Density Gradient Centrifugation for the Separation of Sporulating Forms of Bacteria*

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SUMMARY
Urografin has been shown to be suitable as a supporting medium for isopycnic density centrifugation of Bacillus megaterium spores. The complete separation of vegetative cells and spores can be achieved through the use of such gradients. Vegetative cells were found to have an average density of 1.135 g per cm³, while the average spore density was 1.305 g per cm³. Under normal sporulating conditions the spores formed consisted of a heterogeneous population with a wide spectrum of densities. These density differences could be correlated with such properties as resistance to thermal death and ability to be heat-activated.

Spores formed in the absence of Ca++ are less dense than those obtained under the usual conditions.

The pattern of cell distribution obtained on the Urografin gradient can serve as a gauge of the efficiency of sporulation and degree of synchrony of a sporulating culture.

In certain genera of bacteria, exhaustion of some required nutrient initiates a complex sequence of events that results in the transformation of the vegetative cell into a sporangium, the production of a spore within the sporangium, and its later release. The phenomenon of sporulation represents, therefore, an example of cell differentiation and may serve as a model for this process in higher forms.

However, attempts of defining, at a chemical level, the stages through which a sporulating culture passes are hampered by the extreme asynchrony of such cultures.

At present there is no method for directly synchronizing a sporulating culture. Gillis et al. (1) have used the filtration technique for synchronizing the cells of the vegetative phase just prior to the inception of sporulation. However, the lengthy time required for sporulation permits asynchrony to develop, limiting the effectiveness of such a procedure.

An alternative approach would consist of isolating the different elements of a mixed sporulating population. Isopycnic gradient centrifugation offers a means of separating those elements that differ in density. Sucrose was used as a supporting medium in experiments by Church and Halvorson (2) and also by Howitt (3). Because of the high density of the spores, however, most of the preparation was recovered as a pellet and was therefore less adequately characterized. Lewis, Snell, and Alderton (4) used the lead chelate of N,N'-(dihydroxyethyl)-N,N'-(dicarboxymethyl)ethylenediamine as a supporting medium for a gradient and were able to separate distinct classes of spores. However, the spores were not tested for viability, and thus it is not known whether they were damaged by the high concentration of lead.

It is apparent that, if isopycnic gradient centrifugation is to be used successfully as a method of separation and characterization of the different cellular forms, a supporting medium is needed with a high density, low viscosity, and no toxic effect on the cells.

Schatz, Haslbrunner, and Tuppy (5) have reported the use of Urografin, a compound with high density and relatively low viscosity, as a supporting medium for a density gradient. Their successful isolation and characterization of functional mitochondria from such gradients indicated that this compound might be without toxic effect on bacteria.

This paper describes the separation and characterization of vegetative cells and spores of Bacillus megaterium on a density gradient formed with Urografin. It has been possible to separate completely these different cellular forms in high yield. The gradient pattern also provides a measure of the degree of synchrony of the system, the extent to which sporulation occurred, and the extent to which germination had proceeded at any given time.

METHODS
Growth Condition—B. megaterium (local strain) was grown on a minimal medium modified after Millet and Aubert (6). The modification consisted of omitting the yeast extract from the medium. The components of the medium were: NH₄Cl, 0.2 g; Na₂HPO₄·7H₂O, 0.45 g; KH₂PO₄, 0.3 g; NaCl, 0.3 g; MgCl₂·6H₂O, 0.01 g; NaSO₄, 0.01 g; MnCl₂·2H₂O, 0.001 g; glass-distilled water to 100 ml; glucose, 0.1%; and CaCl₂·2H₂O, 0.015% (pH 6.8). The salt, glucose, and calcium chloride solutions were autoclaved separately and combined after cooling. This minimal medium will be referred to in the text as Medium A.

When defective spores, deficient in their content of dipicolinic acid, were required, the CaCl₂ was omitted. The cells were grown at 34°C with vigorous aeration. Spore yield was markedly dependent on temperature, and so the precise control of this variable was particularly critical. Unless otherwise stated, an inoculum (2 x 10⁷ spores) was added to 200 ml of Medium A.
During the growth phase, harvested after 60 hours, about 45 hours after the end of growth, the glucose limitation, growth of the vegetative cells ceased upon the occurrence of the exponential phase. Medium A was used for the preparation of solid media, the glucose concentration was increased to 0.5%.

Preparation of Spore Suspension—To ensure standard conditions, spores were used for inoculation. The spores were obtained by permitting the bacteria to sporulate in a 6-liter Erlenmeyer flask containing 1.5 liters of Medium A. The culture was harvested after 60 hours, about 45 hours after the end of growth. The spores were collected by centrifugation and washed several times with water. They were then suspended in water at a concentration of about 10^9 cells per ml and stored at -15°C. No heat activation was used before inoculation unless specifically stated. In all experiments, the washing of vegetative cells and all dilutions were carried out with the use of salts media.

Formation and Characterization of Urografin Gradient—Exponential gradients of Urografin were formed by allowing 30 ml of H_2O to drop from an elevated Erlenmeyer flask into a specified volume of Urografin (usually 19 or 37.5 ml) while the Urografin solution was mixed with a magnetic stirrer. The gradually diluted Urografin, 30 ml, was collected through Tygon tubing fitted with a glass capillary, along the side of a cellulose nitrate tube, 1 inch in diameter. The flow rate was regulated to require about 30 min for the formation of a gradient. A closed system was used so that addition of fluid to the mixing chamber displaced an equal volume into the collecting tube.

The Urografin gradients were formed at room temperature and then placed in a cold room at 4°C for 10 to 15 min. The characteristics of the gradient produced by this means were examined on several occasions. Advantage was taken of the fact that Urografin absorbs strongly in the ultraviolet with a maximum absorption at 260 μm. A linear correlation was found between the absorbance at 260 μm and the density for Urografin solutions (Fig. 1). This made possible the facile evaluation of the density of any particular fraction from a gradient. The expected exponential nature of the gradient was confirmed and found to be in good agreement with calculations (discussed in “Results”).

Layering of Samples—Aqueous suspensions of vegetative cells or spores were diluted 1:1 with a Urografin solution corresponding to the lowest concentration in the gradient and kept at 0°C for 10 to 15 min before being layered on top of the gradient. Up to 1 x 10^8 spores corresponding to 50 mg, dry weight, were placed on a single gradient. A sharp interface between the Urografin and cell suspensions was always observed.

Centrifugation and Sampling—The tubes containing the gradients were centrifuged in a Spinco model L centrifuge swinging bucket rotor SW 25.1 at 4°C for 30 min at 15,000 rpm. Centrifugation for 90 min or increasing the speed of centrifugation to 22,000 rpm did not change the location or shape of the bands. Fractions were collected by puncturing the bottom of the tube with a device that allowed minimal shaking. A modified syringe needle No. 20 was used for gradients involved in banding vegetative cells and a No. 19 needle for gradients used for banding spores. These needle sizes were chosen to give similar flow rates with gradients of different viscosities.

Fractions of 0.5 to 1.5 ml were collected in the cold room. A 30-ml gradient was distributed in about 90 min. The fractions were plated after appropriate dilution on minimal agar and incubated at 37°C for 24 hours. The plates were recounted after 48 hours to ensure scoring those spores that germinated slowly. Colonies were counted with the use of the Quebec Colony counter. Heat resistance was evaluated by plating the sample before and after heating at 60°C for 15 min, unless otherwise stated.

When it was desired to reband certain fractions, the Urografin was diluted 1:10 with H_2O, and the cells were collected by centrifugation at 10,000 rpm for 30 min. The pellet was taken up in the appropriate mixture of water and Urografin.

Materials

Urografin (N,N'-diacetyl-3,5-diamino-2,4,6-triiodobenzoate, 1 an x-ray contrast agent manufactured by Schering) was purchased from Squibb as a 76% solution in sterile 20-ml ampules. The 76% solution is the highest concentration obtainable. For purposes of more facile comparison this solution will be regarded as 100% in concentration. Therefore, a 1:1 dilution of the commercial material will be referred to in the text as a 50% Urografin solution.

Results

Effect of Urografin on B. megaterium—Urografin was tested for possible toxic effects on the vegetative and spore forms of B. megaterium. The Urografin had no discernable effect on the viability of the vegetative cells even after 6 hours. Longer periods of exposure did result in a loss of viability, but this was also found in the control tube without Urografin. The spores were found to be viable and their heat resistance unaffected even after 94 hours in Urografin (Table I).

Gradient Formation—Applying the equation,

\[ m_k = (m_0 - m_1) e^{-Ah/2} + m_1 \]

where \( m_0 \) = initial Urografin concentration in the mixing chamber; \( m_1 \) = Urografin concentration in reservoir (usually \( m_1 = 0 \)); \( m_k \) = Urografin concentration at height \( h \) from the bottom of the tube in which the gradient is formed; \( h \) = distance from bottom of tube in which gradient is built; \( A \) = cross-sectional area of the test tube; \( Ah \) = volume at height \( h \) from the bottom of the tube in which the gradient is formed; and \( H \) = volume of solution in mixing chamber, an exponential gradient could be formed with a desired range of densities. A gradient with \( H = 19 \) ml

1 The German trade name for this compound is Urografin. In this country it is sold under the trade name of Renografin.
and $m_0 = 60.5\%$ (density range 1.050 to 1.242 g per cm³) was used for separating spores from vegetative forms; and a gradient with $H = 37.5$ ml and $m_0 = 100\%$ (density range 1.180 to 1.400 g per cm³) was used for resolving different spore forms. Fig. 2 shows the agreement achieved between the experimentally determined distribution of Urografin on a gradient of $H = 37.5$ ml and $m_0 = 100\%$, and the theoretical distribution calculated by means of the above equation.

Vegetative Cells on Gradient—In order to evaluate the density limits of a vegetative population of B. megaterium, a culture was harvested while still in log phase. An aliquot was placed on a gradient of Urografin of $H = 19$ ml and $m_0 = 60.5\%$. The highest concentration of the population of exponential phase cells occurred at 12.5 ml from the bottom of the tube, as can be seen from Fig. 3. The peak fraction had an absorbance at 260 μμ of $3.35 \times 10^4$, corresponding to a density of 1.135 g per cm³. Less than 1% of the population had a density greater than 1.185 g per cm³, and less than 1% of the population a density lower than 1.115 g per cm³. When observed microscopically, no obvious differences could be seen between the light and heavy cells. The vegetative cells of B. megaterium floated on a gradient of $m_0 = 100\%$ and $H = 37.5$ ml which was used for banding spores. The recovery of vegetative cells from the gradient varied from 60 to 80%.

It became of interest to compare the distribution pattern of B. megaterium to another organism which is considerably smaller in size. Fig. 4 shows the banding of B. megaterium and Escherichia coli vegetative cells on a gradient $H = 15$ ml and $m_0 = 100\%$. It appears that, although the pattern of distribution is similar, E. coli formed a somewhat sharper band and the bulk of the population had a higher density than that of B. megaterium.

Banding of Spores on Gradient—Bacteria were allowed to sporulate under the conditions described under “Methods” and were harvested after 60 hours. On a gradient of $H = 37.5$ ml and $m_0 = 100\%$, 1.5 $\times$ 10⁸ colony-forming units were layered and then subjected to centrifugation for 30 min at 15,000 rpm (Fig. 5). Neither the shape of the band nor the position of its peak changed after 60 and 90 min of centrifugation. The highest concentration of spores occurred 9.5 ml from the bottom of the tube with a density of 1.305 g per cm³. Less than 5% of the spore population had a density smaller than 1.275 g per cm³, and less than 5% of the spore population had a density higher than 1.335 g per cm³. In this particular experiment the ratio of spores to vegetative cells was 13.3:1. The vegetative cells which

**Table I**

<table>
<thead>
<tr>
<th>Effect of Urografin on viability and heat resistance of B. megaterium</th>
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<tbody>
<tr>
<td>A washed suspension of bacteria ($7.3 \times 10^7$ per ml) was added to an equal volume of 100% Urografin and kept at 0°. Samples were taken at intervals and distributed on minimal agar plates (Medium A) after appropriate dilutions. To test the effect of Urografin on the viability and heat resistance of spores, a washed suspension ($8.3 \times 10^7$ per ml) was added to 3 volumes of 100% Urografin and maintained at 0°. Samples were taken out, diluted appropriately, and plated after heating at 60° for 15 min.</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Colony-forming units</th>
<th>0 hr</th>
<th>2 hrs</th>
<th>4 hrs</th>
<th>6 hrs</th>
<th>24 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vegetative cells suspended in Urografin ($\times 10^6$)</td>
<td>375</td>
<td>317</td>
<td>380</td>
<td>260</td>
<td>160</td>
</tr>
<tr>
<td>Vegetative cells suspended in buffer ($\times 10^6$)</td>
<td>355</td>
<td>365</td>
<td>165</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spores in Urografin ($\times 10^4$)</td>
<td>267</td>
<td>194</td>
<td>217</td>
<td></td>
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</tbody>
</table>

*Plated after heating the suspension of the spores at 60° for 15 min.*
Sporulation

Fig. 4. Comparison of banding patterns of *B. megaterium* and *E. coli*. *E. coli W* (vegetative cells, 1.5 X 10^8 cells) (A) and *B. megaterium* (vegetative cells, 9.0 X 10^7) (B) were banded on a separate gradient of H = 15 ml and m_o = 100% and centrifuged for 60 min at 22,000 rpm. Fractions were collected and assayed as in Fig. 3. The peak tube contained 4.5 X 10^7 and 2.8 X 10^7 cells, respectively.

Fig. 5. Banding of spores on a Urografin gradient. Washed spores, 1.5 X 10^8, were layered on a gradient of H = 37.5 ml and m_o = 100% and centrifuged for 30 min at 15,000 rpm. Fractions were collected and assayed as in Fig. 3. The peak tube contained 2.9 X 10^8 spores.

Fig. 6. Rebanding of separate fractions from a spore band on Urografin gradients. Two fractions with densities of 1.340 and 1.280 g per cm^3 were obtained as described in legends to Figs. 3 and 5, separately layered on two gradients of H = 37.5 ml and m_o = 100%, and centrifuged for 30 min at 15,000 rpm. The peak tube of the band with a density of 1.340 g per cm^3 contained 6.5 X 10^5 spores, and the peak tube of the band with a density of 1.280 g per cm^3 contained 1.3 X 10^5 spores.

floated on the gradient represented 7% of the whole population. However, it is possible that the original percentage of vegetative forms was higher since vegetative asporogenous forms may have lysed during the long period necessary for sporulation and thus would not be scored. The recovery of spores from the gradient varied from 70 to 90%.

The spore band was also found to be wide, and thus it became important to test whether the different spore densities reflected a true density heterogeneity of the spore population or an artifact created on the gradient. In an effort to distinguish between the two possibilities, two separated spore populations with densities of 1.340 and 1.280 g per cm^3 were selected, diluted with water, and recovered by centrifugation. The pellets were then suspended in 1.0 ml of H_2O, each suspension was placed on a separate gradient of H = 37.5 ml and m_o = 100% and centrifuged at 15,000 rpm for 30 min, and fractions were collected and plated as described under "Methods." One spore band was obtained on each gradient (Fig. 6) with densities of 1.335 and 1.280 g per cm^3, respectively. Microscopic observation showed only spores to be present in the two bands.

This finding clearly indicates that the spores found at different points on the gradient were intrinsically distinct with respect to density. A detailed study of the resistance to heat inactivation of the two separate spore fractions (Fig. 7) showed that there is a marked difference between the heavy and the light spores. Whereas the germination of the heavy spores could be accelerated by heat up to 70°, the germination of the light spores was not affected by heat treatment up to 60°, and at 70° their viability had decreased by 92%.
It became of interest to test the utility of the method in studying spores formed under different conditions.

**Calcium-deficient Spores**—In one experiment, bacteria were allowed to sporulate under the usual conditions except that CaCl₂ was omitted from the medium. It has been shown (2, 7, 8) that spores grown under these conditions contain minimal amounts of dipicolinic acid and are also heat-sensitive. Fig. 8 shows that spores grown under these conditions band differently. Since no attempt was made to free the medium completely of Ca⁺⁺, it is not surprising that some of the spores banded where normal spores do. Those with density of 1.305 g per cm³ and higher were found to be heat-resistant. However, the majority of spores were found to be heat-sensitive. A clear separation from non-sporulating vegetative forms was achieved in this case as well. It is interesting to note that the Ca⁺⁺-deficient spores had the density of the “light” spores (1.305 g per cm³ and lighter). It is clear that under these conditions a homogeneous population is not formed. It was noted by Black, Hashimoto, and Gerhardt (8) that spores produced under conditions of Ca⁺⁺ deficiency are unstable in water. However, no loss of viability was noted when these spores were kept in Urografin at 0° for at least 10 days.

**DISCUSSION**

Isopycnic gradient centrifugation with Urografin as supporting medium was found to be a useful tool for examination of sporulating systems. The advantages of this method are numerous. Urografin was shown to have no toxic effects on either vegetative cells or spores, nor did Urografin induce germination, as judged by the fact that spores retained their heat resistance even after 24 hours in Urografin. The high density of Urografin makes possible the equilibrium centrifugation of spores. The reproducibility of the method as well as its ease and speed makes it useful as a routine method. The complete separation of the spores from the vegetative forms permits a precise evaluation of the efficiency of sporulation. With this method it was possible to examine for the first time the heterogeneity of spore populations formed under normal sporulating conditions, as well as in a Ca⁺⁺-deficient medium. It became obvious from the rebanding experiment that separate fractions of the original spore area differ with respect to density. The lighter spores (density less than 1.305 g per cm³) germinated faster and were not affected by heat activation, whereas the heavy spores were slow in germinating and their germination could be accelerated by heat treatment. This provides some insight into the nature of the heterogeneity of the spores band.

It is not clear whether the formation of light spores is a result of a lytic enzyme in the medium which releases some of the spores before their maturation has been completed. This lytic enzyme could also be located in the cytoplasm of a mature prespore and act on a neighboring cell of the chain, causing it to release an immature spore, as already suggested by Strange and Dark (9). The possibility also exists that these lighter spores were originally denser but were induced by some mechanism to germinate and thus represent spores at different stages of germination. In this connection it is worth mentioning the incipient germination described by Curran and Pallansch (10). The heterogeneity is probably not due to a deficiency in the medium since spores that were formed in Medium A enriched with 2% yeast extract (not shown) manifested an identical wide band.

It is well established that sporulation in the absence of Ca⁺⁺...
results in the production of spores deficient in their content of dipicolinic acid. This is a reasonable consequence of the fact that dipicolinic acid occurs in normal spores as the Ca++ salt. There are probably many changes in the spore structure that might result from the absence of a component that comprises usually 10 to 15% of the dry weight, but these are not known. Therefore, it is not possible to compute what effect the absence of Ca++ will have on spore density. However, in view of the much greater density of calcium dipicolinate (1.71 g per cm³) relative to that of normal spores, it is not surprising that the spores obtained in the absence of Ca++ should be less dense than those obtained on the usual medium.

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