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Washing Bacteria by Centrifugation Through a Water-Immiscible Layer of Silicones

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ABSTRACT

HURWITZ, CHARLES (Veterans Administration Hospital, Albany, N.Y.), CATHERINE B. BRAUN, AND RICHARD A. PEABODY. Washing bacteria by centrifugation through a water-immiscible layer of silicones. J. Bacteriol. **90**:1692–1695. 1965.—A method is described which enables the separation of cells from aqueous suspension without altering the internal aqueous environment of the cells. The method consists of centrifuging the cells from the aqueous environment through a more dense, immiscible solvent consisting of a mixture of two silicones. A thin film of the aqueous environment equal to 7×10^{-13} ml per cell remains with the bacteria during the separation procedure. The method by which this volume was determined is described. The procedure itself has no measurable effect on viability or permeability of the cells and permits recovery of about 90% of the cells from the aqueous environment. With this method, it has been found that the intracellular water volume of *Escherichia coli* ML 35 accessible to sucrose or inositol is 1.96×10^{-12} ml $\pm$ 6%, or 85% of the total volume of the cell determined by visual measurement.

The determination of the intracellular concentrations of diffusible solutes has presented a difficult problem (MacDonald and Gerhardt, 1958; Brown, 1964), arising from the necessity for washing cells to remove external contaminants before determining the intracellular concentration. Since washing will itself change the equilibrium between the intra- and extracellular solutes, a significant degree of uncertainty is introduced by the method itself. The determination of the amount of intracellular water which is accessible to diffusible substrates has also presented a difficult problem for the same reasons.

In the following, we shall describe a procedure which permits isolation of cells in such a way that the intracellular aqueous environment is not altered. The procedure is applicable to determining solutes in manageable volumes (as small as 1 ml) of cells in any desired physiological state.

The procedure is based on separating cells from the external contaminating aqueous environment by centrifugation through a layer of water-immiscible solvent which does not itself injure the cells or alter the permeability barrier.

In addition to its nontoxic properties, the immiscible solvent must be more dense than the aqueous medium, but less dense than the cells. A mixture of two silicones was found to have the desired density characteristics.

MATERIALS AND METHODS

Escherichia coli ML 35, a mutant which is constitutive for β-galactosidase, but lacks the β-galactoside permease, was used. This strain was the gift of B. D. Davis. This "cryptic" mutant hydrolyzes *o*-nitrophenyl-β-D-galactoside (ONPG) at a low rate unless its permeability barrier is damaged, since the rate of hydrolysis is limited by the rate of entry of the substrate past the permeability barrier.

Change in permeability of the bacterial cells was measured as a change in rate of hydrolysis of ONPG, by use of minor modifications of the procedure of Lederberg (1950). To measure recovery of cells, their deoxyribonucleic acid was labeled by growth in the presence of methyl-labeled tritiated thymidine. The cells were grown for about two generations in medium containing the thymidine. The cells were then washed by centrifugation, suspended in medium without added thymidine, and incubated until exponential growth was again achieved. Viable counts were made in quadruplicate after growth on nutrient agar.

A mixture of 1 part of a light silicone (SF-96-5) and 2 parts of a heavier silicone (Versilube F-50) was found to have the desired density characteristics (density 1.02 at 4 C). The silicones were obtained through the courtesy of Abbot Pozefsky of the General Electric Co., Silicone Products Division, Waterford, N.Y.

RESULTS

We first determined the effect of exposure of *E. coli* ML 35 to the silicones on three parameters: viability, permeability, and recovery of cells.

Table 1 illustrates the effect of exposure to the silicones on permeability of the cells to the chromogenic β-galactoside, ONPG. The cells were shaken with the silicone for 10 min before the addition of the substrate. The rates of hydrolysis of ONPG resulting from the addition of toluene are shown as a control to demonstrate what happens when the permeability barrier is destroyed. The experiment shows that permeability is unaffected by exposure to the silicones.

TABLE 1. *Effect of exposure to silicones on permeability of Escherichia coli ML 35**

Silicone	No toluene	Toluene
Control	4	605
Versilube F-50	4	570
SF-96 (5)	4	560
Versilube + SF-96	7	570

* In tubes containing 0.1 ml of the silicones, 0.2 ml of *E. coli* ML 35 cells were shaken for 10 min. The cells were then exposed to the chromogenic substrate, *o*-nitrophenyl-β-D-galactoside, for 15 min, and the amount of *o*-nitrophenol formed was measured in Klett units. Toluene was added to similarly treated samples to demonstrate that the crypticity of this mutant had not been altered by the exposure to the silicones.

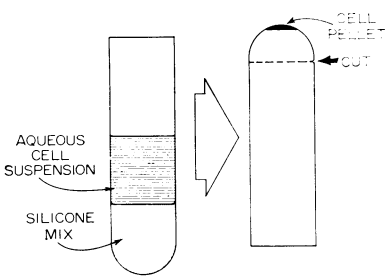

FIG. 1. *Relative position of the aqueous cell suspension, the silicone mix, and the location of the pellet and cut. The 10-ml cellulose nitrate tubes for the HS-2 swinging-bucket rotor were obtained from Sorvall. We have recently found that a 12-place fixed 40°-angle rotor (Sorvall SE-12) is also suitable for this purpose. Silicone-washed cells can also be collected in the Sorvall Continuous Flow System by filling the bottom half of each collecting tube with the silicone wash solution.*

TABLE 2. *Recovery of cells**

Prepn	Viable count/ml	H^3
		count/min
Control	4.7×10^8	1,539
Silicone mix	4.5×10^8	1,454
Per cent recovery	96.0	94.0

* Three 4.0-ml samples of tritiated *E. coli* ML 35 growing in nutrient broth were treated as follows: one (labeled control) was sampled for viable count and then centrifuged to sediment the cells, which were extracted with 5% hot trichloroacetic acid to remove tritium for counting; the other two samples (labeled silicone mix) were centrifuged through silicone. One of these pellets was suspended in water for viable count; the other pellet was extracted with hot 5% trichloroacetic acid to remove tritium for counting.

For the next experiment, the tritium-labeled, aqueous cellular suspension was layered above the silicone mixture in 10-ml cellulose nitrate tubes (Ivan Sorvall, Inc., Norwalk, Conn.), as illustrated in Fig. 1; 3 ml of silicone mix at 4 C were first added, and 4 ml of the aqueous cellular suspension were then layered above the silicone solution. After centrifugation for 15 min at 15,000 × *g* in a swinging-bucket HS-2 Sorvall rotor, the supernatant fraction was removed by aspiration of the aqueous phase and decantation of the silicone phase to avoid contamination of the pellet by the aqueous layer. The tube was then cut just above the bottom curvature, and the pellet was suspended in water for viable count or for extraction of the label for measurement of radioactivity. Table 2 shows that recovery of label and viable count exceeded 90%.

Since the cells, after centrifugation, could not be dispersed in silicone, but could be readily dispersed in water, it appeared that separation of the cells from the aqueous environment was not complete, but that the cells passed through the silicone surrounded by a film of water. In fact, the extracellular aqueous layer could be readily seen by phase-contrast microscopy.

It is probable that the innocuous behavior of the silicones results from the fact that they are prevented from coming in contact with the cellular membrane by the tightly bound film of water. The contamination of the recovered cells by a small amount of the external aqueous environment necessitated a quantitative measure of this aqueous film, which was obtained in the following way. Eighty milliliters of a cell suspension were centrifuged, and the sediment was suspended in 4 ml of nutrient broth. The 20-fold concentrated

TABLE 3. *Effect of growth phase on intra- and extracellular water per cell*

Intracellular water (ml/cell)	Extracellular water (ml/cell)	Viable count per ml
1.96×10^{-12}	0.68×10^{-12}	4.5×10^{8}
—	0.40×10^{-12}	8.3×10^{8}
1.00×10^{-12}	0.27×10^{-12}	1.43×10^{9}

cellular suspension was centrifuged through the silicone layer and separated from the aqueous layer as described previously; 80 μliters of a C^{14}-dextran solution (mol wt, 15,000 to 17,000; New England Nuclear Corp., Boston, Mass.) were then added to the pellet. After thorough mixing, the cellular suspension was transferred to a 1-ml conical centrifuge tube, and the cell mass was separated from the supernatant fluid by centrifugation. Three 10-μliter samples of the supernatant fluid were removed for radioisotope counting, care being taken to avoid contamination of the aqueous samples with the underlying small layer of residual silicone. The cells were then diluted to a known volume for viable counts. The amount of extracellular water was calculated from the decrease in specific activity per unit volume of the C^{14}-dextran solution, since the dilution of the dextran solution could come only from extracellular water. For these cells, the extracellular water was found to be 0.68×10^{-12} ml per cell, from the equation: $x = 0.08\ (A - B)/B$, where x = volume of extracellular water in milliliters, A = counts per minute per milliliter of the original C^{14}-dextran solution, and B = counts per minute per milliliter of the final supernatant fraction.

Since the extracellular water volume was known, we were now in a position to determine the intracellular water volume. At 37 C, we found that sucrose entered, and was also metabolized by these cells, as evidenced by the accumulation of label in the protein fraction. However, at 4 C in the presence of 0.02 M sodium azide, only a trace of accumulation of label in the protein fraction was found. Tritiated cells were therefore exposed to C^{14}-sucrose at 4 C in the presence of azide and centrifuged through the silicone layer. The C^{14} in the pellet was corrected to 100% recovery, by use of the recovery of tritium as a measure of recovery of cells. If the assumption is made that C^{14}-sucrose under these conditions was present intracellularly and extracellularly at the same concentration as in the original external environment, the intracellular water volume can be calculated in the following way. The volume of total accessible water was calculated by dividing the corrected recovered counts per minute by the counts per minute per milliliter, and was found to be 2.65×10^{-12} ml per cell. Similar experiments were performed with C^{14}-inositol, which is not metabolized by these cells. The previously determined volume of the extracellular aqueous film was subtracted from the total accessible water volume (2.63×10^{-12} ml) to determine the intracellular accessible water volume, which was found to be 1.96×10^{-12} ml per cell.

Photographs of these cells, plated on a thin film of nutrient agar, were enlarged 3,000-fold, and the cell volume was estimated to be 2.3×10^{-12} ml from the external linear measurements. The intracellular water volume accessible to sucrose or inositol therefore is calculated to be 85% of the total volume of the cell. This finding appears to validate the original assumption that sucrose and inositol were present intracellularly and extracellularly at the same concentration. The validity of this assumption is further increased by our finding that neither concentration of sucrose nor time of exposure had any effect on the calculated volume.

An example of the potential sensitivity of the method is shown in Table 3. *E. coli* cells decrease markedly in size in early resting stage. Intra- and extracellular accessible water volumes were determined for cells at different stages of the growth cycle. Both intra- and extracellular water volumes calculated by this procedure decreased, as expected, as the cells decreased in size.

DISCUSSION

Two comments concerning limitations of the method should be made. If the method is to be used for measuring density of bacterial cells, the fact that silicones are compressible liquids must be taken into consideration. Although the density of the silicone mixture at 4 C was found to be 1.02, the actual density during centifugation would be higher.

Care must be taken to use uncontaminated tracer compounds, since small amounts of radioactive contaminants can cause serious errors in the calculations if the contaminants are themselves capable of being "fixed" in or on the cell. The possibility of adsorption of charged ions to cell surfaces must also be considered.

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ADDENDUM

It was called to our attention by one of the reviewers that a similar method has been previously developed for separation of *Tetrahymena* from aqueous media, by use of solvent mixtures of the diesters of *o*-phthalic acid as the water-immiscible layer (Ballentine and Burford, Anal. Biochem. **1**:263, 1960).

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