

Liquid Nitrogen Cryo-Impacting: a New Concept for Cell Disruption

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High-efficiency disruption of bacteria can be accomplished in 2 or more min by the new procedure of liquid nitrogen cryo-impacting. Release of the dipicolinic acid-Ca²⁺ chelate paralleled the breakage of *Bacillus megaterium* endospores. Lactate dehydrogenase activity was much better in supernates from liquid nitrogen cryo-impacting-broken *Escherichia coli* cells than in those from sonically treated and broken *E. coli* cells.

The disruption of bacterial cells has been a difficult and time-consuming process. Mechanical equipment most generally used is expensive and may require rather large volumes of culture. Existing methods for disrupting cells also have been inadequate for efficient breaking of some bacteria such as *Streptococcus mutans* (5). Traditional methods for cell disintegration involve some type of sonic treatment, explosive decompression, shearing, or chemical extraction (2, 6, 7). Whereas many of these methods are useful for specific procedures, one difficulty with all of these techniques has been and remains the obvious alteration of normal biological activity (2, 10).

The results of this study indicate a new approach to the rupture of microbial cells by the principle of cryo-impacting. The basic idea is that individual cells or masses of cells when frozen at very low temperatures (-196 C) will crack easily under low-impact force. This principle has been extensively employed in the freeze-etching technique (9).

MATERIALS AND METHODS

During this experiment a ball hammer mill (Dangoumau Ball Mill, Paris, France) (Fig. 1) was used to contain up to 1 g (wet weight) of cell mass. The reciprocal device is designed to hold a jar made of 18% chrome steel (D^o 150 ml, Société Prolabo) in which the frozen cell mass is placed along with a large steel ball (Fig. 2).

Five cell types were used: vegetative cells of *Bacillus megaterium* (OSU no. 125 from ATCC no. 19213), spores of *B. megaterium* (OSU no. 125), *Streptococcus mutans* (OSU no. S126), *Streptomyces coelicolor* A(3)2 (OSU no. 686 from ISP no. 5049), and *Escherichia coli* (OSU no. 933).

Cell pellets were frozen in liquid nitrogen in amounts of 1 g or less by dropping the cell pellet directly into the liquid nitrogen-filled jar. The jar

and ball (the latter placed in the larger jar section) were precooled and filled with nitrogen.

The duration of disintegration needs to last no more than 2 min for vegetative cells, and it was found that the jar would remain cold during this period. It was observed that for the most part more than 90% breakage was achieved in 2 min for vegetative cells and *S. coelicolor* spores and in 8 min (with 2-min intervals of recooling the jar with liquid nitrogen) for *B. megaterium* endospores. Determination of cell breakage was based on examination of control and experimental samples in the phase contrast microscope and in the electron microscope, the latter procedure using preparations shadowed with germanium and preparations negatively stained with 2% uranyl acetate.

To harvest the broken cells from the jar, a precooled spatula (dipped in liquid nitrogen) was used to remove material pooled by post-breakage addition of liquid nitrogen.

Quantitative estimation of breakage was made by inspection of equal volumes of control and test sample using direct counts in the phase contrast microscope. Twenty fields were inspected in duplicate samples.

Release of the dipicolinate (DPA)-Ca²⁺ chelate from *B. megaterium* was monitored at 270 nm in a Beckman DU spectrophotometer (1, 8). Readings were taken from supernatants of samples centrifuged (5 C) at 6,780 × g for 20 min. Absorbance spectra of the released DPA-Ca²⁺ chelates were recorded with a Bausch and Lomb model 505 recording spectrophotometer using double-distilled water as the reference.

Lactate dehydrogenase (LDH) assays were performed using supernatants of disrupted *E. coli* cells. Cells grown at 37 C on nutrient agar (Difco) were subcultured twice for 12 h from a refrigerated stock and then for 8 h in 10 ml of nutrient broth. The 10-ml culture was used to inoculate 500 ml of nutrient broth which was poured over 250 ml of nutrient agar in a 1-liter Erlenmeyer flask. Cells were harvested by centrifugation (5 C) at 6,780 × g for 20 min and washed twice with 0.1 M phosphate buffer (pH 7.2)

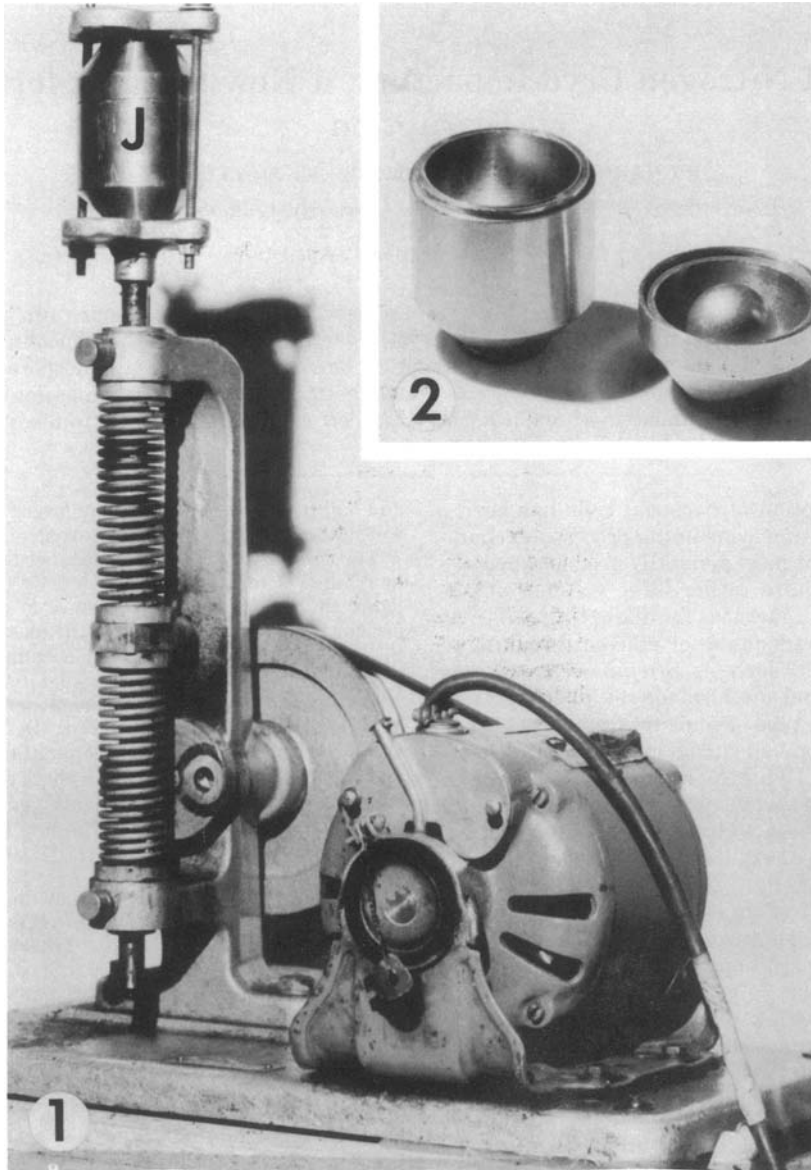


FIG. 1. Prolabo device containing stainless-steel jar (J) mounted at the top. When motor is energized the reciprocation of the spring-mounted arm causes a reciprocal motion of 700 cycles/min.

FIG. 2. Stainless-steel jar components containing the stainless-steel ball which impacts in the chamber during reciprocal motion.

under the same centrifugation conditions. The cells were homogenized in 50 ml of phosphate buffer, and the sample was split into two equal volumes and centrifuged again. One pellet was treated for 2 min by the liquid nitrogen cryo-impacting (LNCI) process, and one pellet was resuspended in phosphate buffer in a stainless-steel tube stored in ice. Sonic treatment of this latter sample was carried out for two 8-min periods, during which the suspension temperature did not exceed 5 to 7 C. Samples from both

treatments were suspended in 30 ml of phosphate buffer (30 mg/ml, dry weight), and cell debris was pelleted (5 C) at $6,780 \times g$ for 20 min. After centrifugation supernatants were stored on ice until assayed for LDH activity. Stock solutions for the LDH assay were as follows: (i) phosphate buffer, 0.1 M, pH 7.2, (ii) cell extracts (undiluted), (iii) reduced nicotinamide adenine dinucleotide (NADH) (Sigma), 7.5 mg/10 ml of phosphate buffer, and (iv) sodium pyruvate, 55 mg/5 ml of buffer.

Using phosphate buffer as absorbance blank, experimental cuvettes (1-cm path length) initially contained 2.0 ml of buffer, 0.5 ml of the respective *E. coli* supernates, and 0.5 ml of NADH solution. Endogenous substrate utilization was permitted to oxidize NADH until 14.1 min elapsed, at which time 0.3 ml of the pyruvate solution was added to each of the experimental cuvettes. NADH oxidation was monitored as a function of decrease in absorbance at 340 nm (4) using a Beckman DU spectrophotometer.

RESULTS AND DISCUSSION

The results of this set of experiments indicate that cell populations of microorganisms are exceptionally easily disrupted with very short treatment times. It was found that 2 min of machine impacting was sufficient to break over 95% of the cells in the microbial cultures examined. This is particularly noteworthy in the case of *S. mutans* and endospores of *B. megaterium* which have always been difficult to rupture. Phase microscope observation of the broken cells reveals that cells were cracked subter-

minally as well as through other regions, and the cell contents were subsequently released. Examination of electron micrographs (Fig. 3) indicates that this was the case. Breakage planes (Fig. 3, arrows) frequently occur across cell wall and cytoplasmic membrane. Thawing of broken samples then releases cytosol contents. These and other observations of broken cells in the electron microscope reveal that cells are not exploded as with the French pressure cell (2).

Figure 4 illustrates the relationship between percentage of breakage and 270-nm absorbance increase for LNCl-broken and sonically treated *B. megaterium* spores. Data for both samples were expressed on the basis of 2 mg (wet weight) of spores per ml. Within the 8-min treatment period, there was essentially no spore breakage by sonic treatment, and absorbance at 270 nm remained 0.06. In contrast, LNCl-broken spores showed straight line breakage kinetics as a function of time and also exhibited proportion-

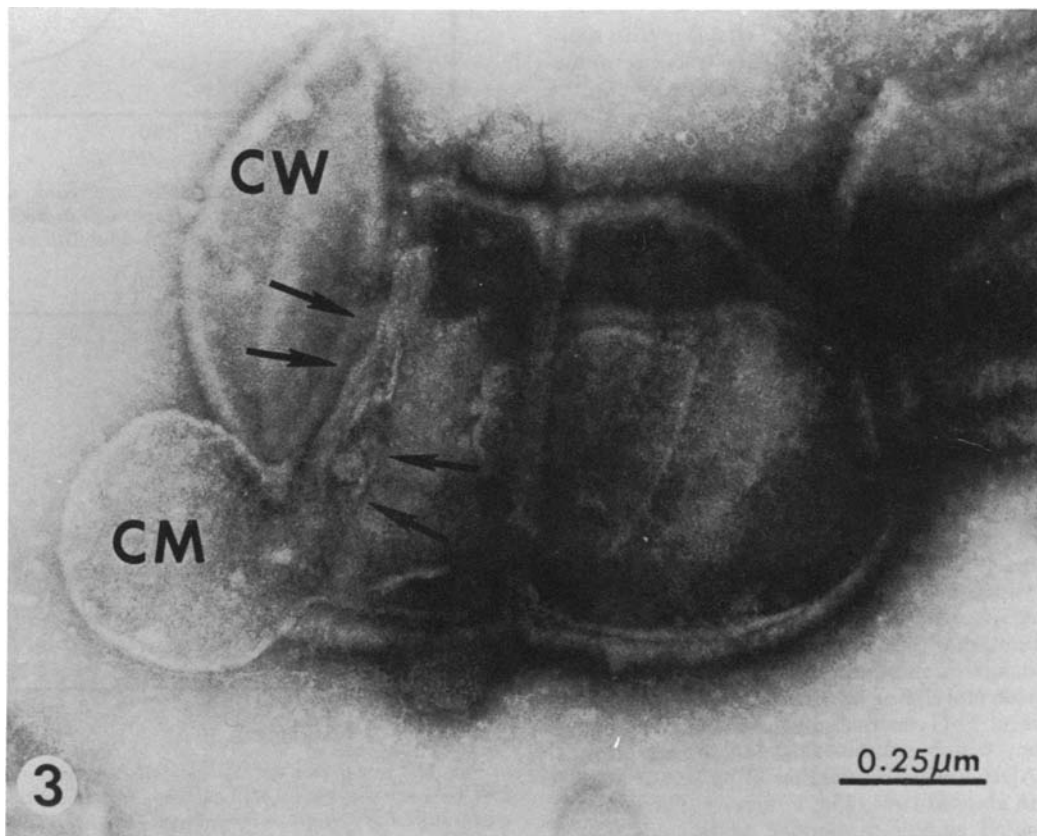


FIG. 3. A typical uranyl acetate negatively stained *S. mutans* cell after 2 min of cryo-impacting. The cell was ruptured, and cell contents were released. The cytoplasmic membrane (CM) and cell wall (CW) components appeared to be intact, with the typical single-fracture zone clearly visible (arrows).

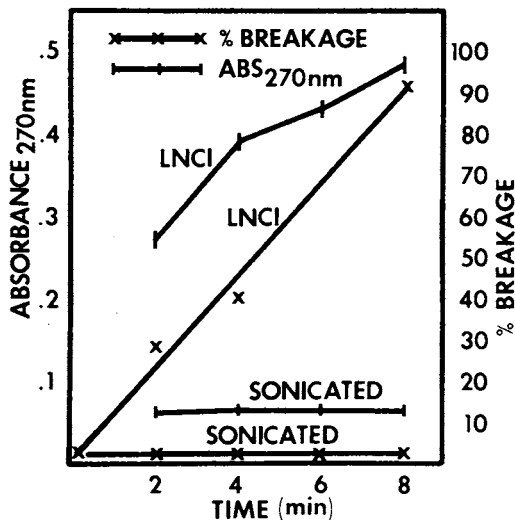


FIG. 4. Relationship between absorbance at 270 nm (Ca^{2+} -DPA) and percentage of breakage of *B. megaterium* spores (2 mg/ml, wet weight).

ate release of the DPA- Ca^{2+} chelate. With spore disruption performed at -196°C being analogous to the *S. mutans* cell breakage (Fig. 3), the DPA- Ca^{2+} chelate was probably released from the spore cytosol. Ultraviolet spectral analysis (Fig. 5) of supernatants of LNCI-broken *B. megaterium* spores correlates very closely with the purified DPA- Ca^{2+} chelate spectra recorded by Slepecky (8). When existing as a chelate with Ca^{2+} , DPA absorbs maximally at 270 nm, and the peak at 278 nm is identified as Ca^{2+} induced (8).

Examination of enzyme activities confirms the contention based upon electron microscopy (Fig. 3) that mechanics of LNCI cell disruption are different from those of conventional methods. In addition to more efficient breakage, the LNCI procedure permitted detection of higher LDH activity than did the sonically treated *E. coli* preparation. Endogenous utilization of substrate and dehydrogenase and oxidase (3) were permitted to function in the presence of added NADH. Under these conditions, NADH consumption (decline in absorbance at 340 nm) was much more rapid in the sonically treated preparation stabilizing at 0.12 absorbance units after 3 min. This contrasted with the LNCI preparation, which exhibited linear consumption of NADH until introduction of pyruvate at 14.1-min elapsed time (Fig. 6, arrow). The sonically treated sample responded to the addition of pyruvate by very rapid reduction of NAD, a process which essentially stopped at 15 min (Fig. 6). These activity curves (Fig. 6) show that,

with the small quantity of cells used for this LDH assay, LDH activity was imperceptible in the sonically prepared *E. coli* supernates and that LDH activity was present in the LNCI-prepared *E. coli* extract.

The potential of use for this simple technique (LNCI) is excellent. Since some enzymes within cell systems are inactivated during the conven-

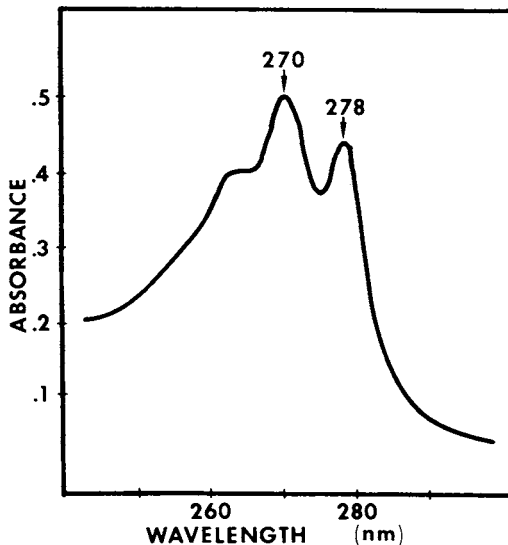


FIG. 5. Representative ultraviolet spectrum of LNCI-broken *B. megaterium* spore supernate indicating release of the Ca^{2+} -DPA at 278 and 270 nm, respectively (8-min LNCI).

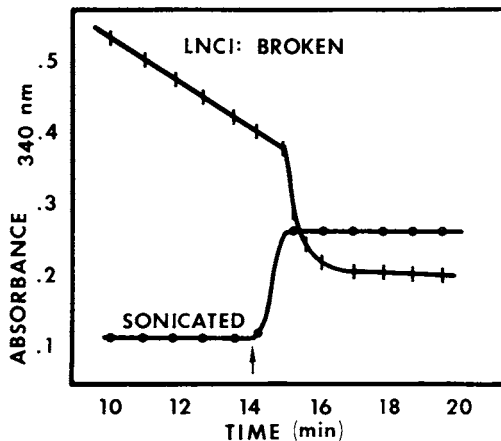


FIG. 6. Comparison of the sonically treated and LNCI-broken supernates of *E. coli* showing the apparent lack of LDH activity in the sonically treated preparation and the LDH activity in the LNCI-prepared enzyme extract. The arrow indicates the time at which pyruvate was added to each experimental cuvette.

tional process of cell breakage (2), the extremely low temperatures used with this technique should assure minimal disruption or denaturation of active proteins. Complicating exogenous materials such as lysozyme are eliminated even for efficient breakage of cells previously considered resistant to breakage. In addition, the simplicity of the system will lend itself readily to any laboratory.

With the type of jar we employed, it must be pointed out that there was obviously not an airtight seal when the jar was assembled and of course gaseous nitrogen was released.

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