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Wet and Dry Bacterial Spore Densities Determined by Buoyant Sedimentation†

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The wet densities of various types of dormant bacterial spores and reference particles were determined by centrifugal buoyant sedimentation in density gradient solutions of three commercial media of high chemical density. With Metrizamide or Renografin, the wet density values for the spores and permeable Sephadex beads were higher than those obtained by a reference direct mass method, and some spore populations were separated into several density bands. With Percoll, all of the wet density values were about the same as those obtained by the direct mass method, and only single density bands resulted. The differences were due to the partial permeation of Metrizamide and Renografin, but not Percoll, into the spores and the permeable Sephadex beads. Consequently, the wet density of the entire spore was accurately represented only by the values obtained with the Percoll gradient and the direct mass method. The dry densities of the spores and particles were determined by gravity buoyant sedimentation in a gradient of two organic solvents, one of high and the other of low chemical density. All of the dry density values obtained by this method were about the same as those obtained by the direct mass method.

Buoyant sedimentation in a density gradient between water and a dense solution or between organic solvents of low and high density (isodensity or isopycnic equilibrium sedimentation) has been used extensively to separate nucleic acids, viruses, and subcellular particles (19). The method also has been extended to bacterial spores, both as a preparative procedure to separate them (2, 7, 8, 11, 12, 13, 15, 17, 18, 20, 21) and as a quantitative procedure to determine a wet or dry density value as a basic biophysical parameter (8, 12, 13, 16). In the wet density determination, however, complications would occur if the gradient medium permeated partially into the spores.

Both the wet densities (grams of wet spores per milliliter of wet spores) and the dry densities (grams of dry spores per milliliter of dry spores) of representative types of dormant spores have been determined previously by direct mass (weight and volume) measurements directly on gram amounts of spores, with correction for interstitial water (3, 5, 14). Consequently, we undertook determinations of the two density values on some of the same spore types by the buoyant sedimentation method with several gradient media, compared the results with those obtained by the direct mass method, and explained the differences by permeability determinations.

MATERIALS AND METHODS

Spores. Various types of clean dormant *Bacillus* spores that varied widely in heat resistance were produced and prepared as previously described (3). Spores of *Clostridium perfringens* 8-6, a mutant that produces coatless spores (7), were generously provided by James T. Lindsay, Division of Food Research, Commonwealth Scientific and Industrial Research Organization, North Ryde, Australia.

Reference and standard particles. Two types of Sephadex beads (Pharmacia Fine Chemicals, Uppsala, Sweden) were selected as permeable reference particles: G-10 has a permeability exclusion limit (700) that is less than the molecular weights of Metrizamide and Renografin, and G-50 has one (30,000) that is greater than these weights. Polystyrene beads (Dow Diagnostics, Indianapolis, Ind.) of about the same size (868 nm) as the spores were selected as impermeable reference particles. Wool fibers obtained directly from sheep, delanolinized with solvents, and cut into small pieces were also used.

Glass beads (Scientific Products, Inc., Detroit, Mich.) with a range of known densities near that of spores were used as standards.

Gradient media. Commercial gradient media that had chemical (dry) densities that were higher than the dry densities of the spores and reference particles were selected.

For the wet density determinations, three media were selected. Metrizamide (Nyegaard and Co., Oslo, Norway) has the following properties: a single compound {2-[3-acetamido-5-(*N*-methylacetamido)-2,4,6-

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triiodobenzamido]-2-deoxy-D-glucose}, chemical density = 2.17 g/ml, molecular weight = 789, nonionic, low osmolarity, high water activity (0.989 when wet density = 1.38), and intermediate viscosity. Renografin-76 (E. R. Squibb & Sons, Princeton, N.J.) has the following properties: a 66:10 mixture of two compounds (methylglucamine diatrizoate, molecular weight = 809, and sodium diatrizoate, molecular weight = 634) plus certain additives, ionic, high osmolarity, high water activity (0.908 when wet density = 1.38), and intermediate viscosity. Percoll (Pharmacia Fine Chemicals) has the following properties: a single material (colloidal silica particles coated with polyvinylpyrrolidone), solution density = 1.13 g/ml, mean particle diameter = 29 to 30 nm in 0.15 M NaCl, nonionic, low osmolarity, high water activity (0.995 for an isoosmotic suspension in saline), and low viscosity.

For the dry density determinations, two miscible organic solvents were selected, one with high and the other with low chemical density: perchloroethylene (molecular weight = 165; chemical density = 1.62 g/ml) and 4-*tert*-butyltoluene (molecular weight = 148; chemical density = 0.85 g/ml). Both were obtained from Aldrich Chemical Co., Milwaukee, Wis.

Wet density determination by buoyant sedimentation. A linear gradient of Metrizamide or Renografin was prepared with degassed solutions in a 13- or 3-ml centrifuge tube by use of a conventional gradient maker. A water suspension of the spores or reference particles was degassed by vacuum, and a small amount was applied to the top of the gradient. The tube was centrifuged in a Sorvall HB-4 swinging bucket rotor at 16,000 × *g* at 25°C for 1 h, at which time equilibrium was reached; however, with *Bacillus stearothermophilus* spores, the tube was centrifuged at 5,000 × *g* for 1.5 h to prevent spore germination. The wet density of the material was obtained by withdrawing a sample and measuring the refractive index of the gradient solution just above and below the band of the material. The average of these two measurements, converted to a density value by use of a calibration curve, was considered to represent the wet density of the material. Successively narrower gradients were used to increase accuracy.

A linear gradient of Percoll was prepared by centrifuging 10 ml of isoosmotic Percoll solution in a 13-ml centrifuge tube in a Sorvall SS-34 fixed-angle rotor at 25,000 × *g* for 1 h. The isoosmotic Percoll solution was made by adding nine parts (vol/vol) of the manufacturer's stock Percoll solution to one part (vol/vol) of 1.5 M NaCl. Once the gradient was formed, it remained stable for 1 week if not disturbed. A water suspension of the spores or reference particles was degassed by vacuum, and a small amount was applied to the top of the gradient. Standard glass beads of known densities were applied at the same time. The tube was centrifuged in an International 221 swinging bucket rotor at 1,800 × *g* for 1 h, at which time equilibrium was reached. The wet density of the spores or reference particles was obtained by interpolation between the positions of the standard glass beads.

Dry density determination by buoyant sedimentation. A linear gradient of butyltoluene and perchloroethylene was prepared in a 100-ml cylinder at 25°C, with precautions to exclude moisture. A sample of the spores or reference particles was lyophilized, heated at 110°C under vacuum, and suspended in butyltoluene. A small amount of the suspension and standard glass beads of known densities were applied together to the top of the gradient and allowed to settle by gravity for 1 or more days until equilibrium was reached. The dry density of the material was obtained by interpolation between the positions of the standard glass beads.

Wet and dry density determinations by direct mass measurements. Direct mass measurements were made as described previously (3).

Determination of spore permeability. The equilibrium permeability of spores to Metrizamide was determined by the space technique, which has been used extensively in our laboratory (9, 10). The results were expressed as the fraction of the spore volume (ml/ml) that was permeated by the solute (R^v).

Accuracy of determinations. Each value reported represents the average of at least three and as many as eight determinations. The standard errors were on the order of ±5%.

RESULTS AND DISCUSSION

Wet density. Table 1 shows values for the wet densities of various spore types and reference particles obtained by centrifugal sedimentation in density gradients of three different media, compared with the values obtained by direct measurement of mass wet weight and volume. With a density gradient of Metrizamide or Renografin, the values for the wet densities of the various spore types were higher than those obtained by the direct mass method. With a density gradient of Percoll, however, the values were about the same as those obtained by the direct mass method.

The differences between the wet densities obtained with a Percoll gradient and those obtained with a Metrizamide or Renografin gradient were explained by differences in the permeation of the gradient media into the spores. The spores were partially permeable to Metrizamide (R^v = 22 and 15% for *Bacillus cereus* and *B. stearothermophilus*, respectively), and the results were consistent with the general permeability properties of bacterial spores for solutes of similar molecular size (3, 10). Each of the two compounds in Renografin has about the same molecular size as the Metrizamide compound and thus permeates the spores to about the same extent. Consequently, the wet density values obtained by use of Metrizamide or Renografin did not represent the entire spore, but instead represented an internal compartment that was not permeated. However, the colloidal particles in Percoll are much too large to permeate into the spore at all. Consequently, the wet density values obtained by use of Percoll represented the entire spore accurately, as did those obtained by the direct mass method.

This explanation was substantiated by values for the wet densities of the reference particles

TABLE 1. Wet densities of various spore types and reference particles obtained by centrifugal buoyant sedimentation with densities gradient of Metrizamide, Renografin, or Percoll, compared with wet densities obtained by direct mass method

Spore type or reference particle	Wet density (g/ml) by following method:			
	Metrizamide gradient	Renografin gradient	Percoll gradient	Direct mass
B. cereus T, calcium deficient	1.31	1.30	1.17	1.12[a]
B. cereus T, calcium sufficient	1.30	1.29	1.12	1.13[a]
B. subtilis subsp. *niger*, band A	1.26	1.26	1.21	1.20[a]
B. subtilis subsp. *niger*, band B	1.30	1.29		
B. subtilis subsp. *niger*, band C	1.35			
B. stearothermophilus, smooth colony	1.34	1.35	1.26	1.22[a]
C. perfringens 8-6, band A	1.26		1.27	1.23
C. perfringens 8-6, band B	1.35	1.34		
C. perfringens 8-6, band C	1.38	1.38		
Sephadex G-50 beads	1.17	1.29	1.04	1.06
Sephadex G-10 beads	1.23	1.24	1.23	1.22
Polystyrene beads	1.05	ND[b]	1.05	1.04

[a] Reference 3.
[b] ND, not determined.

obtained with the three gradient media. Like the spores, Sephadex G-50 beads are partially permeable to Metrizamide or Renografin but not to Percoll; consequently, the wet density obtained with Percoll was lower than that obtained with the other two media but about the same as that obtained by the direct mass method. However, Sephadex G-10 beads and polystyrene beads are impermeable to all three media; consequently, the wet densities obtained about the same with all gradients by the direct mass method.

The Percoll buoyant sedimentation method and the direct mass method have about the same accuracy in determining the wet density of the entire bacterial spore. The Percoll method is useful for small amounts of spores and has also been used successfully with vegetative bacterial cells (22).

Although resulting in inaccurate values for the wet density of the entire bacterial spore, a density gradient with Metrizamide remains useful for the separation of dormant spores from germinated spores, vegetative cells, parasporal crystals, and debris. The technique also is useful for the separation of subpopulations from a heterogeneous population of dormant spores, apparently because of differences in permeability. Our observation of several discrete buoyant density bands in the *Bacillus subtilis* and *C. perfringens* spore populations was similar to previous findings (7, 8, 13, 17, 20). Renografin is less useful because it contains a mixture of salts and additives.

Dry density. Table 2 shows values for the dry densities of various spore types and reference particles obtained by gravity sedimentation in a density gradient of two solvents compared with

TABLE 2. Dry densities of various spore types and reference particles obtained by gravity buoyant sedimentation with density gradient of two solvents, compared with dry densities obtained by a direct mass method

Spore type or reference particle	Dry density (g/ml) by following method:	
	Solvent gradient	Direct mass
B. cereus T, calcium deficient	1.40	1.43[a]
B. cereus T, calcium sufficient	1.47	1.45[a]
B. subtilis subsp. *niger*	1.50	1.45[a]
B. stearothermophilus, smooth colony	1.39	1.44[a]
C. perfringens 8-6	1.42	ND[b]
Sephadex G-10 beads	1.43	1.40
Sephadex G-50 beads	1.43	1.40
Polystyrene beads	1.05	1.04[a,c]
Wool fibers	1.30	1.30[d]

[a] Reference 3.
[b] ND, not determined.
[c] Reference 6.
[d] Reference 1.

values obtained by the direct mass method. It was shown in a separate experiment that the solvents did not extract solids from the spores. Both methods resulted in about the same dry density values (an average of 1.44 g/ml for the various spore types).

With the buoyant sedimentation method, it is assumed that the solvents pass through the several integument layers and completely displace voids in ultrastructural solids throughout the entire dried spore. The largest source of error in the method perhaps is incomplete dehydration of the spores before immersion and thus incomplete displacement by solvent of the voids in the spore solids. Maximum displacement and accuracy should be obtainable by the use of an inert and nonabsorbing molecule of very small size, such as nitrogen or hydrogen gas (4).

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