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Effect of Lipid Materials on Heat Resistance of Bacterial Spores

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The apparent heat resistance of spores of *Bacillus megaterium*, *B. subtilis*, *B. cereus*, *B. stearothermophilus*, and *Clostridium botulinum* type E in lipids was investigated and compared with the resistance of the spores in phosphate buffer solution. The most pronounced increase in heat resistance was noted for *B. subtilis* and *C. botulinum* type E, the increase varying with the type of lipid used. A high water content of the lipids used as heating menstruum lowered the heat resistance of the spores. Possible explanations for the high heat resistance of spores in lipids are discussed.

It is known that spores suspended in lipid materials are generally more resistant to heat than spores suspended in water systems (6, 7, 20, 21). It has also been claimed that the heat resistance of spores in fats approaches their resistance to dry heat (4) or even exceeds it (13).

There is also some evidence that spores or vegetative cells suspended in the lipid phase might survive exposure to temperatures used in the conventional heat sterilization of foods (7, 9, 11, 12, 17). Under certain circumstances, i.e., heavy agitation of food samples, the spores might migrate out of the lipid and cause spoilage after subsequent incubation (9).

Judging from the literature, however, very little is known about the underlying principles of fat protection, the distribution of the spores between lipid and water phases, and the significance of fat-protected spores in heat-sterilized foodstuffs.

This investigation, which is the first of a series dealing with these problems, is concerned with the degree of fat protection of some *Clostridium* and *Bacillus* spores suspended in heated soybean oil, olive oil, triolein, and liquid paraffin.

MATERIALS AND METHODS

Organisms. B. cereus (NCIB 5893), *B. megaterium* (NCIB 8508), *B. subtilis* (NCIB 8054), *B. stearothermophilus* (NCIB 8924), and *C. botulinum* type E (SIK 1537/62) were used.

Culture media for sporulation. B. cereus sporulated completely after 36 hr at 30 C in strongly aerated G-medium, described by Stewart and Halvorson (22) and modified by Hashimoto et al. (8). *B. megaterium* and *B. subtilis* spores were produced at 30 C in a synthetic medium according to Donnellan et al. (5). The spores were harvested after 36 and 48 hr, respectively. *B. stearothermophilus* was cultured at 55 C in a medium consisting of 0.6% nutrient broth, 0.2% tryptone, 0.2% glucose, 0.3% K_2HPO_4, and 0.1% KH_2PO_4. The spores were harvested after 60 hr. *C. botulinum* type E was cultured at 30 C in Robertson's cooked-meat medium, and the spores were harvested after 10 days of incubation.

Preparation of clean spore suspension. Clean stock suspensions of spores of *B. cereus*, *B. megaterium*, and *B. subtilis* were prepared as described in an earlier paper (15). Spores of *B. stearothermophilus* were collected by centrifugation, and the cell mass was then treated with lysozyme (1 mg/ml) in phosphate buffer (*p*H 7.0) for 12 hr at 4 C and then for 2 hr at 30 C. No vegetative cells survived this treatment. The spores were then washed seven times with phosphate buffer (*p*H 7.0). The spore suspensions of *C. botulinum* were prepared according to the method described by Abrahamsson et al. (1).

Preparation of spore suspension in oil. Amounts of 1 or 2 ml of clean spores suspended in distilled water were distributed among screw-cap tubes and lyophilized. Each tube contained about 10^9 *B. cereus* or *B. megaterium* spores and about 10^8 *B. stearothermophilus* or *B. subtilis* spores. The number of *C. botulinum* spores per tube was approximately 5×10^6. After lyophilization, the tubes were stored at −18 C until used. On the day of the experiment, the dry spores were suspended in oils or triglycerides by vigorous shaking with glass beads at room temperature. The suspensions contained no aggregates of spores, as judged from microscopic examination.

Heating menstruum. The spores were heated in phosphate buffer, *p*H 7.0 (10), or in soybean oil, liquid paraffin, triolein, or olive oil.

Heating conditions. Oil suspensions of the spores were heated at 112, 117, and 121 C, and buffer suspensions were heated at 85, 90, and 95 C. The experiments were performed in an oil bath, and the screw-cap tubes (110 × 10 mm) were sealed and immersed in the oil bath to $^{10}/_{11}$ of their length, with only the

TABLE 1. *Heat resistance of Bacillus spores in phosphate buffer (pH 7.0) and in various lipids*[a]

Organism	Heating menstruum														
	Phosphate buffer						Soybean oil			Olive oil, 121 C	Triolein			Liquid paraffin	
	80 C	85 C	90 C	95 C	100 C	112 C	112 C	117 C	121 C		112 C	117 C	121 C	112 C	121 C
B. cereus.....		220	71	13	8		46.5		30	17.5	14		10	21	8
B. subtilis.....		125	54	8		1	275	105	108		65	13	8		
B. megaterium.	175	29	8	4.5		<1	11.5	8	6	7	15	9	6		
B. stearothermophilus....			28						8						

[a] The resistance is expressed as a *D* value calculated from the number of surviving spores after heating for 30 min in the lipids, and after heating for 15 min (or 5 min at 112 C) in the buffer solution.

screw cap above the oil surface. The time necessary for equilibration of the temperature in the liquid phase of the tube, as estimated by a thermocouple, was 3.5 min at 112 C and 4 min at 121 C. The suspensions of the spores in oils were always heated for more than 30 min.

Determination of surviving spores. The number of surviving spores in the oil suspensions was estimated according to Miles et al. (16), with the exception that in the first step in the dilution series 1 ml of oil or triglyceride was transferred to 9 ml of 55% (v/v) ethyl alcohol, which was of about the same density as the lipids. The mixture was sonically treated for 1 min, and the emulsion formed was stable for more than 12 hr. The oil droplets were 1 to 2 μ in diameter. The emulsions were then diluted in the usual way. Neither dilution step with alcohol nor sonic treatment of the suspensions had any adverse effect on the spore number, even when the number of surviving spores was small. On the contrary, the spore count in our controls was generally higher than that obtained with the conventional technique with emulsifiers or with the membrane-filter technique. Growth and toxin production after incubation of heated samples were taken as a criterion for presence of surviving *C. botulinum* spores. The data presented in tables and figures are mostly mean values of three series of experiments.

Subculture conditions for determination of surviving spores. Throughout the investigation, Tryptone Glucose Extract Agar (Difco) was used as recovery medium for all *Bacillus* species with the exception of *B. stearothermophilus*, for which Dextrose Tryptone Bromcresol Purple Agar (2) was used. *C. botulinum* was subcultured in a proteose-peptone medium. The incubation temperature was 30 C for all species except *B. stearothermophilus*, which was incubated at 55 C.

RESULTS

Heat resistance in soybean oil. The survival of *Bacillus* spores in neutral phosphate buffer at 90 C and in soybean oil at 121 C is given in Table 1. In buffer, the least heat-resistant spores were those of *B. megaterium*. Somewhat more resistant were the spores of *B. subtilis* and *B. stearothermophilus*, and most resistant were those of *B. cereus*. The heat resistance of the *B. stearothermophilus* spores in neutral buffer solution was much lower than expected.

In soybean oil, *B. subtilis* was the most resistant species and *B. stearothermophilus* spores were almost as sensitive as those of *B. megaterium*.

The relative heat resistance of *C. botulinum* type E spores in soybean oil and phosphate buffer solution (*p*H 7.0) has also been investigated. The results (Table 2) clearly demonstrate a pronounced increase in heat resistance of *C. botulinum* type E spores in soybean oil. Thus, surviving spores grew and gave rise to toxin when heated in soybean oil for 10 min at 121 C or 60 min at 112 and 90 C, whereas in phosphate buffer no growth was demonstrable after heating of the spores at 121 C for 20 min.

TABLE 2. *Heat resistance of Clostridium botulinum type E spores (10^6 spores/ml) in phosphate buffer (pH 7.0) and in soybean oil*

Temp (C)	Exposure time (min)	Phosphate buffer		Soybean oil	
		Growth[a]	Toxin MLD/ml	Growth[a]	Toxin MLD/ml
80	20	+		+	
	60	+	2×10^4	+	
90	20	+		+	
	60	−		+	
112	5	+		+	
	20	−		+	1.5×10^4
	60	−		+	2×10^4
121	10	−		+	
	20	−		−	

[a] Symbols: + = good growth after 4 days at 30 C; − = no growth after 4 days at 30 C.

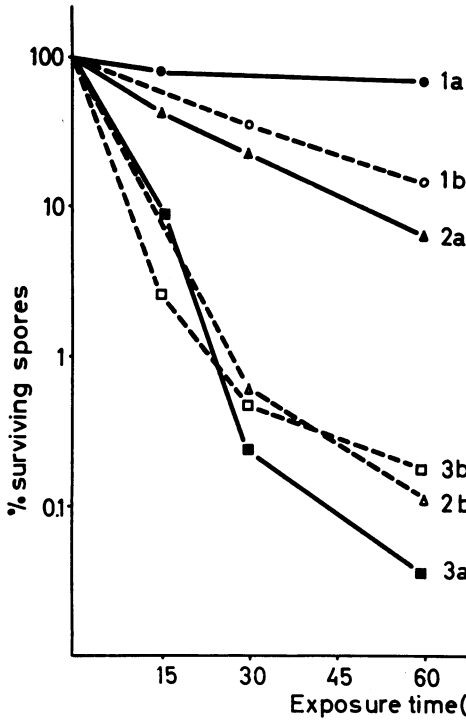

FIG. 1. *Heat resistance at 112 C of some Bacillus spores in soybean oil and triolein. (1a) B. subtilis, soybean oil; (1b) B. subtilis, triolein; (2a) B. cereus, soybean oil; (2b) B. cereus, triolein; (3a) B. megaterium, soybean oil; (3b) B. megaterium, triolein.*

Heat resistance in various kinds of lipids. The heat resistance of *Bacillus* spores (Fig. 1 and 2, Table 3) varied with the type of fat in which they were suspended. Thus, *Bacillus* spores were generally more heat resistant in soybean oil than in triolein, and *B. cereus* was fairly resistant in soybean oil and olive oil but less so in liquid paraffin. Moreover, *B. subtilis* seemed to increase more in heat resistance than did *B. megaterium* and *B. cereus* (Table 1).

Influence of water content of the lipids. The soybean oil, triolein, and olive oil used had a water content of 0.02 to 0.04% as estimated gravimetrically after heating of the oil overnight in vacuum.

A few experiments were performed with soybean oil and triolein, with addition of water. Thus, the heat resistance of *B. cereus* spores was determined in soybean oil and triolein to which 10, 100, and 300 μliters of water per ml of oil had been added at 20 C just before the oil suspensions of the spores was heated in tubes with tight screw caps. Percentages of surviving spores are given in Table 4. Addition of small amounts of water to the oil appeared to have a pronounced effect on the number of survivors, compared with the number in oil with a lower water content. In triolein, the water content should be somewhat higher than in soybean oil to produce the same effect.

Soybean oil autoclaved in open vessels at 121 C was found to have a water content of about 2% and the *D* value for *B. cereus* spores in this menstruum was the same as for spores heated in oil with an addition of 10 μliters of water per ml before the suspension was heated.

DISCUSSION

In the present investigation, the sporulation conditions and the conditions under which heated spores were recovered were standardized. This allowed estimation of the effect of variation of the heating menstruum on the survival of the spores.

In moist environment, the thermophilic spores of *B. stearothermophilus* and *B. coagulans* are more heat-resistant than the spores of most mesophilic organisms (18). In a dry atmosphere, the order of heat resistance of at least some *Bacillus* spores is different. Thus, *B. subtilis* var. *niger* spores were found by Bruch et al. (3) to be much more resistant to dry heat than *B. stearothermophilus*.

That the heat resistance of various types of bacterial spores varies with the relative humidity

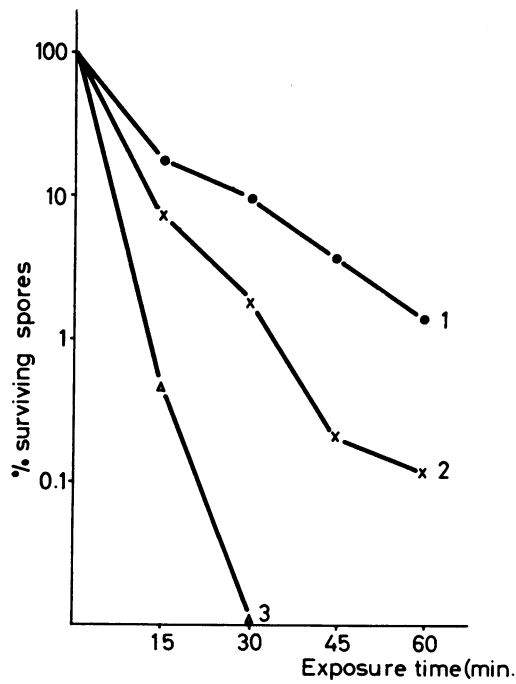

FIG. 2. *Heat resistance at 121 C of Bacillus cereus spores in soybean oil, olive oil, and liquid paraffin. (1) Soybean oil; (2) olive oil; (3) liquid paraffin.*

TABLE 3. *Heat resistance of Bacillus cereus spores in soybean oil and olive oil at different temperatures*

Exposure time (min)	Surviving spores (%)		
	Soybean oil		Olive oil, 121 C
	112 C	121 C	
0	100	100	100
15	43	25.6	7.4
30	23.5	10.8	1.8
45		3.2	0.21
60	6.3	2.2	0.12

TABLE 4. *Heat resistance of Bacillus cereus spores in lipids with addition of various amounts of water*

Added water (μliters per ml of oil)	Surviving spores (%) at 121 C					
	Triolein			Soybean oil		
	0 min	15 min	30 min	0 min	15 min	30 min
0	100	2.4	0.31	100	25.6	10.8
10	100	1.24	0.225	100	0.068	0.0057
100	100	0.0086	0.00146	100	0.04	0.04
300	100	0.0029	0.0002	100	0.017	—

of the atmosphere has also been demonstrated by Murrel et al. (19), who found that the resistance of four of six species studied decreased markedly with increasing humidity in the atmosphere. The two exceptions were spores of *B. stearothermophilus* and *B. coagulans*, whose heat resistance changed only to a smaller degree with increasing humidity.

The fat protection has been attributed, by Lang (14) among others, merely to the poor heat conductivity in lipids. The big differences in heat resistance in buffer and lipids noticed in the present investigation can, however, hardly be explained only by a lower heat conductivity of lipids compared to that in buffer solution.

The protecting effect seems to correlate with the water content of the lipids to a certain extent. Lipids saturated with water still have, however, a high capacity to protect the spores against heat. Our results, therefore, suggest that the high resistance of spores in lipids is not due entirely to the water-free environment. This is also stressed by the fact that the protective effect varied in various lipids with the same water content.

Assuming that the lipids have a specific effect on heat resistance and that this effect is not due to low heat conductivity or water-free environment, one apparent possibility would be that the free fatty acids present in the lipids might have a stabilizing effect on the spores. This is also in accordance with the finding that palmitic acid added to soybean oil had a certain additional protecting effect and that the protecting capacity of water-free, liquid paraffin, which contains no free fatty acids, is less pronounced than that of soybean oil and olive oil.

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