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Influence of Environmental Storage Relative Humidity on Biological Indicator Resistance, Viability, and Moisture Content

R. R. REICH†* AND L. L. MORIEN‡
Sybron, Medical Products Division, Rochester, New York 14692

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The effect of environmental storage relative humidity (RH) on the moisture content, viability, and moist heat and gaseous ethylene oxide (EO) resistance of biological indicators (BIs) was evaluated. No statistically significant difference was observed between the initial *Bacillus stearothermophilus* spore population and the spore population of BIs stored at 20°C and 0, 20, 44, or 55% RH or under ambient, 4°C, or −20°C conditions after 12 months. A statistically significant decrease in moist heat resistance from initial starting levels was found for BIs stored at 20°C and either 0 or 20% RH. There was a statistically significant decrease in the *B. subtilis* BI spore population, compared with initial levels, when the BIs were stored at 20°C and 0% RH concomitant with a significant increase in their EO resistance. BI storage at 20°C and 20 or 44% RH, or under ambient, 4°C, or −20°C conditions, had no significant effect on EO resistance. BIs stored at 20°C and 66% RH demonstrated a significantly lower EO resistance compared with starting levels.

The influence of moisture, usually expressed as water activity, A_w, or percent equilibrium relative humidity (RH), on the thermal resistance of bacterial spores has been evaluated by numerous investigators (1, 2, 8, 15, 16, 19). Murrel and Scott (19) demonstrated that the maximal spore heat resistance occurred between A_w values of 0.2 and 0.4 and that the resistance dramatically decreased at A_w levels of less than 0.2 and greater than 0.4. These findings have been confirmed by other investigators.

The critical influence of moisture on the efficiency of ethylene oxide (EO) sterilization has also been documented by several investigators (7, 9, 11, 17; R. R. Reich, J. E. Whitbourne, and L. L. Morien, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, Q41, p. 201). Maximal spore resistance occurs at low RH values. The resistance decreases as the RH levels are raised to approximately 40% and tends to level off over the higher RH ranges (17).

Gillis (13) recently hypothesized that the stable heat resistance of *Bacillus stearothermophilus* biological indicators (BIs) he observed on ambient temperature storage was a result of the low environmental RH. He further suggested that BIs should be stored in a low-RH environment to maintain acceptable performance characteristics.

† Present address: Ethox Corporation, Buffalo, New York 14210.

‡ Present address: Skyland Scientific, Belgrade, MT 59714.

We have previously reported the loss of moist heat resistance of *B. stearothermophilus* BIs stored under either ambient or freezer conditions (23). In another report, we found that the EO resistance and viability of *B. subtilis* BIs was stable under either ambient or freezer conditions (22). Similar results were observed by Rohn (24). The present investigation was conducted to assess the effect of BI storage, at different RH conditions, on the viability, resistance, and water content of *B. subtilis* and *B. stearothermophilus* BIs.

MATERIALS AND METHODS

BIs. The BIs used in this investigation were UNISPORE BIs (lot A-35; Sybron, Medical Products Division), which are dual-species indicators containing spores of both *B. stearothermophilus* and *B. subtilis*. The initial spore populations were 5.9243 and 6.4150 $\log_{10}$ for *B. stearothermophilus* and *B. subtilis*, respectively. The BIs had the following initial resistance values: a decimal reduction value (*D*-value) at 121°C in saturated steam ($D_{121°C}$) of 1.6 min and a *D*-value at 1,200 mg of EO per liter, 60 ± 10% RH, and 55 ± 1°C of 1.8 min.

Maintenance of constant-humidity environment. Specific humidity environments were prepared at a constant temperature of 20°C, in glass desiccators, using an excess of the chemical substance indicated below, in contact with a saturated aqueous solution of the solid phase of that chemical. At the given RH, the chemicals used were: 0%, $CaSO_4$ (Drierite) or P_2O_5; 20%, $KC_2H_3O_2$; 44%, $KCO_3 \cdot 2H_2O$; and 66%, $NaNO_2$.

BI storage. BIs were stored at 20°C in defined

humidity environments of 0, 24, 44, and 66% RH. The BIs were also stored under non-frost-free freezer (−20°C), refrigerator (4 to 6°C), and ambient (20 to 28°C, 20 to 60% RH) conditions. The BIs were stored undisturbed for 1 year and then evaluated.

BI quantitation. Spore quantitation was performed as previously described (22, 23). The mean colony-forming units per BI value resulted from the analysis of at least nine data values.

EO resistance testing. The testing was conducted in a specially designed 18-liter laboratory test chamber, a system previously described in detail by West (26). The system meets or exceeds the Association for the Advancement of Medical Instrumentation specifications outlined in reference 3. All cycles were conducted with an EO concentration of 1,200 mg/liter, with an up-time to achieve gas concentration of approximately 20 s at 55 ± 1°C and 60 ± 10% RH. Gas concentration was obtained by passing 100% ethylene oxide liquid supplied by Union Carbide-Linde Specialty Gases, Somerset, N.J., through a heat exchanger. The BI temperature come-up time was 4 min. We have previously documented the critical importance of a precisely defined and accurately controlled challenge cycle (Reich et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1978). The challenge cycle sequence used in these investigations was previously described in detail (22). Duplicate sets of 10 BIs were exposed for different time periods for each resistance determination.

Heat resistance testing. The BI steam challenge testing was conducted in a specially designed and constructed laboratory test chamber. The system meets or exceeds all specifications outlined in the AAMI standard for BIER/steam vessels (4). The apparatus consists of an 18-liter chamber equipped with a 1.0-liter automatic plunger. A polypropylene BI holding rack is included in the plunger. Ten BIs are exposed per cycle. Pre- and postcycle 20-inches-of-Hg (ca. 67.5 kPa) vacuums are automatically drawn during each challenge exposure. The temperature is monitored and controlled by a resistance temperature device to ±0.2°C. The exposure time for test samples to achieve and descend from temperature is controlled to ±1.5 s.

Calculation of resistance. The resistances of the BIs were compared by an analysis of *D*-values. The *D*-values were calculated by the most-probable-number or fraction negative procedure of Stumbo et al. (25). At least three sets of quantal data were collected for each resistance determination. Individual *D*-values were calculated for each time period. The final reported *D*-value was the mean of these individual calculations.

Determination of BI moisture content. The moisture content of the BIs was determined by using a Metrohm Automatic Karl Fischer Titrator, distributed by Sybron/Brinkman. Five BIs were titrated for each assay, and each assay was repeated at least three times. The Karl Fischer reagent was standardized so that each milliliter would titrate approximately 2.5 mg of water. The reported milligrams of water/BI was the average of the three individual readings divided by 5.

Determination of the time to achieve water vapor equilibrium between BIs and test chamber environments. The EO BI challenge cycle includes a 5-min, pre-EO gas, 55 ± 1°C temperature dwell followed by a 5-min, pre-EO gas, 60 ± 10% RH dwell at temperature. The RH dwell period was previously recommended based on data obtained from BI weighting after various RH dwell exposure periods (Reich et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1978). The accuracy of this recommendation was reevaluated by using the actual water content per BI obtained with the Automatic Karl Fischer Titrator.

The number of milligrams of water/BI obtained when the test chamber and BIs achieved a water equilibrium condition was determined by conditioning BIs, previously stored at different RH conditions, for 5 days at pre-EO gas test conditions, i.e., 55 ± 1°C and 60 ± 10% RH. This figure was compared with the milligrams of water/BI figures obtained from BIs exposed to various timed RH exposures. The minimal time to achieve the milligrams of water/BI figure established as the equilibrium level was assumed to be the time water-vapor equilibrium conditions were achieved between the test chamber environment and sample BIs.

RESULTS

***B. stearothermophilus* BIs.** Table 1 shows the influence of 12 months' storage at different environmental conditions on the viability and moist heat resistance of *B. stearothermophilus* BIs. *B. stearothermophilus* is the BI organism of choice to monitor moist heat sterilization processes; consequently, only the influence of the storage environment on this performance characteristic

TABLE 1. Influence of storage environments on *B. stearothermophilus* BIs

Storage conditions	Mean mg of water/BI ± 1 SD	Mean ($\log_{10}$) CFU[a]/BI ± 1 SD	Mean $D_{121°C}$[b] (min) ± 1 SD
Initial	ND[c]	5.9243 ± 0.0285	1.57 ± 0.1026
0% RH	0.248 ± 0.046	5.6128 ± 0.0368	0.51 ± 0.0745
20% RH	2.085 ± 0.083	5.9858 ± 0.0475	1.12 ± 0.1257
44% RH	3.044 ± 0.069	5.9956 ± 0.0537	1.52 ± 0.1131
66% RH	4.666 ± 0.040	5.8573 ± 0.0398	1.37 ± 0.1343
Ambient	2.234 ± 0.078	5.9685 ± 0.0410	1.47 ± 0.2003
Refrigerated	2.608 ± 0.102	5.9294 ± 0.0311	1.30 ± 0.0968
Freezer	3.545 ± 0.134	5.9294 ± 0.0285	1.53 ± 0.0636

[a] Colony-forming units.
[b] Time at 121°C to reduce the spore population by 90%.
[c] Not done

TABLE 2. Influence of storage environments on *B. subtilis* BIs

Storage conditions	Mean mg of water/BI ± 1 SD	Mean (log_{10}) CFU[a]/BI ± 1 SD	Mean $D_{121°C}$[b] (min) ± 1 SD
Initial	ND[c]	6.4150 ± 0.0323	1.80 ± 0.2154
0% RH	0.248 ± 0.046	5.9590 ± 0.0419	2.30 ± 0.0566
20% RH	2.085 ± 0.083	6.3802 ± 0.0221	1.56 ± 0.0778
44% RH	3.044 ± 0.069	6.3222 ± 0.0408	1.67 ± 0.0939
66% RH	4.666 ± 0.040	6.4472 ± 0.0311	1.10 ± 0.1556
Ambient	2.234 ± 0.078	6.4314 ± 0.0388	1.76 ± 0.1202
Refrigerated	2.608 ± 0.102	6.4150 ± 0.0684	1.97 ± 0.0603
Freezer	3.545 ± 0.134	6.3802 ± 0.0546	1.85 ± 0.2629

[a–c] See Table 1.

was chosen for evaluation. There was no significant difference at the 95% confidence level in the BI spore viability between the BIs stored at any of the investigated environments and the original starting spore population. The storage environment had a more dramatic effect on the moist heat resistance of the BIs. There was a significant decrease in the resistance at the 95% confidence level of BIs stored at 20°C and either 0 or 20% RH compared with the initial starting BI resistance. No significant difference in resistance was observed between the initial BI resistance and the resistance of BIs stored at 20°C and 44 or 66% RH or under ambient, refrigerated, or freezer conditions. With the 20°C controlled RH environments, maximal resistance was observed with BIs stored at 44% and decreased when the RH was increased or decreased from this level.

The mean water content per BI ranged from 0.248 mg for BIs stored at 20°C and 0% RH to 4.666 mg for BIs stored at 20°C and 66% RH. BIs stored in the ambient laboratory environment had an average water content of 2.234 mg per BI; refrigerator- and non-frost-free freezer-stored BIs had an average water content per BI of 2.608 and 3.545 mg, respectively.

***B. subtilis* BIs.** Table 2 shows the effect of 12 months' storage, under different environmental conditions, on the EO resistance and viability of *B. subtilis* BIs. *B. subtilis* is the BI organism of choice for monitoring the efficiency of EO sterilization processes; consequently, only the influence of the storage environment on this parameter was investigated. There was a significant decrease at the 95% confidence level between the mean spore population of BIs stored at 20°C and 0% RH and the initial mean starting spore population. No other significant differences between the original mean BI spore population and the spore populations of BIs stored at different environmental conditions were observed.

As was the case with *B. stearothermophilus* BIs, the storage environment had a great influence on the BI resistance. Maximal BI EO resistance was obtained with storage at 20°C and 0% RH. The resistance observed from these BIs was significantly higher at the 95% confidence level than the original starting resistance. BI storage at 20°C and 20 or 44% RH had no statistically significant effect on EO resistance. BI storage at 20°C and 66% RH, however, had an adverse effect on resistance: a significantly lower *D*-value at the 95% confidence level was observed (Table 2). BI storage under ambient, refrigerated, or freezer conditions had no significantly observable effect on EO resistance. Moisture content of the BIs as a function of the storage environment was identical to that described above for *B. stearothermophilus* BIs.

Time to achieve water vapor equilibrium conditions in 18-liter EO test chamber. Table 3 shows the influence of pre-EO cycle RH dwells on the attainment of water vapor equilibrium between the 18-liter test chamber environment and the included BIs, as assessed by the BI moisture content. BIs previously stored at 20°C and 20 or 66% RH were equilibrated at test conditions, 55 ± 1°C and 60 ± 10% RH, for 5 days. After this time, the mean milligrams of water/BI value was 2.508 for both sets of BIs. This figure was assumed to be the amount of water per BI at chamber BI equilibrium conditions.

BIs previously stored at 20°C and 20, 44, and 66% were then exposed to challenge cycles consisting of a 5-min temperature dwell at 55 ± 1°C and 60 ± 10% RH dwells of 5, 15, 30, and 60 min (Table 3). Each set of BIs obtained the equilibrium water content within 30 min. RH dwell times beyond 30 min did not alter the results. Therefore, 30 min appears to be the optimal time, in our system, to achieve water vapor equilibrium conditions between the test chamber environment and included BIs.

DISCUSSION

We have previously reported on the influence of storage conditions on the moist heat resistance of *B. stearothermophilus* BIs during a 24-month evaluation period (23). The present results suggest that the heat resistance of *B.*

TABLE 3. Influence of humidification dwell time on BI moisture content

Storage condition (% RH)	Temp dwell, 55 ± 1°C (time)	RH dwell, 60 ± 10% at 55 ± 1°C (time)	Mean mg of water/BI ± 1 SD
20	5 min	5 days	2.508 ± 0.005
66	5 min	5 days	2.508 ± 0.003
0	5 days	0	0.917 ± 0.012
44	5 days	0	0.962 ± 0.020
20	5 min	5 min	2.200 ± 0.064
	5 min	15 min	2.501 ± 0.091
	5 min	30 min	2.494 ± 0.102
	5 min	60 min	2.520 ± 0.076
44	5 min	5 min	2.302 ± 0.007
	5 min	15 min	2.432 ± 0.102
	5 min	30 min	2.522 ± 0.084
	5 min	60 min	2.531 ± 0.052
66	5 min	5 min	3.104 ± 0.083
	5 min	15 min	2.814 ± 0.031
	5 min	30 min	2.491 ± 0.008
	5 min	60 min	2.496 ± 0.074

stearothermophilus BIs can be maintained by storage under ambient, freezer, or 44% RH at 20°C conditions for at least 12 months. Storage at lower RH environments, as suggested by Gillis (13), led to a significant loss of heat resistance without a concomitant loss of spore viability.

Marshall et al. (18) reported that freeze-drying and storage of dried spores damaged a proportion of the spore population so that they became heat labile, but not necessarily nonviable. A similar phenomenon could be occurring with the *B. stearothermophilis* BIs equilibrated in the 0 and 20% RH environments. These environments are dehydrating the spores as shown by their lowered water content.

Ernst (10) found that linen materials, processed in a high prevacuum steam sterilizer, could be dehydrated before steam injection. During steam heating and hydration of these materials, large volumes of steam were condensed, resulting in the release of the heat of vaporization, which resulted in a superheated condition within the linens. If the system contained residual air, charring of the fabric could occur. It was hypothesized that superheated steam energy was generated from this exothermic reaction. An analogous situation might be occurring with dehydrated BIs. During steam processing, small amounts of air might be trapped within the glassine envelope, leading to a similar superheated phenomenon in the spore microenvironment. This hypothesis is consistent with the data of Pflug and Smith (20). They reported that unpackaged spore strips exhibited a greater resistance to steam sterilization than did identical spore strips contained in glassine envelopes. This theory might partially explain the significantly lower observed $D_{121°C}$-values for BIs stored at 20°C and 0 and 20% RH when compared with the original starting values.

Murrell and Scott (19) found that when spores were heated under different A_w conditions, as the A_w values approached zero, all species of spore became more heat labile. Their observed changes were significant, the resistance at 0.00 A_w being only about 1% of the maximum observed spore resistance values. They postulated that the water which hydrated some spore components, probably proteins or protein-containing complexes, produced a marked stabilization against the adverse changes caused by high temperatures. Loss of this stabilizing water of hydration, they felt, made the spore more vulnerable to heat inactivation. These observations are consistent with our data.

Gerhardt and Black demonstrated that spores were freely and rapidly permeable to water molecules (5, 12). This observation apparently conflicts with our observation of lower heat resistance of BIs equilibrated at low RH levels, but challenged in a 100% RH environment (saturated steam). If their findings were valid in our system, it would be expected that the spores would be rapidly rehydrated in the steam environment, thus restoring their initial heat resistance.

A partial explanation of this apparent conflict might be found in the report of Quinn (21), who stated that organic materials exhibit a significant moisture-humidity hysteresis behavior. Several theories have been advanced to explain the failure of materials to return to the same equilibrium level after exposure to wet or dry conditions. Quinn stated that the reason was a true hysteresis phenomenon, explained by actual

changes in the chemical bonding of water, by changes in electrostatic (H bonding) attraction, or by a combination of both. He further stated that hydrated forms and waters of crystallization would be relatively stable compounds and would change only with a large change in vapor pressure differential (extremely low RH or A_w). The molecular attraction of the material constituents, other than water, would vary depending on the interposition of the water molecules. Resistance to interposition by water would be greater for a material which had been dried (desiccated spore), allowing closer attraction among the molecules other than water.

Therefore, a possible explanation for the observed lower resistance in saturated steam of BIs previously dehydrated in a low RH environment might be the loss of the water of hydration from those spore components that stabilize the spore against the effects of elevated temperatures. This loss of hydrated water would result in a true hysteresis effect, due to changes in molecular bonding, and would, therefore, cause a permanent alteration of spore heat resistance. This theory is consistent with our observations.

Rohn has previously demonstrated that storage of *B. subtilis* BIs at temperatures ranging from 2 to 35°C does not significantly affect EO resistance or spore viability (24). Similar results were also reported by Reich (22). The present study supports these conclusions. A total of 12 months of storage at 20 or 44% RH at 20°C, ambient, refrigerated, or freezer conditions had no significant effect on *B. subtilis* BIs' EO resistance or viability. Unlike the results obtained with BIs stored at the above conditions, storage at 0% RH at 20°C significantly affected both spore viability and resistance. The spore population decreased, and the EO resistance increased. The desiccation of the BIs must have damaged a proportion of the spore population so that they became nonviable. If, as hypothesized above, prolonged BI storage under desiccating conditions results in a loss of water of hydration of some spore protein components, this might particularly explain the observed higher EO spore resistance. Water is known to be a necessary reactant in the EO alkylation reaction(s) (6, 14). A chemical rearrangement of some spore components, due to dehydration, might interfere with the ability of water and EO from reaching or reacting with specific cellular components, probably nucleic acids, in the spore protoplast.

In summary, as a result of these investigations, we recommend that ambient temperature storage of BIs below approximately 20% RH be avoided. Storage under these conditions has been shown to significantly affect the resistance of both *B. stearothermophilus* and *B. subtilis* BIs. Ambient temperature storage above approximately 66% RH should similarly be avoided to maintain a stable and predictable pattern of BI resistance.

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