Lack of Influence of Suspending Media on Heat Activation of
*Bacillus subtilis* Spores and Absence of Deactivation¹

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**Abstract**

BUSTA, F. F. (University of Illinois, Urbana), and Z. JOHN ORDAL. Lack of influence of suspending media on heat activation of *Bacillus subtilis* spores and absence of deactivation. Appl. Microbiol. **12**:111–114. 1964.—*Bacillus subtilis* strain 5230 endospores suspended in a variety of suspending media at a concentration of ca. 10⁸ per ml were heated at 95 and 75 C. The effect of the heating at 75 C was measured by plate count, and was reported as the heat-activated decimal fraction of the total viable-sporule population. Thermal inactivation at 95 C was influenced by the suspending medium. No effects on heat activation at 75 C were noted for suspending media containing glucose, xylose, ribose, NaCl, or sodium phosphate; nor was there any marked effect due to a change in pH from 5 to 8. The heat-activation response was retained during postheating storage at 5 C in water up to 215 days. Postheating storage in several suspending media for 7 days also indicated no deactivation.

A number of investigators reported on the influence of media used to suspend bacterial spores for a heat-activation treatment (Curran and Evans, 1945; Brachfeld, 1955; Treadwell, Jann, and Salle, 1958; Hernier, 1958; Splittstoesser and Steinraus, 1962; Finley and Fields, 1962). The reported effects have been varied and numerous. The effects of suspending media on heat activation generally were measured by plate counts, and the differences in heat-activation responses due to the suspending media were based on the differences in plate counts after heating.

Similar plate count analyses were used to report the loss of the heat-activation response during postheating storage. This deactivation and also subsequent reactivation was reported by Curran and Evans (1947), Brachfeld (1955), and Desrosier and Heilgman (1956).

The present investigation was designed to evaluate past reports on the effect of the suspending media on heat activation, and to evaluate the occurrence of deactivation, by employing new methods for the measurement of heat activation, namely, the use of the heat-activated decimal fraction of the total number of viable spores (Busta and Ordal, 1964).

**Materials and Methods**

The test culture, recovery media, method of heating, and especially the formulation and use of CaDPA-TGE were described previously (Busta and Ordal, 1964). Preparation of the spore suspension also was described previously (Busta and Ordal, J. Food Sci. *in press*).

**Measurement of heat activation.** The amount of heat activation is reported as a decimal fraction, i.e., the number of heat-activated spores divided by the total number of viable spores. Standard plate count methods were used throughout. The diluent was sterile deionized distilled water. Spore suspensions were heated at concentrations of ca. 10⁸ spores per ml. Subsequent dilutions were such that ca. 100 spores were deposited into each plate. For each sample, 13 plates were prepared. The average counts from ten plates with fortified TGE enumerated the heat-activated number, and the average counts from three plates with CaDPA-TGE enumerated the total viable number. Plates were incubated for 24 hr at 37 C.

**Suspending media.** In all instances, the water was sterile, deionized, distilled water. The NaCl solutions contained only salt and water. The phosphate buffer was made from the sodium salts and used at pH 7.0 in most instances (Gomori, 1955); however, phosphate buffers were adjusted to pH 5.2, 6.0, 7.2, and 8.0 for one trial. The study of heating at 95 C employed ribose, xylose, and glucose solutions prepared with water and autoclaved prior to use, as were the salt solutions. The sugar solutions used in heating at 75 C were prepared by adding the sugar to sterile phosphate buffer with no additional heat treatment prior to inoculation.

**Results**

**Heating at 95 C.** In addition to numerous reports on the influence of the suspending medium on the apparent thermal resistance of bacterial spores, several studies on heat activation of bacterial spores also indicated significant effects of the heating medium on the apparent heat-activation response.

Heating *Bacillus subtilis* 5230 spores in a variety of suspending media at 95 C resulted in slow rates of thermal inactivation in water, NaCl, or phosphate buffer; however, the presence of glucose, ribose, or xylose appeared to accelerate thermal inactivation. These data (Fig. 1 and 2) confirmed the presence of effects on thermal inactivation due to the suspending media. The erratic pH levels in the

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unbuffered sugar solutions did not permit evaluation of the acceleration since the low pH, rather than the presence of the sugars, may have increased the thermal inactivation.

Nevertheless, when the heat activation at 95 C was measured as the heat-activated decimal fraction of the total viable population, no marked differences were observed among the several systems. Due to the rapid rate of heat activation at 95 C, differences possibly were not evident in the data; therefore, a lower temperature (75 C) was employed to measure the initial rates of heat activation in several suspending media.

**Effect of pH.** Phosphate buffers at pH 5.2, 6.0, 7.2, and 8.0 were employed to determine the effect of the pH on the rate of heat activation (Fig. 3). The influence of pH on heat activation is slight, if there is any influence at all. The buffer at pH 5.2 appeared to yield slightly accelerated heat activation; however, the increase in rate was not as dramatic as might be expected if compared with the corresponding increase in rate that is characteristic of thermal inactivation at low pH.

**Effect of solutes.** The presence of glucose, ribose, or xylene did not noticeably influence the rate of heat activation at 75 C (Fig. 4). Heat-activation rates in water, 8.5 mm NaCl solution, 170 mm NaCl solution, and 25 mm phosphate buffer were equally unaffected by the suspending medium. Within the accuracy of the plate count procedure.

**FIG. 1.** Effect of suspending media on thermal inactivation of endospores of Bacillus subtilis 5230. Symbols: O, water control (range, pH 6.1 to pH 7.5; average, pH 6.8); A, 0.56 mm glucose solution (range, pH 5.7 to pH 6.4; average, pH 6.0); D, 5.6 mm glucose solution (range, pH 5.1 to pH 6.2; average, pH 5.7); 56 mm glucose solution (range, 5.1 to pH 5.8; average, pH 5.4). Recovery medium was CaFPA-TGE.

**FIG. 2.** Effect of suspending media on thermal inactivation of endospores of Bacillus subtilis 5230. Symbols: O, 8.5 mm NaCl solution (range, pH 6.0 to pH 6.8; average, pH 6.5); A, 67 mm ribose solution (range, pH 4.0 to pH 5.2; average, pH 4.9); D, 67 mm xylene solution (range, pH 4.6 to pH 5.7; average, pH 5.4); , 170 mm NaCl solution (range, pH 5.7 to pH 6.8; average, pH 6.0); 25 mm sodium phosphate buffer at pH 7.1. Recovery medium was CaFPA-TGE.

**FIG. 3.** Effect of pH of suspending medium on the heat activation of endospores of Bacillus subtilis 5230. N\textsubscript{R}/N\textsubscript{T} indicates number of heat-activated spores as a decimal fraction of the total viable population. Symbols: A, pH 7.2; D, pH 5.2; , pH 6.0; 8.5 All solutions were 25 mm sodium phosphate buffer.

**FIG. 4.** Effect of sugar solutions used as suspending media on the heat activation of endospores of Bacillus subtilis 5230. N\textsubscript{R}/N\textsubscript{T} indicates number of heat-activated spores as a decimal fraction of the total viable population. Symbols: A, 56 mm glucose; A, 67 mm ribose; 67 mm xylene. All sugars were in 25 mm sodium phosphate buffer (pH 7.0).
procedure, the curves derived from the heat-activation data on all the tested suspending media could be superimposed upon one another.

*Stability of heat-activation response.* B. subtilis 5230 spores were heated at 75 °C in several suspending media and then stored at 5 °C in the same media to determine the stability of the heat-activation response. There were no marked differences among responses in phosphate buffer with or without glucose, and there were no noticeable losses in heat-activation response after storage at 5 °C for 48 hr (Fig. 5). The absence of deactivation during postheating storage was substantiated by data obtained after 7 days of storage; these results are not illustrated because they were essentially the same as those presented in Fig. 5.

Equally pertinent data were obtained when spores, suspended in water or 100 mM NaCl solution, were heated and stored under the same conditions as stated above. No deactivation was observed in any of the four suspending media tested, and the rate of heat activation appeared to be the same in water, 100 mM NaCl, or phosphate buffer with or without glucose.

Extended postheating storage of spores in water at 5 °C further substantiated the absence of deactivation. When samples were heated for 40 min at 75 °C and stored at 5 °C for 0, 95, 144, and 215 days, the heat-activated decimal fractions were 0.60, 0.65, 0.65, and 0.68, respectively. Again, no loss in heat-activation response was observed.

**Discussion**

Thermal inactivation of B. subtilis 5230 spores at 95 °C was affected by the pH or the presence of certain solutes in the suspending medium; however, heat activation under the identical conditions was not noticeably influenced. The effect of the suspending medium on thermal inactivation is well documented (Schmidt, 1957). The influence of the suspending medium on heat activation also has been studied. Heat activation of spores of B. subtilis 15 u (same strain as 5230) suspended in a variety of media was reported by Curran and Evans (1945). Their data indicated marked differences among responses to heating in several media; consequently, they ranked these media according to the magnitude of increase in plate counts that resulted from heating. Other spore suspensions also responded in a manner similar to spores of strain 15 u. Phosphate buffers used as suspending media for heat-activation studies were observed to have definite effects on the resultant response (Brachfeld, 1955; Finley and Fields, 1962).

Hernier’s (1958) study of heat activation of B. subtilis SJ2 spores suspended in several sugar solutions indicated that the chemical nature of the sugar had an effect on the response. He reported dramatic differences in plate counts after heating, and noted the decreases in counts when pentoses were added to the phosphate buffer medium and increases in counts when ketohexoses or aldohexoses were present in the buffered suspending medium.

Contrary to these previous reports, our studies on the effect of the suspending medium on heat activation of strain 5230 spores at 75 °C indicate that no marked influences were evident when the medium was varied. Results of heat-activation studies at 75 °C are representative of heat activations at other temperatures (Busta and Ordal, *in press*). No distinct effect was observed when glucose, ribose, xylose, NaCl, or sodium phosphate buffers at several pH levels were incorporated into the solution used to suspend the spores for heat treatment. Phosphate buffer at pH 5.2 may have enhanced the rate of activation. This observation is in agreement with Murrell’s (1955) report of increased heat activation in solutions at low pH levels.

Contradiction of numerous past reports by the observation of the absence of an effect of the suspending medium on heat activation requires explanation. Some of the earlier reports cited here were based on increases in plate counts after heating the spores in the test solutions at one time and temperature and did not measure total viable-spore populations. Our study not only evaluated a series of exposure times at each temperature, but also evaluated the amount of heat activation as the decimal fraction of heat-activated spores, i.e., the number of spores heat-activated of the number of viable spores present in the suspension. Thermal inactivation occurring simultaneously with heat activation, but at a different rate, could explain the results presented in the earlier reports. A rapid rate of thermal inactivation in one suspending medium and a slow rate in another, but, at the same time, similar rates of heat activation in both media would explain the data in the literature. The use of total viable spores as a measure might account for the discrepancy in results.
counts and the decimal fraction of heat-activated spores eliminates discrepancies in the results due to thermal inactivation. Therefore, with the exclusion of thermal-inactivation influences, the rate of heat activation did not appear to be affected by the medium in which strain 5230 spores were suspended for heating.

Deactivation or the loss of the heat-activation response upon prolonged storage has been reported on a number of occasions. Spores, heat-activated and stored in nutritionally incomplete media at 37 C, rapidly lost viability when measured by plate counts (Curran and Evans, 1947). When the apparent population could be increased by a poststorage heating, the original loss in viability was thought to be deactivation and the secondary increase in plate count was thought to be a reactivation by the heat treatment. Brachfeld (1955) observed deactivation at refrigerator temperatures within 7 days. Desrosier and Heiligman (1956) also observed a deactivation which was reversed partially by a poststorage heating.

Contrary to these early reports on deactivation and reactivation, the heat-activation response observed with B. subtilis 5230 spores was stable during postheating storage at a low temperature. No deactivation was observed after storage at 5 C for 7 days in phosphate buffer, glucose solution, NaCl solution, and water. In addition, extended storage (215 days) after heating in water resulted in no loss of the heat-activation response.

Again, as in the studies on the effect of suspending media, early investigations were based on the enumeration of spores by conventional plate count, and increases or decreases in counts indicated activation or deactivation, respectively. Deactivation was reported during storage at 37 C, room, and refrigerator temperatures. Our storage studies used 5 C exclusively. The determinations of total viable counts with CaDPA-TGE were used for the investigation of deactivation, and the heat-activation response was recorded as the decimal fraction of the total viable-spor e population. Thus, any loss in total numbers during extended storage did not obscure the measurement of the actual response. No deactivation was observed when the response was measured in terms of the decimal fraction of heat-activated spores.

Reactivation obviously was not observed in the present study, because there was no deactivated system to re-activate. Reactivation as indicated by plate count data in the early literature can be explained in the manner Halvorson (1958) interpreted the deactivation and subsequent reactivation of the glucose oxidation system reported by Church and Halvorson (1957). The deactivation may be merely an inactivation process occurring during storage, and the reactivation then would be merely activation of a fraction which previously had not been activated. This explanation would be consistent with the reports in the literature and the data reported here.

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Literature Cited


