THE VIABILITY OF HEAT-ACTIVATABLE SPORES IN NUTRIENT AND NONNUTRIENT SUBSTRATES AS INFLUENCED BY PRESTORAGE OR POSTSTORAGE HEATING AND OTHER FACTORS

HAROLD R. CURRAN AND FRED R. EVANS

Division of Dairy Research Laboratories, Bureau of Dairy Industry, Agricultural Research Administration, U. S. Department of Agriculture, Washington, D. C.

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We have previously reported (Curran and Evans, 1945) that a large proportion of the spores of certain thermotolerant and thermophilic aerobes require a preliminary heat shock before they will germinate; in such cultures usually some of the spores do not require heat activation, but the majority will germinate only after they have been suitably activated. Since activation appears to initiate the germination process, it seemed reasonable to suppose that activated spores maintained under conditions inimical to completion of the germination process might lose their vitality rather rapidly. Subsequent observations proved the accuracy of this supposition and revealed that devitalization of heat-activated spores is causally connected with environmental conditions. The present paper deals with some of these conditions and indicates their relationship to viability in heat-activated spores.

METHODS AND MATERIALS

The organisms used and their sources were *Bacillus subtilis*, strains LB (Bureau of Dairy Industry), 6 (American Can Co.), FDA (Food and Drug Administration), 6634 (American Type Culture Collection); *Bacillus circulans*, 7049 (N. R. Smith); and thermophilic flat sour cultures, 1518 (National Canners Assn.), C_2P_3, C_1P_4, 07, M_{17} (American Can Co.). With two exceptions (6634 and 7049), all of the cultures were isolated from spoiled, commercially processed canned foods.

The spores were produced on standard beef extract tryptone agar and were incubated at the optimum temperature of the organism. The technique used in their collection and preparation has been described previously (Curran and Evans, 1945). The plate method of enumeration was employed; the plating medium was that used for the production of spores with 0.5 per cent glucose added. Plates were counted after 48 hours of incubation at temperatures optimal for the organism. The counts represent the averages of triplicate plates.

Heat treatments were conducted in a thermostatically controlled glycerol bath (±0.5 C). The buffers consisted of mixtures of 1 per cent K_2HPO_4 and KH_2PO_4 solutions in proportions suitable to give the desired pH values; all determinations of the latter were made with a Beckman glass electrode apparatus. Baker's cp glucose was used except where otherwise noted. Pyrex glassware was used throughout. After storage, sample tubes were closed with tightly

fitting, sterile rubber stoppers and shaken for several minutes to ensure resuspension of the sedimented spores.

EXPERIMENTAL

In the first experiment, washed spores were suspended in sterile distilled water or 1 per cent glucose solution and thoroughly mixed. Aliquots of each were then dispensed into each of 4 sterile test tubes. Plate counts were made before

TABLE 1

Viability of heat-activatable spores in distilled water and 1 per cent glucose solution as influenced by pre- or poststorage heating

(Storage temperature 37 C)

ORGANISM	DISTILLED WATER					1 PER CENT GLUCOSE SOLUTION			
	Time held	No heat	95 C—15 min before storage	95 C—15 min after storage	95 C—15 min before and after storage	No heat	95 C—15 min before storage	95 C—15 min after storage	95 C—15 min before and after storage
	days	*per ml*	*per ml*	*per ml*	*per ml*	*per ml*	*per ml*	*per ml*	*per ml*
B. subtilis (LB)	0	8,600	45,000	(45,000)	45,000	8,600	56,000	(56,000)	56,000
	2	8,000	23,000	45,000	33,000	6,000	170	44,000	420
	10	9,900	17,000	45,000	34,000	4,300	28	39,000	112
	30	10,400	18,600	39,400	37,900	3,800	8	33,000	72
B. subtilis (FDA)	0	4,800	84,000	(84,000)	93,000	4,900	88,000	(88,000)	103,000
	2	5,900	42,000	79,000	89,000	3,300	29,000	98,000	68,000
	10	5,000	19,000	75,000	71,000	2,200	15,000	72,000	46,000
	30	5,400	20,300	77,000	86,000	3,500	8,700	86,000	59,000
B. subtilis (6634)	0	106,000	109,000	(109,000)	109,000	115,000	97,000	(97,000)	97,000
	2	101,000	96,000	94,000	82,000	99,000	79,000	99,000	74,000
	10	96,000	91,000	86,000	80,000	86,000	81,000	86,000	75,000
	30	99,000	66,000	80,000	72,000	89,000	57,000	91,000	56,000
B. circulans	0	11,000	95,000	(95,000)	50,000	11,500	80,000	(80,000)	47,000
	2	9,900	440	80,000	8,900	7,600	2,080	73,000	8,100
	10	4,700	82	67,000	9,800	5,200	1,330	66,000	6,700
	30	5,000	134	57,000	10,300	4,500	2,750	53,000	8,500

( ) = potentially viable spores.

and after heating at 95 C for 15 minutes, and again after storage at 37 C for varied periods with and without poststorage heating. The results are shown in table 1. Three of the cultures used were activated by heat; one, 6634, showed no direct response to heating but is included to furnish a basis for comparison. Neither the decrease of glucose to 0.1 per cent nor the substitution of an especially purified grade (U. S. Bureau of Standards) materially affected the recorded results.

It is clear from these results (table 1) that the activation of spores by heat materially affects their subsequent viability when they are maintained under

conditions unfavorable for their normal development. When the spores were activated and stored in distilled water, the number of cells which produced colonies decreased substantially during the first 10 days of storage. This loss of apparent viability was especially marked in *B. circulans*, with which the number of viable spores was reduced 99 per cent in 2 days. When the activatable spores were heated both before and after storage, the viable count was substantially increased over that in which only prestorage heating was employed. When the spores were heated after, but not before, storage, the count did not change greatly throughout the period of observation; likewise the spores receiving no heat either before or after storage underwent comparatively little change in the number of viable spores. The nonactivatable culture (6634) evidenced little change under similar conditions of manipulation. With this nonreactive culture, however, prestorage heating of the spores, though not affecting their capacity to germinate immediately, diminished appreciably their period of maximum viability.

When the spores were heated and stored in 1 per cent glucose solution, the losses in viability were greater and more rapid in the activatable *B. subtilis* cultures. The viability losses in *B. circulans* were, however, somewhat less in glucose than in water solutions. In both unheated suspensions and in those heated only after storage rather slow but progressive losses in viability occurred in the heat-activated spores on storage. It is of interest to observe that the culture showing greatest activation (*B. subtilis*, FDA) did not show the greatest or most rapid diminution in viability. Comparable results were obtained with culture 1518, a heat-activatable, flat sour type. Fluctuations in pH for LB were within the range 7.04 to 6.83, which indicates that acidity per se was not responsible for the observed mortality. The fact that poststorage heating substantially increased the plate count in those suspensions which received prestorage heating poses a question as to the cause of this reaction. It should be noted that even two initial heatings do not appreciably increase the count with the activatable *B. subtilis* cultures but reduce considerably the count of *B. circulans*.

Microscopical examination of hanging drop and stained (aqueous crystal violet) film preparations lead to the conclusion that some of the heat-activated spores, after pregerminative changes involving swelling and partial loss of refractility, deactivate—that is, revert to their original inactivated condition and thus require a second heating to condition them for germination. There was no indication that the increased count resulted from germination and resporulation. In the stained preparations the spores of the activatable *B. subtilis* cultures in both water and glucose solutions stained ringwise before, and similarly or slightly tinted throughout after, preliminary heating at 95 C for 15 minutes, and also throughout the period of rapid death (2 and 10 days). Similarly treated spores of *B. circulans* stained ringwise before initial heating, and before heating at 2 and 10 days, but stained solidly after heating before storage and after storage for 2 and 10 days.

It is apparent from the foregoing that spores activated and stored in certain

nutritionally incomplete substrates may become nonviable with surprising rapidity. The described results were obtained in mediums approximately neutral and with storage at 37 C. The influence of these factors (pH and temperature) upon the mortality of stored heat-activated spores was next considered.

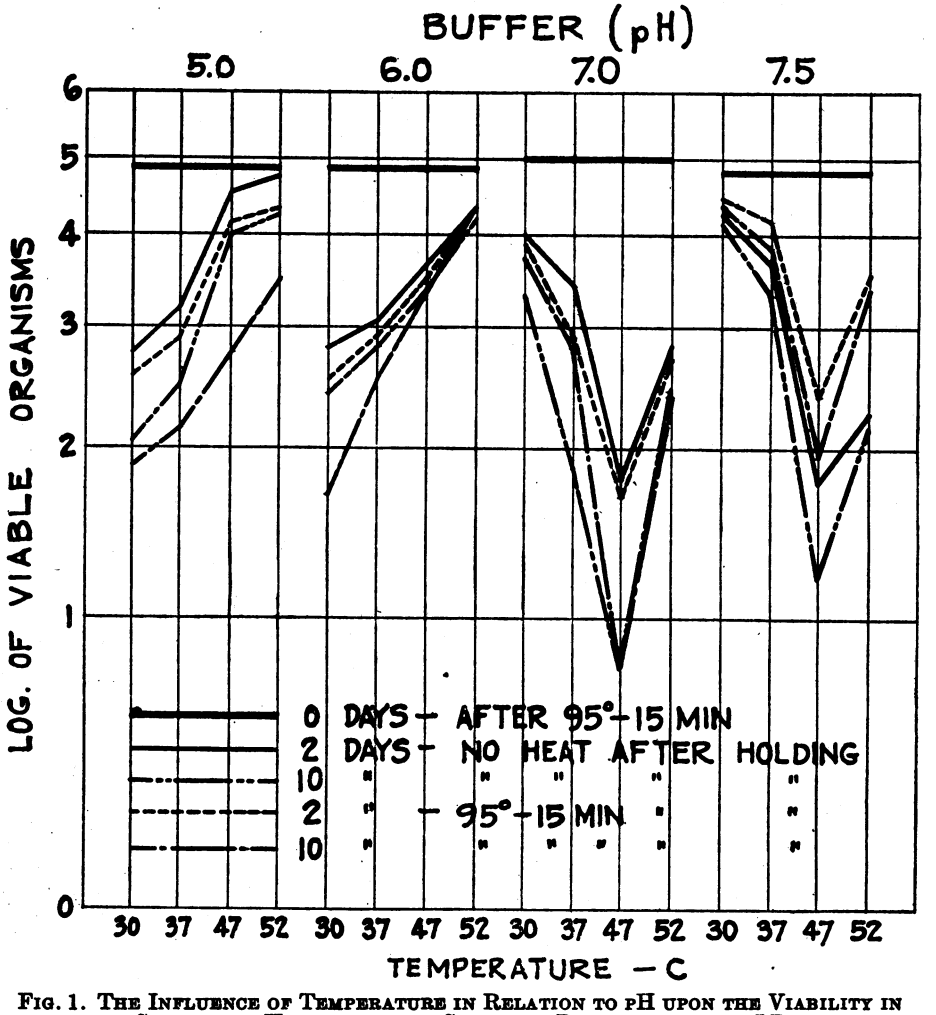

FIG. 1. THE INFLUENCE OF TEMPERATURE IN RELATION TO pH UPON THE VIABILITY IN STORAGE OF HEAT-ACTIVATED SPORES OF BACILLUS SUBTILIS LB

To accomplish this, phosphate buffer solutions containing 0.1 per cent glucose were so combined as to yield 4 buffer mixtures having pH values ranging from pH 5.0 to pH 7.5. Tubed sterile solutions of these buffer-glucose mixtures were seeded uniformly with diluted aqueous suspensions of washed spores, their reactions checked, and the suspensions heated at 95 C for 15 minutes, cooled, and stored at each of 4 temperatures. Plate counts were made initially (after

95 C for 15 minutes) and after 2 and 10 days of storage with and without post-storage heating. The results obtained with 3 activatable cultures are recorded in figures 1 to 3.

Each figure consists of a series of separate curves in which the logs of viable organisms (ordinates) are plotted against temperature (abscissae) in substrates of varying pH. The initial viable counts after activation at 95 C for 15 minutes

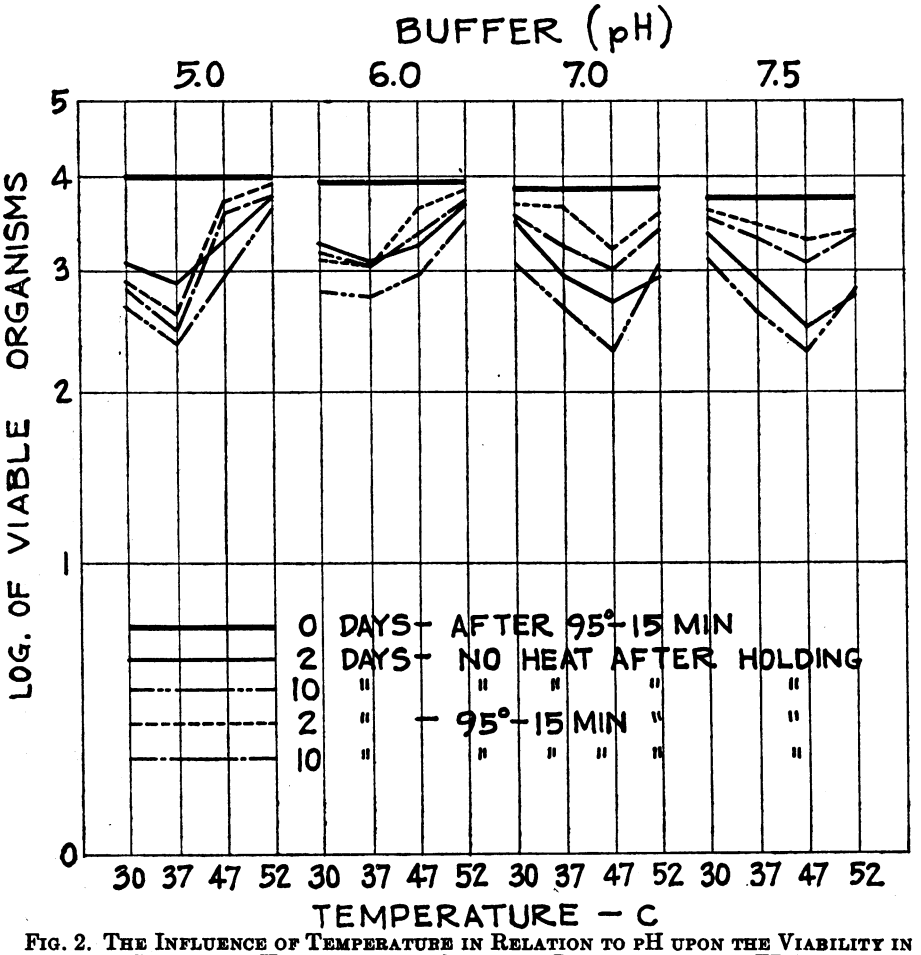

FIG. 2. THE INFLUENCE OF TEMPERATURE IN RELATION TO pH UPON THE VIABILITY IN STORAGE OF HEAT-ACTIVATED SPORES OF BACILLUS SUBTILIS FDA

are represented by the solid parallel lines. The curves show culture differences and also some areas of general agreement. Thus the counts after poststorage heating were generally higher than those not heated after storage; the margin of this difference in comparable samples is a measure of deactivation. This phenomenon was most pronounced at intermediate temperatures of storage (37 to 47 C), and usually greater in the neutral or alkaline substrates than in those definitely acid. Of interest also is the fact that with poststorage heating,

the greatest mortality occurred not at the highest temperature of storage but at intermediate temperatures, as with deactivation. These intermediate temperatures approximate, in general, the optimum growth temperature of the organism. However, in *B. subtilis* LB in acid substrates devitalization was greatest at suboptimum growth temperature. In the two *B. subtilis* cultures minimum viability with or without poststorage heating occurred at a lower temperature

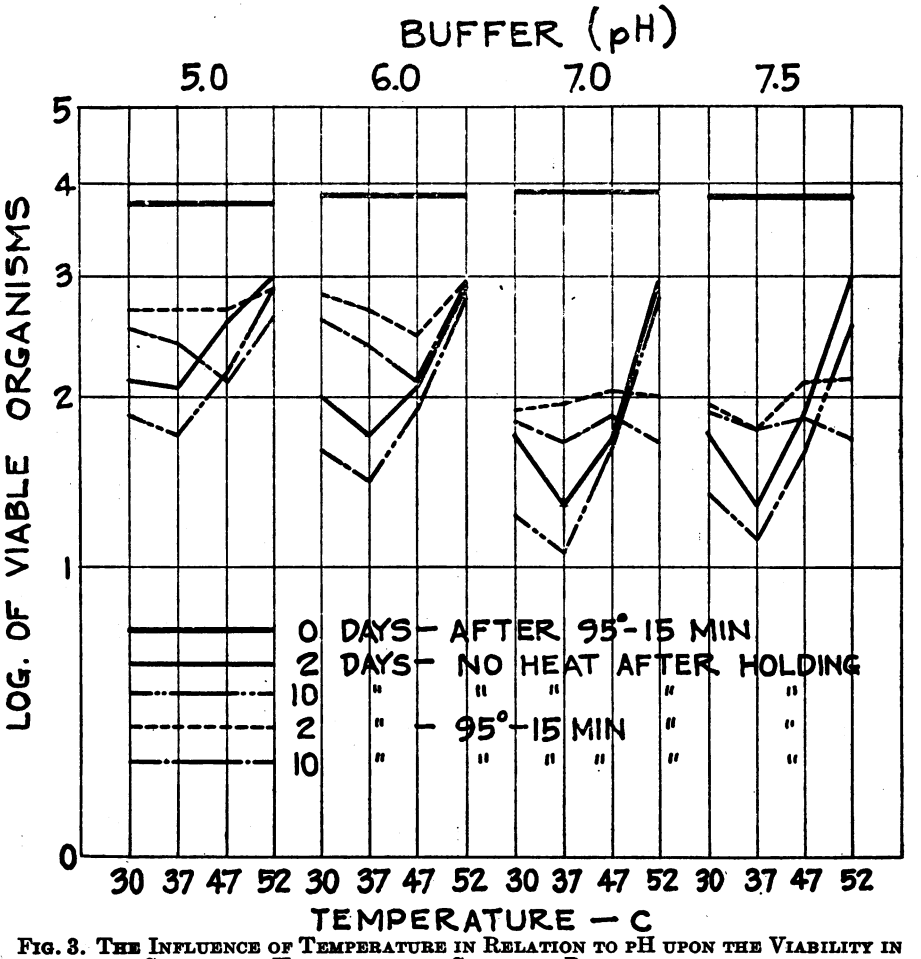

FIG. 3. THE INFLUENCE OF TEMPERATURE IN RELATION TO pH UPON THE VIABILITY IN STORAGE OF HEAT-ACTIVATED SPORES OF BACILLUS CIRCULANS

in acid than in neutral or alkaline medium. Some observations were made upon samples subjected to fluctuating storage temperatures in the range of ±30 to 52 C. However, the losses in viability were in no instance more rapid or greater than those obtained under selected constant temperatures. In buffer suspensions, temperature-pH relationships are necessarily modified by a specific effect of the salt, although this factor is constant for any given pH.

It has been shown that spores activated and stored in a nonnutritional or incomplete medium tend to lose viability rapidly with storage temperature

within the normal range of growth of the organism. It next became of interest to study the behavior of spores activated and stored in a nutritionally complete medium but subsequently maintained at subminimum growth temperatures. In this instance, the block to complete germination becomes purely physical rather than physicochemical in nature. In table 2 are recorded the results obtained when the usual pre- and poststorage heating and storage were conducted

TABLE 2

*The viability of heat-activated spores in milk as influenced by pre- and poststorage heatings**

ORGANISM	STORAGE		NO HEAT	PRESTORAGE HEAT ONLY	PRE- AND POST-STORAGE HEAT
	Period	Temperature			
	mo	*C*	*per ml*	*per ml*	*per ml*
B. subtilis	0		19,000	84,000	(84,000)
	1	3	21,000	53,000	35,000
	2	3	20,000	42,000	31,000
	4	3	24,000	27,200	24,600
C_2P_3	0		42,000	42,000	(42,000)
	1	16	46,000	12,100	
	2	16	44,000	9,900	8,700
	4	16		7,800	6,700
C_1P_4	0		45,000	46,000	(46,000)
	1	16	57,000	18,300	
	2	16	49,000	18,800	15,000
	4	16		14,700	13,900
07	0		54,000	70,000	(70,000)
	1	16	77,000	33,000	33,000
	2	16	62,000	36,600	30,800
	4	16		32,900	32,700
1518	0		62,000	66,000	(66,000)
	1	37	65,000	36,900	27,300
	2	37	73,000	24,300	21,100
	4	37		4,000	7,600

* 95 C for 15 minutes.

in a milk substrate. The storage temperature for each culture was below that of normal germination and growth. The data (table 2) show that spores activated in milk and then stored at a temperature below that permissive of complete germination tend to lose their viability rather rapidly, though at a substantially slower rate than that which occurred in nutritionally incomplete mediums. The drop in count was greatest during the first month of storage, after which the rate of decline was very slow. There is very little evidence of deactivation in this medium; however, in a nutritionally complete substrate deactivation may be masked by a very gradual development of some of the spores with accompanying loss of heat stability.

In table 3 are shown changes in the viability of spores in relation to storage

after their exposure to severe heating in milk—the heating being sufficient to kill all but a very small proportion of the initial population. As may be seen, the highly resistant fraction of the spores of *B. subtilis* LB underwent little

TABLE 3

The viability of spores in milk after heating at 115 C without poststorage heating at 95 C for 15 minutes

ORGANISM	INITIAL CONCENTRATION	HEATED AT 115 C	STORAGE		VIABLE SPORES
			Period	Temperature	
	per ml	*min*	*mo*	*C*	*per ml*
B. subtilis LB	121,000	20	0		20,900
			1	3	33,100
			2	3	36,400
			4	3	32,000
	121,000	32	0		0.6
			1	3	0.6
			2	3	0.3
			4	3	0.3
C_2P_3	250,000	15	0		2,570
			1	16	260
			2	16	108
			3	16	44 (7)
C_1P_4	234,000	15	0		1,280
			1	16	380
			2	16	270
			3	16	248 (173)
07	334,000	20	0		370
			1	16	8.6
			2	16	9.0
			3	16	0.6 (2.6)
M 17	49,000	20	0		710
			1	16	690
			2	16	360
			3	16	150 (43)
1518	900,000	60	0		620
			1	16	4.3
			2	16	1
			3	16	0.6 (0)

( ) = after heating at 95 C for 15 minutes.

change in viability; the surviving flat sour spores in contrast died rather rapidly with storage. Wheaton and his associates have observed a similar destruction of thermophilic flat sour spores in creamed corn when this product was inoculated before processing and subsequently stored at subminimum growth temperatures;

complete sterility was attained in about 6 months (unpublished). The foregoing observations suggest possibilities for the elimination of flat sour spores by manipulation of heat treatment and storage temperatures.

DISCUSSION

Heat activation in bacterial spores has its counterpart in certain of the fungi, notably in the ascospores of *Ascobolus* and *Neurospora* (Dodge, 1912; Shear and Dodge, 1927; Goddard, 1935) and in *Phycomyces* (Robbins, Kavanagh, and Kavanagh, 1942). The process of activation in *Neurospora tetrasperma* (Goddard, 1935) was shown to be completely reversible (deactivatable). Since activation in bacterial spores has been observed only in certain aerobic species, a relationship between aerobic sporeformers and the true fungi is suggested. Similarities between the sporeforming bacilli and the *Mucorini* and other fungi have been pointed out by Cook (1932).

The observed effects of heat activation upon the viability of spores and the influence thereon of various physical and chemical factors encourage speculation as to the nature of the devitalizing process. In nutritionally incomplete substrates providing a utilizable carbohydrate, the development of sufficient acidity might conceivably induce a rapid death of the activated spores, but with *B. subtilis* LB in glucose solution, over 99 per cent of the spores became rapidly nonviable without appreciable change in reaction. Phage virus is transmissible by spores (Dooren de Jong, 1931; Cowles, 1931; Adant, 1932) and presumably may attack spores; however, there was no evidence that lysis of the spores occurred in our experiments.

The rapid mortality of spores following their activation and storage in water or glucose solutions and the slower mortality rate of similarly treated spores in milk suggest the presence in milk of a substance which protects the organisms from rapid lethal action. In this connection the findings of Winslow and Brooke (1927) appear to have some relevance. These authors found that young vegetative cells of *Bacillus cereus* and *Bacillus megatherium* died with extreme rapidity when suspended in distilled water and centrifuged twice to remove substances carried over from the agar slope on which they had previously been grown. A high degree of protection was afforded by extreme dilutions of nutrient broth, or its component ingredients, but not by sugar or balanced salt solutions. We may assume that heat activation provides a definite impulse toward germination; concomitant with or soon after activation spores become perceptibly less spore-like and more like vegetative cells. It might be reasoned, therefore, that heat-activated spores become in effect very young vegetative cells and in consequence react in pure water and glucose solutions as did the vegetative cells of the sporeformers described by Winslow and Brooke (1927). However, this interpretation is contraindicated, first because in both *B. subtilis* cultures rapid losses in viability were not accompanied by greatly increased permeability of the spores to crystal violet—a change which occurs in the process of normal germination and which is associated with or preceded by a material decrease in heat stability. Moreover, young vegetative cells of *B. subtilis* and *B. circulans* have not in

our hands shown susceptibility to pure distilled water and glucose solutions comparable in degree to that described for *B. cereus* and *B. megatherium*.

In the spores of *Phycomyces*, heat activation seems to effect a diffusion of essential growth substances from the cells into the surrounding medium (Robbins *et al.*, 1942). Whether this reaction is in any way responsible for the devitilization of such spores is not known, nor is it yet known whether a similar leaching out of cell contents occurs following the heat activation of bacterial spores. This point will be the subject of further investigation.

It might be postulated that spores as a result of activation are stimulated to intense metabolic activity. In the absence of an extraneous source of nitrogen or energy, the contained supply of these essential food elements would be rapidly exhausted. If the nitrogen-containing substances are depleted and a source of energy, either intra- or extracellular, is still available, a situation may be conceived in which catabolic processes gain the ascendancy and lead to cell death from internal causes. An enzymic reaction of this nature might be expected to have rather definite temperature optima varying with environmental conditions. By this hypothesis glucose or other utilizable carbohydrate would greatly *accelerate* the death process of activated spores in an incomplete medium, provided an excess of available energy was not present in the cells, and would have no such effect if sources of energy were available within the cell. The reaction with *B. subtilis* LB accords with the first condition, that with *B. circulans* with the second. Knaysi (1945) demonstrated considerable nitrogen-containing reserve material in the spores of a culture of *Bacillus mycoides* but found no utilizable sources of energy. Just why the activation process is reversible for some spores under particular conditions (deactivation), yet for other individuals under similar conditions is irreversible, is not apparent. In earlier observations on heat activation of spores in water and glucose solutions (Curran and Evans, 1945) we found no evidence for deactivation, probably because of the very low storage temperature (3 C).

SUMMARY

The spores of aerobic, mesophilic and thermophilic, heat-activatable cultures undergo profound and rapid alteration in viability when activated and stored under conditions unfavorable for complete germination.

When activated and stored in nutritionally incomplete substrates (distilled water or glucose solution), spores die rapidly or undergo deactivation with retention of viability; when activation is conducted in a nutritionally complete medium (milk) with storage at submininum growth temperatures, the spores die more slowly and there is little evidence of deactivation. Unheated, activatable or nonactivatable spores undergo little or no change in viability over long periods; in glucose solution the rate of deterioration is measurably increased.

The reaction associated with the rapid death of heat-activated spores has a temperature optimum ranging with the organisms studied from 30 to 47 C, depending upon the organism and the pH of the substrate.

A theory for the mechanism of the observed reactions is advanced.

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