Fallacy of "Crushing Death" in Frozen Bacterial Suspensions. (22334)

ARTHUR P. HARRISON, JR. AND ROSE E. CERRONI.
(Introduced by Charles C. Randall.)
Department of Biology, Vanderbilt University, Nashville, Tenn.

It is well known that when a bacterial culture is frozen and thawed a portion of the cell population is killed. It has been generally accepted that death results from some mechanical effect produced as a result of the solidification of the suspension—either from crushing by ice crystals or from pressure resulting from ice formation(1-6). It has been demonstrated repeatedly that different species possess different susceptibilities to the lethal effect of freezing(7-9). If the lethal factor is mechanical, the differences in susceptibility to freezing must reflect differences in susceptibility to this mechanical factor. It becomes necessary, therefore, to conclude that the cells of species less susceptible to the lethal effect of freezing are physically stronger than the cells of species more susceptible. In fact, this conclusion has been presented on at least one occasion(5).

With experiments summarized in this paper we have sought to correlate the physical strength of cells with their susceptibility to freezing. In this manner, evidence for or against the ice crushing theory could be obtained. To determine the relative physical strength of different bacteria we have subjected them to violent shaking with small glass beads in the Mickle Tissue Disintegrator (MTD). Here the lethal factor is certainly mechanical, since very little death occurs in the absence of beads or abrasive, even upon prolonged shaking.

Materials and methods. Each experiment consisted of subjecting 2 different organisms to the 2 lethal conditions—successive freezings and vigorous shaking with beads. From each experiment, therefore, 4 sets of data were obtained. In every experiment *Escherichia coli* strain 69L-15 was one of the organisms employed; it was always the "reference organism" to which the behavior of a second organism was compared. The bacteria were cultivated as already outlined(9). The 2 cultures were centrifuged, the culture broth decanted, and the cells resuspended in broth of the following percentage composition: yeast extract (Balt. Biol. Lab., dehydrated), 1.0; Tween 80 (Atlas Powder Co.), 0.1; K_2HPO_4, 0.2; and $MgSO_4 \cdot 7H_2O$, 0.01. The cell concentration was adjusted (using broth) so that the viable cell count of each suspension would be approximately the same. An aliquot (7 ml) of the reference suspension (*E. coli*) was placed into a sterile vial containing 3 g of glass beads (0.057 mm average diam.).* The vial was stoppered and placed in its receptacle on one of the steel reeds of the MTD. A second aliquot (5 ml) of the suspension was placed into a sterile test tube (15 mm by 150 mm) fitted loosely with an aluminum cap. Aliquots of the "comparison suspension" were then dispensed in like manner. (Comparison suspensions were prepared using the following species: *Microbacterium flavum* strain 0J 10, *Lactobacillus fermenti* strain 69L-3, and *Bacillus pumilus* strain NRS 236.) The contents of the 2 test tubes were frozen simultaneously by placing the tubes in a −22°C alcohol bath. After 25 minutes at −22°C they were thawed simultaneously by transferring them to a +35°C water bath. A 0.1 ml sample was removed from each tube and the tubes were placed again at −22°C. In this manner, the suspensions were frozen and thawed 8 or more successive times. The 0.1 ml samples were diluted and plated in order to determine the number of cells which remained viable after a given number of successive freezings. Concurrent with the freezing-thawings, the suspensions in the MTD were being vigorously shaken. Periodically, the instrument was stopped for a time just sufficient to remove a 0.1 ml sample from each vial and to interchange the vials between the 2 receptacles. Switching the vials after each sampling was done in order to nullify the

* Minnesota Mining and Manufacturing Co., Saint Paul, Minn.

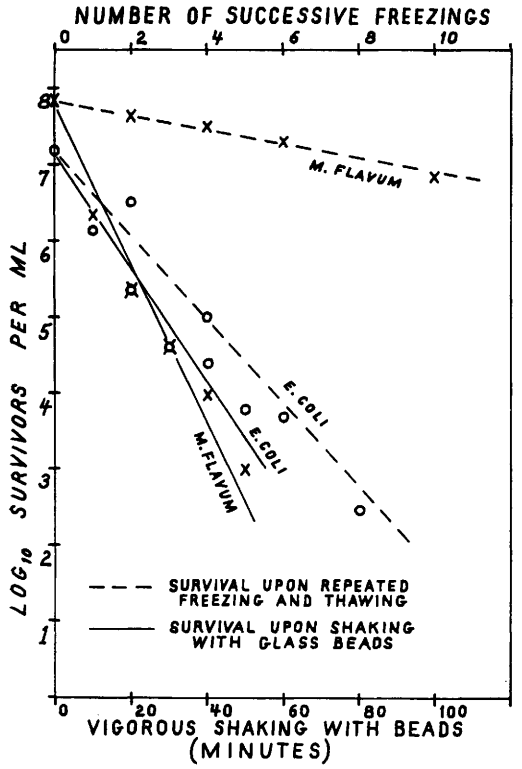

FIG. 1. Comparison of survival of *Microbacterium flavum* and *Escherichia coli* when subjected to successive freezings and when subjected to shaking with beads in the Mickle Tissue Disintegrator.

effect any small inequality in the amplitude of vibration of the 2 reeds would have upon survival of the bacteria in the respective vials. During shaking the vials were kept cool by means of a jet of CO_2. The 0.1 ml samples were diluted and plated in order to determine the number of cells which survive shaking for various periods of time. The dilution and plating technics already have been described (9,10).

Results. Fig. 1 illustrates that the susceptibility of *E. coli* to freezing is much greater than that of *M. flavum,* in spite of the fact that, as evidenced by rate of destruction in the MTD, the cells of *E. coli* appear to be physically stronger than those of *M. flavum.* In an experiment comparing *L. fermenti* with *E. coli,* little difference in the susceptibility to freezing of these 2 species was detected, although the cells of the latter species manifested a greater susceptibility to destruction in the MTD. On the other hand, in an experiment comparing *B. pumilus* with *E. coli* the cells of the former organism were less susceptible both to freezing and to shaking. There is no doubt that if sufficient pairs of species had been tested every possible combination of relative susceptibilities to the 2 lethal conditions would have been obtained. It is apparent that there is no correlation between the physical strength of cells and their susceptibility to freezing.

The susceptibility to freezing manifested by any given species is affected by both its remote and recent history. Species that are facultative toward oxygen are much less susceptible to the lethal effect of freezing when cultivated under forced aeration(9,10). In Fig. 2 it is readily apparent that cells of *E. coli* which had been aerated during growth are extremely resistant to freezing, whereas those cultivated in the customary manner show the usual susceptibility. It is important to note that the cells from the 2 cultures do

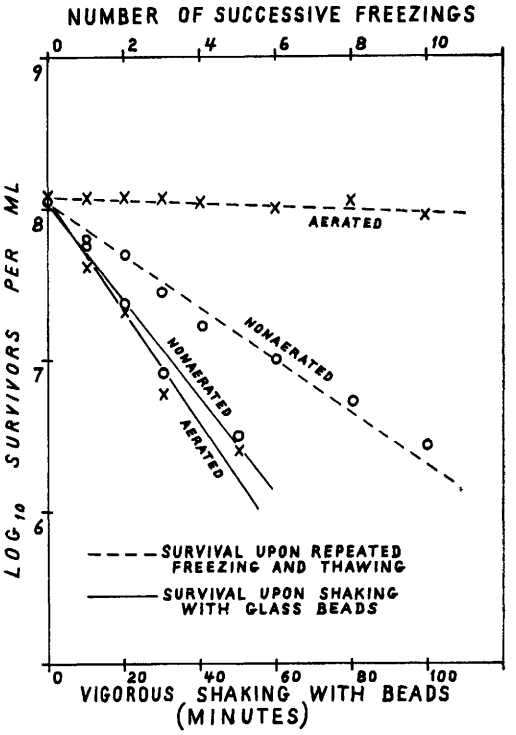

FIG. 2. Comparison of survival of aerated and nonaerated cultures of *Escherichia coli* when subjected to successive freezings and when subjected to shaking with glass beads in the Mickle Tissue Disintegrator.

not differ appreciably with respect to their physical strength. Indeed, it hardly would be expected that aeration would render cells physically stronger; rather, it might be expected that the cells would be altered in permeability and/or concentration of intracellular metabolites and enzymes. The greater resistance of aerated cells is undoubtedly due to such alterations. It is suggested that the actual cause of death upon freezing and thawing is not due to a mechanical factor but is due to some chemical or physicochemical phenomenon. The effect of solutes and initial cell concentration upon survival, as well as the very shape of the survival curves(9,10) supports this contention.

Summary. There appears to be no correlation between the physical strength of cells and their susceptibility to the lethal effect of freezing. It is therefore unlikely that the lethal factor of freezing-thawing is mechanical.

1. Keith, S. C., Jr., *Science,* 1913, v37, 877.
2. Hilliard, C. M., and Davis, M. A., *J. Bact.,* 1918, v3, 423.
3. Berry, J. A., *ibid.,* 1933, v26, 459.
4. Weiser, R. S., and Osterud, C. M., *ibid.,* 1945, v50, 413.
5. Proom, H., *Symposium on Freezing and Drying.* Edited by R. J. C. Harris, Hafner Publishing Co., New York, 1952, p122.
6. Hollander, D. H., and Nell, E. E., *Appl. Microbiol.,* 1954, v2, 164.
7. Hilliard, C. M., Torossian, C., and Stone, R. P., *Science,* (new series), 1915, v42, 770.
8. Tanner, F. W., and Williamson, B. W., PROC. SOC. EXP. BIOL. AND MED., 1928, v25, 377.
9. Major, C. P., McDougal, J. D., and Harrison, A. P., Jr., *J. Bact.,* 1955, v69, 244.
10. Harrison, A. P., Jr., *ibid.,* 1955, v70, 711.

Received March 8, 1956. P.S.E.B.M., 1956, v91.

Resistance of Specifically-Sensitized *Treponema pallidum* to Methylene Blue Stain.* (22335)

BARBARA J. ROSENAU AND JOHN F. KENT. (With assistance of Robert W. Sanders.)
Department of Serology, Walter Reed Army Institute of Research, Washington, D.C.

During studies of toxoplasmosis Sabin and Feldman(1) developed a serodiagnostic test based on the observation that *Toxoplasma gondii,* which is normally stained by methylene blue, lost affinity for the dye when incubated with homologous antiserum and a thermolabile factor in normal serum. Recent experiments(2) showed that virulent *Treponema pallidum* becomes similarly resistant to methylene blue when incubated with syphilitic serum and complement. The purpose of the present studies was to explore possible applications of the reaction in the diagnosis of syphilis.

Materials and methods. The treponeme stain consisted of 0.10 g methylene blue† and 0.025 g Na_2CO_3 in distilled water to make 100 ml. It was prepared by diluting 1 ml of 2.50% Na_2CO_3 with 89 ml water, then adding rapidly 10 ml of a stock 1% solution of the dye. Both stock and dilute dye solutions were stored at 3-6°C, care being taken however to discard any showing sediment or contamination. New glass slides and coverslips, used in microscopic examination of test mixtures, were cleaned in ethanol (70%) and wiped dry with clean gauze. All other glassware was cleaned scrupulously with sulfuric-chromic acid mixture. The basic test procedure was the treponemal immobilization test(3,4). To 0.05 ml of serum in 13 x 100 mm test tube were added 0.40 ml of suspension containing 7-9 x 10^6 treponemes/ml and 0.05 ml guinea pig complement (C'); 0.05 ml of inactivated guinea pig C' was substituted in otherwise identical serum control. The mixtures were incubated 18 hours at 35°C in an atmosphere of nitrogen (95%) and carbon

* Presented in part at Symposium on Recent Advances in the Study of Venereal Diseases, April, 1955, Washington, D.C.

† Methylene blue chloride (dye content, 87%), National Aniline Division, Allied Chemical and Dye Corp., New York City.