# Bacterial Spores: Chemical Sensitization to Heat

Abstract. *Spore heat resistance is largely an inducible property, chemically reversible between a sensitive and resistant state. Therefore, the constitutive assumption and common practices based on it, such as direct testing of spores for heat resistance without prior treatment and the assumption of logarithmic death for spores in general, are no longer appropriate. A new approach is reported to the reduction of heating severity for a given survivor reduction of bacterial spores suspended in complex biological mixtures at their ordinary* p*H. Heating time advantages amount to severalfold and do not involve antimicrobial additives.*

Investigation and measurement in the field of bacterial spore heat resistance have been dominated by the assumption that heat resistance is an intrinsic or constitutive fixed property of the spore. Two general classes of influences on spore heat resistance have been recognized and widely documented. One of these consists of variables exerting their effect during growth and sporulation of the parent bacterial culture. Such factors include temperature, strain, and nutrition of the sporulating culture (culture effects). The second refers to effects of different heating media whose action has been observed to occur during the heat resistance test itself (heating medium effects). There are reviews of these two classes of effects on spore heat resistance (*1*).

In addition, that is, between sporulation and spore heat testing, there exists a reversibly manipulatable variable of large magnitude, that of heat resistance state (*2, 3*). The phenomenon of heat resistance in mature bacterial spores can be controlled reversibly by chemical means between a heat-sensitive and a heat-resistant state. The degree to which spore heat resistance thus can be turned off and on is substantial. For example, the temperature at which equal survivor rates are obtained changes about 28°C between the heat-sensitive and heat-resistant states of *Bacillus stearothermophilus* spores. This amounts to a change of several hundredfold in resistance, exceeding many of the previously observed between-species differences.

These changes of state are brought about by treating mature isolated spores before testing for heat resistance. The spores are not tested for heat resistance in the environments which produce the changes of heat resistance state. In other words, these treatments induce a changed heat resistance which persists when the spore is transferred to a new environment for testing. Tests of heat resistance of the two states are carried out in water after thorough removal of the reagents ($H^+$ and buffered metal cations) used in the treatments. Of course, unless the heat tests are carried out in a medium relatively inert with respect to capacity to induce change of state, the new or latest effect exerted by a noninert medium will be confounded with the effect of change of state. This situation occurred in many reported heating-medium effects. Such testing artifacts due to lack of inertness of the testing medium may occur inadvertently through alkali leakage from glass tubes and through flame combustion products in tube sealing (*4*).

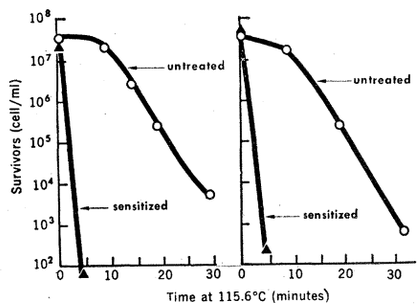


Fig. 1. Survival of bacterial spores *Bacillus stearothermophilus*, strain 1518, in complex biological substrates, with and without a heat-sensitizing treatment. (Left) Survival in liquid culture medium, *p*H 5.95; (right) survival in strained beef liver, *p*H 5.85. For the untreated samples, spore concentrations were $10^8$ per milliliter in both the culture medium and the liver. Before inoculation, the liver was diluted with an equal volume of water to facilitate homogenization. For the sensitized samples, the spores, thoroughly dispersed in water, were mixed with the substrates previously adjusted to *p*H 3 with HCl, to result in a concentration of $10^8$ spores per milliliter in the same concentration of substrate used for the untreated mixtures. After 1 hour at 70°C the acidified mixtures were neutralized with NaOH to the original *p*H's listed. Care was taken that no portion of the spore population missed sensitization through imperfect mixing. Heat resistance tests by the tube method were then run on both the sensitized and untreated samples. Survivors were defined as colony outgrowth on agar plates (tryptone, 1 percent; glucose, 0.5 percent, soluble starch, 0.1 percent) incubated at 53°C for 2 days. An arbitrary temperature lag of 1 minute was assumed for borosilicate tubes (9 mm) in a stirred oil bath at 115.6°C.

The existence of separate spore heat resistance states not only requires change of the constitutive assumption of the nature of spore heat resistance, but also requires change of the common experimental designs and practices based on that assumption. Probably the most important of such practices has been that of testing the heat resistance of spores directly in an "as received" condition with the purpose of later using such test results as a model for the behavior of presumably similar spores in another situation. Spores subjected to such a direct test may or may not, depending on their prior treatment, actually express a significant part of their potential heat resistance. Such disregard of heat resistance states at best would usually amount to testing a mixed flora and could result in a serious underestimation of the heat resistance capacity of a spore sample.

To view the resistance capability of a spore lot, a preparation of the resistant form should be made and then tested for resistance in water and in the substrates of interest. Since valence of the cation is important (*3*), the calcium form (divalent) resistant state would be preferred. The kinetic considerations (*3*), which form the basis of the heat adaptation phenomenon should not be neglected in preparing the calcium form of spores.

Another common practice based on the assumption of a constitutive rather than a reversibly inducible nature for heat resistance is the general assignment of a logarithmic order of heat survival for spores. A third is the designation of single or narrow ranges of numerical values for the heat resistance of species, strains, or even batches of spores.

The problem of structural, compositional, and nutritional heat damage resulting from the sterilization of nonacid or low-acid substrates has been a major stimulus to spore investigation. We now report a new approach to lessening the severity of heating required for a given degree of survivor reduction in such low-acid spore-bearing substrates.

The basis of this approach lies in the unexpected persistence of the heat-sensitive state in the presence of natural biological mixtures in their ordinary *p*H range. Although the kinetics of calcium-hydrogen exchange and heat adaptation (*3*) would tend, qualitatively, to favor this approach, its success apparently is due primarily to the absence or very low concentrations of free calcium ion in natural biological mixtures.

Application of this approach involves first sensitizing the spores to heat by converting them to the hydrogen form by acid treatment. The substrate containing live but heat-sensitized spores is then neutralized back to its original *p*H before the lethal heat treatment. Since the rate of the heat-sensitizing conversion of the spore to the hydrogen form varies inversely with *p*H but positively with temperature, a wide range of conditions of *p*H and temperature are available for the sensitizing step. A choice would be guided by such factors as the time available, the stability of the substrate, and the metal form of the spores. Spores in the divalent (calcium) form require a lower *p*H than do spores in the monovalent form.

To demonstrate this new approach to heat severity reduction (Fig. 1) two complex biological mixtures were chosen, liver and tryptone culture medium, each inoculated with a large amount of the heat-resistant spores of *Bacillus stearothermophilus*.

With this approach, heating time advantages for a given degree of survivor reduction are large, amounting to several-fold. For example, the normal heating times without such a sensitizing treatment were longer by 8.5-fold for liver and 11-fold for the culture medium at a 100,000 × survivor reduction.

Because an acid and a base are added in sequence, the net additive in this approach is a small amount of a salt of an acid, in this case sodium chloride. The added sodium chloride amounted to 0.15 percent for the culture medium and about 1.0 percent for the liver on an undiluted basis. Inasmuch as this approach to heating severity reduction does not involve the use of antimicrobial additives, the attendant questions of additive persistence, toxicity, and ecological effects are avoided.

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### References and Notes

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3. G. Alderton, P. A. Thompson, N. Snell, *Science* **143**, 141 (1964).
4. Well-cleaned quartz tubes, enlarged to a thin wall at one end, and vacuum-sealed to avoid flame products, appear to be reliably inert. Alkali leakage from preconstricted borosilicate glass tubes can be minimized by vigorous leaching with water twice (122°C for 1 hour).
5. We thank Mrs. P. A. Thompson for plating and counting the tests.

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## Cell Microfluorometry: A Method for Rapid Fluorescence Measurement

Abstract. *A high-speed flow system for quantitative determination of fluoresence of cells containing fluorochrome has been developed. Feulgen-DNA distributions in populations of tissue culture cells and human leukocytes have been measured at a rate of $10^4$ to $10^5$ cells per minute and compare well with results of other independent methods.*

Fluorescent dyes have found widespread use in biology and medicine, examples being nucleic acid cytochemistry, fluorescent antibody studies, exfoliative cell diagnosis, cancer cell detection, and fluorochromasia (*1*). A method for rapid, quantitative measurement of fluorescent light emission from cells containing fluorochrome has been developed. A flow system is used; cells in aqueous suspension are measured at a rate of $10^4$ to $10^5$ cells/min. When stained by the fluorescent Feulgen procedure, normal human leukocytes show a Feulgen-DNA distribution with a single peak, while Chinese hamster ovary (*2*) cells growing asynchronously or synchronously show distinctive bimodal distributions which depend upon cell distribution around the life cycle.

The principle of the method is simple. Cells containing fluorochrome flow in a narrow stream (diameter 50 $\mu$) across a beam of exciting light (diameter 100 $\mu$); the resultant fluorescent light pulses are viewed perpendicularly to both cell stream and light beam by a photomultiplier (that is, dark field illumination). The cell stream is formed in a laminar flow chamber developed from a design by Crosland-Taylor (*3*); the light source is an argon ion laser (Spectra-Physics model 140) operated at 488 nm and a beam power of about 1 watt. No microscope and a minimum of optics are used. A simple lens of 20 cm focal length focuses the laser beam to the desired 100-$\mu$ diameter at the cell stream, and a pair of *f*1.6 movie projection lenses transfer an image of the cell stream-laser beam intersection at unity magnification to a low-noise photomultiplier (ITT type FW-130) with enhanced long wavelength response (S-20). A yellow filter (Corning CS 3/69) between the projection lenses is the barrier filter. A pinhole 400 $\mu$ in diameter in the image plane of the projection lenses serves as a limiting aperture to reduce optical noise. A cell takes about 15 to 20 $\mu$sec to cross the light beam, which is also the duration of the fluorescent light pulse. The resulting electrical pulses from the photomultiplier have this same duration, and their amplitude is proportional to fluorescence intensity. They are amplified, analyzed for amplitude, and stored in the memory of a multichannel pulse-height analyzer (SCIPP 1600, Victoreen Instrument Co.) at a typical rate of 50,000 per minute. The contents of analyzer memory are thus a frequency distribution histogram of fluorescent light emission per cell which is displayed and read out for further analysis.

The fluorescent Feulgen reaction described by Kasten and by Ruch (*4*) is used to stain for DNA. Modifications involving centrifugation were necessary to permit the several steps in the staining procedure to be carried out with cells in suspension rather than on slides. The procedure takes 2 hours. When viewed in a fluorescence microscope, the stained cells show little or no cytoplasmic fluorescence, while nuclei (or chromosomes in mitotic cells) fluoresce bright green. Centrifugation is conducive to cell clumping, an undesirable effect which is reduced by shearing at each step and sonication at the end of the procedure. A method which avoids the possibility of clumping would be advantageous.

Figure 1 shows both Feulgen-DNA and volume distributions for Chinese hamster ovary cells growing asynchronously. The volume distribution (before staining) measured with a modified Coulter volume spectrometer and a long aperture [diameter 80 $\mu$, length 260 $\mu$ (*5*)] is broad, unimodal, and typical of a cell population in exponential growth with cells of all ages present. The DNA distribution as indicated by fluorescence shows two peaks: the first represents cells with diploid DNA content ($G_1$ phase cells), and the second represents cells with tetraploid DNA content ($G_2$ and M phases). The region between peaks represents cells synthesizing DNA (S phase). The coefficient of variation