

EFFECT OF DIVALENT CATIONS IN THE SPORULATION MEDIUM ON THE
THERMAL DEATH RATE OF *BACILLUS COAGULANS* VAR.
*THERMOACIDURANS*¹

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In a previous paper, El-Bisi and Ordal (1956a) reported that increased levels of phosphate, in the growth and sporulation medium, significantly reduced the thermal resistance of spores of *Bacillus coagulans* var. *thermoacidurans*. It was postulated that the phosphate anion lessened the availability of divalent cations to the sporulating cells and that spores so produced possessed a reduced thermal resistance. A relationship between the calcium content and the degree of thermal resistance of bacterial spores has been suggested by Curran (1952) and by Sugiyama (1951). Williams (1929) reported that the addition of magnesium to peptone containing media yielded spores of *Bacillus subtilis* of increased thermal resistance.

In this investigation, the effect of the divalent cations, calcium, magnesium, and manganese, added to the sporulation medium, was studied in more detail for their effect on the thermal resistance of spores of *B. coagulans* var. *thermoacidurans*.

MATERIALS AND METHODS

Test culture. *B. coagulans* var. *thermoacidurans*, American Type Culture Collection no. 8038, was used in this investigation. Stock cultures were maintained on thermoacidurans agar (Difco) containing additional 1 ppm MnSO₄.

Media. Spore crops were produced either on agar surface cultures or in broth shake cultures. Basal media used were thermoacidurans agar (proteose-peptone, 0.5 per cent; yeast-extract, 0.5 per cent; glucose, 0.5 per cent; K₂HPO₄, 0.5 per cent; and agar, 2 per cent) and thermoacidurans broth (the same components omitting the agar).

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The K₂HPO₄ was either eliminated or the concentration reduced as indicated for the particular experiment. Reagent grade CaCl₂(2H₂O), MgCl₂(6H₂O), and MnSO₄(H₂O) were used to supply the divalent cations added to the basal medium. The final pH value of all media was adjusted to 7.2 with NaOH or HCl prior to sterilization.

Preparation of spore suspensions. For the production of spores on agar media, petri plates were inoculated from an 18-hr thermoacidurans broth culture and incubated at 45 C for 6 days. For their production in shake culture, Erlenmeyer flasks, each containing 50 ml broth, were similarly inoculated and shaken on a rotary shaker at 45 C for 6 days. After incubation, spores were harvested and washed four times in cold distilled water by centrifugation. The spore concentration was determined both by a direct microscopic count, using a Petroff-Hausser chamber, and by plate count. Prior to plating, the aliquot used was heated at 80 C for 5 min to destroy the vegetative cells. The plating medium was thermoacidurans agar (Difco) fortified with 1 ppm MnSO₄. There was a good correlation between the two methods of evaluating the spore numbers in all spore preparations as the two counts agreed within 5 to 15 per cent of each other. Prepared spore suspensions were stored in the refrigerator at about 4 C. There was no apparent change in either the count or the thermal resistance of such spore suspensions over a two-month storage period.

Determination of thermal death rates. The apparatus and procedure used have been described (El-Bisi and Ordal, 1956a). Spores were heated in M/40 phosphate buffer of pH 7 at 96.4 C (±0.02 C). At the end of the 5 min, following the inoculation of the spores into the heating menstruum, the first sample was withdrawn to represent the initial count of viable spores present in the heating menstruum. Following this sample, 10 to 12

more samples were taken at 5- or 10- min intervals. Therefore, each survivor curve was constructed from data obtained from 11 to 13 samples taken at the appropriate intervals. This number of samples was deemed necessary for establishing the complete shape of the survivor curves. The number of survivors was determined by plating the sample, in triplicate, on thermoacidurans agar (Difco) fortified with 1 ppm $MnSO_4$. The addition of 1 ppm $MnSO_4$ to the recovery medium appeared to stimulate the development of colonies by the surviving spores. Maximum counts were obtained after incubation for 40 to 48 hr at 45 C. Duplicate thermal death rate determinations were made on each spore crop.

Statistical analysis of data. The logarithms of the mean plate counts for each thermal death run were plotted against the heating time in minutes in order to obtain a rough estimate of the linear relationship between the two. Most of the curves thus obtained showed a definite shoulder or a sharp break. The linearity test was then made on every survivor curve in order to determine whether the break was real (Snedecor, 1946). In almost every case, the break in the curve was found to be significant. The points on the initial shoulder, and those on the remaining portion of the curve were, therefore, fitted separately to a least-squares-deviation straight line.

The thermal death rate constant D (time in min required for 90 per cent destruction) was computed from the corresponding regression coefficients. The D value for the shoulder portion and that for the latter portion of each survivor curve were designated as D_1 and D_2 , respectively. The mean values of duplicate D_1 and D_2 determinations were then subjected to the statistical "t" test (Snedecor, 1946) in order to define the order of significance of the differences in thermal resistance, within each tested group.

RESULTS

Effect of the phosphate concentration added to the sporulation medium. This experiment was carried out in order to obtain further information on the effect of the phosphate concentration in the sporulation medium on the subsequent thermal resistance of the spores produced. Spore crops were cultivated on the basal thermoacidurans agar (containing 0.5 per cent K_2HPO_4), the same agar fortified with 1 ppm $MnSO_4$ to enhance

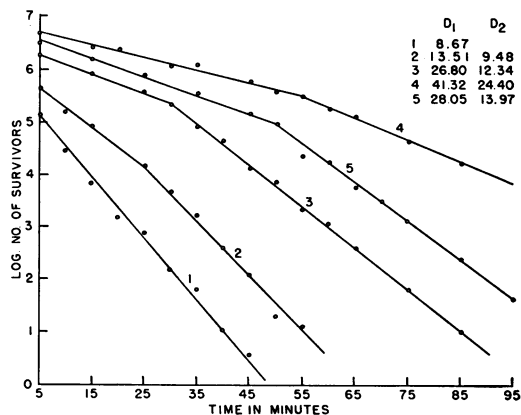


Figure 1. Effect of the phosphate concentration in the sporulation medium on the thermal death rate of *Bacillus coagulans* var. *thermoacidurans*. Curves represent the computed death rates of spores produced on a given medium. The D values reported are the arithmetic mean of duplicate thermal death rate determinations. Curve 1: basal thermoacidurans agar (containing 0.5 per cent K_2HPO_4). Curve 2: the same agar fortified with 1 ppm $MnSO_4$ (fortified agar). Curve 3: fortified agar, phosphate reduced to 0.2 per cent K_2HPO_4 . Curve 4: fortified agar, phosphate reduced to 0.05 per cent K_2HPO_4 . Curve 5: fortified agar without K_2HPO_4 .

sporulation (Amaha *et al.*, 1956), and fortified agars in which the K_2HPO_4 concentrations were reduced to 0.2, 0.05, and 0 per cent, respectively. Results of the thermal death rate determinations made on these spore crops are illustrated in figure 1.

With all spore crops except the one produced on the basal thermoacidurans agar (curve 1), a definite break in the survivor curves was established at the end of initial heating periods ranging from 25 to 55 min and after a death total ranging from 85 to 95 per cent. The statistical analysis of the variance of regression for the respective data (Reynolds and Lichtenstein, 1952) revealed that the log survivors and heating time were not linearly related over the entire heating period. However, when the data for the initial portion or the latter portion of such curves were analyzed separately, the relationship was linear. Therefore, it was assumed that the initial shoulders in all survivor curves were real, and thus the thermal resistance value for each spore crop should be indicated by two consecutive death rates; one as the initial death rate, D_1 , and the

other as the secondary death rate, D_2 . All of the D_1 and D_2 values were computed from their respective regression coefficients. Duplicate values were obtained in each case, and their arithmetic means are the ones reported in figure 1.

On application of the "t" test to the D_1 and D_2 values belonging to the different spore crops, significant differences were found to be due to the change in the phosphate concentration added to the sporulation medium. Lower phosphate levels enhanced the thermal resistance of the spores produced. These results are in general agreement with those previously reported by El-Bisi and Ordal (1956a). With reduction of the K_2HPO_4 concentration from 0.5 (curve 2) to 0.2 per cent (curve 3), the increase in the D_1 value was significant at the 0.01 level, and in the D_2 value at the 0.02 level. Further reduction of K_2HPO_4 to 0.05 per cent (curve 4) produced spores with even greater thermal resistance; the increase in both D_1 and D_2 values, as compared to either curves 2 or 3, was significant at the 0.01 level. When the K_2HPO_4 was omitted from the sporulation medium (curve 5), the spores so produced possessed a thermal resistance which was significantly greater than that of those produced on a medium containing 0.5 per cent K_2HPO_4 (curve 2) but were less resistant than those produced on the 0.05 per cent medium (curve 4).

It was also interesting to note some increase in the thermal resistance of the spores caused by the addition of 1 ppm $MnSO_4$ to the basal sporulation medium. The increase was only in the D_1 value and was significant only at the 0.05 level (curves 1 and 2).

When the thermal resistance of different spore preparations is compared, it is also important to consider the duration of the initial death rate. For example, although the difference in the D_1 and D_2 values obtained from curves 3 and 5 was not statistically significant, the initial death rates persisted for 30 and 50 min, respectively. This would indicate that a greater portion of the latter spore population possessed the higher thermal resistance represented by the D_1 rate.

Effect of divalent cations added to the sporulation medium. (1) Agar-surface cultures:—Divalent cations have been reported to be capable of combining with proteins in a manner which adds new strength to their intramolecular linkages and increases their thermal stability (Sugiyama, 1951; Gorini, 1951; Gorini and Felix, 1953). It is pos-

sible that divalent cations could exert a similar effect on bacterial spores. If we accept the theory that the death of a bacterial cell is due to the denaturation of a critical proteinaceous molecule (Rahm, 1943), then we could assume that the presence of excess divalent cations in the sporulation medium would stimulate the incorporation of such cations in the spore proteins with the result that such spores would have an increased thermal resistance. We could further assume that the unfavorable effect of high phosphate levels in the sporulation medium upon the subsequent thermal resistance of the spores produced would be due to the interference of the phosphate anions with the availability of such divalent cations to the sporulating cells.

The following experiment was carried out to examine the above assumptions, i. e., to test for the possible effect of divalent cations such as Ca^{++} , Mn^{++} , and Mg^{++} , when added to the sporulation medium, upon the subsequent thermal resistance of the spores produced. Different spore crops were produced on the basal thermoacidurans agar fortified with different concentrations of different divalent cation salts. The K_2HPO_4 was omitted from the basal medium in order to avoid the interference of the phosphate anion with the availability of the tested cations. Thermal death-rate determinations were then made on the resulting spore crops and the data statistically analyzed in the manner described above. Results are illustrated in figure 2.

It may be seen from figure 2 that the addition of certain divalent cation salts to the sporulation medium did have a definite effect on the spores' thermal resistance. Such an effect was apparently a function of either the kind or the concentration of the added cation. The statistical evaluation of the differences in both D_1 and D_2 values of the different spore crops revealed the following. The addition of 60 ppm $MgSO_4$ to the basal medium did not significantly affect the D_1 value but caused some increase in the D_2 value which was significant only at the 0.05 level (curves 6 and 7). The addition of 1 ppm $MnSO_4$ had a slight effect, the increase in the D_1 and D_2 values was also significant only at the 0.05 level (curves 6 and 8). However, increasing the level of $MnSO_4$ to 50 ppm produced the spore crop of the highest thermal resistance; the increase in both the D_1 and D_2 values was significant at the 0.01 level (curves 6 and 9). The addition of 50 ppm $CaCl_2$

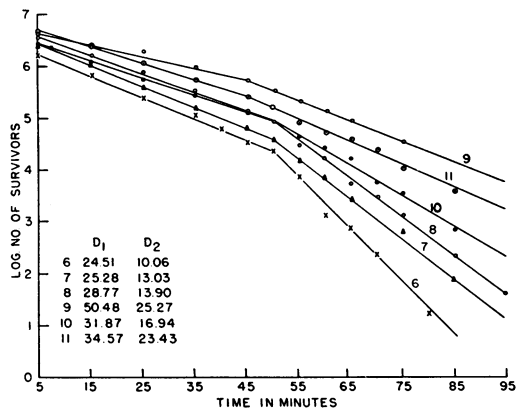


Figure 2. Effect of divalent cations added to the sporulation agar medium on the thermal death rate of *Bacillus coagulans* var. *thermoacidurans*. Curves represent the computed death rates of spores produced on a given medium. The D values reported are the arithmetic mean of duplicate thermal death rate determinations. Curve 6: basal thermoacidurans agar without the phosphate (Medium I). Curve 7: Medium I plus 60 ppm $MgCl_2$. Curve 8: Medium I plus 1 ppm $MnSO_4$. Curve 9: Medium I plus 60 ppm $MnSO_4$. Curve 10: Medium I plus 50 ppm $CaCl_2$. Curve 11: Medium I plus 50 ppm $MnSO_4$ and 50 ppm $CaCl_2$.

also caused an increase in thermal resistance, but it was not as marked as that caused by 50 ppm $MnSO_4$; the difference in both the D_1 and D_2 values was again significant at the 0.01 level (curves 6 and 10). When both (50 ppm $MnSO_4$ and 50 ppm $CaCl_2$) were added to the sporulation medium, the resulting spores acquired a thermal resistance which was greater than that of spores produced on the medium containing only 50 ppm $CaCl_2$ (curves 10 and 11), but less than that of spores produced on the medium containing only 50 ppm $MnSO_4$ (curves 9 and 11).

(2) Broth shake cultures:—The analytical data reported for the basal thermoacidurans agar medium (personal communication from H. W. Schoenlein of Difco Laboratories, Detroit, Michigan) were approximately 3 μ moles calcium, 2 μ moles magnesium, and 0.03 μ moles manganese ml, over 98 per cent of which was contributed by the agar alone. It was, therefore, obvious that the basal thermoacidurans broth would be almost free from the above cations and thus would furnish better conditions for checking the effect of such divalent cations on the thermal resistance of the spores produced. Accordingly, it was thought

desirable to demonstrate such an effect in the basal thermoacidurans broth with the shake culture technique.

Spore crops were, therefore, produced in the basal thermoacidurans broth and broth fortified with different concentrations of magnesium, manganese, and calcium salts. The phosphate was again omitted throughout to avoid its interference with the availability of such cations, except for one case where a spore crop was produced in basal broth with the phosphate (K_2HPO_4 , 0.5 per cent) for comparison purposes. Duplicate thermal death-rate determinations were then made on these different spore crops and the D_1 and D_2 values computed and compared in the manner described above. Results of this experiment are illustrated in figure 3.

The data in figure 3 show an important difference in the shape of the survivor curves between cases where excess calcium was added to the sporulation medium and where either other cations or no cations were added. All the curves belonging to the spore crops produced in the presence of excess calcium showed a definite shoulder, whereas other curves did not.

The statistical "t" test applied to the differences in the D_1 values revealed the following. The addition of 60 ppm $MgSO_4$ to the basal broth gave spores of slightly higher thermal resistance; the increase in D_1 value was significant only at the 0.05 level (curves 13 and 14). The addition of 1 ppm $MnSO_4$ caused some but not a significant increase in the D_1 value (curves 13 and 15). Increasing the concentration of $MnSO_4$ to 50 ppm, however, produced spores with a much higher thermal resistance; the increase in the D_1 value was significant at the 0.01 level (curves 13 and 16). Spores produced in broth fortified with 5 ppm $CaCl_2$ had almost the same resistance as those produced in the presence of 60 ppm $MgSO_4$ (curves 17 and 14); the increase in the D_1 value over that of the control crop was significant only at the 0.05 level (curves 13 and 17). Raising the $CaCl_2$ concentration to 45 ppm increased the spores' thermal resistance considerably; the increase in the D_1 value was significant at the 0.01 level (curves 13 and 18). However, further increasing the $CaCl_2$ level to 450 ppm did not materially affect the thermal resistance as both spore crops exhibited similar D values (curves 18 and 19). The combined effect of 50 ppm $MnSO_4$ and 50 ppm $CaCl_2$ was the most striking, and the

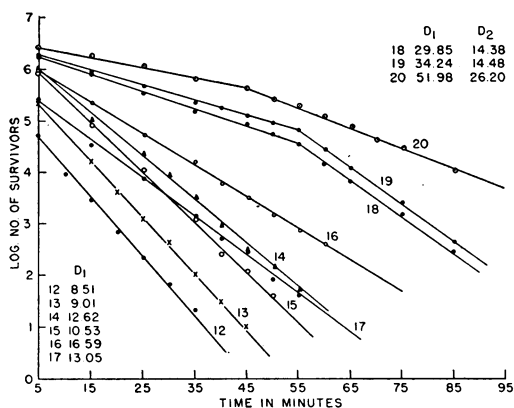


Figure 3. Effect of divalent cations added to the sporulation shake-culture medium on the thermal resistance of *Bacillus coagulans* var. *thermoacidurans*. Curves represent the computed death rates of spores produced in given media. The *D* values reported are the arithmetic mean of duplicate thermal death rate determinations. Curve 12: basal thermoacidurans broth (containing 0.5 per cent K_2HPO_4). Curve 13: same broth without the phosphate (Medium II). Curve 14: Medium II plus 60 ppm $MgSO_4$. Curve 15: Medium II plus 1 ppm $MnSO_4$. Curve 16: Medium II plus 50 ppm $MnSO_4$. Curve 17: Medium II plus 5 ppm $CaCl_2$. Curve 18: Medium II plus 45 ppm $CaCl_2$. Curve 19: Medium II plus 450 ppm $CaCl_2$. Curve 20: Medium II plus 50 ppm $MnSO_4$ and 45 ppm $CaCl_2$.

spores produced possessed the highest thermal resistance (curve 20). It is interesting to compare these results with those previously obtained when the spores were produced on agar media. On agar media, the calcium and manganese exhibited a somewhat competitive effect in regard to the increase in the spores' thermal resistance, whereas in broth shake culture, when appropriate concentrations were used, the effect appeared to be additive. This is probably due to the fact, previously pointed out, that the agar itself contains enough calcium to account for part of the increase in the thermal resistance of the resulting spores.

The thermal resistance exhibited by spores produced in the basal broth containing 0.5 per cent K_2HPO_4 (curve 12, figure 3) was consistent with that of the crop produced on the basal agar also containing 0.5 per cent K_2HPO_4 (curve 1, figure 1). When the phosphate was omitted from both media, the spores produced on agar increased significantly in their thermal resistance (curve 6, figure 2), whereas those produced in

broth did not show any significant change (curve 13, figure 3). This is consistent with our explanation of the effect of an increased phosphate concentration in the sporulation medium. In agar media where the calcium is in excess to that needed for growth and sporulation, the added phosphate would serve to bind the extra calcium so that it would not be incorporated into the spore. In broth media, the amount of calcium is probably only enough to allow normal growth and sporulation. In such media the effect of additional phosphate would be greatly reduced.

Effect of different buffering and chelating compounds in the heating menstruum. It was assumed that the enhancing effect of calcium and manganese was due to their incorporation into the spore material in a manner which increased their thermal resistance. It was also assumed that the phosphate effect was that of a chelating agent and that when it was present in the medium it served to bind the cations with the result that they were essentially unavailable to the sporulating cells. It was of interest, therefore, to determine whether or not phosphate or other chelating agents would affect the thermal resistance of preformed spores if the spores were exposed to such agents during the thermal resistance determination.

The spore crop used was produced on thermoacidurans agar, lacking the KH_2PO_4 but fortified with 1 ppm $MnSO_4$. Duplicate thermal death rate determinations were then made in the following heating menstrua: m/40 phosphate buffer (our standard heating menstruum); m/100 phosphate buffer; m/15 phosphate buffer; m/1,000 ethylenediaminetetraacetic acid (EDTA); m/200 trihydroxymethylaminomethane (tris buffer); and m/100 glycylglycine. In each case the pH of the heating menstruum was adjusted to pH 7 with HCl or NaOH. The data obtained are presented in figure 4. It is apparent that the composition of the heating menstruum had a pronounced effect on the thermal death rate of these spores. When the phosphate concentration was increased from m/40 to m/15 or when the phosphate was replaced by any of the other solutions, there was not only a significant reduction in both *D* values but also a marked decrease in the duration of the initial rate, *D*₁, (curves 23, 24, 25, and 26 as compared with curve 22). The reduction in the *D* values was most pronounced when glycylglycine or tris buffer were contained in the heat-

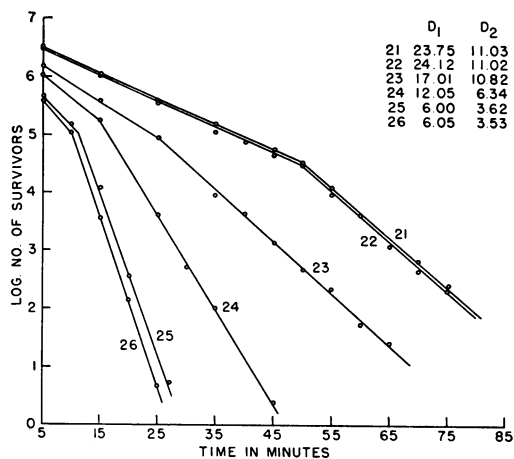


Figure 4. Effect of buffering and chelating compounds added to the heating menstruum on the thermal death rate of *Bacillus coagulans* var. *thermoacidurans*. The spores used were produced on thermoacidurans agar, without phosphate, but fortified with 1 ppm MnSO₄. Curves represent the computed death rates of spores heated in the indicated menstruum adjusted to pH 7 ± 0.05. The D values reported are the arithmetic mean of duplicate thermal death-rate determinations. Curve 21: m/100 phosphate. Curve 22: m/40 phosphate. Curve 23: m/15 phosphate. Curve 24: m/1000 EDTA. Curve 25: m/200 tris buffer. Curve 26: m/100 glycylglycine.

ing menstruum. The EDTA and the m/15 phosphate, likewise, produced significant effects. However, when the phosphate concentration was reduced to m/100 the resulting thermal death rates were similar to those obtained in the standard phosphate solution (m/40) (curves 21 and 22).

In order to rule out the possibility that the carry-over of the above-mentioned buffering and chelating compounds to the plating medium exerted an inhibitory action toward the germination and/or the growth of the surviving spores and thus produced the effect of an apparent higher thermal death rate, the following experiment was carried out. Spores from the same spore harvest were heated in m/40 phosphate buffer for 30 min at 94.6 C. Appropriate dilutions of this heated suspension were then plated out in thermoacidurans agar to which was added m/150 phosphate, m/10,000 EDTA, m/2000 tris buffer, or m/1000 glycylglycine, respectively. Differences in the survivor counts were evaluated and found to be insignificant. It was, therefore, con-

cluded that the above reported differences in thermal resistance, when the spores were heated in the different menstrua, were real and occurred primarily because of their presence in the heating menstruum and not because of any effect of carry-over into the recovery medium. Williams and Hennessee (1956) reported a somewhat similar effect of phosphate on the thermal resistance of *Bacillus stearothermophilus*. They found that an increased molarity of phosphate in the heating menstruum, over the range m/120 to m/15, lead to an apparent reduction in the thermal resistance of the spores. However, they also demonstrated that the phosphate had an inhibitory effect, if it were present in the recovery medium at a concentration of m/120 or more.

The marked differences in the thermal resistance characteristics of the spores when they were heated in the different menstrua suggested that these changes were related to the loss of one or more of the cations which contributed to the thermal resistance of the spores. The rate of loss should be greater in the presence of an increased phosphate concentration or in the presence of more powerful or more specific chelating agents. To determine whether or not treatment with one of these agents prior to heating would affect the thermal resistance of the spores, portions of the same spore crop were suspended and shaken in EDTA solutions under the following four conditions: (a) 1 per cent at pH 7.5 for 3 hr at room temperature, (b) the same solution for 48 hr at 45 C, (c) 1 per cent at pH 10.5 for 48 hr at 45 C, and (d) 3 per cent at pH 10.5 for 48 hr at 45 C. In each case the spores were removed from the solution by centrifugation and were washed four times in distilled water. The treated spores were then heated in m/40 phosphate buffer, and their survivor curves were established and compared. No significant differences in thermal resistance were detected for the spores receiving the different treatments.

Sussman (1954), working with mold spores, found that such spores became sensitive to EDTA after a mild heat shock. In order to examine this possibility, an aliquot of our spore suspension was heat-shocked at 85 C for 5 min and then treated with 1 per cent EDTA at pH 10.5 for 3 hr at room temperature. After centrifugation and washing, the thermal resistance characteristics of the treated spores were compared to those of the untreated spores. Again, no differences

were detected. These experiments, therefore, suggest that if the effect of the EDTA is that of chelating the appropriate cations, then a fairly drastic heat treatment is necessary for releasing such cations from the spore before they can be bound by the chelating agent.

DISCUSSION

The data presented demonstrate that the thermal resistance of spores of *B. coagulans* var. *thermoacidurans* can be markedly altered either by subjecting the sporulating cells or the spores to different environmental conditions. It would appear that the divalent cations, particularly calcium and manganese, have a definite effect on the thermal resistance of the spore, but the exact role of these cations in the mechanism of thermal resistance is yet to be elucidated.

The addition of extra calcium or manganese to the sporulation medium caused a significant increase in the thermal resistance of the spores. Increasing the phosphate level in the sporulation medium caused a significant drop in the spores' thermal resistance. It is postulated that the phosphate anion interfered with the availability of such divalent cations to the sporulating cell. When the spores were heated in menstrua which contained certain chelating compounds or had the phosphate level increased, a significant increase in the thermal death rate was obtained. Here again it is believed that such active anions enhanced the rate at which the divalent cations were removed from the heated spores. Further experimentation, however, is necessary to evaluate more specifically the role of these cations in the sporulation medium, in the spores produced, their rate of release into the heating menstruum, and the residual amounts in heat killed spores. We are currently attempting to develop a chemically defined medium which will support abundant sporulation in shake culture in order that the role of these cations may be evaluated under more defined and more controllable conditions.

Thermal death curves for bacterial spores are conventionally assumed to be of exponential nature. However, during the course of this study, the spores of *B. coagulans* var. *thermoacidurans* produced in media rich in divalent cations, especially calcium and manganese, showed a break or an initial shoulder in the subsequent survivor curve. This phenomenon has been encountered by several other investigators

(Reynolds and Lichtenstein, 1952; Malin, 1952; El-Bisi and Ordal, 1956a). Kaplan, *et al.* (1953) confirmed that the initial shoulder in their survivor curves of putrefactive anaerobe no. 3679 was real and not due to experimental error. Malin (1952) demonstrated that the shoulder disappears with an increase in the heating temperature. This was also true in our case; when the spores of *B. coagulans* var. *thermoacidurans* were heated at a relatively low temperature, namely 93 C, the initial slow death rate persisted so long, longer than 100 min, that it could have been easily overlooked if the over-all heating time had not been long enough to detect the breakpoint.

This phenomenon seems to be related to more than one factor and could be attributed to sporulation, heating, and recovery conditions.

El-Bisi and Ordal (1956b) offered a discussion of the main types of abnormalities likely to occur in such survivor curves. According to them, the occurrence of the initial shoulder could be due, totally or partially, to the dormancy of the spores, and that during the initial heating period, while death commences, increasing numbers of survivors become heat activated, thus manifesting a false decrease in the rate of death. Once the heat activation effect comes to an end, the spores will respond only to the inherent death rate and will thus produce a break in the survivor curve. However, this explanation seems not to apply, since all through the present investigation the initial numbers of spores were checked by both plate count and direct microscopic count, and no significant differences were found between the two counts.

The second possibility is the multiple-hit effect. In this case, either more than one molecule in the same spore must be inactivated to cause death or the spores are clumped and more than one spore must be destroyed before a single colony would be eliminated from the survivor plate count. Again this seems not to be true in our case, since thorough microscopic examination of our spore suspensions revealed no clumps. Furthermore, our survivor curves showed a break and both segments of the curve still conformed to the logarithmic order of death. Such a curve is different from the shape expected through the multiple-hit mechanism.

The third possibility, advanced by Malin (1952), is the production of death accelerating materials in the heating menstruum during the

heating process. In order to examine this assumption, spores were heated in M/40 phosphate buffer at the level of 4×10^8 spores/ml for 120 min until almost all the spores were dead. Two similar spore inocula were then heated; one in the previously used buffer and the second in a new batch of the same buffer, and both survivor curves established. No significant differences were found between the two curves. Therefore, the idea of toxic principles developed in the heating menstruum was also disproved.

That the spores become more exacting in their nutritive requirements or more sensitive to certain inhibitors as they are heated (Curran and Evans, 1937; Murrell *et al.*, 1950; and Amaha, 1952) is a fourth possibility. Murrell *et al.* (1950) showed that the addition of starch to nutrient agar markedly increased the recovery of heated spores, the effect being more pronounced as the heating time increased. However, in our case, the thermoacidurans agar (pH 7.0) fortified with 1 ppm $MnSO_4$ gave the highest count, and the use of 0.1 per cent starch did not significantly affect the number of survivors. The use of other complex media, such as liver infusion agar and brain-heart infusion agar, or the addition of eight kinds of vitamins to our recovery medium, was also found to be ineffective in obtaining higher survivor counts.

We believe that the increased death rate, following the initial rate, is due to a change in the thermal resistance of the spores being heated because of some unknown mechanism and that this change is related to the removal of calcium and manganese from such spores. It was repeatedly found that the spores produced in the presence of excess calcium or manganese exhibited a shoulder in their survivor curves. It was assumed that the excess cations were taken up by the sporulating cells and were incorporated somehow into an unknown protective mechanism which contributed to the increase in thermal resistance presented by that initial low death rate (D_1). The initial death rate would persist as long as such a protective mechanism was maintained. After exposure to sufficient heat, the protective effect would be reduced, as a result of the loss of the divalent cations, or for some other reason, and the secondary or higher death rate (D_2) would be manifested. The facts that the initial break disappeared or that the duration of the initial rate was reduced with the increase of

the phosphate level in the sporulation medium as well as the fact of the addition of strong chelating agents to the heating menstruum, all of which contribute to the unavailability or loss of divalent cations, are the main factors which led us to consider the above assumption. However, the fact that the spores produced in the presence of 0.05 per cent K_2HPO_4 acquired higher thermal resistance than those in the medium to which no K_2HPO_4 was added suggests that the mechanism of thermal resistance is still too complicated to be related to a single factor, such as that of excess divalent cations, and that further investigations must be performed before a definite explanation can be accomplished.

SUMMARY

The thermal death rate of spores of *Bacillus coagulans* var. *thermoacidurans* is markedly affected both by the composition of the sporulation medium and by the composition of the heating menstruum. In the sporulation medium, an increase in the phosphate concentration reduced thermal resistance, whereas the addition of the divalent cations, calcium and manganese, caused an increase. Magnesium was without apparent effect.

When the spores of a given harvest were heated in the presence of M/15 K_2HPO_4 , M/1000 ethylenediaminetetraacetic acid (EDTA), M/200 trihydroxymethylaminomethane (tris buffer), or M/100 glycylglycine in place of the standard heating menstruum, M/40 K_2HPO_4 , the thermal death rate was significantly increased. It was also demonstrated that treating the spore crop, prior to heating, with different concentrations of EDTA at different temperatures and pH levels, was without effect on their subsequent thermal resistance. Likewise, the addition of the heating-menstruum components to the recovery medium did not significantly affect the survivor plate counts.

The occurrence of an initial shoulder in the survivor curve of our more heat resistant spore preparations was discussed. It was postulated that this phenomenon could be due to a change in the mechanism of resistance and that it probably involved the removal of the divalent cations, calcium and manganese.

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