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Akinetes of the cyanobacterium *Nostoc* PCC 7524: morphological changes during synchronous germination

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Abstract. Following dilution into fresh medium in the light, akinetes of *Nostoc* PCC 7524 germinated synchronously. Synchrony was maintained at a high level during the first 24 h, at which time the young filaments were composed either of three cells (with N_2 as nitrogen source) or four cells (with NO_3^- or NH_4^+), and at a slightly lower level during the next 24 h of growth. The pattern of cell division was similar in media containing the different nitrogen sources although the timing of the major events varied. In the presence of N_2 or NO_3^-, heterocysts differentiated synchronously; the first developed invariably from a terminal cell of the young filament at approximately 19 h, the second from the other terminal cell after further vegetative cell division. Heterocyst differentiation did not occur in the presence of NH_4^+. In the absence of nitrogen (gas phase argon: CO_2) akinete germination initially followed the same pattern as that observed in N_2, this early stage probably occurring at the expense of intracellular reserve materials.

During germination, a new laminated layer, similar in structure and position to that found in the heterocyst envelope, appeared in the akinete envelope. This layer was not present in the germinating akinetes of a mutant which was incapable of forming heterocysts.

Key words: *Nostoc* – Akinetes – Germination – Cyanobacteria

The synchronous differentiation of akinetes (spores) by the heterocystous cyanobacterium *Nostoc* PCC 7524 in batch culture in response to light energy limitation was described by Sutherland et al. (1979). These differentiated cells exhibited pronounced changes in structure and macromolecular composition, were resistant to desiccation, low temperatures and freezing (Sutherland et al. 1979). A detailed study of viability of vegetative cells and akinetes during freezing and thawing was described by Chauvat and Joset-Espardellier (1981). In contrast, akinetes were sensitive to temperatures above the temperature maximum for growth (Sutherland et al. 1979) and may therefore be considered as overwintering structures, a function very different to that of the heat-resistant bacterial endospore.

Although cyanobacterial akinetes were first reported by Carter (1856) and Rabenhorst (1865), long before Cohn (1877) and Koch (1877) described the bacterial endospore, and have subsequently been described in several species of cyanobacteria (see Nichols and Carr 1978; Nichols and Adams 1982, for reviews), little information is available concerning the structural and macromolecular changes which occur during germination. Akinete germination has been reported to be induced in some cyanobacteria by such easily controlled means as transfer to fresh medium (Miller and Lang 1968) and increased light intensity (Roelofs and Oglesby 1970; Yamamoto 1976; Braune 1979). In other species, akinetes either germinated too readily (Fay 1969; Rother and Fay 1977) and were not amenable to experimental analysis, or could not be induced to germinate (Wildman et al. 1975). The morphological changes occurring during germination of akinetes of *Cylindrospermum* (Miller and Lang 1968), *Aphanizomenon flos-aquae* (Wildman et al. 1975) and *Anabaena variabilis* (Braune 1980) have been described. Such studies were often difficult to interpret, because the non-synchronous populations of germinating akinetes contained cells in different stages of development. Stulp and Stam (1982) demonstrated, by light microscopy, a relatively well synchronised germination of akinetes of *Anabaena* strain 1617. Germination commenced between 25 and 40 h after transfer to fresh medium, with 90% of the akinetes having germinated at 43 h; morphological changes, however, were not examined in detail.

Techniques for the induction of synchronous growth have been in use for many years in the study of the biochemistry, physiology and morphogenesis of other organisms but, in cyanobacteria, only in unicellular strains has synchrony been maintained over several cell generations (Venkataraman and Lorenzen 1969; Asato and Folsome 1970; Herdman et al. 1970; Gleason and Ooka 1978). In this communication we describe the morphological changes which occur during the synchronous germination of akinetes of *Nostoc* PCC 7524 and during the subsequent synchronous growth of the young multicellular filament.

Methods

Organism. Nostoc PCC 7524 was obtained from the Culture Collection of Cyanobacteria, Institut Pasteur, Paris (Rippka et al. 1979) and grown as described by Sutherland et al. 1979.

Purification of akinetes. Stationary phase cultures containing akinetes (Sutherland et al. 1979) were maintained at 0 to 4°C for 7 days. During this period, the majority of the

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remaining vegetative cells lysed and their proportion was reduced from 40% to less than 10%. Akinetes were collected after centrifugation (1,200 × *g*, 15 min), which reduced the vegetative cells to less than 5% of the total population.

Akinete germination. Akinete preparations containing about 10^8 akinetes per ml were diluted 10-fold into medium BG-11_0 (Rippka et al. 1979), modified by the addition of $NaHCO_3$ (1 g/l), in the culture vessels described by Sutherland et al. (1979). In some experiments, $NaNO_3$ (1.5 g/l) or $(NH_4)_2SO_4$ (0.7 g/l) were added as sources of combined nitrogen. Cultures were gassed with air/CO_2 (95:5, v/v) at 34 ± 0.5°C with a light intensity of 130 μE/m^2/s (Osram warm white) at the surface of the vessel.

Electron microscopy. Samples were prepared and examined by electron microscopy as described by Sutherland et al. 1979.

Results and discussion

The pattern of akinete germination as seen by light microscopy

Akinetes could be distinguished from vegetative cells by their larger size and by the presence of an outer envelope and numerous phase-bright cyanophycin granules. Such akinetes, when diluted in batch cultures under continous light, germinated with a high degree of synchrony. This synchrony was maintained at a high level during the first 24 h and at a slightly reduced level during the following 24 h. The early pattern of germination was similar irrespective of the nitrogen source provided (N_2, NO_3^-, NH_4^+) although the timing of the major events varied.

When diluted into medium lacking combined nitrogen and provided with N_2 (as air: CO_2, 95:5 v/v) as sole nitrogen source, synchronous division of the akinetes commenced after 12 h (Fig. 1a). In the resulting two-celled germlings,

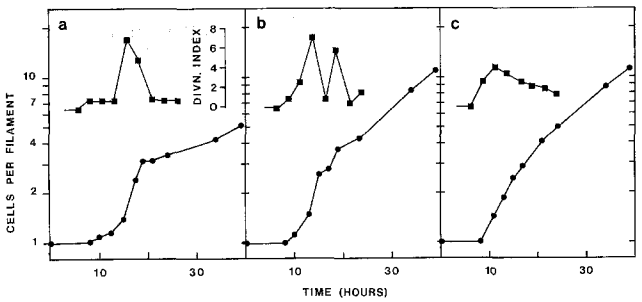

Fig. 1. Cell division during germination of akinetes of *Nostoc* PCC 7524 with **a** N_2 as sole nitrogen source **b** NO_3^- and **c** NH_4^+. The mean filament length (●) increased from 1.0 (the individual akinete) by successive vegetative cell division. The division index (■) represents the fraction of the cell population dividing at the indicated times; a value of 10 would represent 100%. All values are the means of cell counts performed on 100 individual akinetes (in the early stages of germination) or young filaments

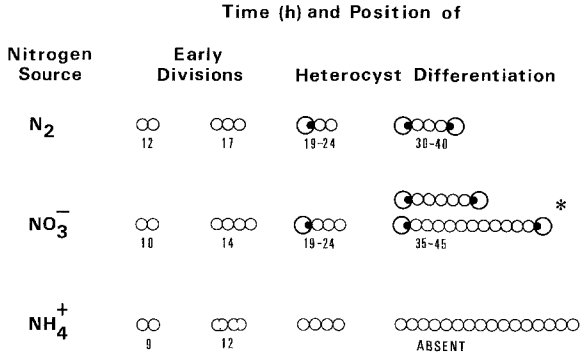

Fig. 3. Pattern and timing of major events during early germination of akinetes of *Nostoc* PCC 7524. The large cells with dark polar granules represent heterocysts. Under N_2, both vegetative cells in the short heterocystous filament divide, producing a filament containing the terminal heterocyst and four vegetative cells; the terminal cell differentiates into a heterocyst. In the presence of NO_3^-, the second heterocyst may develop either immediately after the division of the three vegetative cells or following the next round of cell division (*)

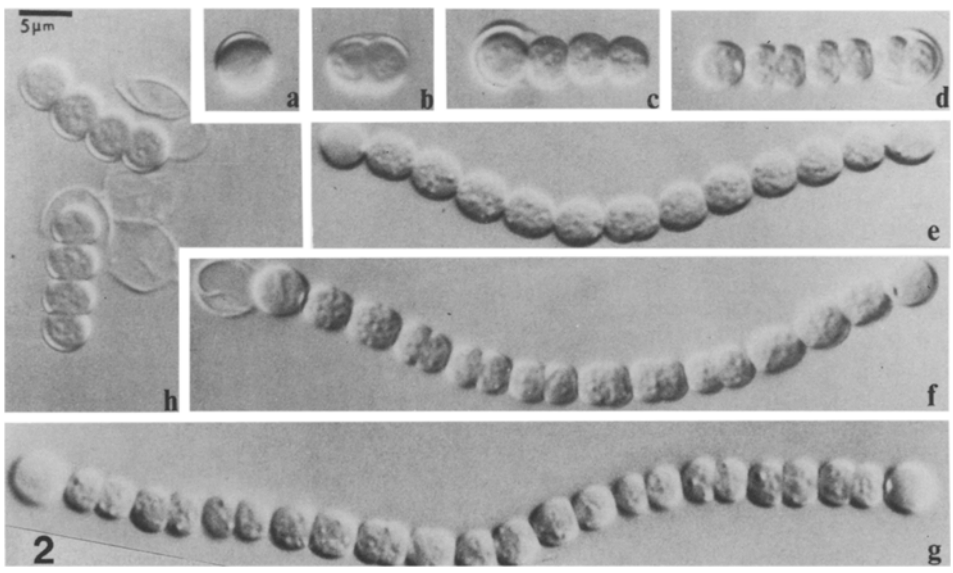

Fig. 2
Nomarski differential interference contrast micrograph showing various stages of akinete germination and filament growth in medium containing NO_3^-. The mature akinete (*a*) swells and the first division often takes place inside the akinete envelope (*b*); with subsequent divisions the envelope ruptures but may remain attached to the developing filament (*c, d, f, h*). The attached envelope does not appear to influence the position of development of the first heterocyst (*c, d*). Empty akinete envelopes retain their shape (*f, h*). Heterocysts first differentiate from the terminal cells of the young filament (*e, f, g*)

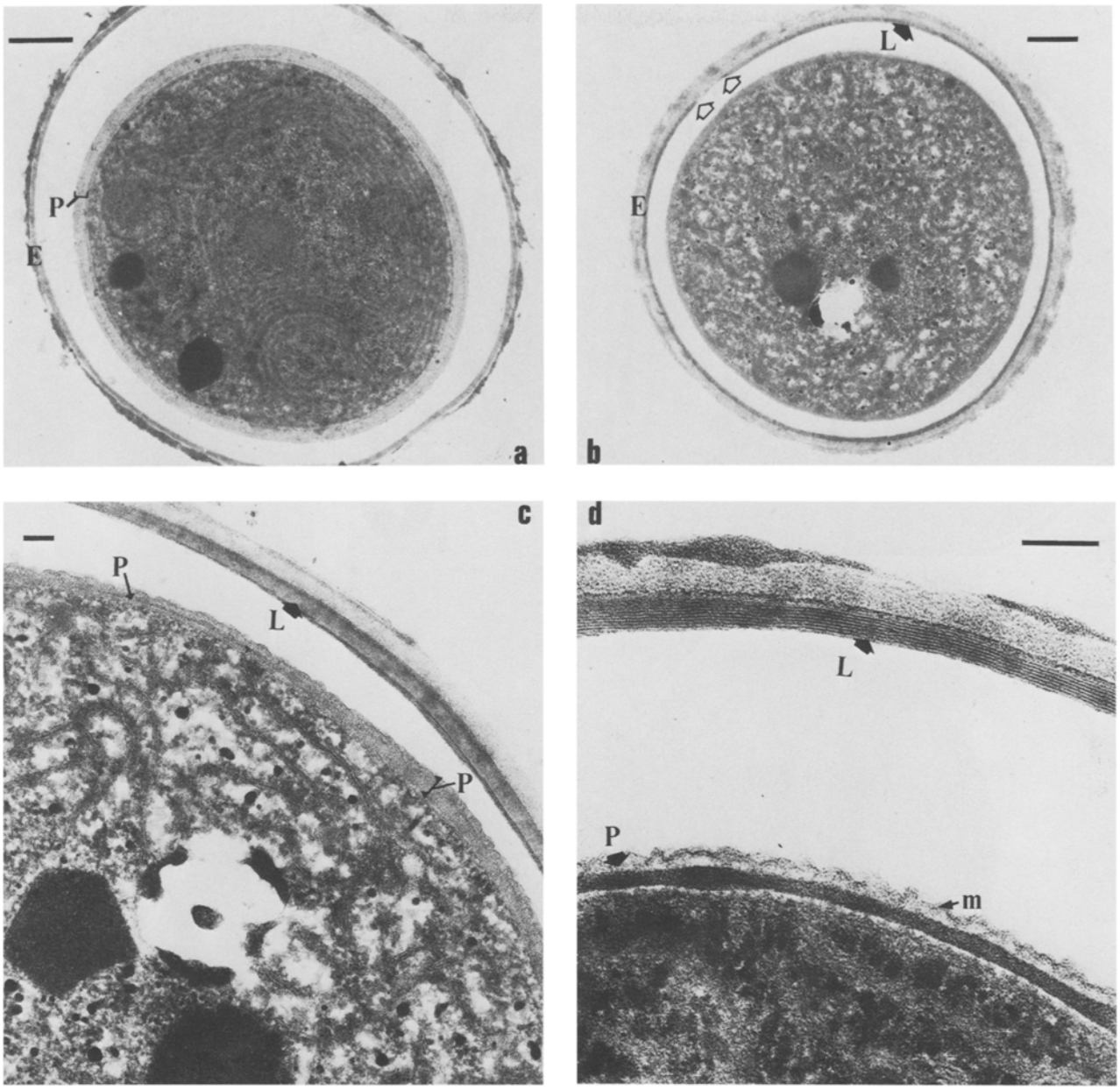

Fig. 4. a Transmission electron micrograph of a mature akinete showing a two-layered envelope (*E*) and a thick peptidoglycan layer (*P*) in the cell wall. Two electron-dense cyanophycin granules are visible; **b** Transmission electron micrograph of an akinete at an early stage of germination. A new layer (*L*) has appeared on the inside of the envelope (*E*). This layer is of uniform thickness and is more electron-dense than the other two layers of the envelope; it contains a small gap (indicated by arrows); **c** Transmission electron micrograph of part of a germinating akinete showing the three layers of the envelope and the uneven thinning of the peptidoglycan layer (*P*) of the cell wall; **d** Transmission electron micrograph of part of a germinating akinete showing the cell wall with wavy outer membranous layer (*m*) and the peptidoglycan layer (*P*) which is thinner than in mature akinetes but has not yet reverted to the thickness of the vegetative cell. The three layers of the envelope and the individual laminations of the *L* layer are clearly visible. The light and dark layers appear to be of about equal width (2 nm); Bar markers represent 500 nm in **a** and **b**, 100 nm in **c** and **d**

one cell divided again at approximately 16 h while the other differentiated into a heterocyst, first observed in a mature form (in the light microscope) at 19 h, producing a germling comprised of three cells. Heterocyst differentiation was well synchronized; 50% of the filaments possessed a terminal heterocyst at 19 h and 100% by 24 h. Thereafter, vegetative cell growth commenced at the normal rate for the culture conditions employed. A second heterocyst developed from the other terminal cell of the filament between 30 and 40 h. The early pattern and timing of akinete germination in the absence of a source of nitrogen (gas phase argon: CO_2, 95:5 v/v) was identical to that observed under N_2, except that vegetative cell division did not occur after 19 h. Thus akinete germination can occur in the absence of exogenous nitrogen sources, presumably at the expense of intracellular reserves.

In medium containing NO_3^-, akinete germination was accelerated; two rapid, successive cell divisions (Fig. 1b) produced a four-celled filament. Heterocyst differentiation was slightly retarded; only 20% of the filaments contained these cells after 19 h, and 90% at 24 h. Again, heterocysts

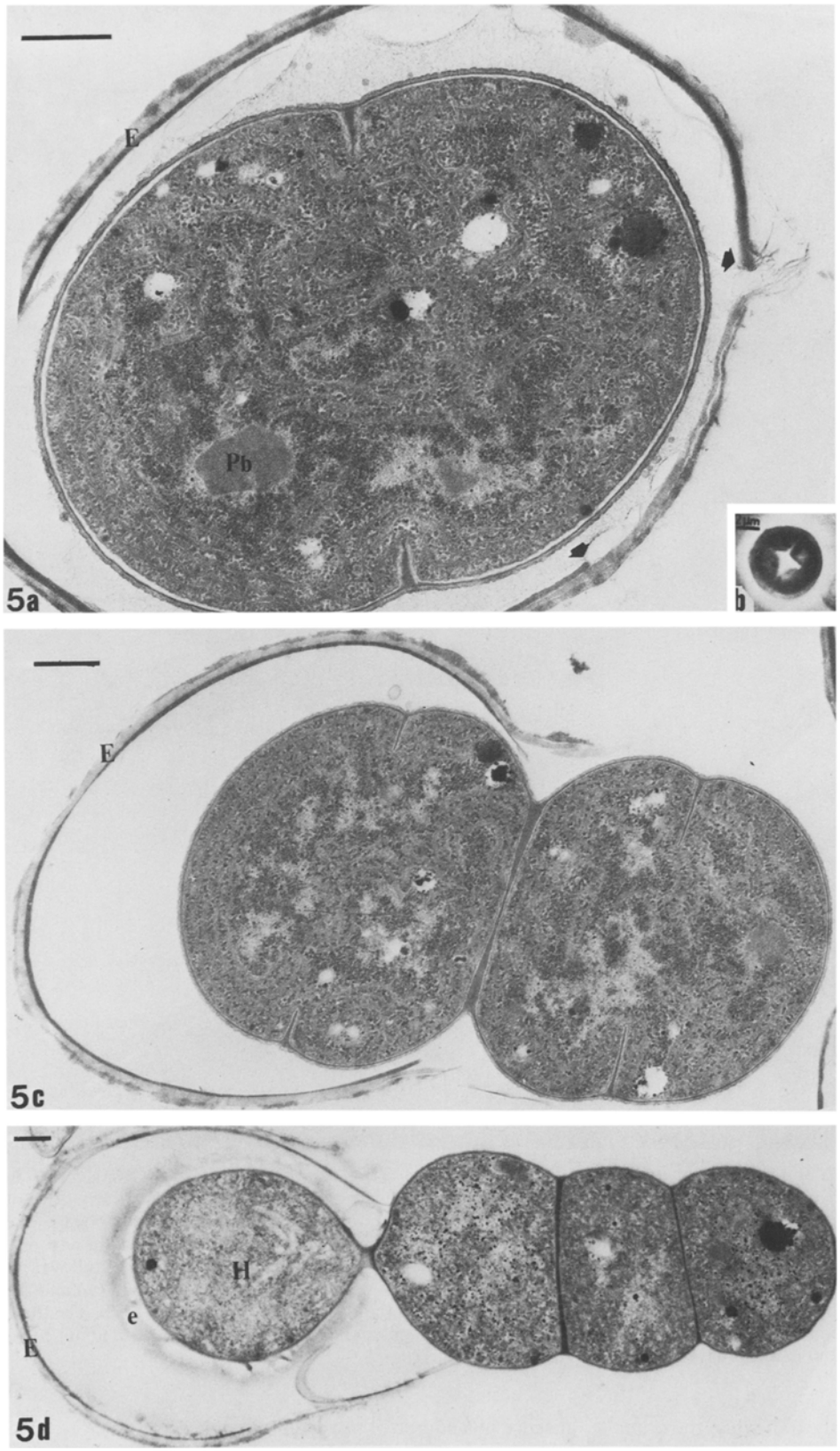

Fig. 5. **a** Transmission electron micrograph of a germinating akinete showing an early stage of formation of the cross wall of the first division. There is a wider break in the laminated layer of the envelope (arrows) than in Fig. 4b. The outer layers of the envelope have also split and pulled apart. Polyhedral bodies (*Pb*) are visible. Few cyanophycin granules remain; **b** Phase contrast micrograph of a germinating akinete. The appearance of the split in the envelope suggests that the germling breaks through it by mechanical pressure; **c** Transmission electron micrograph of a germinating akinete at the second division stage. The division is well synchronized in both cells. The envelope (*E*) has retained its rigidity; **d** Transmission electron micrograph of a filament at the four-celled stage. The akinete envelope (*E*) has been retained and a heterocyst (*H*) has begun to differentiate from the terminal cell of the filament inside the akinete envelope. Some formation of the heterocyst envelope (*e*) has taken place and a neck-like connection is visible between the developing heterocyst and the adjacent vegetative cell. Bar markers represent 500 nm

always differentiated from one of the two terminal cells, the second terminal cell producing a heterocyst between 35 and 45 h (peak at 40 h). The pattern of akinete germination and cell differentiation in the presence of NO_3^- is shown in Fig. 2.

In the presence of NH_4^+, the timing of germination was similar to that described above, except that heterocysts did not differentiate and cell division continued at a rapid rate (Fig. 1c). These results are consistent with the repressive effect of NH_4^+ on heterocyst differentiation in other cyanobacteria.

The timing and pattern of the major developmental events in media containing the different nitrogen sources are summarized in Fig. 3.

Electron microscopy of akinete germination

As described previously (Sutherland et al. 1979) the envelope of the mature akinete consists of two layers of different electron densities. An electron translucent area separates the outer envelope from the cell wall. The akinete cell wall differs from that of the vegetative cell in having a greatly thickened (approximately 10-fold), and less electron dense, peptidoglycan layer (Fig. 4a).

Following the transfer of mature akinetes to fresh medium in the light, with N_2 as sole nitrogen source, changes were apparent after 2 h. There were fewer cyanophycin granules, the peptidoglycan layer of the cell wall was thinner and a new electron dense layer was present on the inside of the envelope (Fig. 4b). The formation of the first transverse cell wall (Fig. 5a) generally occurred while the germinating akinete was still inside the envelope and in the light microscope the envelope was seen to fit tightly around the resulting two-celled filament (Fig. 2). This supports our previous suggestion (Sutherland et al. 1979) that the electron translucent area between the envelope and the cell wall is an artifact of the preparation for electron microscopy.

An early stage in the formation of the cross walls of the second cell division, after germination with N_2 as sole nitrogen source, is shown in Fig. 5c, division occurring synchronously in both daughter cells. The young filament has partly emerged from the akinete envelope, few cyanophycin granules remain within the cells and numerous glycogen granules are present. The retention of the akinete envelope at this stage is variable, since a large proportion of the young filaments are not associated with such structures.

Figure 5d shows a four-celled filament with the akinete envelope still attached to a terminal cell of the filament. The differentiation of this terminal cell into a heterocyst in the absence of exogenous combined nitrogen, is shown by the early stages of development of the outer wall layers of the heterocyst, reorganization of the cytoplasm and appearance of a neck-like connection between the young heterocyst and the adjacent vegetative cell.

Changes in the akinete cell wall and envelope during germination

The electron dense layer which appeared inside the envelope during the first 2 h of akinete germination was seen at high magnification to be composed of alternating light and dark laminae with a combined width of 4 nm (Fig. 4d). The formation of this layer was accompanied by a decrease in thickness of the peptidoglycan layer of the cell wall to that of a vegetative cell. The diminution of this wall layer usually occurred unevenly around the cell (Fig. 4c).

As germination proceeded the laminated layer ruptured and the broken edges separated (Figs. 4b, 5c). The overlying envelope material was frequently seen to stretch across the gap left by the separating laminated layer before finally itself rupturing (Fig. 5a), suggesting that the laminated layer is composed of less elastic material than the outer layers. The rupture of the envelope prior to emergence of the young filament is also shown under phase contrast microscopy in Fig. 5b.

This laminated layer is similar in appearance and apparent inelasticity to that observed by Braune (1980) in germinating akinetes of *Anabaena variabilis*, although the spacing between the electron dense layers (5 nm) in the latter organism was slightly greater than that described here.

The 4 nm spacing between laminae observed in *Nostoc* PCC 7524 is the same as that found in the heterocyst wall of *Anabaena cylindrica* by Winkenbach et al. (1972). This layer does not occur in the germinating akinetes of an aheterocystous mutant of *Nostoc* PCC 7524, suggesting that this component is common to both types of differentiated cells and may, as in the heterocyst, be composed of glycolipid.

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