

The Effect of Water Activity, Solutes and Temperature on the Viability and Heat Resistance of Freeze-dried Bacterial Spores

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SUMMARY

Freeze-dried spores of *Bacillus megaterium*, *B. stearothermophilus*, *Clostridium bifermentans* and *C. botulinum* type E suffered little or no loss in viability after storage at 25° at water activity (a_w) values between 0.2 and 0.8. When stored over P_2O_5 (0.00 a_w) the spores of all four species showed a marked loss in viability. The above results were similar for spores whether stored in air or in vacuum. With spores stored over distilled water (1.00 a_w) the *Bacillus* spores underwent a large loss of viability in vacuum, but not in air; for spores of the clostridia the reverse was true. The addition of DL-glyceraldehyde, diacetyl or ribose (0.05 M) to the spore suspensions before drying caused increased death during storage at 0.50 a_w and to a lesser extent at 0.20 a_w . Death was greater at 30° than at 10°. The addition of sucrose, glutamate or semi-carbazide did not decrease the viability. When the dried spores were resuspended in dilute phosphate buffer after storage for 2-6 years their resistance to heating was greatest after storage at a_w values of 0.4, 0.6 and 0.8.

INTRODUCTION

Information about the retention of viability and heat resistance of spores under various storage conditions is important in culture preservation, in food and medical microbiology, and to increase our knowledge of the bacterial spore and its role in microbial ecology. It is known that spores retain well their viability and heat resistance under many conditions, both in the dry and wet state, but data about precise conditions for storage over extended periods are meagre and in many cases not very critical. Evans & Curran (1960), who made a comprehensive study of the effects of preheating, storage pH values and storage temperature on viability of spores in dilute phosphate buffer reviewed the early literature. In their studies Evans & Curran observed considerable loss in viability at pH 6.0 or less and at 30° and 37° by spores of aerobic species. Bullock & Lightbown (1947) studied the effects of moisture on the viability of *Bacillus subtilis* spores in dried peptone powder. In 32 days at a_w values of 0.0 and 0.32, no decrease in viability occurred. Further work (Bullock & Tallentire, 1952) showed that the spores stored at 0.0 a_w and 20° for 2 years retained 100% viability and did not begin germination on rehydration. At 0.66 a_w the spores still retained their viability after 250 days and did not begin germination on rehydration. At 0.78 a_w 80% of the spores in the rehydrated suspension began germination and were still viable. With storage at higher a_w values

the spores began germination within 20 days and became non-viable in peptone (0.90 and 1.00 a_w). Under very moist conditions, and depending on the media, the partially germinated spores multiplied (in peptone+lactose powder), died (in lactose powder) or remained viable (kaolin powder). Hawrylewicz, Gowdy & Ehrlich (1962) observed that spores of *Clostridium botulinum*, freeze-dried in the presence of crushed lava, showed greater decreases in viability in air and in vacuum than in a nitrogen atmosphere. The effect of storage conditions on the retention of the original heat resistance of spores (not the change to the heat-labile state as studied by Bullock & Tallentire (1952)) is not clear. Drying and then storage in the dry state or in liquid media have been reported either to increase or to decrease the heat resistance of the spores (Magoon, 1926; Williams, 1929). Magoon (1926) stored thin layers of sporulated cultures of *Bacillus mycoides* for up to 180 days at 0, 0.50 and 1.00 a_w at 10, 20 and 30°. Up to four-fold increases in heat resistance were observed during storage at 20° at these three a_w values. Sometimes this increase occurred within 30 days, but on continued storage the heat resistance decreased. The high degree of heat resistance was retained best at 20° at the 0.5 and 1.0 a_w values. In the present work the viability of freeze-dried spores of four organisms stored for periods up to 6 years in air or in vacuum at a_w values from 0.00 to 1.00 has been followed. The opportunity was also taken to determine in several cases the degree of heat resistance of the spores after rehydration.

METHODS

The following organisms were used: *Bacillus megaterium* strain c1 (Knaysi), *B. stearothermophilus* ATCC 7958, *Clostridium bifermentans* Weinberg 226, *C. botulinum* Type E, ATCC 9564.

Spores of *Bacillus megaterium* were grown in potato infusion (Robinow, 1951) in shake flasks at 30°, and those of *B. stearothermophilus* at 50° in this medium at half-strength supplemented with a salts mixture (Ohye & Murrell, 1962). Spores of *Clostridium bifermentans* were grown at 30° in cooked meat medium (Dubovsky & Meyer, 1922), and *C. botulinum* type E at 25° in a mixture of 4 vol. papain meat digest broth (Asheshov, 1941) + 1 vol. of peptic meat digest (Dubovsky & Meyer, 1922) + 0.05% (w/v) sodium thioglycollate. On the completion of spore formation, the spores were harvested on the centrifuge, washed 6 times with water, and any remaining viable vegetative forms were killed by heating for 10–20 min. at a temperature sublethal to the spores (15–20° above the maximum growth temperature of the particular species).

Samples (0.2 ml.; containing about 10^7 spores) of the spore suspensions were dispensed in lightly plugged Pyrex tubes (9 × 88 mm.), freeze-dried, and the tubes sealed inside larger tubes (150 × 16 mm.) containing about 0.5 ml. of a salt solution or a saturated salt solution of known water activity a_w (Robinson & Stokes, 1955) or P_2O_5 . In some experiments ampoules were prepared as described by Murrell & Scott (1957). The tubes were either sealed without evacuation or after evacuation to about 0.02 mm. Hg pressure. The tubes were stored in the dark at 25°. The effect of solutes was studied by adding to the spore suspension before drying, aqueous solutions to give the desired concentration in the undried suspension. Where necessary solutions were neutralized with NaOH.

At intervals during storage single tubes of each treatment were removed, opened, the spores rehydrated with 2 ml. 0.05 M-phosphate buffer (pH 7) and viable counts made. Viable counts of *Bacillus megaterium* and *B. stearothermophilus* were made on glucose Tryptone agar plates (in triplicate) at 30° and 50°, respectively. Viable counts of the clostridia were made at 30° in oval tubes containing pork infusion agar (Brewer, 1940) + 0.1% (w/v) sodium mercaptoacetate (thioglycollate).

The heat resistance of the spores was determined by diluting the 2 ml. sample of rehydrated suspension from one ampoule to 20 ml. with phosphate buffer and heating 1.5 ml. samples of this in ampoules (9 × 75 mm.) sealed in air for suitable times at selected temperatures controlled to ± 0.05°. The survivors were determined by viable counts of appropriate dilutions of the heated suspensions, and survivor curves were plotted (see Fig. 5). The reproducibility of estimates of heat resistance of spore suspensions from replicate ampoules is shown in Table 4. The rate of decrease in viability was expressed as decimal reduction times (D values) both for the storage-viability and heat-resistance studies. The average D values were calculated from the regression coefficients for the linear regressions of log. survivors against time.

RESULTS

The moisture contents of the spores during storage at the various a_w values are given in Table 1. These values are the ranges for spores of six bacterial species obtained from water sorption isotherms at 25° (Marshall, in preparation).

Table 1. *The moisture content of spores at various a_w values at 25°*

The figures for the moisture content ranges are for the spores of six bacterial species (Marshall, in preparation).

a_w value	Moisture content (% dry wt.)	a_w value	Moisture content (% dry wt.)
0.00	0	0.60	9.5–16.0
0.10	4.8–8.2	0.70	12.2–19.8
0.20	5.5–10.2	0.80	12.1–25.5
0.30	6.0–11.7	0.90	38.5–57.0
0.40	7.3–12.4	1.00	40–88
0.50	8.5–12.6		

Viability of spores during 25° storage at various a_w values

The results with four species are given in Figs. 1–4; in Fig. 4 the results for two species mixed together are recorded.

Bacillus megaterium. During a storage period of more than 6 years at a_w values of 0.22, 0.43, 0.62 and 0.80, in air or in vacuum there was no significant decrease in viability (Fig. 1). However, at 0.00 a_w the rate of death was substantial both in air and in vacuum. During the first 40 weeks the viable counts decreased by about four log. units, subsequently the rates of destruction decreased appreciably. In other experiments *B. megaterium* spores were stored in vacuum at six a_w values between 0.75 and 1.00 for about 120 weeks, but at the high a_w values the spores were only slightly less stable than between 0.22 and 0.80 a_w . The viable counts of spores stored over pure water, however, decreased by a little over two log. units after two years in

vacuum. Of the spores stored at a_w values above 0.8 60–90% usually stained with dilute crystal violet added after rehydration (see Bullock & Tallentire, 1952).

Bacillus stearothermophilus. The spores of this organism showed only small decreases in viability with any storage treatment, except in the treatments in vacuum at a_w 1.00, where the viable counts decreased rapidly (Fig. 2). At 0.00 a_w in air the viable counts decreased by over one log. unit in the first 40 weeks but thereafter remained constant; at 0.00 a_w in vacuum the initial decrease was less marked.

Clostridium bifermentans. Under conditions of extreme dryness (a_w 0.00) both in

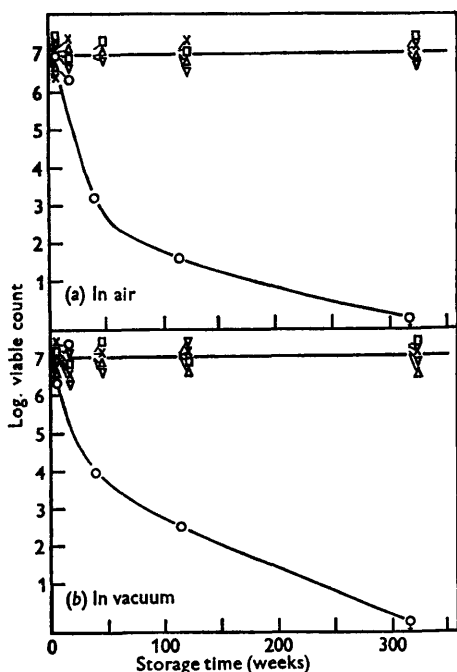


Fig. 1

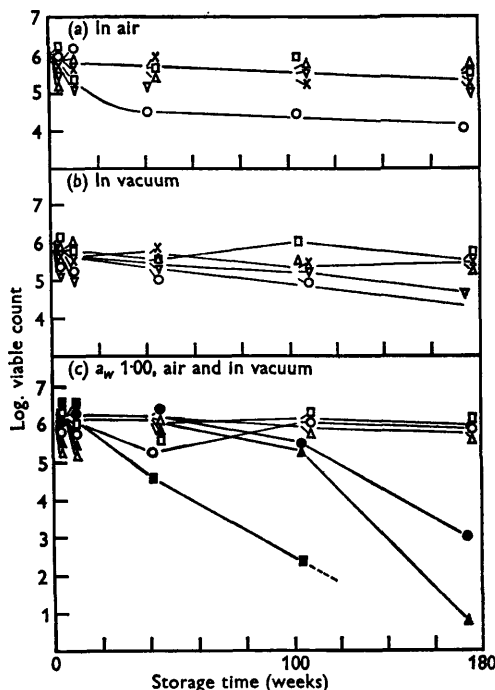


Fig. 2

Fig. 1. Survival of spores of *Bacillus megaterium* during storage in air and in vacuum at 25° at various a_w values. \circ 0.00 a_w ; \triangle , 0.22 a_w ; \square , 0.43 a_w ; \times , 0.62 a_w ; ∇ , 0.80 a_w . The regressions for a_w values (excluding 0.00 a_w) were not significantly different, and the means for air and in vacuum were not significantly different from each other or from zero.

	Mean regression coefficient ($\times 10^3$)	S.E. ($\times 10^3$)	D.F.
Air	-0.021	0.096	18
In vacuum	-0.028	0.039	29

Fig. 2. Survival of spores of *Bacillus stearothermophilus* during storage at 25° at various a_w values. In (a) and (b), \circ , 0.00 a_w ; \triangle , 0.22 a_w ; \square , 0.43 a_w ; \times , 0.62 a_w ; ∇ , 0.80 a_w . In (c) open symbols, storage in air; closed symbols, storage in vacuum; \square , 1.00 a_w , undried; \triangle , 1.00 a_w , rehydrated in water; \circ , 1.00 a_w , equilibrated through vapour phase. The regressions for air treatments (excluding 0.00 a_w) were not significantly different; their mean value was significant (-0.00034 ± 0.00016), giving a D value of 2900 days with 95% fiducial limits of 1500 and 50,000 days. The regressions for 0.22, 0.43, 0.62 and 0.80 a_w values in vacuum were not significantly different, and their mean value was not significantly different from zero.

air and in vacuum the decrease in viability was rapid (Fig. 3). Little killing occurred at the 0.22, 0.43, 0.62 and 0.80 a_w values in air or in vacuum although the viable counts were generally slightly less after storage in air than after storage in vacuum. The death rate in air was slightly greater than that in vacuum but not significantly so (see legend, Fig. 3). At a_w 1.00 the decrease in viability was more rapid in air than in vacuum, particularly when the spores were suspended in liquid water.

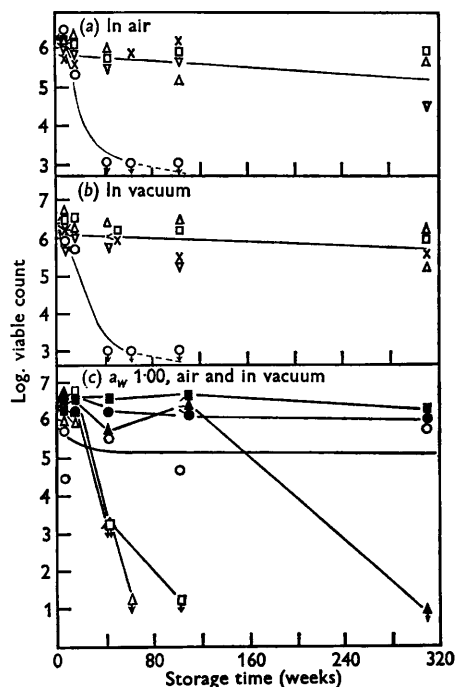


Fig. 3

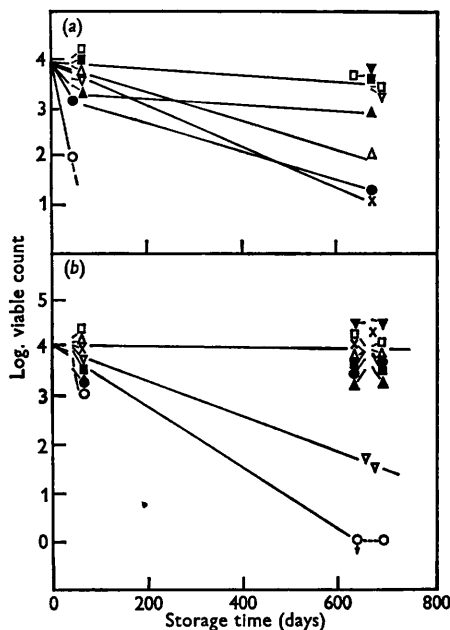


Fig. 4

Fig. 3. Survival of spores of *Clostridium bifermentans* at 25° at various a_w values. In (a) and (b), O, 0.00 a_w ; Δ , 0.22 a_w ; \square , 0.43 a_w ; \times , 0.62 a_w ; ∇ , 0.80 a_w . In (c) open symbols, in air; closed symbols, in vacuum; \square , 1.00 a_w undried; Δ , 1.00 a_w rehydrated in water; O, 1.00 a_w equilibrated through vapour phase. The regressions at the a_w values 0.22, 0.43, 0.62 and 0.80 in air or in vacuum were not significantly different and the mean value in air was not significantly different from that in vacuum.

	Mean regression coefficient ($\times 10^3$)	S.E. ($\times 10^3$)	D (days)	Fiducial limits (95%)
In air	-0.310	0.097	3,226	1970-8850
In vacuum	-0.198	0.087	5,051	2670-47,600

Fig. 4. Survival of spores of (a) *Clostridium botulinum* type E and (b) *Bacillus stearothermophilus* dried together, at 25° in vacuum at various a_w values: O, 0.00; \bullet , 0.05; Δ , 0.10; \blacktriangle , 0.20; \square , 0.40; \blacksquare , 0.60; \times , 0.80; ∇ , 0.90; \blacktriangledown , 1.00 (liquid phase).

Clostridium botulinum Type E with *Bacillus stearothermophilus*. Spores of these two species were mixed together in suspension and, therefore, freeze-dried and equilibrated under identical conditions. A considerable decrease in viability of the *C. botulinum* type E spores occurred in 2 years, especially at the very low a_w values

(Fig. 4). On the other hand, the spores of *B. stearothermophilus* showed little decrease in viability except at a_w values 0.00 and 0.90.

Effect of storage at various a_w values on the heat resistance of spores. The effect of storage on the heat resistance of rehydrated spores was tested with three species (Tables 2–4). It is evident from Table 3 that storage for 6 years at the a_w values of 0.22, 0.43, 0.62 and 0.80 had very little adverse effect on the heat resistance of

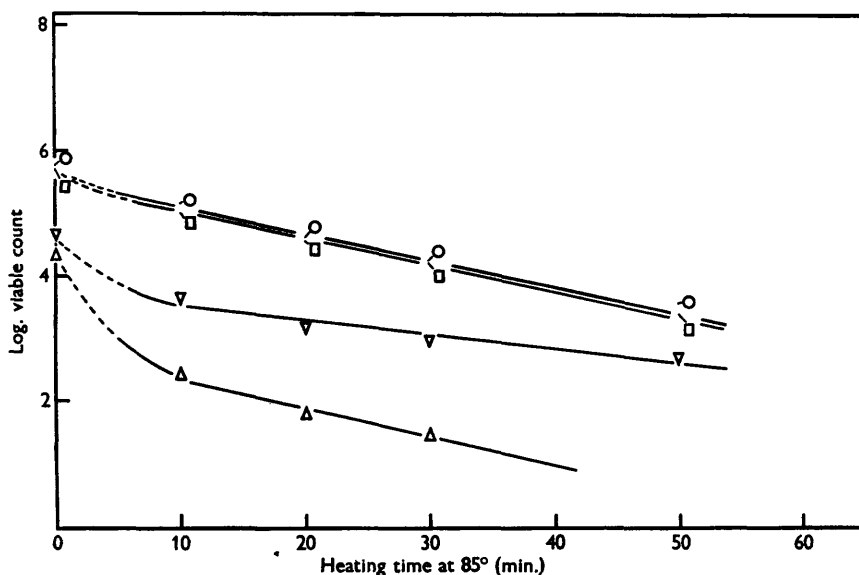


Fig. 5. Survival curves of rehydrated spores of *Bacillus megaterium*. Spores stored for 320 weeks at various a_w values before rehydration. Storage in vacuum at a_w values: O, 0.22; □, 0.43; ▽, 0.62; in air: △, 0.62.

Table 2. Heat resistance of rehydrated spores of *Clostridium bifermentans* after storage at various a_w values for 310 weeks
At a_w values of

Storage atmosphere	At a_w values of					Original suspension		
	0.22	0.43	0.62	0.80	1.00			
	D _{95°} values*					Vapour phase	Undried	
Air	8.5 ± 0.8	9.9 ± 0.2	—	26.3 ± 7.6	2.9 ± 0.2	—	12.5 ± 0.5	
Vacuum	14.4 ± 0.2	17.6 ± 0.7	20.8 ± 0.2	8.2 ± 0.2	9.8 ± 0.3	3.2	—	

* Mean D values (decimal reduction time) in min. at temperature indicated of spores re-suspended in 0.05 M-phosphate buffer (pH 7) sealed in air. Mean D values calculated from the regression coefficient ± s.e. for the regression of log. viable count on time.

Bacillus megaterium spores. Storage in vacuum, however, showed a slight but definite superiority over storage in air. The survival curves for *B. megaterium* (Fig. 5) showed an initial rapid decrease in several instances, which was due to the death of the spores in which germination had been initiated. This was confirmed by micro-

scopic observations of the presence of stained spores. The viable counts of unheated preparations, therefore, were excluded from the estimations of D values.

With *Clostridium bifermentans* spores also, storage in vacuum resulted in better retention of heat resistance (Table 2). In several instances with this species and with *Bacillus megaterium* (Table 3), an increase in heat resistance was observed, which was either a result of the drying process or of changes during storage (see Magoon, 1926). *B. stearothermophilus* spores in the mixed preparation with *C. botulinum* spores did not retain their heat resistance nearly as well as spores of the above two species (Table 4). This may, however, have been a consequence of their admixture with spores of *C. botulinum*. The viable counts of spores of *C. botulinum* type E had here decreased too much to enable satisfactory determinations of their heat resistance.

Table 3. Heat resistance of rehydrated spores of *Bacillus megaterium* after storage at different a_w values for 320 weeks

Storage atmosphere	Storage a_w values				Original suspension
	0.22	0.43	0.62	0.80	
	D _{85°} values				
Air	18.0 ± 2.7	24.8 ± 7.1	21.9 ± 3.8	9.10 ± 0.66	23.0 ± 2.4
Vacuum	22.9 ± 1.9	24.4 ± 1.0	47.3 ± 9.5	33.8 ± 12.1	—

Table 4. Heat resistance of rehydrated spores of *Bacillus stearothermophilus* after storage of 96 weeks in vacuum at various a_w values

	Storage a_w values												Original suspension†			
	0.05		0.10		0.20		0.40		0.60		0.80 0.90			1.00		
	(i)*	(ii)	(i)	(ii)	(i)	(ii)	(i)	(ii)	(i)	(ii)	(i)	(ii)	(i)	(ii)		
D _{115°} value‡	11.5	7.0	8.7	9.7	13.6	12.2	16.8	12.4	9.7	6.7	7.7	8.9	11.3	11.0	22.2	(31.7)§
Limits (±)	0.9	2.2	1.0	1.0	1.0	1.9	2.1	0.4	0.9	1.2	—	0.8	1.2	0.5	0.6	(0.0)

* (i) and (ii) refer to suspensions derived from separate storage ampoules. † Suspension before storage. ‡ D value, mean, min. at 115°. § Heat resistance of the rehydrated freeze-dried spores before storage. —, Regression not significant.

Effect of solutes and storage temperatures on the viability of spores stored at 0.20 and 0.50 a_w . Various solutes have been shown to affect the viability of freeze-dried vegetative forms during storage and rehydration (Scott, 1958; Leach & Scott, 1959; Scott, 1960). It is not known what effect such substances may have on spore viability. Two experiments with two species were carried out to test the effect of some of these substances. In the first experiment, sucrose (0.25 M), glutamic acid (0.25 M), semicarbazide (0.05 M) and ribose (0.05 M) were added separately and in various mixtures before freeze-drying, to suspensions containing spores of both *Bacillus megaterium* and *B. stearothermophilus*. These solutes had no apparent effect on the viability of the spores during storage for 81 weeks at 10° and 30°.

In the second experiment the solutes DL-glyceraldehyde, ribose and diacetyl were tested (each at 0.05 M) again with both species of spore dried together. Glyceraldehyde decreased the viable count of both organisms by 6 log. units in 81 weeks at 30°

(Figs. 6, 7). The rate of decrease in viability was most rapid at the higher storage temperature and at the higher a_w value with both organisms. After the initial phase of rapid decrease in viability the rate of decrease in viability was less at the higher temperature. Diacetyl caused a rapid decrease in viability of up to 2 log. units within 10 days at the higher a_w value and temperature, with spores of both species; thereafter the viable count remained fairly constant (Figs. 6, 7). Ribose caused a definite decrease in viability, which was more pronounced at the higher temperature and with spores of *Bacillus megaterium* (Figs. 6, 7).

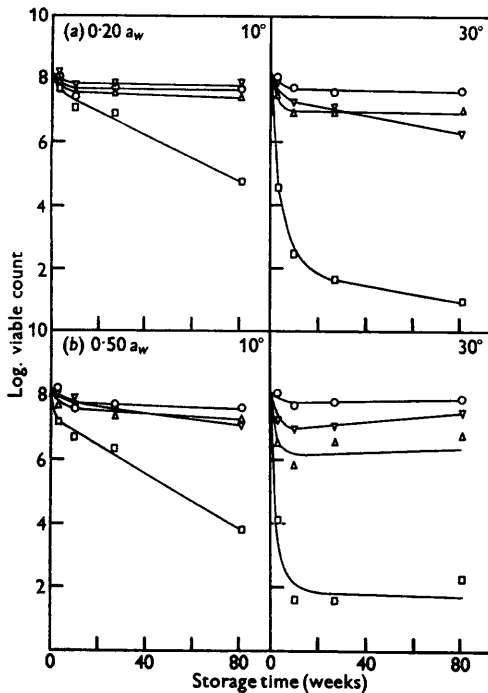


Fig. 6

Fig. 6. The effect of solutes, storage temperature and a_w value on the viability of dried spores of *Bacillus stearothermophilus* stored in vacuum. \circ , water; \square , DL-glyceraldehyde; \triangle , diacetyl; ∇ , D-ribose.

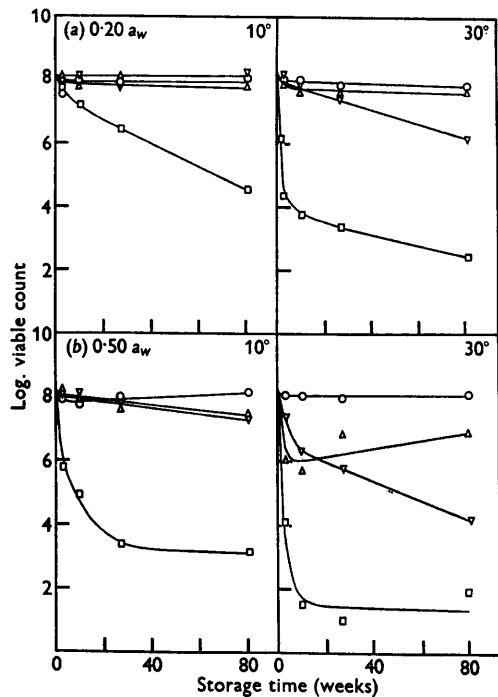


Fig. 7

Fig. 7. The effect of solute, temperature and a_w value on the viability of dried spores of *Bacillus megaterium* stored in vacuum. \circ , water; \square , DL-glyceraldehyde; \triangle , diacetyl; ∇ , D-ribose.

DISCUSSION

Three points emerge from these experiments. First, spores of four bacterial species when stored under conditions of extreme dryness in air or in vacuum showed a marked decrease in viability. The cause of this instability has not been investigated, but it is interesting to note that the spores differed from vegetative organisms which at 0.00 a_w lost viability much more rapidly in air than in vacuum (Scott, 1958; Lion & Bergmann, 1961). Spores heated or irradiated under very dry conditions have also been shown to be less resistant to heat (Murrell & Scott, 1957) and to irradiation (Tallentire, 1958) than when similarly treated under moister conditions.

Secondly, the aerobic species showed instability in vacuum at a_w 1.00 and the anaerobic organisms instability in air at this a_w value (Figs. 2, 3). The understanding of this observation is complicated by the fact that under these moist conditions a high proportion of the spores showed initiation of germination in the rehydrated suspension. The possibility, therefore, exists that the aerobic spores which had begun germination lost viability in the absence of oxygen, and the spores of the anaerobes in which germination had been initiated became non-viable because of the presence of oxygen. An alternative but less likely interpretation is that the ungerminated aerobic spores under these moist conditions needed oxygen to respire and to retain viability. This interpretation may be more applicable to the facultative anaerobic thermophile (*Bacillus stearothermophilus*) which would be much less likely to begin germination at 25° at a_w 1.00 in vacuum.

Thirdly, it is evident that certain low molecular weight solutes were able to induce death of bacterial spores stored at water activities which were otherwise favourable for retention of viability and heat resistance. The active solutes were carbonyl compounds: this suggests that the Maillard reaction postulated by Scott (1960) may also be a mechanism which causes death of dry spores. In the case of spores the permeability of the spores to the solutes may be of considerable importance. If the solutes do not reach the spore protoplasm before freeze-drying, then they may have no effect on the retention of viability in the dry state. This may explain why ribose was less active than glyceraldehyde. The reaction involved in loss of viability in the presence of these solutes was accelerated at the higher temperature and a_w values.

It may be concluded from these studies that freeze-dried spores of some species do not survive well under extremely dry or very moist conditions, and that for maximum retention of viability and heat resistance storage in air or in vacuum at a_w values of 0.2–0.8 is recommended. In general at these a_w values storage in vacuum was superior to storage in air for retention of the original heat resistance of the spores.

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