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Calcium Accumulation during Sporulation of *Bacillus megaterium* KM

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Accumulation of Ca^{2+} in *Bacilli* occurs during stages IV to VI of sporulation. Ca^{2+} uptake into the sporangium was investigated in *Bacillus megaterium* KM in protoplasts prepared in stage III of sporulation and cultured to continue sporulation. These protoplasts and whole cells exhibit essentially identical Ca^{2+} uptake, which is compared with that of forespores isolated in stage V of sporulation. Ca^{2+}, uptake into both sporangial protoplasts and isolated forespores occurs by Ca^{2+}-specific carrier-mediated processes. However, protoplasts exhibit a K_m value of 31 μM, and forespores have a K_m value of 2.1 mM. Sporangial protoplasts accumulate Ca^{2+} against a concentration gradient. In contrast, Ca^{2+} uptake into isolated forespores is consistent with downhill transfer in which both rate and extent of uptake are affected by the external Ca^{2+} concentration. Dipicolinic acid has no effect on Ca^{2+} uptake by isolated forespores, apart from decreasing the external Ca^{2+} concentration by chelation. A model for sporulation-specific Ca^{2+} accumulation is proposed, in which Ca^{2+} is transported into the sporangium, resulting in a concentration of 3–9 mM in the mother-cell cytoplasm. This high concentration of Ca^{2+} enables carrier-mediated transfer down a concentration gradient into the forespore compartment, where a low free Ca^{2+} concentration is maintained by complexing with dipicolinic acid.

In contrast with exponentially growing cells of *Bacilli* (Silver *et al.*, 1975; Bronner *et al.*, 1975) and *Escherichia coli* (Silver & Kralovic, 1969; Rosen & McClees, 1974; Silver, 1977), which are thought to maintain a low Ca^{2+} concentration in the cytoplasm by metabolically active efflux, sporulating *Bacilli* are found to accumulate Ca^{2+} from stage IV to VI of sporulation, concomitant with biosynthesis of dipicolinic acid (Young & Fitz-James, 1962; Eisenstadt & Silver, 1972). On a dry-weight basis, spores of most bacterial species contain 2–4% of Ca^{2+} and 5–15% dipicolinic acid in an approx. 1:1 molar ratio. The available evidence favours a spore cytoplasm location for these compounds (Leanz & Gilvarg, 1973; Gould & Dring, 1974). Several proposals have been made implicating these high spore concentrations of Ca^{2+} and dipicolinic acid in the acquisition of heat resistance and the imposition and maintenance of dormancy (Gould & Dring, 1974).

During spore morphogenesis the forespore becomes a discrete cell within the mother-cell compartment, containing at least one complete genome and bounded by a double membrane, comprising the forespore inner and outer membranes [for a description of the overall process, including delineation of the various sporulation stages, see Ellar (1978)]. From morphological considerations, the outer surface of the forespore outer membrane arises from what was previously the inner (cytoplasmic) surface of the mother-cell plasma membrane, and the forespore inner membrane retains the polarity of the mother-cell membrane. This results in a reversal of membrane polarity not previously observed in bacteria (Ellar, 1978; Wilkinson *et al.*, 1975). The forespore outer membrane can be shown to have reversed surface polarity with reference to both the forespore inner membrane and the mother-cell plasma membrane (Wilkinson *et al.*, 1975; Andreoli *et al.*, 1975). This reversed polarity has important implications when transport and biosynthesis during sporulation are considered (Hanson *et al.*, 1970; Freese, 1972; Silver, 1977). Thus, if each of the forespore membranes retained the normal mechanisms for active transport, these could conceivably act in opposite directions (Freese, 1972). With the development of techniques for the isolation of forespores at all stages of development (Andreoli *et al.*, 1973; Ellar & Posgate, 1974), it became possible to compare transport of solutes into the isolated forespore and into the intact sporangium.

In earlier studies with *Bacillus megaterium* KM, the proportion of the sporangial Ca^{2+} associated with the developing spore increased during the period of Ca^{2+} accumulation (La Nauze *et al.*, 1974), and dipicolinic acid was found only in the forespore compartment (Ellar & Posgate, 1974). Eisenstadt & Silver (1972) demonstrated Ca^{2+}-specificity and

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saturation kinetics for sporulation-specific Ca^{2+} accumulation by intact *Bacillus subtilis* cells, and Bronner & Freund (1972) also argued in favour of a sporulation-specific Ca^{2+}-pumping mechanism. La Nauze *et al.* (1974) showed that forespores released from the mother-cell sporangium could not continue Ca^{2+} accumulation when incubated under conditions identical with those resulting in sporangial Ca^{2+} accumulation, thus indicating a requirement for the structural integrity of the mother-cell compartment. The object of these experiments was therefore to investigate the mechanism of Ca^{2+} uptake into the mother-cell compartment and its subsequent transfer into the forespore. The sporulating protoplast system described by La Nauze *et al.* (1974) was found to be suitable for continued investigation of Ca^{2+} accumulation during sporulation and in addition there could be little interference by the cell wall on the observed accumulation properties.

This paper describes the kinetics of Ca^{2+} uptake by sporangial protoplasts of *B. megaterium* KM and by forespores isolated from protoplasts in stage V of sporulation. A model for sporulation-specific Ca^{2+} accumulation is suggested. Some of these results have been presented in preliminary form (Hogarth *et al.*, 1977).

Experimental

Cultivation of the organism

The organism used was a sporogenic strain of *B. megaterium* KM that remains lysozyme-sensitive throughout sporulation and was cultivated synchronously in CCY medium as described by Ellar & Posgate (1974).

Preparation and culture of protoplasts

Sporulating cells were harvested in late stage III [at this stage in sporulation, cells in culture samples treated briefly with lysozyme (100 μg/ml) showed ovoid phase-dark forespores when examined by phase-contrast microscopy (Ellar & Posgate, 1974)]. Protoplast preparation and culture were carried out by the method of La Nauze *et al.* (1974) with minor modifications. Cells were harvested from a measured volume of sporulating CCY cultures in late stage III by centrifugation (8000*g* for 30s at 25°C), washed in an equal volume of prewarmed sucrose/salts buffer and the pellet was resuspended in an equal volume of prewarmed sucrose/salts buffer, to which lysozyme (100 μg/ml) had been added. Sucrose/salts buffer consisted of: sucrose, 0.6M; Tris/HCl, pH 7.3, 25 mM; $MgCl_2$, 15mM; K_2SO_4, 1.0mM; $MnSO_4$, 0.01 mM; $CaCl_2$, 0.0915mM: 25ml samples of this suspension were then distributed to 250ml Erlenmeyer flasks or 10ml samples into 100ml Erlenmeyer flasks and these were incubated at 30°C in a Grant reciprocal shaking water bath at 50 cycles/min with a 5 cm displacement.

Isolation of forespores from protoplast cultures

Forespores were prepared from protoplasts in stage V of sporulation, essentially by the method of Ellar & Posgate (1974), by selective disruption of the mother-cell plasma membrane by mild sonication for up to six 1s pulses with a 1.2cm sonic probe (Dawe Instruments Ltd., London W.3, U.K.) operating at maximum output at 4°C. Forespores, separated from the mother-cell cytoplasm and membrane fragments by differential low-speed centrifugation (11600*g* for 3min at 4°C), were resuspended in a volume of sucrose/salts buffer equal to the volume of protoplast culture from which they were derived.

Uptake of Ca^{2+}

In all Ca^{2+}-uptake studies the concentrations of protoplasts and forespores in the suspensions were adjusted to be equivalent to the concentration of cells in the original cell culture, resulting in a final concentration of approx. 2×10^8–2.5×10^8 protoplasts or forespores/ml of culture. Ca^{2+} uptake was determined by rapid filtration of 0.2ml samples of protoplasts and 0.5ml samples of forespores on Millipore filters (0.45 μm) and extensive washing with sucrose/salts buffer. The filters were dried and radioactive counts determined in scintillant containing 4g of 2,5-bis-(5-t-butylbenzoxazol-2-yl)thiophen per litre of toluene.

In general, carrier-free ^{45}Ca was added to protoplast cultures in stage III to give a final Ca^{2+} concentration of 9.15 μM and 0.5 μCi/ml.

Forespore Ca^{2+}-uptake assay systems consisted of: 5ml of isolated forespores in sucrose/salts buffer; 0.05ml of $CaCl_2$ containing 27.8 μCi of ^{45}Ca to give a final Ca concentration as indicated in the text; 0.1ml of 25mM-Tris/HCl, pH 7.3, or various additions. Assays were incubated with shaking in 50ml Erlenmeyer flasks at 30°C for 3–4min before the initiation of the Ca^{2+} uptake by the addition of $^{45}CaCl_2$. Experiments showed that forespores could be stored for at least 300min at 4°C before assay without affecting their ability to accumulate Ca^{2+} subsequently.

Chemicals

Carrier-free $^{45}CaCl_2$ (483mCi/nmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All other reagents were of analytical grade.

Results

Ca^{2+} accumulation by sporulating protoplasts

La Nauze *et al.* (1974) showed that Ca^{2+} uptake and the sequence of sporulation was essentially

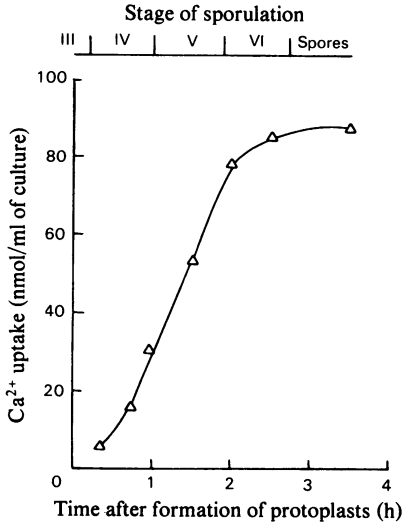

Fig. 1. *Ca^{2+} uptake by sporulating protoplasts and corresponding stages of sporulation*

Protoplasts were prepared in stage III and cultured in sucrose/salts buffer, and Ca^{2+} uptake was determined as described in the Experimental section.

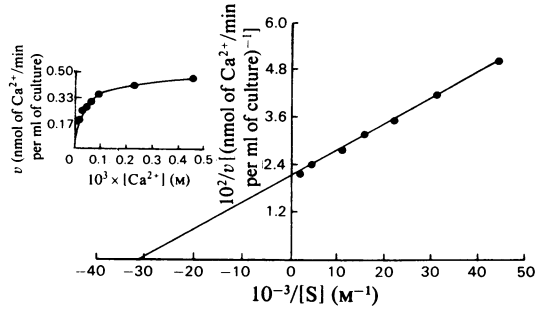

Fig. 2. *Effect of Ca^{2+} concentration on protoplast Ca^{2+} uptake*

Protoplasts were prepared in stage III and were incubated in various concentrations of $CaCl_2$. Ca^{2+} uptake was determined as described in the Experimental section and the rate of Ca^{2+} uptake calculated from the linear region (stage IV–early V) of the uptake curves. The large Figure shows a Lineweaver–Burk plot for Ca^{2+} uptake against Ca^{2+} concentration, and the inset the Michaelis–Menten plot.

identical in whole cells and protoplasts, which were prepared in stage III and cultured to allow continued sporulation. Fig. 1 shows the Ca^{2+} uptake by a typical sporulating protoplast culture and compares the corresponding stages of sporulation. Ca^{2+} accumulation commenced in stage IV of sporulation, coincident with the change from phase-dark to phase-grey forespores, and continued until early stage VI sporulation. Ca^{2+} accumulation by sporulating protoplasts in the presence of various concentrations of external Ca^{2+} demonstrated saturation kinetics (Fig. 2), with a K_m value of 31 μM and $V_{max.}$ of 0.5 nmol/min per ml of culture. Protoplast Ca^{2+} accumulation showed similar specificity to that reported by Eisenstadt & Silver (1972) for *B. subtilis*. When non-radioactive cations were included in the assays to a final concentration of 0.25 mM, $^{45}Ca^{2+}$ accumulation was decreased in the order $Sr^{2+} > Mn^{2+}$. The addition of Na^+ at this concentration had no effect on Ca^{2+} uptake. Ca^{2+} uptake by whole cells in CCY medium (0.5 mM-Mg^{2+}) was similar to Ca^{2+} uptake by cells in sucrose/salts buffer (15 mM-Mg^{2+}), indicating that Mg^{2+} has little significant effect on Ca^{2+} accumulation.

Ca^{2+} uptake by isolated forespores

As noted in the introduction, La Nauze *et al.* (1974) demonstrated some requirement for mother-cell integrity for Ca^{2+} entry into the forespore compartment. In subsequent experiments with isolated forespores, attempts have been made to explore this requirement by manipulation of the incubation-medium composition. Experiments of the type described by La Nauze *et al.* (1974) were carried out, in which ^{45}Ca was added to protoplasts prepared at stage III of sporulation. These were then cultured to stage V and the forespores released by sonication. During subsequent incubation of the forespores in sucrose/salts buffer neither dipicolinicacid, nor malate and ATP [substrates for the intact isolated forespore, because of the reversed polarity of the forespore outer membrane (Wilkinson *et al.*, 1975)] stimulated Ca^{2+} uptake by forespores.

The concentration of Ca^{2+} in the mother-cell cytoplasm is estimated to be 3–9 mM (see the Discussion section). Experiments were therefore carried out to determine whether this high concentration of Ca^{2+} is necessary to allow it to enter the forespore. Fig. 3 shows the result of incubating forespores, isolated in stage V, in the presence of 9.7 mM-$^{45}CaCl_2$ at 30°C. After 20 min 8 nmol of Ca^{2+} had accumulated/ml of forespore culture, the Ca^{2+} accumulation plateauing after 15 min. Thus forespores released from the sporangium and separated from the mother-cell constituents could be induced to incorporate Ca^{2+} merely by the provision of a high external Ca^{2+} concentration. The results from an extensive series of control experiments suggested that the observed Ca^{2+} incorporation by forespores was not a result of non-specific binding to the outer surface of the forespore. Firstly, the incorporated Ca^{2+} was not removed by extensive washing of forespores with buffer of 100-fold lower Ca^{2+} concentration than the Ca^{2+}-uptake assay concentration; secondly, the incorporated Ca^{2+} was not removed by treatment with EDTA after resuspension of the forespores in a

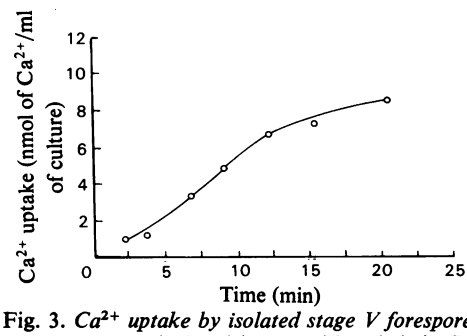

Fig. 3. *Ca^{2+} uptake by isolated stage V forespores*
Forespores were isolated in stage V and their Ca^{2+} uptake in the presence of 9.7mM-$CaCl_2$ was determined as described in the Experimental section.

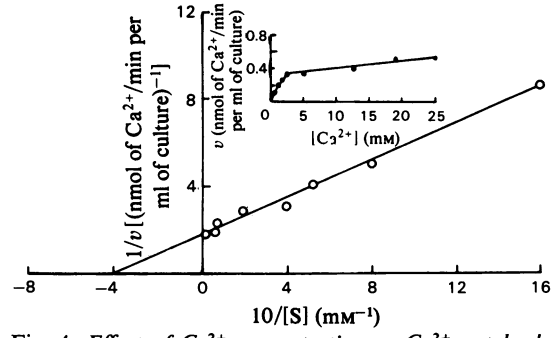

Fig. 4. *Effect of Ca^{2+} concentration on Ca^{2+} uptake by isolated stage V forespores*
Ca^{2+} uptake by forespores, isolated in stage V and incubated with various concentrations of $CaCl_2$, was determined as described in the Experimental section. The rate of Ca^{2+} uptake was calculated from the initial rate of uptake. The large Figure shows a Lineweaver–Burk plot for Ca^{2+} uptake against Ca^{2+} concentration, and the inset the Michaelis–Menten plot.

Mg^{2+}-free sucrose/salts buffer; thirdly, Ca^{2+}-uptake assays carried out at 4°C resulted in only a very low incorporation of Ca^{2+} compared with uptake at 30°C, and finally similar rates of Ca^{2+} uptake were observed for forespores prepared from protoplasts at different stages of maturation during a 1h period centred on stage V. Since this is the period of formation of spore cortex and coat, it suggests that changes in the nature of the forespore outer integuments do not influence Ca^{2+} uptake.

When isolated forespores were incubated at 30°C in the presence of various concentrations of $^{45}CaCl_2$, Michaelis–Menten kinetics were observed, and a Lineweaver–Burk plot (Fig. 4) yielded a K_m of 2.1 mM and $V_{max.}$ of 0.51 nmol of Ca^{2+}/min per ml of culture. The experiments showed that the total amount of Ca^{2+} incorporated by the forespores depended linearly on the assay Ca^{2+} concentration up to 2.5mM and increased with concentration up to at least 7.5mM-$CaCl_2$.

When isolated forespores were incubated in saturating concentrations of Ca^{2+} (7.5mM), they accumulated Ca^{2+} at a rate comparable with that of the sporangial protoplasts from which they were prepared. The rate and extent of forespore Ca^{2+} uptake was unaffected by inclusion in the assay of dipicolinic acid up to concentrations of 2.25mM (Table 1). However, at a dipicolinicacid concentration of 4.5mM, the rate of Ca^{2+} uptake was decreased to 72% of the control value and the total Ca^{2+} incorporated was reduced to approx. 55% of the control (Table 1).

Ca^{2+} uptake by isolated forespores was found to be specific. It was decreased by the inclusion in the assay of various non-radioactive cations (e.g. Sr^{2+}) to a final concentration of 25mM. Mg^{2+} and Na^+ has no effect on uptake, however.

Table 1. *Effect of dipicolinic acid on Ca^{2+} uptake by isolated stage V forespores*
Various concentrations of dipicolinic acid, as its sodium salt at pH 7, were included in isolated-forespore Ca^{2+}-uptake assays. Uptake was determined in the presence of 6.9mM-$CaCl_2$ as described in the Experimental section.

Dipicolinic acid (mM)	Rate of Ca^{2+} uptake (nmol of Ca^{2+}/min per ml of forespore culture)	Total Ca^{2+} incorporated in 40min (nmol/ml of forespore culture)
0	0.36	6.5
0.9	0.36	7.0
2.25	0.36	6.8
4.5	0.26	3.6

Ca^{2+} exchange in isolated forespores

To determine whether Ca^{2+} incorporated by isolated forespores could exchange with external Ca^{2+}, forespores were first preloaded with ^{45}Ca by incubation in 9.7mM-$^{45}CaCl_2$, as in the Ca^{2+}-uptake experiments. After centrifugation and resuspension in sucrose/salts buffer at 4°C, these preloaded forespores lost only approx. 6% of their ^{45}Ca during a 40min incubation at 4°C in the presence or absence of 9.7mM-$CaCl_2$ (Fig. 5). Preloaded forespores incubated at 30°C in sucrose/salts buffer (91.5μM-$CaCl_2$) lost up to 20% of their ^{45}Ca, whereas addition of non-radioactive 9.7mM-calcium resulted in marked loss of ^{45}Ca at this temperature, which varied from 50 to 60% between experiments (Fig. 5). Thus extensive exchange of the Ca^{2+} incorporated into forespores *in vitro* only occurred on challenge with high external Ca^{2+} concentrations at 30°C.

Similar exchange experiments were carried out on forespores isolated from sporulating protoplast

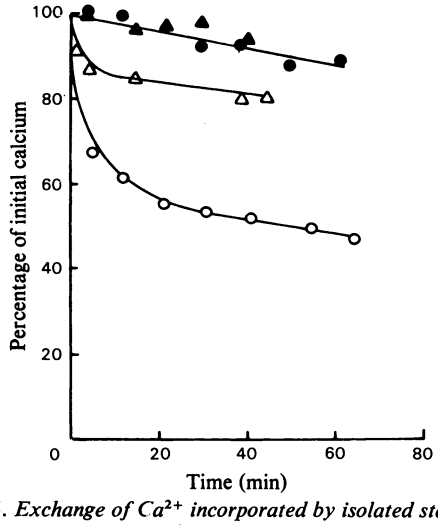

Fig. 5. *Exchange of Ca^{2+} incorporated by isolated stage V forespores*

Isolated forespores were preloaded with ^{45}Ca by incubation for 45min at 30°C with 9.7mM-$^{45}CaCl_2$. The forespores were harvested by centrifugation at 4°C and resuspended in sucrose/salts buffer at 4°C. Addition of non-radioactive 9.7mM-$CaCl_2$ was made in some cases and the forespores were incubated at either 4°C or 30°C. The ^{45}Ca content of the forespores was determined during this incubation and the results are expressed as percentages of the ^{45}Ca content in the forespore at zero time. △, No addition of Ca^{2+}, incubated 30°C; ▲, no addition of Ca^{2+}, incubated 4°C; ○, 9.7mM-$CaCl_2$ added, incubated 30°C; ●, 9.7mM-$CaCl_2$ added, incubated 4°C.

cultures that had been incubated with ^{45}Ca from stage III of sporulation. ^{45}Ca in such forespores was therefore incorporated *in situ*, that is, while they were contained within the sporangium. By contrast, when these forespores were incubated in the presence or absence of 9.7mM-$CaCl_2$ at 30°C, only 5–10% of the ^{45}Ca was lost over a period of 40min.

Discussion

In the present study, Ca^{2+} accumulation has been investigated in sporulating protoplast cultures and in the forespores isolated from these protoplasts. Ca^{2+} accumulation by sporulating protoplasts was observed to obey Michaelis–Menten kinetics, with a K_m value for Ca^{2+} of 31μM. This value was determined for 'steady-state' Ca^{2+} accumulation during late stage IV of sporulation, rather than for initial uptake rates. The present K_m for sporulating protoplasts of *B. megaterium* KM has a value tenfold less than that found by Eisenstadt & Silver (1972), who measured initial Ca^{2+}-uptake rates in *B. subtilis*. However, there are reports that these measurements of K_m and $V_{max.}$ in *B. subtilis* have not proved reproducible (Silver *et al.*, 1974, 1975; Scribner *et al.*, 1975).

La Nauze *et al.* (1974) showed that the Ca^{2+} content of forespores, released from the sporangium in stage IV to stage VI of sporulation, was 10–15 nmol/ml of culture lower than that of the corresponding protoplasts. Since under similar conditions no dipicolinic acid was found in mother-cell cytoplasm (Ellar & Posgate, 1974), the Ca^{2+} released did not result from forespore damage and must therefore have been located in the mother-cell compartment before sonication. If it is assumed that all of the Ca^{2+} exists free in the cytoplasm, then the mother-cell cytoplasmic Ca^{2+} concentration may be estimated. By using a volume of the mother-cell cytoplasm calculated from the dimensions of the sporulating cell and forespore, a concentration range of 3–5mM was calculated. By using a value of 4μl of cell water/mg dry weight (Silver *et al.*, 1975) and assuming that in stage V the cell water is distributed evenly between forespore and mother-cell compartments in proportion to their respective volumes, a range of 6–9mM was obtained.

In experiments to reconstitute the Ca^{2+}-transporting system in isolated forespores, it was shown that the only component of the mother-cell necessary to demonstrate Ca^{2+} incorporation in the isolated forespore was this high intracellular Ca^{2+} concentration. In saturating concentrations of external Ca^{2+}, the rate of Ca^{2+} uptake into isolated forespores *in vitro* was comparable with that into the sporangium *in vivo*. However, the extent of incorporation into isolated forespores was low and the period during which the incorporation was observed was correspondingly short, compared with the situation *in vivo*, where 95% of the medium Ca^{2+} becomes located in the forespore and mature spore in the period from stage IV to VI of sporulation.

Ca^{2+} incorporation by isolated forespores showed saturation kinetics with a K_m value of 2.1mM, which agrees well with the estimated mother-cell cytoplasmic concentration. The data suggest that Ca^{2+} uptake by forespores is by a carrier-mediated system, where the total uptake is influenced by the external Ca^{2+} concentration. Both protoplast and forespore Ca^{2+}-uptake systems were specific.

Models in which the transfer of dipicolinic acid into the forespore is in some manner linked to the uptake of Ca^{2+} have been suggested (Eisenstadt & Silver, 1972). Dipicolinic acid (0.9 and 2.25mM) had no effect on Ca^{2+} incorporation by isolated forespores. However, concentrations of 4.5mM-dipicolinic acid decreased both the rate of forespore Ca^{2+} uptake and the total Ca^{2+} incorporated. This effect can be readily explained by the formation of a Ca^{2+}– dipicolinic acid complex, thereby reducing the effective concentration of Ca^{2+} available to the forespore. With 4.5mM-dipicolinic acid, the free Ca^{2+}

concentration is reduced to approx. 2.5mM which should result in a 21% decrease in the rate of Ca^{2+} accumulation (Fig. 4). The observed decrease was consistent with this prediction. Thus apart from decreasing the external concentration of Ca^{2+} by chelation, dipicolinic acid had no effect on the forespore Ca^{2+} uptake *in vitro* described here.

Dipicolinic acid has been shown to be exclusively located in the forespore compartment (Ellar & Posgate, 1974) and its site of synthesis in the sporulating cell is thus of considerable significance in evaluation of models for Ca^{2+} transport during sporulation. The participation of part of the lysine-biosynthetic pathway in the biosynthesis of dipicolinic acid is now well established, although the mechanisms controlling the biosynthesis of dipicolinic acid and diaminopimelic acid remain unclear (Forman & Aronson, 1972; Hoganson & Stahly, 1975). Dihydrodipicolinate synthase has been shown to be located in the mother-cell cytoplasm (C. Hogarth, unpublished observations). However, the usefulness of this enzyme in predicting the site of biosynthesis of dipicolinic acid is complicated by its role in the production of diaminopimelate for biosynthesis of cortex and cell wall. There is one report that dipicolinate synthase, the only enzyme specific for dipicolinic acid biosynthesis, is present in the mother-cell cytoplasm and not in the forespore (Andreoli *et al.*, 1975). However, this enzyme is not yet well characterized and the assay is extremely difficult (Forman & Aronson, 1972). Interpretation of results is further complicated by the extreme instability of the enzyme (Chasin & Szulmajster, 1969) and the extensive non-enzymic conversion of dihydrodipicolinic acid into dipicolinic acid (Chasin & Szulmajster, 1967). Thus these studies do not rigorously exclude the possibility that dipicolinate synthase may be present in the forespore cytoplasm or outer integuments. Because of this uncertainty about dipicolinate synthase, further studies on its distribution within the sporangium are essential and it would be of interest to determine directly whether isolated forespores are capable of uptake of either dihydrodipicolinic acid or dipicolinic acid.

The possibility that dipicolinic acid or dihydrodipicolinic acid may be made in the mother-cell cytoplasm and rapidly transported to the forespore, where it chelates with Ca^{2+}, cannot be excluded. However, the results described here do indicate that the isolated forespore is capable of Ca^{2+} uptake without an obligatory requirement for dipicolinic acid.

One of the typical characteristics of transport systems is that efflux from the cells is not simply by leakage but is carrier-mediated (Kepes & Cohen, 1962; Silver & Kralovic, 1969). At 30°C loss of ^{45}Ca from preloaded forespores was greatly accelerated by high external Ca^{2+} concentrations, indicating an exchange process. If efflux is coupled to influx, then conditions that inhibit influx will also prevent efflux. Ca^{2+} incorporation by forespores occurred at exceedingly low rates at 4°C. High concentrations of external Ca^{2+} at 4°C did not increase the rate of loss of radioactivity, indicating that efflux may be coupled to influx and may occur by a carrier-mediated process. Similar demonstrations of efflux coupled to influx have been described for other ion-transporting systems (Lusk & Kennedy, 1969; Silver, 1969; Silver & Kralovic, 1969).

In these exchange experiments 40–50% of the ^{45}Ca remained unavailable for exchange. In marked contrast, 90% of the calcium, which was incorporated into the forespores while they were contained within the sporangium, remained unavailable for exchange, indicating tight binding to intracellular components.

It is possible that the differences observed between forespore Ca^{2+} uptake *in situ* and *in vitro*, namely the extent and duration of the incorporation and the exchangeability of the incorporated Ca^{2+}, may be attributed to dipicolinic acid. Considering the equimolarity of Ca^{2+} and dipicolinic acid found in forespores and mature spores (J. M. La Nauze, unpublished observations), the equilibrium constant for the formation of the Ca^{2+}–dipicolinic acid complex and its solubility assuming an aqueous environment, a 'free' Ca^{2+} concentration in the forespore of around 0.6mM may be estimated. Since this value is considerably less than the Ca^{2+} concentration in the mother-cell cytoplasm it could allow movement of Ca^{2+} into the forespore down a concentration gradient. The continued synthesis of dipicolinic acid, simultaneously with Ca^{2+} accumulation, together with the localization of dipicolinic acid in the forespore, could therefore provide a mechanism for prolonged net accumulation of Ca^{2+} into the forespore *in vivo*. Chelation and precipitation of calcium with dipicolinic acid could account for the low cation exchangeability observed here. Preliminary results (not given) suggest that dipicolinic acid synthesis does not continue in the isolated forespore during the period of Ca^{2+} accumulation *in vitro*. According to the above model this would seriously limit the extent of Ca^{2+} accumulation *in vitro*. Estimation of the free ^{45}Ca concentration in the forespore cytoplasm after forespore incorporation *in vitro* confirms that Ca^{2+} is not accumulated into the isolated forespore against a concentration gradient. In contrast, it is accumulated into the mother-cell cytoplasm to 3–9mM when the medium Ca^{2+} concentration is 0.1mM.

Thus Ca^{2+} uptake into both sporangial protoplasts and isolated forespores occurs by a calcium-specific carrier-mediated process, with the protoplast system demonstrating a 100-fold lower K_m for Ca^{2+}. Protoplasts accumulate Ca^{2+} against a concentration gradient, whereas Ca^{2+} moves into the forespore by a downhill transfer mechanism in which both the rate and extent of uptake are affected by the external

Ca^{2+} concentration. However, these data are in themselves insufficient to distinguish between active and passive transport, and the energy-dependence of protoplast and forespore Ca^{2+}-transport systems need to be investigated. In the model of sporulation-specific Ca^{2+} transport described here dipicolinic acid is postulated to act as a Ca^{2+} sink, maintaining a low free Ca^{2+} concentration within the forespore compartment, thereby ensuring continued transfer from mother-cell to forespore.

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References

Andreoli, A. J., Suehiro, S., Sakiyama, D., Takemoto, J., Vivanco, E., Lara, J. C. & Klute, M. C. (1973) *J. Bacteriol.* **115**, 1159–1166

Andreoli, A. J., Saranto, J., Baecker, P. A., Suehiro, S., Escamilla, E. & Steiner, A. (1975) in *Spores VI* (Gerhardt, P., Sadoff, H. L. & Costilow, R. N., eds.), pp. 418–424, American Society for Microbiology, Washington, D.C.

Bronner, F. & Freund, T. S. (1972) in *Spores V* (Halvorson, H. O., Hanson, R. & Campbell, L. L., eds.), pp. 187–190, American Society for Microbiology, Washington, D.C.

Bronner, F., Nash, W. C. & Golub, E. E. (1975) in *Spores VI* (Gerhardt, P., Sadoff, H. L. & Costilow, R. N., eds.), pp. 356–361, American Society for Microbiology, Washington, D.C.

Chasin, L. A. & Szulmajster, J. (1967) *Biochem. Biophys. Res. Commun.* **29**, 648–654

Chasin, L. A. & Szulmajster, J. (1969) in *Spores IV* (Campbell, L. L., ed.), pp. 133–147, American Society for Microbiology, Washington, D.C.

Eisenstadt, E. & Silver, S. (1972) in *Spores V* (Halvorson, H. O., Hanson, R. & Campbell, L. L., eds.), pp. 180–186, American Society for Microbiology, Washington, D.C.

Ellar, D. J. (1978) *Symp. Soc. Gen. Microbiol.* **28**, 295–326

Ellar, D. J. & Posgate, J. A. (1974) in *Spore Research 1973* (Barker, A. N., Gould, G. W. & Wolf, J., eds.), pp. 21–40, Academic Press, London and New York

Forman, M. & Aronson, A. (1972) *Biochem. J.* **126**, 503–513

Freese, E. (1972) *Curr. Top. Dev. Biol.* **7**, 85–123

Gould, G. W. & Dring, G. J. (1974) *Adv. Microbial Physiol.* **11**, 137–164

Hanson, R. S., Peterson, J. A. & Yousten, A. A. (1970) *Annu. Rev. Microbiol.* **24**, 53–90

Hoganson, D. A. & Stahly, D. P. (1975) *J. Bacteriol.* **124**, 1344–1350

Hogarth, C., Deans, J. A. & Ellar, D. J. (1977) in *Spore Research 1976* (Barker, A. N., Wolf, J., Ellar, D. J., Dring, G. J. & Gould, G. W., eds.), pp. 243–264, Academic Press, London

Kepes, A. & Cohen, G. N. (1962) in *Bacteria IV* (Gunsalus, I. C. & Stanier, R. Y., eds.), pp. 179–222, Academic Press, New York and London

La Nauze, J. M., Ellar, D. J., Denton, G. & Posgate, J. A. (1974) in *Spore Research 1973* (Barker, A. N., Gould, G. W. & Wolf, J., eds.), pp. 41–46, Academic Press, London and New York

Leanz, G. & Gilvarg, C. (1973) *J. Bacteriol.* **114**, 455–456

Lusk, J. E. & Kennedy, E. P. (1969) *J. Biol. Chem.* **244**, 1653–1655

Rosen, B. P. & McClees, J. S. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 5042–5046

Scribner, H., Mogelson, J., Eisenstadt, E. & Silver, S. (1975) in *Spores VI* (Gerhardt, P., Sadoff, H. L. & Costilow, R. N., eds.), pp. 346–355, American Society for Microbiology, Washington, D.C.

Silver, S. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **62**, 764–771

Silver, S. (1977) in *Microorganisms and Minerals* (Weinberg, E. D., ed.), pp. 49–103, Marcel Dekker, New York

Silver, S. & Kralovic, M. L. (1969) *Biochem. Biophys. Res. Commun.* **34**, 640–645

Silver, S., Toth, K., Bhattacharyya, P., Eisenstadt, E. & Scribner, H. (1974) in *Comparative Biochemistry and Physiology of Transport* (Bolis, L., Bloch, K., Luria, S. E. & Lynen, F., eds.), pp. 393–408, North-Holland Publishing Co., Amsterdam

Silver, S., Toth, K. & Scribner, H. (1975) *J. Bacteriol.* **122**, 880–885

Wilkinson, B. J., Deans, J. A. & Ellar, D. J. (1975) *Biochem. J.* **152**, 561–569

Young, I. E. & Fitz-James, P. C. (1962) *J. Cell Biol.* **12**, 115–133