Effects of Various Gases on the Survival of Dried Bacteria During Storage

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Salmonella newport and Pseudomonas fluorescens were dried together in papain digest broth and sucrose-glutamate, and stored in several gases at various water activities (a_w) between 0.00 and 0.40 at 25 C for various periods up to 81 weeks. Both S. newport and P. fluorescens, dried in papain digest broth and stored in air, died rapidly if the conditions were very dry (0.00 a_w) or moist (0.40 a_w). Storage in carbon dioxide and argon gave greater survival than storage in air but lower survival than did storage in nitrogen or in vacuo. When the organisms were dried in a sucrose-glutamate mixture the differences between the gases were very small, and variations in residual water were less important. Of the inert gases, argon gave the best survival when the organisms were dried in papain digest broth, especially at 0.00 a_w; the survival in neon and krypton was lower and in xenon and helium it was much lower.

The stability of dried bacteria during storage is dependent on a number of factors, the more important ones being the organism itself, the composition of the suspending fluid in which the organism is dried, the residual moisture, the temperature of storage, and the atmosphere of storage (2).

It has been well established by earlier workers that storage in vacuo gives better survival than storage in air (4, 5–8). However, there is no agreement about the relative merits of other gases and whether or not use of these gases is as good as storage in vacuo. Rogers (6) was one of the first workers to compare storage in vacuo with storage in other gases. He found that survival was highest in cultures stored in vacuo and lowest in those stored in air or oxygen. Atmospheres of nitrogen, hydrogen, and carbon dioxide were intermediate in rates of survival. The observations of Naylor and Smith (4) with Serratia marcescens are in agreement with these findings. Proom and Hemmons (5), working with Neisseria, also found that storage in vacuo gave the best survival, although in this case storage in nitrogen was just as good as storage in vacuo.

In this earlier work there had been no control of residual moisture during storage except to dry the particular gas, and a variety of suspending media had been used. In no instance was more than one suspending medium studied in any one experiment. The work of Scott (7) showed that the effect of atmosphere was dependent upon the suspending medium and the moisture level. He found with Salmonella newport that only under the driest conditions and in the absence of sugars was there a marked difference in viability between dried cells stored in air and in vacuo.

In the experiments to be described in this paper, the survivals of two organisms, namely, Pseudomonas fluorescens and S. newport, have been determined when they were dried in two suspending fluids and stored in various gases under conditions of controlled moisture levels.

MATERIALS AND METHODS

Organisms and suspending media. The organisms used were P. fluorescens and S. newport. The two suspending fluids were papain digest broth (7) and a mixture of 0.25 M sucrose and 0.25 M glutamate, Marshall and Scott (3) having shown the protective effects of this combination.

Preparation for and freeze-drying of organisms. Both organisms were grown in brain-heart broth medium for 20 h at 30 C with aeration. Equal volumes of each culture were mixed. The resulting suspension was centrifuged and the cells were resuspended to half their original volume in the particular suspending fluid to give approximately 10^9 cells of each organism per ml. In this way possible variations in the drying, storage, and rehydration of each organism were eliminated.
Replicate ampoules containing 0.2-ml samples of the suspending fluids and organisms were prepared. The ampoules were supported in a rack so that each tube was radially mounted about 20° from horizontal, with its mouth some 2 cm from the surface of the central condenser which was filled with solid CO₂ and ethanol. The apparatus accommodated up to 160 ampoules so that all ampoules for a particular experiment were dried together. Cooling was purely evapative and heating was by radiation from the walls of the steel vacuum chamber. It was found convenient to de-gas the suspensions by evacuation until they had cooled to about 0 to 2 °C before the CO₂ plus ethanol mixture was added to the condenser. Immediately, the condenser was cooled, the rate of evaporation increased, and the suspending fluids and organisms were promptly cooled to −30 to −35 °C. No measurements of temperature were made during drying which was for 4 h, the time found by Scott (7) sufficient to remove almost all the water from all solutes. The dried cells were prepared for storage immediately after drying.

Storage conditions. The ampoules were placed within larger tubes containing phosphorus pentoxide (P₂O⁵) for 0.00 aₜ and 2 ml of the appropriate sulfuric acid solutions (9) for all other aₜ levels. The larger tubes were first drawn out and then sealed in groups of five on a manifold for individual suspending fluids and water activities.

The following conditions for storage were used. (i) In air, the tubes were not evacuated before the final sealing. (ii) In vacuum, the tubes were evacuated in groups of six with a two-stage mechanical pump until the solutions controlling aₜ boiled, or for at least 60 s before the final sealing. (iii) In nitrogen, the tubes were first evacuated and then flushed to atmospheric pressure with oxygen-free nitrogen (supplied in a cylinder containing alkaline pyrogallol). This was repeated twice before the tubes were finally sealed. (iv) In carbon dioxide, the tubes were treated as for nitrogen, carbon dioxide being flushed into the tubes after the third evacuation and the tubes sealed. (v) For storage in inert gases (argon, helium, neon, krypton, and xenon), the spectrally pure gases were supplied in 1-liter flasks at slightly above one atmosphere pressure (British Oxygen Gases Ltd.). A manifold built of capillary tubing was used to conserve the gases for the filling and sealing of groups of six tubes. For each gas, the manifold was evacuated and filled with oxygen-free nitrogen, re-evacuated and filled with the nitrogen, re-evacuated, and then filled to one atmosphere with the particular gas, and the tubes sealed. As the pressure at sealing was controlled with a mercury manometer, the possibility of slight contamination by mercury vapor cannot be excluded.

Storage and viable counts. Immediately on the completion of sealing, the tubes were stored in the dark in an insulated cabinet at 25 °C. Five replicate ampoules for each treatment were stored and after 1, 3, 9, 27, and 81 weeks one ampoule of each was randomly selected for the determination of viable numbers.

The contents of each ampoule were rehydrated with 2 ml of saline. Where necessary, decimal dilutions were prepared in saline. At each dilution four plates were poured using brain-heart-infusion agar. One duplicate set of plates was incubated for 17 to 20 h at 37 °C for the enumeration of S. newport and the other set was incubated for 6 days at 7.5 °C for the enumeration of P. fluorescens. Separate experiments showed that the incubation conditions used completely suppressed colony formation by one organism and permitted full development of the other.

S. newport and P. fluorescens were dried together in the two suspending fluids and stored at 25 °C in five gases (air, vacuum, nitrogen, carbon dioxide, and argon) at three moisture levels (0.00, 0.20, and 0.40 aₜ). There were two replicates.

In a further experiment (not replicated) storage of both organisms was studied in seven gases (vacuum, nitrogen, helium, neon, argon, krypton, and xenon) at four water activities (0.00, 0.10, 0.20, and 0.30) after drying in papain digest broth.

The viable numbers per milliliter in terms of the original undried suspension were expressed as two-figure logarithms derived from the mean counts of duplicate plates.

RESULTS

P. fluorescens was more sensitive to both the drying and storage treatments. Forty percent of the cells of S. newport were still viable immediately after drying in papain digest broth compared with 100% for sucrose-glutamate. Survival of P. fluorescens after drying in papain digest broth was 25% and 95% in sucrose-glutamate. These survivals form the basis for evaluating changes associated with storage treatments. Figure 1 shows the changes in viability of P. fluorescens for all treatments during 81 weeks of storage. The effects on viability became more evident with time and, except for storage in air, were not apparent until the organisms had been stored for at least 9 weeks. Storage in vacuo and in nitrogen was significantly better than storage in air; carbon dioxide and argon gave intermediate results. The rate of death for storage in any gas and in either suspending fluid was least at 0.20 and generally was greatest at 0.40 aₜ. However, the effects of aₜ on survival were very much greater for cells dried in papain digest than for cells dried in sucrose-glutamate. When P. fluorescens was dried in papain digest, the most significant difference between the gases occurred when the cells were stored at 0.00 aₜ. In air, storage at 0.00 aₜ led to death equal to that at 0.40 aₜ, whereas there were very small differences between 0.00 and 0.20 for vacuum and nitrogen.

S. newport was less sensitive than P. fluorescens to all the storage treatments. Table 1 shows levels of survival for S. newport after storage for 81 weeks. The differences between the organisms were more marked when dried in
that for *P. fluorescens* papain digest gave very low viability in air in comparison with sucrose-glutamate; viability for air at 0.00 \( a_w \) was much lower than for 0.20 \( a_w \) in all the other gases and viability was low for the papain digest–0.00 \( a_w \) combination; further, the decrease in viability associated with papain digest as compared with sucrose-glutamate was much greater at 0.40 than at 0.20 \( a_w \). Similar inconsistencies occurred for *S. newport*. Comparison of the marginal means for average main effects shows that sucrose-glutamate and 0.20 \( a_w \) gave the greatest viabilities; as regards gases there was no significant difference between vacuum and nitrogen which were clearly better than the others; these conclusions are true for both organisms.

The differences between the seven gases used in the second experiment increased with time to their greatest values at 81 weeks of storage. 

Table 3 shows the viabilities of both organisms after storage for 81 weeks at all water activities. For *P. fluorescens* when very dry (0.00 \( a_w \)) there were no significant differences in storage between vacuum, nitrogen, and argon; xenon was very destructive and the other three gases gave intermediate survival patterns. At 0.30 \( a_w \) the differences between gases were substantially reduced. Even smaller differences between gases were found after storage at 0.10 and 0.20

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**Table 1. Survival of *S. newport* dried in two suspending fluids and stored for 81 weeks at 25°C in five gases at three water activities**

| Storage conditions | Suspending fluids* |
|--------------------|---------------------|
| **Gas**            | **Papain digest (i)** | **Sucrose-glutamate (ii)** |
| Air                | 0.00                | 1.00                 | 9.12                |
|                    | 0.20                | 7.48                 | 9.88                |
|                    | 0.40                | 2.02                 | 8.61                |
| Vacuum             | 0.00                | 8.56                 | 9.52                |
|                    | 0.20                | 9.50                 | 10.04               |
|                    | 0.40                | 7.30                 | 9.30                |
| Nitrogen           | 0.00                | 8.28                 | 9.60                |
|                    | 0.20                | 9.46                 | 10.12               |
|                    | 0.40                | 7.17                 | 9.43                |
| Carbon dioxide     | 0.00                | 6.88                 | 9.37                |
|                    | 0.20                | 9.28                 | 9.75                |
|                    | 0.40                | 5.65                 | 8.44                |
| Argon              | 0.00                | 5.81                 | 9.14                |
|                    | 0.20                | 8.11                 | 9.96                |
|                    | 0.40                | 6.71                 | 9.14                |

* Numbers in brackets (n') are numbers of observations averaged over two replicates.

* Log viable counts per milliliter immediately after drying: (i) 9.79; (ii) 10.25. Standard errors of means: \( n' = 2, \pm 0.56; n' = 4, \pm 0.39 \) on 29 degrees of freedom.

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**Fig. 1. Graph of means (single ampoules from each of two replicates) of \( \log_{10} \) viable count per milliliter of *P. fluorescens* during storage in five gases at three water activities \( (a_w) \) at 25°C after drying in two suspending fluids; standard error of plotted means: \( \pm 0.44 \) on 29 degrees of freedom. Log count immediately after drying (0 wk) (means of four ampoules): papain digest, 9.83; sucrose-glutamate, 10.37; S.E. of means, \( \pm 0.31 \) on 29 d.f. Symbols: O, 0.00 \( a_w \); [], 0.20 \( a_w \); o, 0.40 \( a_w \).**

papain digest, especially with storage at 0.40 \( a_w \).

Analysis of variance of the data showed that the average main effects of all factors and all their two-factor interactions were highly significant (at \( P = 0.001 \)) for both organisms. The three-factor interaction for *P. fluorescens* was significant at \( P = 0.01 \). This was because the means for papain digest and 0.40 \( a_w \) were very low in relation to those of the other water activities and all the water activities for sucrose-glutamate in all gases. Table 2 shows entries for means over one factor classified in rows and columns for combinations of the remaining two factors in all three possible ways for each organism. The marginal entries for each factor are means over both other factors. The study of means in the two-way tables shows...
### Table 2a. Two-way table of means and standard errors for the log₁₀ viable counts per milliliter of *P. fluorescens* after drying in two suspending fluids and storage for 81 weeks at 25°C in five gases at three water activities ($a_w$)

| Suspending fluids (SF) | Air   | Vacuum | $N_2$  | $CO_2$ | Ar   |
|-----------------------|-------|--------|--------|--------|------|
| Papain digest (PD)    | 2.34 (6)* | 6.55 | 6.08  | 5.25  | 4.84 |
| Sucrose-glutamate (S/G) | 8.14 | 8.76 | 8.56 | 7.48 | 8.31 |

* Numbers in brackets ($n'$) are numbers of observations averaged: standard errors of means: $n' = 4, ±0.31; n' = 6, ±0.25; n' = 10, ±0.02; n' = 12, ±0.18; n' = 20, ±0.14; n' = 30, ±0.11 on 29 degrees of freedom. The interactions $SF \times a_w$, $SF \times gas$ and $a_w \times gas$ were each significant at 0.1%. The interaction $SF \times a_w \times gas$ was significant at 1%.

### Table 2b. Two-way table of means and standard errors for the log₁₀ viable counts per milliliter of *S. newport* after drying in two suspending fluids and storage for 81 weeks at 25°C in five gases at three water activities ($a_w$)

| Suspending fluids (SF) | Air   | Vacuum | $N_2$  | $CO_2$ | Ar   |
|-----------------------|-------|--------|--------|--------|------|
| Papain digest (PD)    | 3.50 (6)* | 8.45 | 8.30  | 7.27  | 6.88 |
| Sucrose-glutamate (S/G) | 9.20 | 9.62 | 9.72 | 9.19 | 9.41 |

* Standard errors of means: $n' = 4, ±0.39; n' = 6, ±0.32; n' = 10, ±0.25; n' = 12, ±0.23; n' = 20, ±0.18; n' = 30, ±0.14 on 29 degrees of freedom. The interactions $SF \times a_w$, $SF \times gas$ and $a_w \times gas$ were each significant at 0.1, 0.1 and 5%, respectively.

### Table 3. Survival of *P. fluorescens* and *S. newport* after drying in papain digest and storage for 81 weeks at 25°C in seven gases at four levels of $a_w$

| Gas            | **P. fluorescens** (i) ($a_w$) | **S. newport** (ii) ($a_w$) |
|----------------|--------------------------------|------------------------------|
|                | 0.00  | 0.10  | 0.20  | 0.30  | Mean | 0.00  | 0.10  | 0.20  | 0.30  | Mean |
| Vacuum         | 7.62  | 9.12  | 8.20  | 6.40  | 7.84 (4) | 8.28  | 9.29  | 8.44  | 7.04  | 8.26 (4) |
| Nitrogen       | 7.27  | 8.57  | 8.10  | 6.22  | 7.54 | 7.92  | 8.81  | 8.06  | 6.90  | 7.92 |
| Helium         | 4.57  | 8.00  | 7.88  | 4.74  | 6.30 | 5.62  | 8.00  | 8.15  | 7.58  | 7.34 |
| Neon           | 5.70  | 8.89  | 7.47  | 4.42  | 6.62 | 6.99  | 9.04  | 8.01  | 7.44  | 7.87 |
| Argon          | 7.80  | 9.29  | 7.97  | 6.20  | 7.82 | 8.14  | 9.37  | 8.43  | 6.92  | 8.22 |
| Krypton        | 6.07  | 8.51  | 7.78  | 3.60  | 6.49 | 7.25  | 9.05  | 8.44  | 6.20  | 7.74 |
| Xenon          | 1.18  | 8.03  | 7.07  | 4.75  | 5.26 | 5.26  | 8.44  | 7.35  | 7.34  | 7.10 |
| Mean           | 5.74 (7) | 8.63 | 7.78 | 5.19 | 6.84 (28) | 7.07 (7) | 8.87 | 8.13 | 7.06 | 7.78 (28) |

* $Log_{10}$ viable counts per milliliter immediately after drying: (i) 10.26, (ii) 10.30. Numbers in brackets ($n'$) are numbers of observations averaged. Standard errors of means: (i) $n' = 2, ±0.82; n' = 4, ±0.58; n' = 7, ±0.43$ on 27 degrees of freedom; (ii) $n' = 2, ±0.50; n' = 4, ±0.35; n' = 7, ±0.26$ on 27 degrees of freedom.
at which levels mortality in all gases was low. Survival of both organisms was greatest at 0.10 $a_w$ and the differences between the gases were substantially suppressed. The only gas giving survival equal to storage in vacuo was argon.

**DISCUSSION**

The main aim of these experiments has been to study the changes during prolonged storage and to see whether any gases were different from storage in vacuo. The techniques used ensure that $a_w$ is, in fact, controlled during storage and enable valid comparisons to be made between different atmospheres. Because of the small amounts of water hydrating specimens at 0.10 to 0.40 $a_w$, equilibrium would have approached to within 0.01 $a_w$ of the appropriate values after storage for 1 week (7).

The results emphasize the overriding importance of controlling the level of residual water at about 0.10 $a_w$ irrespective of the nature of the atmosphere or suspending fluid. The results of workers where $a_w$ is unknown are of limited value. Greiff and Rightsel (1) studied the survival of dried influenza virus when stored in dried gases but the order of stability does not correspond with that reported here for *P. fluorescens* and *S. newport*. However, although they used dried gases, the exact values of $a_w$ during storage were unknown and indeed may have changed after sealing because of the production of water by the Maillard reaction. Our results do provide, for the first time, unequivocal evidence of differences between gases which are clearly a function of the level of residual water. Because of the interactions between the various factors, greatest survival of dried cells after prolonged storage will only be achieved when careful attention is given to selecting the appropriate level of all conditions concerned.

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