentration 5%, w/v). The precipitates were dissolved in NaOH (0.1 M) and protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) with dry bovine serum albumin as a standard. For measurement of nucleic acids the precipitates were extracted with 5% (w/v) trichloroacetic acid at 90°C for 30 min. DNA and RNA were measured in the supernatant by the methods of Burton (1956) and Schneider (1945).

Determination of DPA. In experiments with the wild-type organism, samples (40 ml) were taken from resuspension medium and DPA was determined after autoclaving

Scheme 1. Comparison of morphological and biochemical changes during sporulation. The seven stages of sporulation (Roman numerals) represent schematically the appearance during normal development in the wild-type. Stage I is not shown, and stages V and VI are combined. Nuclear material is indicated only when it can be seen clearly in electron micrographs. The spore cortex is shown stippled. Black bars indicate transitional states at which mutants are blocked. Some of the mutants develop aberrant forms, shown on the right of the diagram (see also the Plates). The number of the mutant strain is given and the marker events it exhibits are indicated by numbers in parentheses, thus 0, no development of any of the marker events of sporulation; 1, exo-protease; 2, antibiotic; 3, alkaline phosphatase; 4, glucose dehydrogenase; 5, refractility. (For quantitative values see Table 1.)


NG 82 (1,2)
N 25 (1,2)
A24, E21(1,2,3)
E34(1,2,3,4)
A23(1,2,3,4, 5±)
II
N38 (1,2)
III
NG13 (1,2,3)
IV
A8, E31, E33 (1,2,3,4,5,±)
O
E22, T20(0) U42(1)
GERMINATION
VII
V, VI

Scheme 1.

the cells with the reagents of Janssen, Lund & Anderson (1958). When testing for DPA in mutants that appeared to lack it, the sample size was increased to 200ml. Also, the possibility was considered that the mutants might be making DPA but losing it to the surrounding medium. Accordingly, samples (200ml) of the supernatant taken at 7h were acidified by addition of conc. HCl (2ml) and extracted three times with butan-1-ol (60ml, 30ml and 30ml). The combined extracts were evaporated to dryness and the residue was dissolved in 0.5ml of water, which was then treated with the reagent of Janssen *et al.* (1958).

RESULTS

Morphological sequence of events in wild-type and in asporogenous mutants in resuspension medium: bizarre cell divisions and formation of 'pygmy' cells

Mutations to the asporogenous state produced morphological changes that can best be considered in relation to normal spore development. In some instances the mutation simply prevented development beyond a certain point; in others development proceeded normally to the blocked stage and then diverged in one of a variety of aberrant ways. Similar mutants have been found in this and other bacterial strains (see, e.g., Young, 1964; Schaeffer, 1969).

The stages of development are illustrated in Scheme 1. The end of each stage is indicated, and the times (t_1, t_2, etc.) at which the stages are reached by wild-type cells are given in parentheses below. It should be stressed that this is an idealized picture because the sporulation time may vary from one experiment to another and the sporulating population is, in any case, unsynchronized, so that the appearance of any one stage is spread out over a period of about 2h.

Stage O. This is the normal vegetative form. Mutants E22, U42 and T20 were blocked between this stage and stage I, and the cells, even after several hours in resuspension medium, retained the characteristic appearance of the vegetative state, although some of them had begun to lyse.

Stage I ($t_{1\frac{1}{2}}$): *formation of chromatin filament.* This stage is not always easily distinguished from some stages of the normal vegetative cell cycle. Nevertheless, we concluded that there were no mutants blocked immediately after this stage because, if there had been, resuspension experiments should have led to the accumulation of a large number of cells with filaments.

Stage II ($t_{2\frac{1}{2}}$): *formation of spore septum.* Five mutants (N38, N25, NG82, E21 and A24) appeared to be blocked after completion of this stage and failed to develop pre-spores. One of these, N38, showed spore septa at each end of the cells that did not enlarge to give a bulge (Plate 1*a*). This mutant could be described as abortively di-sporic and often grew in chains.

Mutant N25 (Plate 1*b* and 1*c*) developed spore septa in excessive amount so that the cells contained a convoluted membrane of considerable length which was still attached to the semi-permeable membrane of the mother cell.

Mutant NG82 (Plate 2*a*) resembled mutant N38 (Plate 1*a*) in that it was abortively di-sporic, but in addition it appeared to be able to lay down cell-wall material between the twin double membranes that form the spore septum. In the normal sporulating cell this septum is distinguished from the cell-division septum not only by its position in the cell but also because it does not permit the laying down of mucopeptide in the inter-membrane space. In

EXPLANATION OF PLATE 1

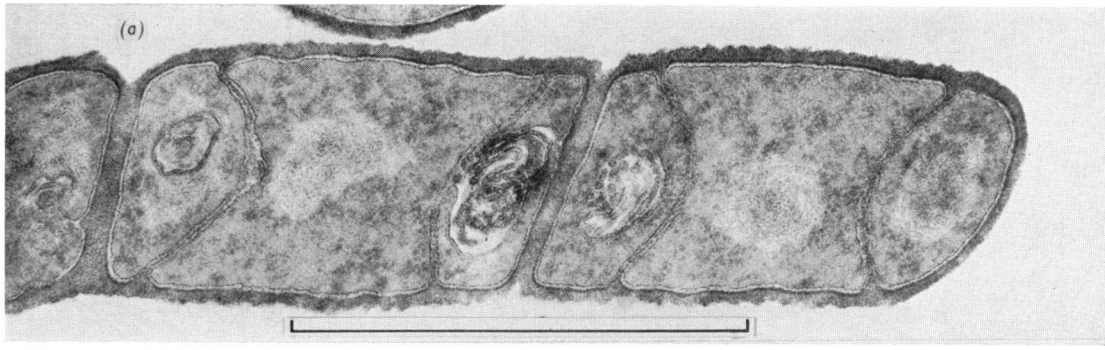
(*a*) Mutant N38. Part of a filament of four cells each of which contained two spore membranes. Development is blocked at stage II. (*b*) and (*c*) Mutant N25. This mutant is blocked at stage II but produces a large amount of spore membrane which does not detach from the cell membrane. The bars represent 1 μm.

EXPLANATION OF PLATE 2

(*a*) Mutant NG82. This mutant begins to form two spores like mutant N38 (Plate 1*a*) but these develop into 'pygmy' cells. The mother cell lyses. (*b*) and (*c*) Mutant E21. This mutant is blocked at stage II. It may form 'pygmy' cells and then form further division septa at intermediate positions. Some of the cells become irregular in shape and produce division septa at an angle to the axis of the cell. (*d*) Mutant NG13. This mutant is blocked at stage III. Pre-spores are released within the cell, but do not develop further. The bars represent 1 μm.

EXPLANATION OF PLATE 3

(*a*) Mutant E34. This, like mutant NG13 (Plate 2*d*), is blocked at stage III, but it tends to lyse easily. The empty spore membrane and the cell membrane are visible. (*b*) Mutant E33. This develops normally to stage IV and a large proportion of cells in this stage accumulate in the culture. The cortex is just visible around the spore membrane. A mesosome is pressed against the cell wall by the developing cortex. (*c*) Mutant A23. Like mutant E33 this is blocked at stage IV, but the cells show a tendency to lyse, losing both cytoplasm and cortical material. The bars represent 1 μm.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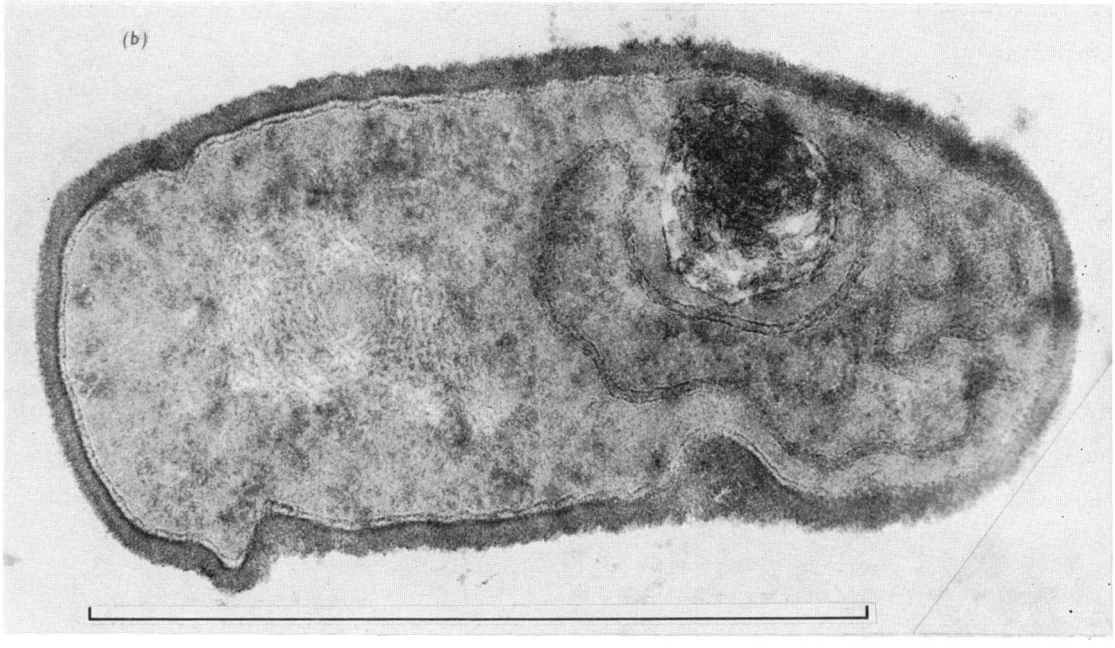
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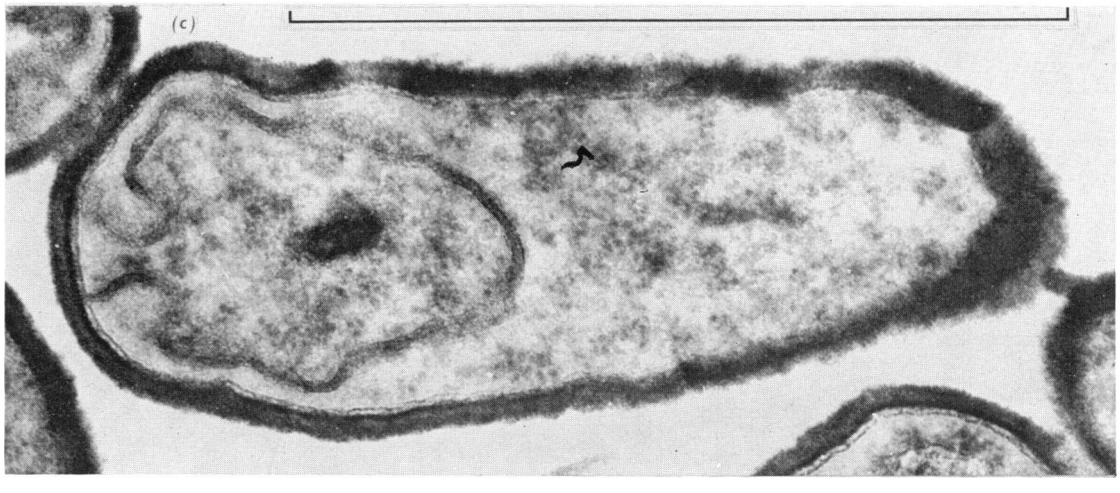
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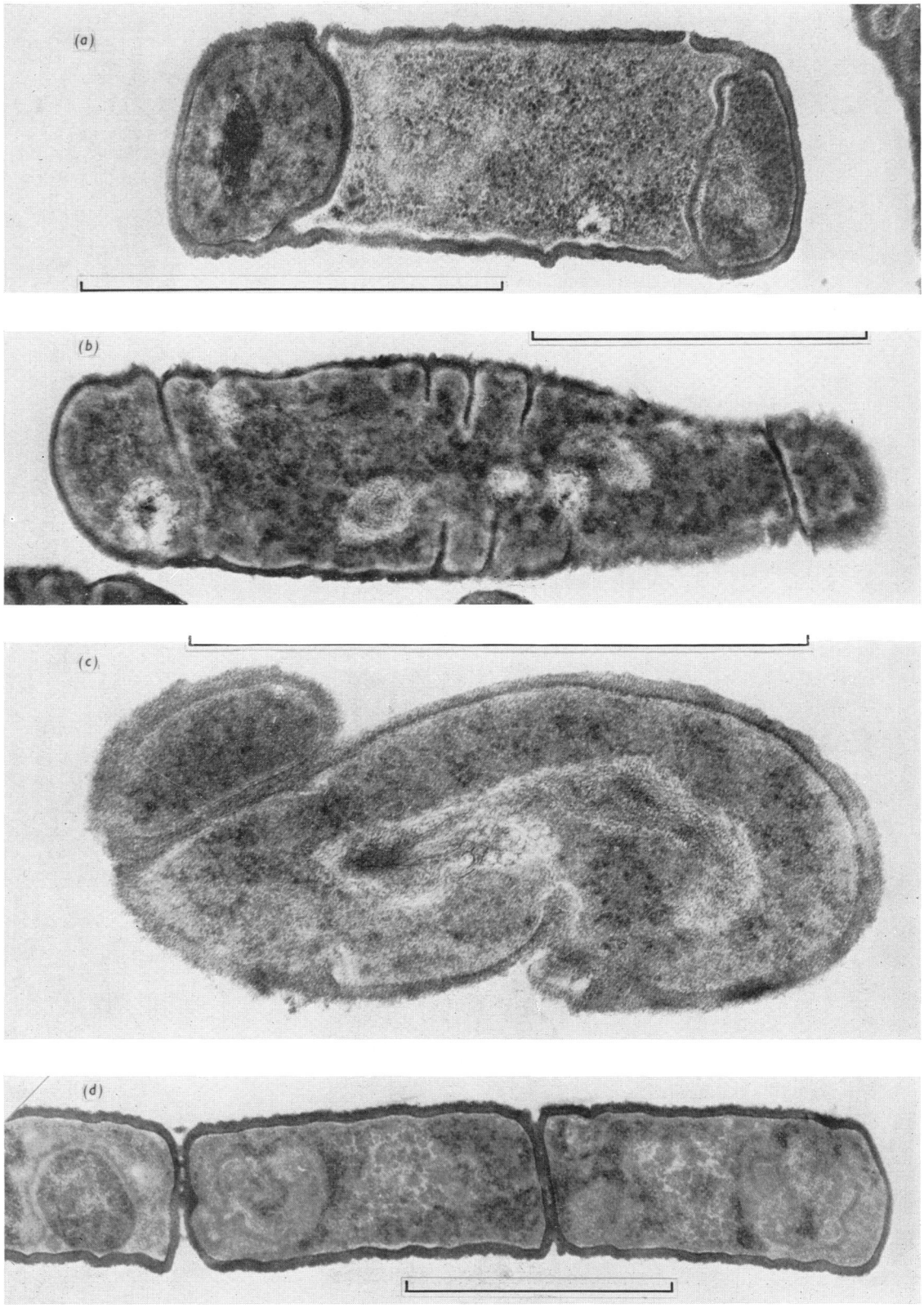

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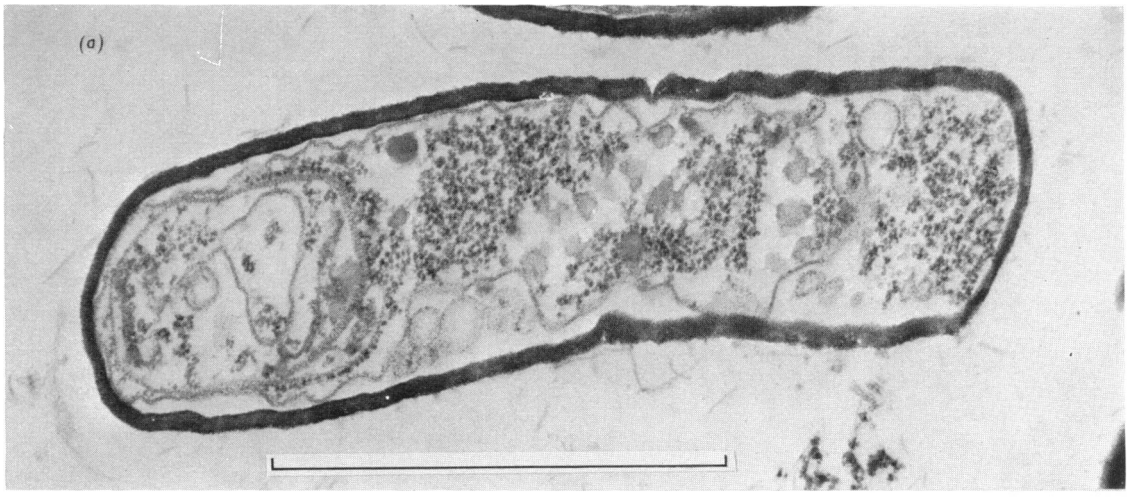
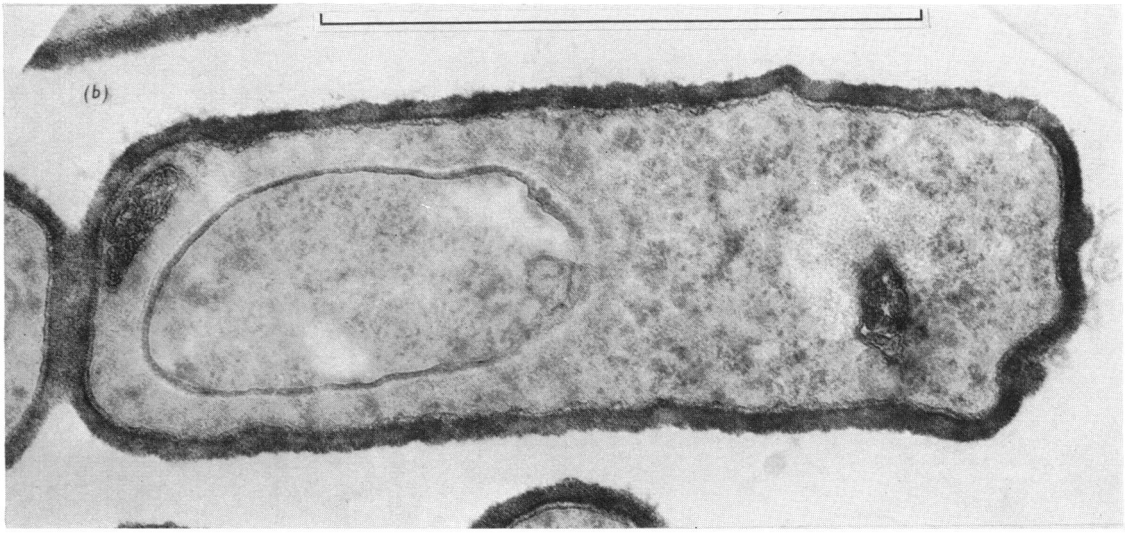

(a)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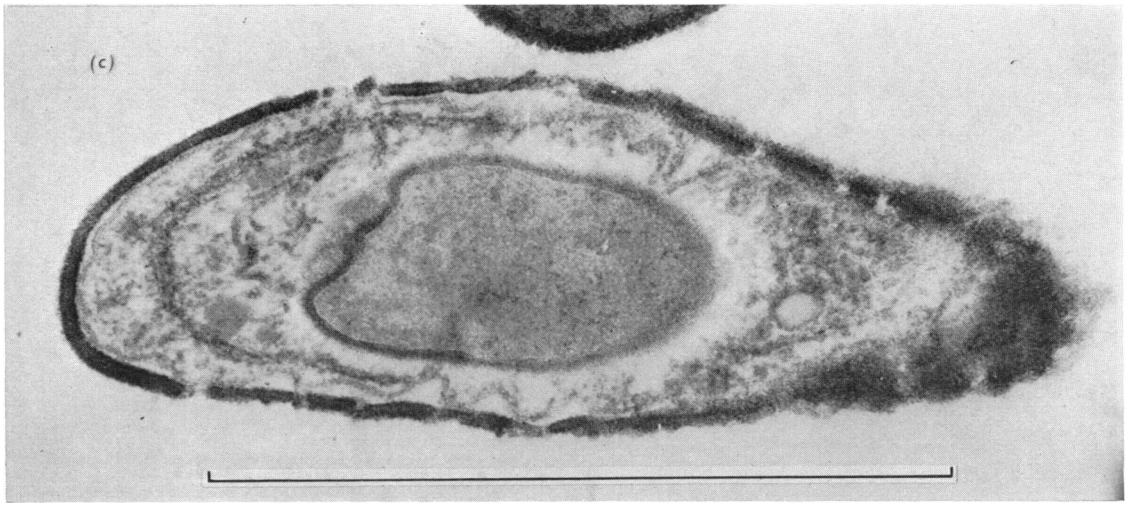
(c)

mutant NG82 densely staining material, continuous with and of similar thickness and appearance to the cell-wall material, was laid down along the spore septum, so that a 'pygmy' cell was produced at one or both ends of the mother cell. The central portion of the cell then lost electron density and eventually lysed. It is not known whether the 'pygmy' cells were viable, but in *Bacillus cereus*, in which a similar phenomenon has been reported (Young, 1964), the small cells as well as the central part were able to grow. Very similar asporogenous mutants of *Bacillus subtilis* have been described by Ryter, Ionesco & Schaeffer (1961).

In the third type of stage II mutant (A24 and E21) aberrant forms of cell division took place. In some cells several cross walls were seen (Plate 2*b*), whereas in others the shape of the cells became distorted and daughter cells developed at angles to the long axis of the cell (Plate 2*c*). These cells are said to show 'bizarre cell division'.

Stage III (t_4): *formation of complete spore protoplast.* In mutant NG13 spore formation appeared to reach stage III in which a detached pre-spore was formed within the cell (Plate 2*d*) but there was no development of cortex or spore coat. The cells tended to lyse. Another mutant of this type is E34 (Plate 3*a*), in which detached pre-spores can be discerned. Again lysis of the mother cell and pre-spore was liable to occur, leaving a cell envelope containing membranous elements among which the pre-spore envelope and the mother cell semi-permeable membrane could be seen.

Stage IV ($t_{5\frac{1}{2}}$): *formation of cortex.* Mutants A8, A23, E31 and E33 when observed in the light microscope could be seen to produce phase-grey (feebly refractile) spores.

Electron microscopy showed that many cells contained pre-spores surrounded by cortical material but that the characteristically dark-staining spore coat material was never laid down. A typical cell from mutant E33 is shown in Plate 3(*b*), where the development of the cortex is suggested by the fact that the mesosome at the end of the cell is closely pressed against the cell membrane.

Whereas mutants A8, E31 and E33 all showed many well-developed stage IV spores and remained stable after 7h in resuspension medium, mutant A23 quickly lysed after stage IV had been reached. The resulting cells (Plate 3*c*) showed the pre-spore still intact, surrounded, at a distance, by another membrane, which might have been the initial layer of the spore coat. Both the cortical area and the cell cytoplasm showed advanced signs of lysis and disintegration.

Stages V and VI (t_8). These stages include coat-formation and maturation of the spore. Specific attempts were made to find mutants blocked at these stages, but without success.

Biochemical sequence of events during sporulation of the wild-type in resuspension medium

The sequence of events during sporulation in resuspension experiments was similar to that described by Warren (1968), who allowed the bacteria to exhaust the glucose of the medium. The main difference was that the time-scale in resuspension experiments was shorter (see Sterlini & Mandelstam, 1969). The times of appearance of marker events in the wild-type are shown in Fig. 1 and summarized in Table 1. Although there were day-to-day variations the normal course of events was as follows. First, exoprotease and antibiotic appeared. These were easily detectable at the end of 40min, sometimes earlier, and their production may be presumed to have begun soon after resuspension. Because of variability in the quantitative assay the appearance of these two substances is indicated by an arrow instead of a curve. Alkaline phosphatase began to be synthesized at the end of 2h, rose to a peak and sometimes began to fall after 5–6h. The decline in activity was probably due to the active intracellular proteolysis, which is known to occur in this strain during sporulation and to which we have already referred. Glucose dehydrogenase synthesis began about 1½–2h later, rising to a peak at about 6h. Its appearance shortly preceded that of refractile spores, which could first be seen in the phase-contrast microscope at 4–4½h and which then increased rapidly in number. Roughly parallel curves show the successive appearance of DPA and the acquisition of heat-resistance (Fig. 1).

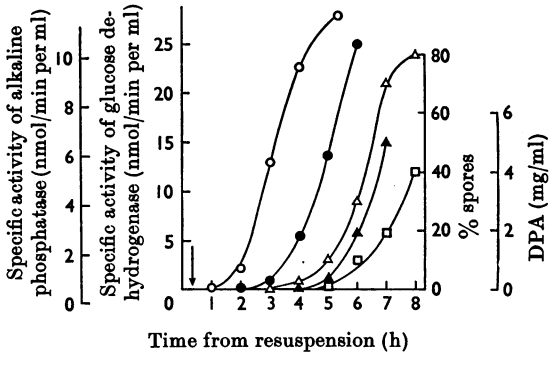

Fig. 1. Appearance of sporulation marker events in wild-type cells. Bacteria were transferred from growth medium to resuspension medium and sampled at intervals as described in the Methods section (see also Table 1). The arrow indicates the probable time at which synthesis of protease and antibiotic began. ○, Alkaline phosphatase; ●, glucose dehydrogenase; △, refractile spores; ▲, DPA; □, heat-resistant spores.

Table 1. *Comparison of sporulation marker events in wild-type and in asporogenous mutants*

Bacteria were transferred from growth medium to resuspension medium to induce sporulation (see the Methods section). Samples were taken hourly for measurement of seven marker events. Protease and antibiotic were measured qualitatively only. Values for alkaline phosphatase and glucose dehydrogenase are those found at 5 h and are given as units/mg of protein. In those experiments in which the enzyme activity was falling at this stage (see Fig. 2) the highest values reached are given in parentheses. DPA values (given in μg/mg of protein) and the values for refractility and heat resistance (given as percentages of the total population exhibiting the property concerned) are those at 7 h. Poor refractility (grey appearance in phase-contrast) is indicated by ±.

Bacterial strain	Stage blocked	Protease	Antibiotic	Alkaline phosphatase	Glucose dehydrogenase	Refractility	DPA	Heat-resistance
Wild-type	None	+	+	13.6	29	80	28	40
E22	0	−	−	<1	<2	<0.5	<0.4	<0.1
T20	0	−	−	<1	<2	<0.5	<0.4	<0.1
U42	0	+	−	<1	<2	<0.5	<0.4	<0.1
N38	II	+	+	<1	<2	<0.5	<0.4	<0.1
N25	II	+	+	<1	<2	<0.5	<0.4	<0.1
NG82	II	+	+	<1	<2	<0.5	<0.4	<0.1
E21	II	+	+	10.5 (12.0)	<2	<0.5	<0.4	<0.1
A24	II	+	+	6.5 (7.0)	<2	<0.5	<0.4	<0.1
NG13	III	+	+	6.3 (14.0)	<2	<0.5	<0.4	<0.1
E34	III	+	+	11.3	14.1	~10±	<0.4	<0.1
A8	IV	+	+	8.3 (10.0)	9.7 (36)	~10±	<0.4	<0.1
E33	IV	+	+	5.7 (7.0)	18 (28)	~10±	<0.4	<0.1
E31	IV	+	+	6.9 (10.7)	5.6	~10±	<0.4	<0.1
A23	IV	+	+	7.6 (8.5)	9.0 (21)	~10±	<0.4	<0.1

Biochemical sequence of events in asporogenous mutants in resuspension medium

The biochemical properties of the mutants are listed in Table 1. Among those blocked at stage 0, mutants E22 and T20 showed none of the characteristics of sporulating cells and need no further description. However, mutant U42 produced exoprotease but not antibiotic. This type of mutant was classified as sp_o^- by Schaeffer (1969). Those blocked at stage II produced protease and antibiotic, but no alkaline phosphatase except for the two mutants, A24 and E21, that underwent abnormal cell division. All mutants in this group were negative for later biochemical events. Of the two mutants classified on the basis of electron micrographs as blocked at stage III, one, E34, produced glucose dehydrogenase but the other did not. All signs of further development were absent. The four mutants blocked at stage IV were not distinguishable biochemically. All characters up to and including glucose dehydrogenase activity were exhibited and they all produced poorly refractile spores, i.e. phase-grey instead of phase-bright. The numbers of cells showing even this feeble refractility was usually 5–10% and never more than 20%. None of the mutants produced measureable quantities of DPA, nor were the spores heat-resistant.

Many of the quantitative values for phosphatase and glucose dehydrogenase given in Table 1 are low. One possible reason for this is that the enzymes seemed to be more susceptible to inactivation or proteolysis in the mutants than in the wild-type and sometimes the peak value had been passed by the time the 5 h sample was taken. For each of these instances the highest value reached is shown in parentheses. A typical result for a stage IV mutant, A8, is shown in Fig. 2. In this experiment phosphatase made its appearance at about the same time as in the wild-type (cf. Fig. 1). However, it reached a peak at 4 h and then began to decline rapidly. In some of the experiments glucose dehydrogenase was similarly affected. The final refractility values for this mutant and for all others in this class were invariably low.

Rates of initiation to sporulation in continuous cultures. A low rate of initiation to sporulation would explain the fact that in late-blocked mutants the proportion of cells reaching the stage of refractility was lower than in the wild-type and so were enzymic activities both for these and for earlier-blocked mutants. Continuous cultures were expected to provide a more sensitive way of detecting a defect in the spore initiation mechanism than the resuspension technique (see the Discussion section). Accordingly, the wild-type, three mutants blocked between stages II and III (N25, A24 and E21) and one blocked after stage IV (A23) were grown in a chemostat under conditions of nitrogen-

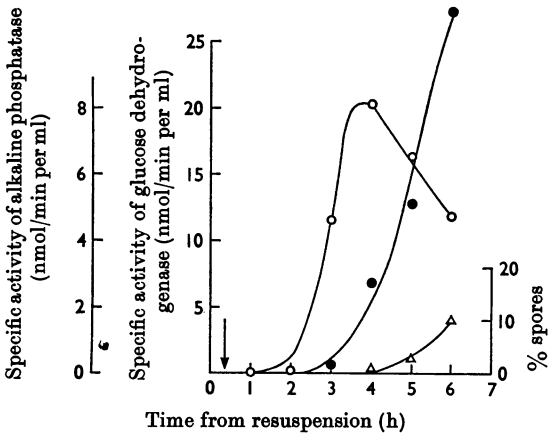

Fig. 2. Appearance of sporulation marker events in a mutant (A8) blocked at Stage IV. Experimental details are as described in Fig. 1. This mutant develops feeble refractility and fails to produce DPA or heat-resistant spores. The arrow indicates the probable time at which synthesis of protease and antibiotic began. ○, Alkaline phosphatase; ●, glucose dehydrogenase; △, refractile spores.

Table 2. *Initiation to sporulation in wild-type and in sporulation mutants*

Bacteria were grown in a chemostat under conditions of nitrogen-limitation with a doubling-time of 5 h. When the cultures were in the steady state samples were taken for electron microscopy. These were examined to obtain the relative incidence of vegetative cells and cells exhibiting some definable stage of sporulation. With mutant strain E21 so few cells were initiated that a reliable estimate could not be obtained.

Bacterial strain	No. counted	No. abnormal*	No. initiated*	% initiated
Wild-type	434	none	167	38
N25	189	51	23	39
A24	74	15	30	61
A23	142	None†	55	39
E21	—	—	—	(<1?)

* Morphological abnormalities are shown in Plates 1, 2 and 3 and in Scheme 1. These develop in resuspension cultures after initiation to sporulation has taken place. It has been assumed that this is true also in continuous cultures and the number of initiated cells therefore includes those with abnormal characteristics.

† Mutant A23 tended to lyse more than the other strains, but this has not been counted as 'abnormal'.

limitation with a doubling time of 5 h. When the culture had reached a steady state (2 days) a sample of the cells was prepared for electron microscopy. Cells that had been cut in longitudinal section were then examined to determine how many still presented the appearance of vegetative cells and how many exhibited some stage of sporulation. Cells that had been cut obliquely were disregarded.

At this growth rate the proportion of wild-type cells that had been initiated and were clearly in some stage or other of spore development was 38% (Table 2). A detailed analysis of this population has been made and is described elsewhere (Dawes *et al.* 1969). In three of the mutants the proportions of initiated cells were very similar, and therefore insufficient to account for the low enzymic activities shown in Table 1. Only in the fourth mutant (E21) was there a definite effect on the rate of initiation. The number of cells exhibiting development to stage II, where this mutant was blocked, was so low that a reliable value could not have been obtained without counting an impracticably large number of cells. Accordingly no values for this mutant are shown in the Table.

DISCUSSION

When the morphological changes found in mutants are compared with those in wild-type cells the following four general observations can be made. (1) In mutants the development sequence may be arrested at one of four different stages, which correspond to the inability to progress beyond stage 0 or the completion of stages II, III or IV. (We have not so far found mutations involving later stages.) (2) As a consequence of a block, cells that have reached that stage of development may accumulate. This type of response is shown particularly well by mutants N38, A8, E31 and E33. (3) Other mutants fail to remain in the comparatively stable situation described in (2) and begin to carry out a particular sporulation function in excess. This is shown in mutant N25 (Plate 1*b*), which continues to produce spore membrane, and in mutant E21 (Plate 2*b*), which repeatedly lays down membranes across the cell. (4) As an alternative to (3) blocked cells may undergo changes that are not characteristic of the sporulation sequence but that sometimes appear to be the result of vegetative cell functions superimposed on cells that have partially completed spore formation. This situation arises notably in the stage II mutants (NG82, A23 and E21), and it seems that formation of the membrane constituting the septum is a critical stage in spore development which, from the lesions encountered, suggests that several types of structural determinants are involved. These, in principle at any rate, should be susceptible to biochemical analysis. There must, for example, be a factor that initiates septum formation at one pole of the cell. An error here can result in membranes being laid down at both ends of

the cell. There must also be a control mechanism that prevents laying down of cell-wall substance between the membranes of the spore septum. If the fault is here, material resembling that of the wall is formed so that the spore septum becomes effectively a cell-division structure enclosing a 'pygmy' cell (Plate 2*a*). Cells of this type have been described in mutants of *Bacillus cereus* and they can grow out into normal bacillary forms (Young, 1964).

There is also presumably a factor to determine the position at which the membrane is placed. This is indicated by the fact that some cells blocked at stage II make several cross walls, most of which resemble cell-division septa. Up to five septa have been observed in one cell (Plate 2*b*). They are usually parallel to one another, closely spaced, and their plane is normal to the long axis of the cell. This 'bizarre cell division' is developed in an extreme manner in mutants E21 and A24 (Plate 2*c*), where the plane of cell division in the latter is at an angle to the long axis of the cell. This gives rise to irregular chains of misshapen cells.

Scheme 1 shows that the biochemical characteristics of the mutants are consistent with the results obtained by electron microscopy, i.e. when a mutation causes the morphological sequence to be blocked at a particular stage the biochemical sequence stops at the corresponding point. Thus two of the mutants blocked at stage O produced neither protease nor antibiotic; the third (U42) produced protease alone. Mutants unable to produce both protease and antibiotic do not generally progress beyond stage O (see Schaeffer, 1969). All mutants developing as far as stage II, i.e. those able to develop the normal spore septum, produced protease and antibiotic, but the next biochemical event, the formation of alkaline phosphatase, appears to be associated with the transition to stage III. Of the five mutants blocked at this stage two (A24 and E21) produced the enzyme and three (N38, N25 and NG82) did not. Glucose dehydrogenase is not found in any mutant blocked at this stage, but it does seem to be associated with transition from stage III to stage IV since one of the two mutants blocked here produced the enzyme whereas the other did not (see Scheme 1).

Considering next the mutants blocked between stages IV and V we find that biochemically they proceed normally as far as glucose dehydrogenase, but they all lack the ability to make DPA. However, as mentioned above, they undergo at least some of the physical changes associated with the appearance of refractility. Although this is an undefined chemical event it is known to be inhibited by chloramphenicol (Fitz-James, 1965; Ryter & Szulmajster, 1965; Sterlini & Mandelstam, 1969), and may be presumed to involve protein synthesis.

It is of note that in resuspension experiments only about 10% of the cells in a mutant population attained even the feeble degree of refractility they were capable of developing. There are two obvious explanations. The first is that the genetic blocks are, for some unknown reason, having the additional effect of lowering the rates of initiation to sporulation. If this were so, it would account not only for the low refractility values of the stage IV mutants but also for the generally low values for enzymic activity (see Table 1). We considered that this possibility would be better tested in continuous culture than in a resuspension medium. In the latter the starvation is so acute that it initiated almost quantitative sporulation in the population. In continuous cultures, however, growth rates can be chosen at which only a proportion of the wild-type cells will be initiated (i.e. the starvation is marginal). It is reasonable to suppose that if there were a deficiency in the initiating mechanism it should be more apparent under these conditions than during total starvation. However, three out of four mutants tested showed no significant difference from the wild-type, so it seems that differences in rates of initiation will not serve as a general explanation for the results shown in Table 1.

Another and more likely reason for the low values in Table 1 is that the incipient refractility is the manifestation of an unstable physical state. This explanation is based on the fact that, if incubation is prolonged beyond 7 h, the number of observably refractile spores begins to fall. It is reasonable to believe that if the proteins necessary for refractility cannot be protected by coat formation, they will be digested by the proteolytic enzymes in the cytoplasm and the spore body will revert to phase-darkness. The low value for refractility (10% of the cells) would then be the result of active formation and destruction of proteins responsible for refractility. If this assumption is correct it is probable that the total number of cells reaching this stage of development is much higher than would appear from the count. Intracellular proteolysis coupled with a tendency of the mutants to lyse more readily than the wild-type would also account for the low values for phosphatase and glucose dehydrogenase (cf. also Fig. 2).

Theoretical implications. From the results (see Table 1 and Fig. 1) we can conclude that the correlation between biochemical events and morphological stages found in the wild-type is maintained in the mutants. This fact provides some assurance that the five biochemical markers we have used really are functions of the sporulating cell and not just the response of the cell's vegetative genome to the change in medium.

The results also make it clear that we are dealing with a dependent sequence as defined in the introduction. This conclusion has implications for the

types of model that have been proposed for the sequential process that produces the spore. On the basis of our present biochemical knowledge three basic models are consistent with the fact that there is an ordered sequence of biochemical events. The first and the third have been discussed elsewhere (see Halvorson, 1965; Mandelstam, 1969).

Model A: simple sequential transcription. In this model the order of the spore genes on the chromosomes corresponds to the biochemical sequence and the latter is established by unidirectional transcription.

Model B: sequential transcription mediated by σ factors. After infection of *Escherichia coli* by phage T4 there are changes in the specificity of the RNA polymerase that are produced by proteins called σ factors (Bautz, Bautz & Dunn, 1969). The result is that the genome of the host ceases to be transcribed; instead, the genes of the infecting phage are transcribed in the correct sequence. Later, Losick & Sonenshein (1969) showed that, in sporulating cultures of *Bacillus subtilis*, there were analogous changes in the specificity of the RNA polymerase that apparently rendered it incapable of transcribing the DNA of infecting phage and, by implication, the genes responsible for the vegetative functions of the host cell. It was further suggested that the changed polymerase would then begin to transcribe the genes responsible for sporulation.

Model C: sequential induction. In its simplest form (see Stanier, 1951) the order is determined because the substrate of the first enzyme gives rise to a product that acts as an inducer and also a substrate of the second enzyme, etc.

Model A is inconsistent with the genetic evidence, which shows that the sporulation genes lie in a number of widely separated places on the chromosome and that this may even be the case when mutations in them produce blocks at the same stage of development (Takahashi, 1965, 1969; Rouyard, Ionesco & Schaeffer, 1967; Hoch & Spizizen, 1969). Of the mutants described in this paper, nine have been 'mapped' together with about 30 others and again there is no discernible correlation between the position of a mutation and the stage of sporulation at which it is expressed (P. Piggot, personal communication). For these reasons the idea of a simple sequential transcription is not tenable.

Model B based on successive alteration of the specificity of RNA polymerase by σ factors satisfactorily accounts for a sequence. However, it does not account for a temporal sequence that is also dependent. To account for this one would have to postulate that the σ factors are themselves expressed as part of the temporal sequence they control. This could be the case if all the mutations we have examined were either mutations of genes for σ factors or polar mutations affecting the expression of these genes. Both suppositions are inherently unlikely, and therefore model B will also have to be rejected unless some other way can be found to explain how the σ factors could themselves become part of the temporal sequence.

The third model could, in principle, account for the fact that the sequence is dependent. On the analogy of sequential induction in the pseudomonads there would be a succession of operons each of which determines a set of enzymes. The end-products of the action of each set of enzymes would induce the next set of enzymes etc. In such a system damage to any one enzyme would result in a failure to produce the proper end product and the whole process would therefore stop. If the sequence were determined in this way one might expect that late-blocked mutants could cross-feed those blocked earlier. However, although the model seems to be the most acceptable of the three no evidence of cross-feeding except of a trivial type has so far been found (see Schaeffer, 1969; Mandelstam, 1969).

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