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Effect of Carbon Source on Size and Associated Properties of *Bacillus megaterium* Spores

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The size of the spores produced by *Bacillus megaterium* ATCC 19213 depended upon the nature of the carbon source present in the defined medium in which they were produced. Homogeneous preparations of small (0.38 μm^3), nearly spherical spores were produced after batch culture in the presence of 2.8 mM citrate, and large (1.17 μm^3), oblong spores were produced by replacement culture in the presence of 7.35 mM acetate. Large and small spores had approximately the same deoxyribonucleic acid content, density, and heat resistance. Large spores contained about 2.5 times the dipicolinic acid, glucosamine, ribonucleic acid, Mn^{2+}, and lipid and about 1.5 times the Mg^{2+}, Fe^{2+}, Ca^{2+}, and dry weight of small spores. Large spores were especially enriched in Zn^{2+} (4.5-fold). More protein (1.5-fold) was extracted from small spores with 1 N NaOH than from large spores, possibly indicating a difference in the spore coats, but large spores contained about twice the Kjeldahl nitrogen of small spores. A difference in the coats may account for the fact that, unlike small spores, large spores showed improved germination with increased times and temperature of heat shocking. The possibility of determining the location of some of these substances within the spore by comparing the compositional ratios with estimated volumes of specific spore layers is discussed.

Both the size and chemical composition of bacterial cells are altered when the growth rate is varied by changing the carbon source in the growth medium (33). Similarly, the size and chemical composition of bacterial spores are affected by the type of complex medium in which the cells grow and sporulate (6, 11, 13). This paper describes a variation in the size of *Bacillus megaterium* spores due to the nature of the carbon source present in a defined medium and the effect of the size variation on certain spore properties.

MATERIALS AND METHODS

Organism and culture media. *B. megaterium* ATCC 19213 was grown in a defined sucrose-salts (SS) medium (35). SS medium was modified by the substitution of other carbon sources for sucrose as indicated.

Preparation of acetate and citrate spores. A standard spore suspension was prepared according to Imanaka et al. (21) and stored at −10 C. A portion of the standard suspension was heat shocked at 70 C for 30 min and used as the inoculum for SS medium supplemented with 100 μg each of L-alanine (Calbiochem) and inosine (Sigma) per ml. This culture was incubated at 30 C on a New Brunswick rotary shaker (180 rev/min) to a turbidity of 70 Klett units (about 10^8 cells per ml) measured in a Klett-Summerson photoelectric colorimeter with a no. 54 filter. At 70 Klett units, 2 ml of the preliminary culture was added to 50 ml of SS medium and again grown to 70 Klett units. This culture was used as a 1% (v/v) inoculum for the final sporulation medium [SS medium modified by the substitution of either 7.35 mM sodium acetate (Fisher) or 2.8 mM sodium citrate (Baker and Adamson) for sucrose]. The final cultures (400 to 800 ml) were incubated on a platform shaker (193 rev/min) at 30 C until sporulation was complete.

To eliminate vegetative cells from preparations, spores were harvested at 2000 × *g* at 4 C in a Sorvall RC-2 refrigerated centrifuge, suspended in distilled water containing 200 μg of lysozyme per ml (Calbiochem), and incubated at 37 C for 1 hr. Preparations were then washed sequentially with 17.3 mM sodium dodecyl sulfate, 1 M NaCl, 0.14 M NaCl, three times with distilled water and were suspended in distilled water. Examination with the microscope showed no loss of refractility during this process, and plating on nutrient agar (Difco) demonstrated the viability of the preparations.

Electron microscopy. A suspension of spores was prefixed in 5% glutaraldehyde in 0.1 M potassium phosphate buffer (pH 7.25) overnight at 4 C. The

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glutaraldehyde was poured off, and the spores were embedded in buffered Ionagar (Oxoid). Cubes (approximately 1 mm^3) were made and fixed overnight in 1% (w/v) OsO_4 in Kellenberger's buffer. Cubes were washed with Kellenberger's buffer and transferred to 0.5% (w/v) uranyl acetate in Kellenberger's buffer for 2.5 hr. The cubes then were moved through an acetone series (70, 90, 95, anhydrous 100%) before being embedded in a mixture of Epon 812 and 815. After polymerization, the samples were sectioned with a diamond knife (Ge-Fe-Ri, Frosinone, Italy) on a Porter-Blum MT-2 ultramicrotome (Ivan Sorvall, Inc., Norwalk, Conn.). Sections were mounted on 300-mesh copper grids, poststained with 2% uranyl acetate, and viewed with a Philips 300 electron microscope at an accelerating voltage of 60 kv.

Deoxyribonucleic acid, ribonucleic acid, protein. Spores were fractionated for chemical analyses by the method of Schmidt and Thannhauser (34) as modified by Hutchison and Munro (20).

The pooled perchloric acid (PCA) extracts were analyzed for ribonucleic acid (RNA) by an orcinol method (8) using yeast sRNA (Calbiochem) as a standard. Deoxyribonucleic acid (DNA) was analyzed in the NaOH extracts by the indole method of Cerrioti (4) using salmon sperm DNA (Calbiochem) as a standard. Protein was assayed in the NaOH extracts by the method of Lowry et al. (28) with bovine serum albumin (Pentex) as a standard.

DPA. Dipicolinic acid (DPA) was assayed in autoclaved samples by the method of Janssen et al. (22) using DPA (Aldrich) as a standard.

Glucosamine. Whole spores were hydrolyzed with 6 N HCl at 100 C in sealed ampoules for periods up to 24 hr. Glucosamine was estimated by the method of Cessi and Piliego (5) using D-glucosamine-hydrochloride (Eastman) as a standard, and the results were extrapolated to zero hydrolysis time.

Kjeldahl nitrogen. The procedure of Lang (26) was followed, with ammonium sulfate (J. T. Baker) as a standard.

Metals. Metals were determined in acidified samples using a Perkin Elmer Model 303 atomic absorption spectrophotometer and the methods described by Crosby et al. (7).

PHB. Poly-β-hydroxybutyrate (PHB) was determined by the method of Law and Slepecky (27).

Lipids. Spores were ground with 120-μm glass beads in a Gifford-Wood Mini-Mill at a clearance of 0.305 mm. The temperature of the suspension was maintained below 10 C by means of an ice-salt bath. After being ground for 30 min, the beads were allowed to settle 10 min, the supernatant fluid was decanted, and the beads were washed with water. The combined supernatant fluids were lyophilized.

The residue was extracted with acetone for 1 hr, twice with chloroform-methanol (2:1) for 2 hr, and once with chloroform-methanol (1:1) for 1 hr. Between extractions, the solvents were removed by filtration through Whatman no. 40 filter paper in coarse sintered-glass filters. The combined extracts were pooled and evaporated to dryness at 60 C in a Rinco flash evaporator.

The residue was suspended in chloroform-methanol (2:1), and the solution was washed twice with 0.3% NaCl (14). One volume of absolute alcohol was added, and the solution was chilled and then centrifuged at 10,000 $\times$ g. The dry weight of the supernatant fraction was determined.

Dry weight. Samples were dried at 80 C in tared containers. Dry weights were measured using a Mettler model H balance at 24 hr intervals until three successive weighings indicated that a constant weight had been achieved.

Spore germination. Heat-shocked spores were germinated at 30 C in 0.055 M potassium phosphate buffer (*p*H 7) containing 100 μg each of L-alanine and inosine per ml. The decrease in optical density was monitored with a Klett-Summerson photoelectric colorimeter with a no. 54 filter.

Heat resistance. Spore suspensions sealed in glass ampoules were totally immersed in an 85-C water bath (Precision Scientific Co.). Ampoules were withdrawn at intervals and cooled in ice water. Heated spore suspensions were serially diluted in distilled water for colony counts in nutrient agar (Difco) after incubation at 37 C for 2 days.

Spore size. Spore size was measured on photomicrographs taken using a Zeiss dark-contrast, phase-contrast microscope. One hundred spores of each type were measured in two dimensions. Volumes were calculated from measured radii, assuming the actual spore shape to be a prolate spheroid or a sphere, depending on the measurements. Dimensions of T_0 cells were measured in a similar manner.

Volumes of spore structural layers. The width of each structural layer on a single spore was measured in five places (using an ocular micrometer calibrated to 0.127 mm units) on electron micrographs such as those in Fig. 3A and B. Layers of uneven thickness were measured in two dimensions on each section. The outer forespore membrane was not visible on all sections, in which case it was assumed to be a layer of thickness equal to that of the inner forespore membrane, lying between the cortex and the coat layers. Ten apparently medial sections were measured, and the results were averaged for each type of spore. The volume of each layer was determined by assuming a series of concentric prolate spheroids, and the results were converted to percentage of the total volume. For comparison, the total volumes were assumed to be in the ratio found when determined as described above.

Terminology. Replacement culture: cells were transferred to fresh medium and responded by sporulating in the absence of cell division. Batch culture: cells were transferred to fresh medium and responded by sporulating after cell division occurred in the new medium. Acetate spores: spores formed by replacement culture in SS containing 7.35 mM acetate. Citrate spores: spores formed by batch culture in SS containing 2.8 mM citrate.

RESULTS

Spore production. The size of the spores formed by *B. megaterium* in the presence of various carbon sources depended on the nature of the carbon source. Large spores were produced in replacement culture with 7.35 mM

acetate, 6.95 mM α-ketoglutarate, or 3.8 mM succinate, or in batch culture with 9.08 mM pyruvate or 11.2 mM L-alanine. Small spores were produced in replacement culture in the absence of a carbon source in basal salts medium (but in poor yield) or in a batch culture with 2.8 mM citrate. Spores produced with other carbon sources (2.9 mM sucrose, 5.0 mM isocitrate, 7.5 mM malate, 7.6 mM oxaloacetate, and 13.5 mM glyoxylate) were of intermediate size.

The behavior of sucrose-grown cells in acetate replacement medium or basal salts medium varied when the growth medium supernatant fluid was removed from the inoculum. Washed inoculum cells gave spores of variable sizes in acetate and very few spores in basal salts medium. The washed cells grew and sporulated normally in fresh SS medium. The effect of the inoculum carry-over was not investigated further. In subsequent experiments, the inoculum cells were not washed.

No growth occurred during production of large spores by replacement sporulation in 7.35 mM acetate (Fig. 1). As the acetate concentration was increased from 7.35 to 117.6 mM, the percentage of cells undergoing replacement sporulation to yield large spores decreased. At 29.4 mM or greater concentrations, growth occurred with a mean generation time of 6 hr, and the spores produced were variable in size. At less than 29.4 mM acetate, the cells underwent replacement sporulation, vegetative growth becoming noticeable only after very prolonged incubation, and the spores produced were uniformly large. Similar concentration effects were observed with succinate and with α-ketoglutarate.

Although growth occurred before the production of small spores in batch culture with 2.8 mM citrate (Fig. 1), small spores were produced with no growth when sporulation occurred in basal salts medium (i.e., in the presence of only those carbon and energy sources which were carried over with the inoculum). Due to the homogeneity of the preparations, citrate and acetate spores were studied further.

Spore appearance. Observation of both types of spores in phase-contrast (Fig. 2) and electron microscopy (Fig. 3) showed differences in size and shape.

Spore size. Acetate spores had a mean volume of 1.17 μm^3, in comparison with a mean volume of 0.38 μm^3 for citrate spores. The ratio of mean volumes was 3.08. The distribution of volumes in populations of each type of spore is shown in Fig. 4.

Resistance. Measurement of the heat resistance of citrate and acetate spores showed no significant differences (Fig. 5). Preliminary results suggest no major difference in survival rate during exposure to ultraviolet radiation.

Germination. Germination studies indicated a difference between the two types of spores in their response to a heat shock (Fig. 6). Both types of spores required a heat shock as well as physiological germinants. However, citrate spores gave an identical germination pattern with heat shocks from 60 to 70 C for 30 to 60 min, whereas acetate spores showed improved germination after heat shocking at higher temperatures and for longer times. With a heat shock at 70 C for 60 min, acetate spores germinated faster than citrate spores, but, at lower temperatures, or shorter times, or both, the reverse was true.

Chemical properties. Determination of the chemical composition of acetate and citrate spores indicated differences between the two (Table 1). Acetate spores contained about the same amount of DNA but about 2.5 times as much RNA, glucosamine, lipid, DPA, and Mn^{2+} and about 1.5 times as much Mg^{2+}, Fe^{2+}, Ca^{2+}, and dry weight as citrate spores. Acetate spores contained about 4.5 times as much Zn^{2+} and about twice as much Kjeldahl nitrogen as citrate spores, but citrate spores contained about 50% more extractable protein than acetate spores. No detectable PHB was found in either type of spore.

Location of substances within spore layers. The following discussion rests on two assumptions. (i) Substances which are located in a particular layer of spores of one size will be located in the same layer in spores of another size. There is no evidence for or against this assumption. (ii) The concentration of a substance in a spore layer does not vary. Thus differences in the total amount of a substance in spores of different sizes result from different volumes of the layers rather than different concentrations of the substances. If the concentration of a substance in its layer varies with spore size, it cannot be located by this method.

If these two assumptions are made, some conclusions about the location of some of the substances in Table 1 within particular spore layers can be drawn on the basis of volume ratios of spore layers of large and small spores.

Table 2 shows the results of calculations of relative volumes of specific layers of acetate and citrate spores. The calculations were based on measurements made from electron micrographs of apparently medial thin sections. For the most reliable measurements, sections

should be selected which are demonstrably medial (e.g., selected from a series of serial sections). Our subjective procedure for the selection of sections for measurement introduces some error into the calculations, although this is partially compensated for by the selection of longitudinal sections for the large-spore measurements. Bearing this limitation in mind, a consideration of the most obvious relationships may prove fruitful.

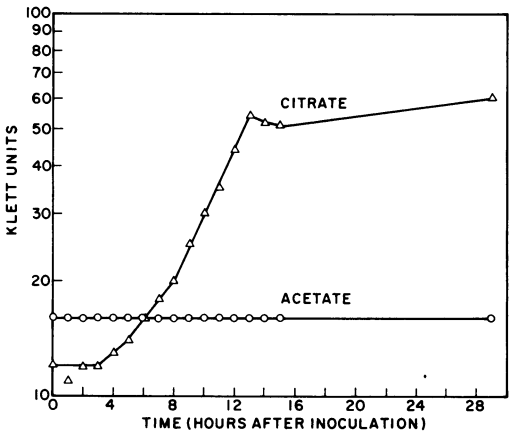

FIG. 1. *Turbidity measurements of cultures producing citrate (△) and acetate (○) spores. Cultures in acetate sporulate immediately upon inoculation. Cultures in citrate sporulate after the end of growth.*

Immediately evident are the relationship between the ratio of spore protoplast or membrane volumes and the ratios (Table 1) of the RNA, lipid, DPA, and Mn^{2+} compositions of large and small spores. That the RNA should be present in a ratio consistent with a location in the spore protoplast is not surprising. Similarly, the expected major location for lipid would be the forespore membranes and the membranous inclusions typically found in spores (Fig. 3D; reference 29). However, the data do not allow a distinction to be made between a protoplast and a membrane location.

That the DPA and Mn^{2+} ratios should indicate a protoplast or membrane location is more unexpected, although the possibility of a Mn-DPA chelate in spores, especially *B. megaterium* spores, has been previously mentioned (2, 39), and some data consistent with a core location for DPA have been reported (10, 32). Fitz-James has recently suggested (12) that the DPA may be located in the spore protoplast. Mn^{2+} incorporation and DPA synthesis in this organism are correlated both in time and quantitatively when spores are formed in SS medium (7).

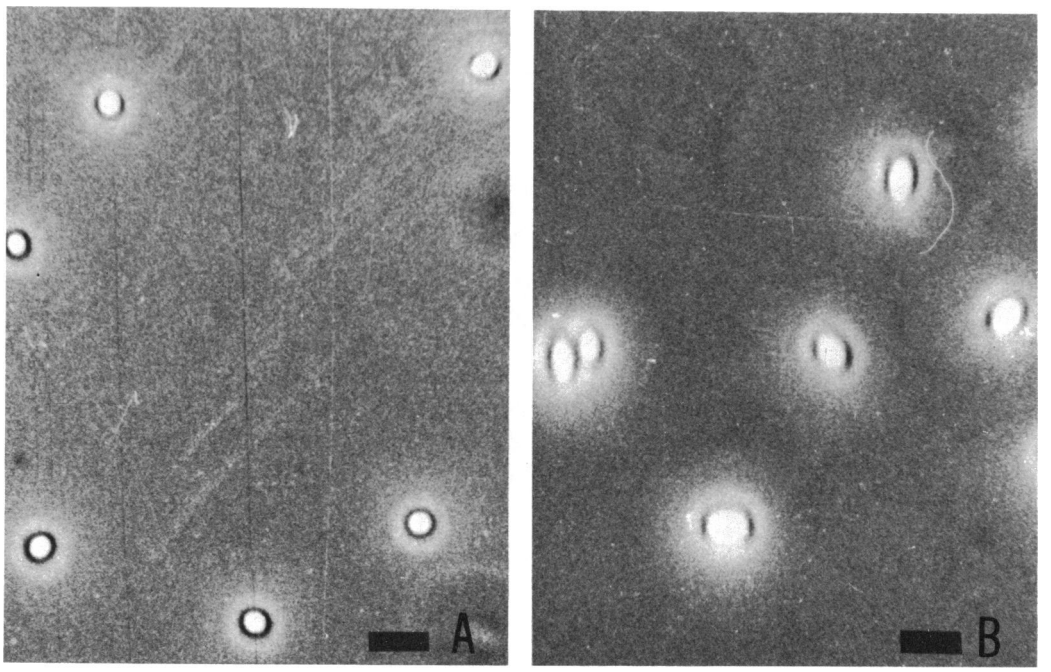

FIG. 2. *Appearance of spores by phase-contrast microscopy. A, citrate spores; B, acetate spores. Bars indicate 2 μm.*

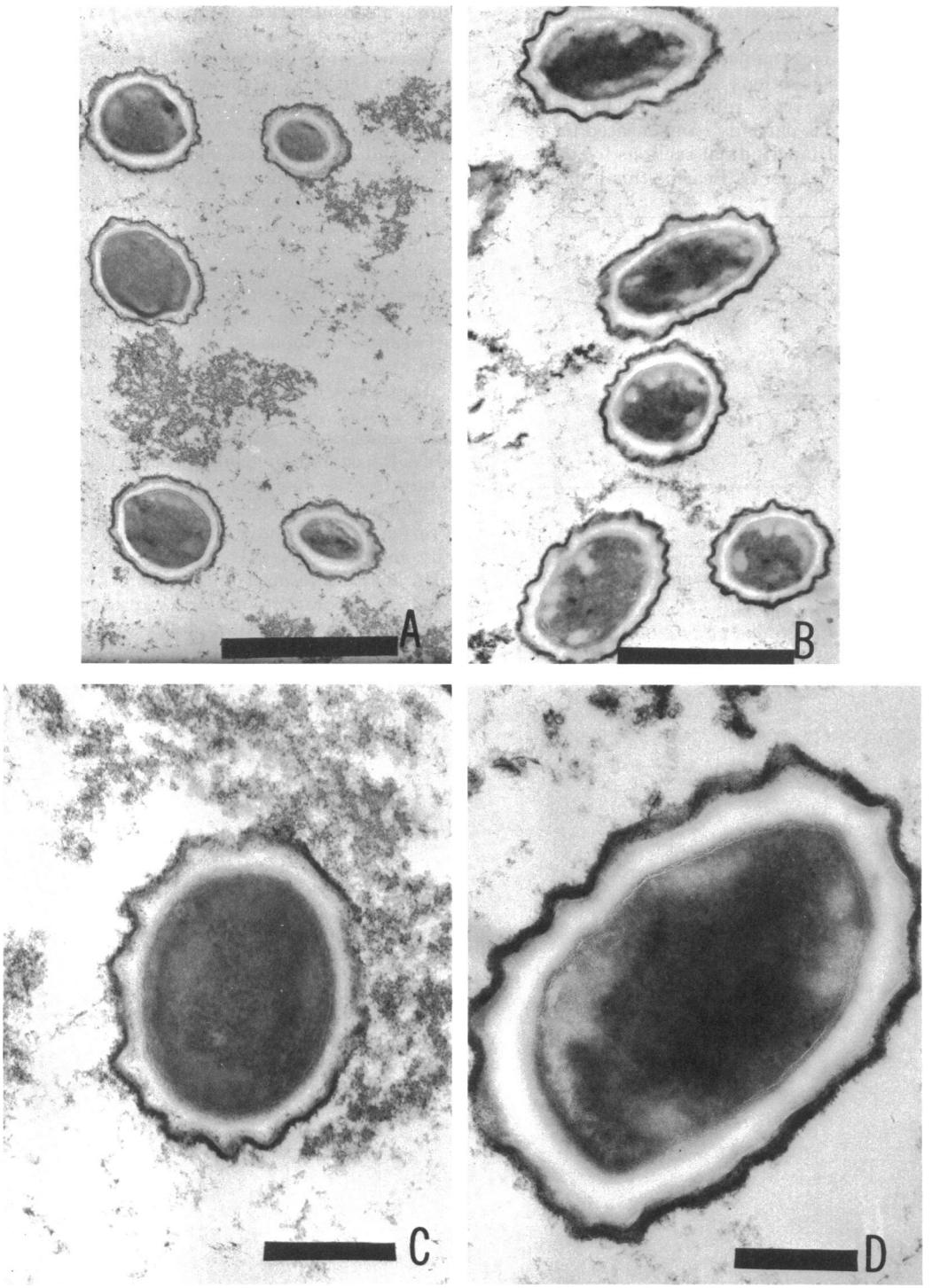

FIG. 3. *Appearance of thin sections of spores by electron microscopy. (A, C) citrate; (B, D) acetate. Bars in A and B indicate 1 μm. Bars in C and D indicate 0.25 μm.*

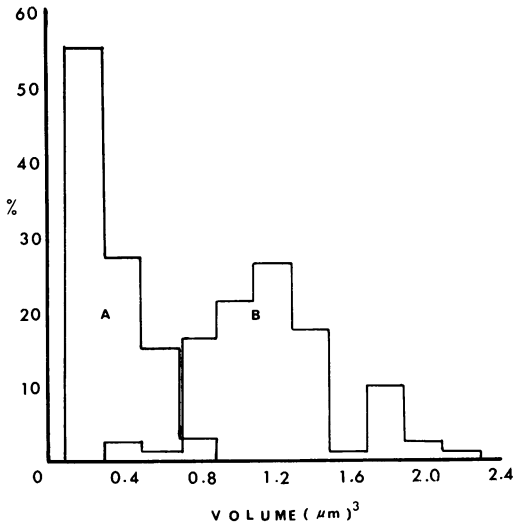

FIG. 4. *Frequency histograms of the percent of citrate (A) and acetate (B) spores with volumes in the indicated ranges. Volumes were calculated assuming a spherical shape for the citrate spores and a prolate spheroid for the acetate spores. (see Fig. 2).*

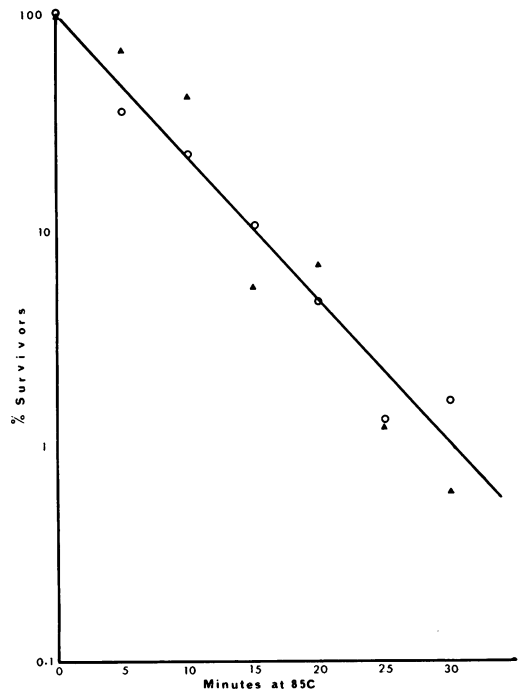

FIG. 5. *Heat resistance of spores as indicated by percentage of spores surviving 85 C for the indicated time. Symbols: (▲) acetate spores; (○) citrate spores.*

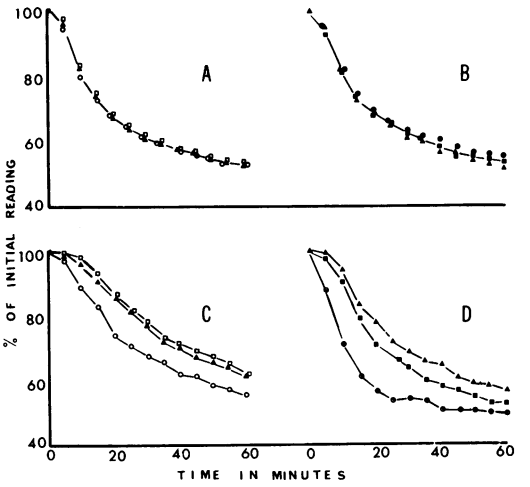

FIG. 6. *Germination kinetics of spores pretreated by various heat shock treatments. Symbols: (□) 60 C, 30 min; (△) 65 C, 30 min; (○) 70 C, 30 min; (▲) 60 C, 60 min; (■) 65, 60 min; (⊙) 70 C, 60 min. A and B citrate spores; C and D acetate spores.*

The possibility that only Ca^{2+} is associated with DPA in these spores is rendered less likely by the Ca/DPA molar ratios. For the citrate spores the ratio is 0.94, near to the expected ratio of 1.00, but for acetate spores the ratio is only 0.61. Since the acetate spores are formed in replacement culture (i.e., in a medium in which the cells do not divide before sporulation occurs), excess Ca^{2+} is available during the sporulation process, and Ca^{2+} limitation is thus not responsible for the low Ca/DPA ratio of acetate spores.

Significant in this regard is the fact that there is no apparent difference in heat resistance of acetate and citrate spores despite the large difference in the Ca/DPA ratios. Murrell and Warth (31) have suggested that heat resistance results from a complex interaction among several spore components rather than a simple dependence of the Ca^{2+} and DPA contents. To test this hypothesis, acetate and citrate spores were washed as described by Murrell and Warth (31) and reassayed for Mg^{2+} and Ca^{2+}. These results and those previously obtained for glucosamine (Table 1) were used to calculate a decimal reduction time at 100 C according to "equation a" (31). Predicted decimal reduction times were 3.7 min for acetate spores and 4.1 min for citrate spores, in much better agreement with the observed similarity in heat resistance at 85 C than the Ca/DPA ratios.

Glucosamine should be located primarily

TABLE 1. *Chemical composition of acetate and citrate spores*

Constituent	Acetate	Citrate[a]	Acetate/citrate
DNA	1.00	1.00	1.00[b]
Extractable protein[c]	8.54	11.36	0.74
Kjeldahl nitrogen	5.06	2.76	1.83
PHB	None	None	
Glucosamine	2.31	0.83	2.79
RNA	3.41	1.39	2.46
Lipid	3.49	1.23	2.84
DPA	11.85	5.06	2.34
Mn^{2+}	1.89	0.77	2.45
Zn^{2+}	4.37	0.94	4.67
Mg^{2+}	0.19	0.17	1.10
Fe^{2+}	3.99	2.78	1.43
Ca^{2+}	1.71	1.12	1.53
Dry weight	76.40	47.85	1.60

[a] Values expressed as micrograms per microgram of DNA.

[b] DNA per spore ratios of acetate spores to citrate spores were in the range 0.90 to 0.99. An error was presumed to lie in the determination of spore numbers and a ratio of 1.00 was assumed. Absolute DNA values in a typical experiment were 1.02×10^{-8} and 1.12×10^{-8} μg/spore, respectively.

[c] Refers to protein solubilized by three extractions with 1 N NaOH at room temperature for 1 hr.

within the cortex and cell wall primordium layers (31). The glucosamine ratio is within 0.5 of the cortical volume ratio.

Mg^{2+}, Fe^{2+}, Ca^{2+}, and dry weights are present in these spores in ratios of about 1.5. From Table 2, it can be seen that none of the spore structural layers considered in the table exhibit this volume ratio. Neither does any combination of these layers result in a ratio of 1.5. Thus, the physical significance of the 1.5 ratio is not clear. The literature (24, 38) indicates that most of the dry weight of spores resides in the cortex and coat layers. If this is the case here, a cortex, coat location, or both are indicated for these metals.

Calculations of the surface areas of each layer in these spores indicate that in each case the ratio of the surface area of the layers is near 2.0. This is much closer to the 1.5 ratio than any volume comparison but not close enough to be convincing.

The location of these metals is a point which might be resolved by further study of this phenomenon. The result would be particularly interesting since the possibility exists of determining a location for Ca^{2+} which is physically distinct from the location of DPA in the spore.

The data are inconsistent in that if 1.5 times as much material is placed in 3 times the volume, a density difference between the spores would be expected. However, by equilibrium centrifugation in Renografin gradients (37) acetate and citrate spores each have a density of 1.352 ± 0.009. Further, the Fe^{2+} concentration may not be physiological since ferric salts precipitate in SS medium, and the results may be affected by the chelating properties of citrate.

TABLE 2. *Volumes of spore layers*

Layer	Acetate[a]	Citrate[a]	Acetate/citrate[b]
Core	33.37	39.70	2.59
Membranes	4.85	6.40	2.33
Cell wall primordium	4.30	3.51	3.77
Cortex	42.19	39.81	3.26
Coats	15.29	10.59	4.45

[a] Values expressed as percentage of the total volume. Based on 10 sections.

[b] Based on a total volume ratio (acetate/citrate) of 3.08.

The data for extractable protein may indicate that the coat proteins of citrate spores are partially solubilized by 1 N NaOH, although spore coat proteins are fairly resistant to 1 N NaOH (29), and washes with NaOH are commonly used as preparative procedures (1) with seemingly little effect. However, Kondo and Foster (24) reported some solubilization by this procedure.

If spore proteins are assumed to be 16% nitrogen by weight, a value for total spore protein can be obtained by multiplying the Kjeldahl nitrogen content by 6.25. The results indicate that 66% of the citrate spore proteins are extractable with 1 N NaOH, but only 27% of the acetate spore proteins are extractable. However, the ratio of the nonextractable proteins is 3.92 compared to a calculated coat volume ratio of 4.45, indicating that the citrate spore coat proteins are not significantly more soluble in NaOH than the acetate spore coat proteins. Our interpretation is that citrate spores contain an elevated concentration of protein in the spore protoplast.

The idea of a difference in the coat layers is supported by observations with the phase-contrast microscope (Fig. 2) in which the refractile appearance of the two types of spores differs significantly. Further, many workers have postulated (for review, see 23) that the heat shock has an effect on the coat layers of spores, and recently (18) a morphological change in the

coat layers has been associated with activation.

Zn^{2+} is present in these spores in a ratio which suggests a location in the coat layers of the spore. Crosby et al. (7) have reported the likely peripheral nature of Zn^{2+} binding in spores of this organism and the fact that Zn^{2+} seems to be the only metal studied which is significantly removed from the spores by a heat shock.

Genetic consideration. Colonies formed by large and small spores on nutrient agar were similar in morphology, as were the constituent vegetative cells. Several colonies derived from each size of spore by plating on nutrient agar were subcultured in nutrient broth and gave identical populations of intermediate-size spores when transferred to SS medium. When transferred from nutrient broth to acetate medium, identical populations of large spores were produced. Since no growth occurs in acetate medium, the variation in size and chemical composition of these spores does not seem to be due to selection of large and small spore mutant strains.

DISCUSSION

Some of this work demonstrated the possibility of varying spore size and chemical composition, apparently without altering the genetic makeup of the spore. Comparison of chemical and structural data suggested that RNA, lipid, Mn^{2+}, and DPA may all be located within the spore protoplast or in the membranes of the spore. A protoplast location for DPA has been suggested by Pearce and Fitz-James (32) on the basis of studies with a cortexless mutant.

The possibility is raised by the data that at least some of the DPA in these spores exists as the Mn^{2+}, not the Ca^{2+}, chelate and that the chelate is located in the spore protoplast or the forespore membranes. Perhaps additional work on this phenomenon using measurements from electron micrographs of serial sections of spores to determine relative volumes of spore layers in a more rigorous manner could resolve this point.

If a Mn-DPA chelate does exist in these spores, it seems not to be essential for the formation of normal spores. Endotrophically formed spores accumulate DPA (3) in the absence of Mn^{2+}, and Mn^{2+} is therefore not required for DPA synthesis. Further, no significant amount of Mn^{2+} is required to obtain normal spores (Greene and Slepecky, *unpublished data*).

Similar analysis suggested that Zn^{2+} may be located in the coat layers of these spores. The possibility exists that acetate and citrate spores differ in their response to heat shocking as a result of differences in the coat layers of the spores. The effect of a greater heat shock may be to remove the larger quantity of Zn^{2+} bound to the coat layers of the larger spores. This is suggestive of a role for Zn^{2+} in the maintenance of the dormant state in bacterial spores, a suggestion certainly worthy of further study.

Some substances were found in concentrations which clearly disagree with assumption two stated above. DNA content per spore remained constant, although spore volume varied threefold. The acetate spores were formed from vegetative cells grown on sucrose with a doubling time of about 1 hr. The citrate spores were formed from vegetative cells grown on citrate with a doubling time of about 3.8 hr. According to Schaechter et al. (33), this should cause a definite difference in DNA per cell at the initiation of sporulation. The apparent equality of DNA content per spore in the citrate and acetate spores indicates that the DNA per spore is a reflection of the mechanism of spore formation, rather than a reflection of the DNA per cell when sporulation is initiated.

The amount of extractable protein was higher in the smaller spores, and it was therefore present in higher concentration in them. The results obtained for the location of Mg^{2+}, Fe^{2+}, and Ca^{2+} may indicate a location at a surface within the spore but could also be explained by a change in concentration with spore size.

Thus, for some substances, study of this phenomenon cannot establish a location. Until the validity or lack of validity of the assumptions stated above can be established for the other substances, the conclusions concerning location of spore components within the spore should be regarded as tentative.

The cytological evidence (Fig. 2 and 3) indicates that acetate and citrate spores differ in shape as well as size. The difference in shape is seen in Fig. 3 to result primarily from the shape of the spore protoplasts. The elongation in shape with increase in spore size is probably a reflection of the rod-shaped nature of the vegetative cells.

Citrate has been previously reported to affect the levels of tricarboxylic acid cycle enzymes through chelation of ferrous ions (15). That this effect is not the cause of the phenomena studied here is shown by the production of small spores in the presence or the absence (SS medium with no carbon source) of

citrate and the production of large spores in media containing both citrate and acetate. However, the specific activities of some tricarboxylic acid cycle enzymes have been shown to vary with the carbon source used for growth (17). We have not eliminated the possibility that a similar mechanism controls the size differences here.

The forespore is known to increase in size between stages II and IV during sporulation (16, 30). It seems reasonable to assume that this growth of the forespore can be encouraged or discouraged by the nutrient environment.

This hypothesis is supported by the observation that large spores are produced on acetate only under conditions in which acetate is present to be utilized as an energy source for growth of the forespore. When conditions allow prior utilization of the acetate for an increase in cell numbers, the spores produced are variable in size. Thus, cells adapted to acetate, either by prior growth on acetate or by exposure to concentrations of acetate above 29.4 mM, produce spores of variable size due to depletion of the medium by prior cell division, leaving conditions less favorable for an increase in forespore size. In addition, small spores are produced by cells which sporulate in replacement culture on limiting concentrations of acetate (Greene and Slepecky, *unpublished data*). When the nutrient environment is limited to carry-over from the inoculum, sucrose-size cells produce small spores. Similarly, cells inoculated into media containing both citrate and acetate proceed to divide, as on citrate alone, but the spores formed after the end of growth were large. This indicates that cells grown in citrate can yield large spores if the nutrient environment is enriched.

It is possible that the forespore increases in size as a result of utilizing the PHB reserves of the mother cell as was reported for endotrophically-formed spores (Komineck, Ph.D. Thesis, University of Illinois, Urbana, 1964). Media yielding large spores would affect spore size by increasing the PHB level of the sporangium. We have not investigated this specific point, but cells in SS medium as late as T_1 can be influenced to form large spores through supplementation of the medium. Ordinarily PHB reserves would be completely utilized by this time (36).

The size and chemical composition of the spores produced by *B. megaterium* depend upon the carbon source present in a defined medium. This seems to be an effect distinct from that of the genetically determined variation in spore size recently shown (9, 25) to occur in *B. subtilis*. It is possible, however, that the effect on spore size and shape which results from mutation of the DNA-dependent RNA polymerase (9, 25) may be due to an alteration of the nutritional balance in the sporangium, leading to effects similar to those described here. Our data agree with the more limited results previously obtained in complex media with *B. cereus* (11, 13). Studies of this type are important in that they offer a possible means of determining the location of some spore constituents within specific layers of the spore and because variation of spore composition due to the nature of the nutritional environment may be important in regulating the distribution of sporeforming organisms in nature.

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