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Some factors affecting preservability of freeze-dried bacteria

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INTRODUCTION

Among the environmental factors affecting the survival of freeze-dried bacteria, the type and concentration of the drying medium, the residual moisture of the dried product and oxydation are considered to be the most important. As to the effects of these factors studies have been made on a variety of bacteria and, in consequence, several improvements have been made in increasing the preservation of freeze-dried bacteria, but much of the underlying mechanism is not understood. For instance, the presence of sodium glutamate in the drying medium has been observed by many workers to improve the viability of freeze-dried bacteria, but no satisfactory explanation for this enhancing effect has been forthcoming. Indeed there are many points relating to the other factors mentioned above which still require clarification, and further studies are obviously indicated in order to promote improvements in technique which will lead to better preservation of dried micro-organisms.

Obayashi (1955, 1960) and Obayashi & Cho (1957) studied some of these factors in relation to the drying of BCG, and later Ota (1959) made similar studies with lactic acid bacilli. These two types of bacteria have many features in common when considering the factors which affect the survival of bacteria both during the process of freeze-drying and the after period. The present study was made for the purpose of examining some of these factors, mostly as they affect the drying of *Lactobacillus bifidus*. This particular organism is considered as playing an important role in the nutrition of suckling infants, and problems connected with its oral administration seemed worth investigating.

MATERIALS AND METHODS

Lact. bifidus, strain no. 22, used in this study, was isolated by Ota (1959) from the faeces of a suckling and it belongs, according to Dehnert's method of classification (1957), to his fifth group. The organism is cultured in a yeast-extract medium (see Table 1) for 24 hr., and the growth harvested by centrifugalization and washed with physiological saline. According to Ota this method of treatment ensures the maximum survival both immediately after freeze-drying and long-term preservation. The washed bacilli are then resuspended in the drying medium in a volume which is one-tenth of that of the original culture. The pH is adjusted to 7·0 with NaOH and the bacterial suspension is dispensed in 1 ml. amounts in ampoules.

Freeze-drying is carried out in a chamber type of desiccator by the same method as is used for the routine production of BCG vaccine (Obayashi, 1955), with initial freezing at $-30°$ C. for 1 hr., 8 hr. desiccation in a vacuum of $\frac{1}{100}$ mm. Hg and for the last 3 hr. heating the product at approximately 30° C.

Except in a few specified instances the ampoules were sealed under ordinary atmospheric conditions; after the completion of desiccation air, after passing through silica-gel filters, was admitted into the chamber to release the vacuum and the ampoules were sealed.

For the anaerobic culture test of *Lact. bifidus*, the pipette culture method devised by Ota (1959) was used throughout this study. Modified liver extract agar (1·2–1·5 %) was dispensed in 4·5 ml. amounts into test-tubes which were kept at a temperature of about 40° C. until ready for use. The dried bacteria were reconstituted with sterile distilled water and then subjected to serial tenfold dilutions in order to obtain suitable bacterial concentrations and 0·5 ml. of each of these

Table 1. *Culture medium of* Lactobacillus bifidus

Yeast extract	1000 ml.
Glucose	10 g.
Lactose	20 g.
Polypeptone	20 g.
Sodium chloride	5 g.
Monobasic potassium phosphate	4 g.
L-cystin	2 g.
Sodium glutamate	2 g.
Sodium acetate	20 g.

pH was adjusted to 6·8.

dilutions were then added to the tubes of culture media. These tubes containing the just-fluid media and the bacterial suspensions were well agitated to ensure thorough mixing, and an appropriate amount of the mixture was sucked up to beyond the top graduation of a 1 or 2 ml. graduated pipette and then set aside to allow the medium to cool and gel. The pipettes were then incubated at 37° C. for 48 hr. after which all the colonies appearing within the 1 or 2 ml. graduation marks were counted. The method is economical in media and aerobic saprophytes do not invade the area to be counted.

RESULTS

Relation between the concentration of the bacilli, the drying medium and the viability of the dried product

When Ota (1959) was studying the effect of the drying medium on the preservation of *Lact. bifidus* he first screened a number of different media—glucosamine, skim-milk, sodium glutamate, glucose, lactose, sucrose, soluble starch and polyvinyl pyrrolidon K 90 (PVP). It was found that the best survival was obtained with sodium glutamate; the next best media appeared to be skim-milk, sucrose and glucose, in that order.

Our first experiment on varying the concentration of the bacillary suspension and the drying medium (sodium glutamate) was as follows. Bacillary suspensions of *Lact. bifidus* were put up in two concentrations, one identical with that of the original culture and the other as a tenfold concentration. The sodium glutamate drying medium was put up in concentrations of 0·1–5 %, which was increased to 10 % for the range of tests with the tenfold bacillary concentration. All the freeze-drying was done in the same drying chamber. The results are set out in graphic form in Fig. 1 and show, in the case of the tenfold bacillary concentration, an increasing viability of the dried products as the concentration of sodium

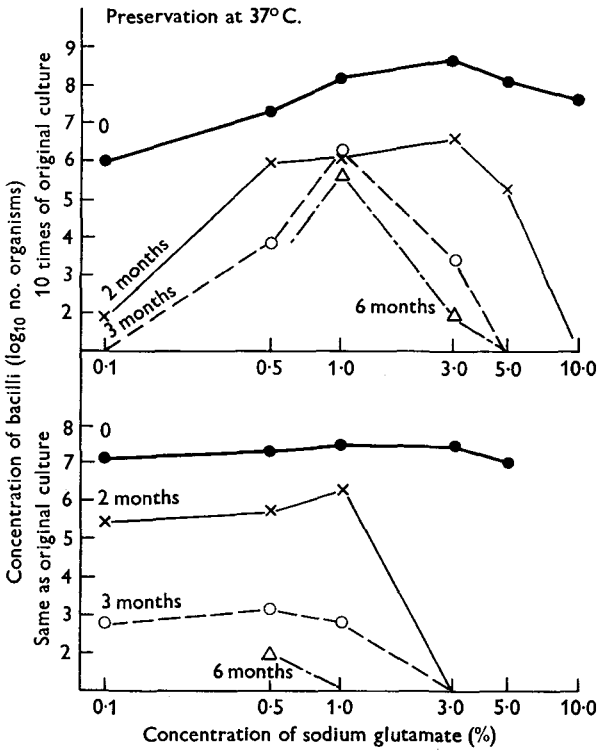

Fig. 1. Effect of concentration of bacilli and adjuvant on preservability of dried *Lact. bifidus*.

glutamate was increased from 0·1 up to 3·0 % both in samples tested immediately after lyophilization and after storage at 37° C. for 2 months. Above this concentration, however, the viability commenced to fall off; the peak of viability shifted back from 3 to 1 % when the sampling was done after storage for 3 and 6 months.

With the lower bacillary concentration the peak viability was with the sodium glutamate at the 1 % level for storage up to 2 months, but beyond this period the peak shifted back to the 0·5 % concentration of sodium glutamate. In contrast to the results obtained with the thick bacillary suspension the viability curves with the weak suspension, as will be seen from the lower half of Fig. 1, were almost

flat as the concentration of sodium glutamate was increased from 0·1 to 0·5% and the differences between the 0·5 and the 1·0% concentrations were not marked. It appears, therefore, that the optimal concentration of the drying medium in relation to the survival of the dried bacteria decreases as the concentration of the bacillary suspension is reduced. This optimal concentration also depends on storage conditions such as time and temperature, so that in general it can be said that the varying factors which affect the survival of dried *Lact. bifidus* are very similar to those observed by Obayashi & Cho (1957) in relation to the freeze-drying of BCG.

Improving preservability of dried bacteria by combining sodium glutamate with other drying media

We had recently observed that the addition of soluble starch to sodium glutamate increased the stability of dried *Lact. bifidus* as compared with sodium glutamate alone. In England, Muggleton (1960) for the freeze-drying of BCG used

Table 2. *Correlation between damaging effect of boiling and that of preservation at a high temperature*

(*a*) *Decrease in viability during preservation*

Type and concentration of drying medium	Preservation at (° C.)	Log_{10} no. organisms: Before drying	After drying	Period of preservation (months): 1	3	6	9	12
Soluble starch 5%	5	9·6	0 (10^6)	4·7	3·9	3·3	—	3·0
	37			—	0	0	0	—
Soluble starch 5%, sodium glutamate 5%	5	9·7	8·7	8·7	8·9	8·9	—	8·7
	37			8·0	7·1	6·8	6·7	—
Sodium glutamate 5%	5	9·4	9·1	8·8	9·3	8·9	9·4	9·1
	37			8·7	6·6	3·1	1·0	—
Lactose 5%	5	10·4	9·2	8·7	8·3	7·7	7·8	6·7
	37			7·6	2·8	—	0	—
PVP 5%	5	10·5	0 (10^6)	6·5	3·9	3·1	4·1	3·0
	37			0 (10^4)	0	0	0	—

Note. Figures in parentheses represent the degree of dilution in respective case.

(*b*) *Decrease in viability after boiling*

Type and concentration of drying medium	Log_{10} no. organisms: Control*	Time of boiling (min.): 10	15	30
Soluble starch 5%	3·0	—	0	0
Soluble starch 5%, sodium glutamate 5%	8·7	—	4·3	3·2
Sodium glutamate 5%	9·1	—	1·0	0
Lactose 5%	6·7	3·0	0	0
PVP 5%	3·0	—	0	0

* Products preserved for 12 months at 5° C.

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combinations of glucose, sucrose and sodium glutamate with dextran, and Greaves (1960) reported an increase in the heat stability of freeze-dried paracolon bacilli and gonococci when he combined dextran and sodium glutamate.

In view of these results we decided to examine the properties of various combinations of sodium glutamate with other drying media in so far as they affected the preservation of the dried bacteria. Polysaccharides and synthetic polymers were the substances chosen for the trials. The usual temperatures of storage were 37° or 45° C., but in addition part of the product was subjected to heat treatment at 100° C. for varying periods in accordance with the method devised by Greaves (1960).

Table 3. *Survival of dried* Lactobacillus bifidus *after boiling and after preservation at* 45° C.

	Log_{10} no. organisms									
			Time of boiling (min.)					Period of preservation at 45° C. (months)		
Type and concentration of drying medium	Before drying	After drying	1	5	10	30	60	1	2	3
Sodium glutamate 3%	10·5	9·9	7·3	6·7	5·6	0	—	4·9	2·3	1·2
Soluble starch 3%	10·5	8·5	0 (10^4)	2·7	0	0	—	0	0	—
Soluble starch 3%, sodium glutamate 3%	10·5	9·9	7·8	7·5	6·3	4·8	3·0	7·8	7·5	7·4
Sodium glutamate 5%	10·4	10·0	6·8	6·8	4·8	0	—	5·0	0	—
Soluble starch 5%, sodium glutamate 5%	10·3	9·9	7·9	7·2	6·1	4·1	3·0	8·0	7·8	7·2
Mannite 3%	10·4	6·5	4·1	0·7	—	0	—	0 (10^2)	0	—
Mannite 3%, sodium glutamate 3%	10·3	9·7	7·1	5·5	3·0	0	—	2·7	1·5	0
PVP 3%	10·3	9·5	5·0	2·7	0 (10^2)	0	—	0 (10^2)	0	—
PVP 3%, sodium glutamate 3%	10·5	9·9	7·8	7·8	7·8	7·1	6·4	8·5	8·1	8·0
Sodium alginate 3%	10·2	8·9	5·2	4·4	2·9	0	—	0 (10^3)	0	—
Sodium alginate 3%, sodium glutamate 3%	10·3	9·5	7·0	6·3	5·6	5·1	4·1	6·3	7·2	6·3
Dextran 3%	10·5	9·4	7·4	3·9	0·3	0	—	0 (10^3)	0	—
Dextran 3%, sodium glutamate 3%	10·4	9·9	7·9	8·1	6·0	4·8	0	8·0	7·4	5·2

Note. Figures in parentheses represent the degree of dilution in the respective case.

Table 2 shows the correlation between the results of these two methods for testing preservability. *Lact. bifidus*, freeze-dried in various drying media, was tested for viability after storage at 37° C. for periods up to 10 months. A duplicate series which had been stored in an ice chamber and then heated at 100° C. for 10–30 min. was similarly tested. The drying medium which gave the highest survival rate after preservation at 37° C. was a combination of sodium glutamate and soluble starch; this also gave the highest survival after the heat treatment at 100° C. for 30 min. With most of the other preparations too there was a correlation between the results obtained with heat treatment at 100° C. and preservation at 37° C. This is further considered later in the paper.

Table 3 shows the effect on the survival of the dried bacteria of adding a second substance to sodium glutamate. This second substance included soluble starch, mannite, PVP, sodium alginate and dextran. Bacteria dried with these substances used alone or in combination with sodium glutamate for the drying media were tested for their viability both after boiling for 1–60 min. and after storage at 45° C. for 1–3 months. When used alone all these substances gave a far lower survival rate than sodium glutamate whether the tests were done immediately after lyophilization or after storage. The results with *Lact. bifidus* were very similar to those reported by Cho & Obayashi (1956) for BCG. Mannite, alone or in combination, was not very satisfactory, but when any of the substances, except mannite, was combined in the drying medium with sodium glutamate the heat stability of the dried product was better than with sodium glutamate alone. The effect with PVP was outstanding. In a detailed study of the effect of heat on the viability of the organisms dried with the different combinations of drying media little difference was observed with up to 5 min. boiling, but with boiling for 10–20 min. the effect became appreciable and with boiling for 30–60 min. it was very markedly in favour of the combination, especially when PVP was combined with sodium glutamate.

The effect of using different concentrations of the constituents in combined drying media

In these experiments, in addition to the various combinations with sodium glutamate already mentioned, two new ones were tested—the sodium salt of carboxymethylcellulose (SCMC) and gelatin. The results of these tests are summarized in Table 4, and it will be seen that whereas SCMC conferred marked stability gelatin was without any such effect. In the tests of preservation after 1 and 3 months storage at 45° C. the combination of 3% PVP and 3% sodium glutamate gave the best survival figures. When sodium glutamate was used in a concentration of 1% the addition of 1% PVP gave the best result followed by 3% and then 5%. Results of the same order but at a somewhat lower level were obtained with dextran and also soluble starch.

In studying the possible correlation between the number of viable organisms after boiling for 5 and 10 min. and also after storage at 45° C. for 1 month graphs were prepared from the results in Table 3, and it will be seen in Figs. 2 and 3 that there is in fact a high positive correlation, r being 0·91 (5 min. boiling) and 0·947 (10 min. boiling). It, therefore, would appear that the boiling test yields results of considerable value for the estimation of probable viability on storage of the dried bacteria, especially when examining different types of drying media in relation to the preservation of the dried bacteria.

Influence of the drying medium on the degree of desiccation of the organism being dried

In order to study another mechanism concerned with the enhancement of the stability of the dried product we examined the effect of different drying media on

Table 4. *Survival of dried* Lactobacillus bifidus *after preservation at* 45° C.

Type and concentration of drying medium	Log_{10} no. organisms: Before drying	After drying	Period of preservation at 45° C. (months): 1	3
PVP 5 %, sodium glutamate 3 %	9·6	9·2	6·6	6·0
PVP 3 %, sodium glutamate 3 %	9·8	8·9	7·2	6·9
PVP 1 %, sodium glutamate 3 %	9·9	9·0	7·1	6·7
PVP 5 %, sodium glutamate 1 %	9·6	9·0	4·4	2·1
PVP 3 %, sodium glutamate 1 %	9·5	8·9	4·5	2·5
PVP 1 %, sodium glutamate 1 %	9·7	9·0	6·5	4·8
Sodium glutamate 3 %	9·6	9·5	0	—
Soluble starch 5 %, sodium glutamate 3 %	9·9	9·1	6·5	5·2
Soluble starch 3 %, sodium glutamate 3 %	9·9	9·1	6·6	5·9
Soluble starch 1 %, sodium glutamate 3 %	9·6	9·3	6·0	2·0
Dextran 5 %, sodium glutamate 3 %	9·9	9·6	7·6	2·0
Dextran 3 %, sodium glutamate 3 %	10·0	9·5	7·2	1·0
Dextran 1 %, sodium glutamate 3 %	9·9	9·4	0	—
SCMC 2 %, sodium glutamate 3 %	9·8	8·8	6·9	4·3
SCMC 1 %, sodium glutamate 3 %	9·8	9·0	7·0	5·5
SCMC 1 %	9·6	8·6	0	—
Gelatin 3 %, sodium glutamate 3 %	10·0	9·2	3·0	0
Sodium alginate 3 %, sodium glutamate 3 %	9·5	8·7	6·7	4·3
Sodium alginate 1 %, sodium glutamate 3 %	9·7	8·9	7·6	6·8

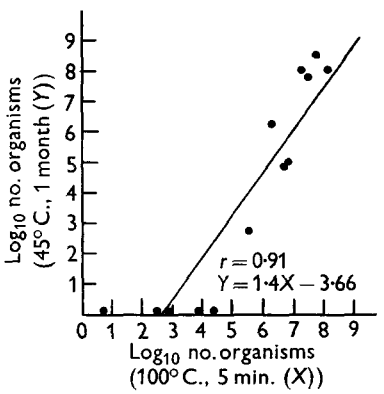

Fig. 2

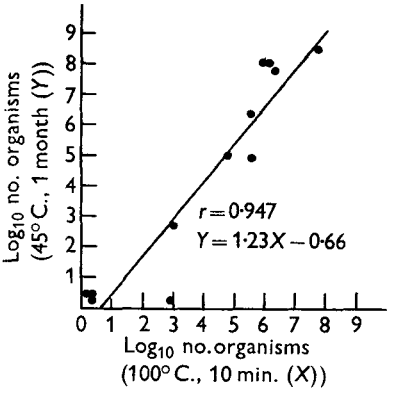

Fig. 3

Fig. 2. Correlation between number of living organisms of dried *Lact. bifidus* after boiling and after preservation at a high temperature (1).

Fig. 3. Correlation between number of living organisms of dried *Lact. bifidus* after boiling and after preservation at a high temperature (2).

the degree of desiccation of the organisms obtained in the drying process and its relation to the stability of the dried product.

Bacterial suspensions mixed with the various drying media were dispensed in weighing-bottles in 2 ml. amounts. They were then freeze-dried for 6 hr. in a chamber desiccator after which they were weighed. They were then heated at

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Table 5. *Effect of type and concentration of drying medium on rate of sublimation*

Type and concentration of drying medium	Sublimation rate (%)
Sodium glutamate 1 %	99·58
Sodium glutamate 5 %	99·21
Sodium glutamate 10 %	98·48
Glucose 1 %	99·55
Glucose 5 %	99·02
Glucose 10 %	98·71
Glucose 1 %, sodium glutamate 1 %	99·07
Glucose 5 %, sodium glutamate 1 %	97·99
Glucose 10 %, sodium glutamate 1 %	96·58
Glucose 1 %, sodium glutamate 10 %	97·74
Glucose 5 %, sodium glutamate 10 %	96·15
Glucose 10 %, sodium glutamate 10 %	94·87
Soluble starch 1 %, sodium glutamate 10 %	98·87
Soluble starch 10 %, sodium glutamate 10 %	98·83
Soluble starch 1 %, glucose 10 %	98·88
Soluble starch 10 %, Glucose 10 %	99·09
PVP 1 %, sodium glutamate 5 %	99·51
PVP 5 %, sodium glutamate 5 %	99·53
PVP 1 %, glucose 10 %	99·08
PVP 5 %, glucose 10 %	99·28
Distilled water	99·80

100° C. for 3 hr., again weighed and the sublimation rate* calculated from the results obtained (see Tables 5 and 6). From their affinity for water the media can be divided into two types. With the first group the rate of sublimation decreases markedly as the concentration of the medium increases; sodium glutamate and glucose belong to this type. No such decrease of sublimation occurs with the second group of drying media as their concentration is increased; PVP, soluble starch, dextran, etc., belong to this second group. Sucrose and lactose appear to stand between the two groups. When two media belonging to the first group are combined the sublimation rate is found to be lower than when either is tested singly. The combination of a medium in the first group with one in the second group results in a sublimation rate which is intermediate between the values obtained when the two media are tested singly.

Table 6 shows the results of culture tests of products, dried in different concentrations of the various drying media, immediately after drying and after boiling for 10, 30 and 60 min. With regard to the viability figures immediately after freeze-drying it will be noted that whereas with sodium glutamate, glucose, sucrose and

* The sublimation rate was calculated from the following formula:

$$\frac{\text{Weight of water vapour sublimated}\dagger}{\text{Weight of water contained in the original material}\ddagger}$$

† The difference between the weight of the material before drying and after drying for 6 hr.

‡ The difference between the weight of the material before drying and its weight after drying for a further 3 hr. at 100° C.

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lactose increasing their concentration resulted in improved viability, with drying media belonging to the second group, with the exception of dextran, the viability decreased as the concentration of the medium was increased. This effect appears to be related to the differences of the respective media in their affinity for water. Once again it will be noted that sodium glutamate-containing drying media were the most effective and of these the combination of PVP with sodium glutamate took first place.

Table 6. *Effect of type and concentration of drying medium both on rate of sublimation and on survival of bacteria*

Type and concentration of drying medium	Sublimation rate (%)	Log_{10} no. organisms		Time of boiling (min.)		
		Before drying	After drying	10	30	60
Sodium glutamate 1 %	99·89	—	7·7	5·0	4·1	2·6
Sodium glutamate 5 %	99·54	9·3	8·7	0	0	—
Glucose 1 %	99·76	—	7·8	0	0	—
Glucose 5 %	99·64	—	8·2	1·2	0	—
Sucrose 1 %	99·89	—	7·8	3·7	2·8	0
Sucrose 5 %	99·82	—	8·7	5·1	0	—
Lactose 1 %	99·90	—	7·4	1·0	0	—
Lactose 5 %	99·84	—	8·2	5·0	0	—
Lactose 10 %	99·80	9·5	8·6	0	0	—
PVP 1 %	99·88	—	7·5	0	0	—
PVP 5 %	99·88	—	6·9	0	0	—
Soluble starch 1 %	99·89	—	6·8	0	0	—
Soluble starch 5 %	99·91	—	6·2	0	0	—
Dextran 1 %	99·88	9·2	6·3	0	0	—
Dextran 5 %	99·87	—	7·3	2·7	0	—
PVP 1 %, sodium glutamate 5 %	99·89	—	8·5	6·1	5·8	3·0
PVP 5 %, sodium glutamate 5 %	99·86	—	8·2	5·8	5·8	4·6
Sucrose 5 %, sodium glutamate 5 %	99·13	—	8·8	0	0	—
Soluble starch 5 %, glucose 5 %	99·80	—	8·3	0	0	—
Lactose 10 %, sucrose 5 %	99·58	—	7·2	0	0	—
Lactose 10 %, soluble starch 5 %	99·85	9·2	8·2	0	0	—
Soluble starch 5 %, sodium glutamate 5 %	99·68	9·3	8·5	6·2	4·8	3·7
Distilled water	99·87	—	7·1	0	0	—

Streptococcus faecalis is considered to be more resistant to the freeze-drying process and to subsequent preservation than *Lact. bifidus*. We thought it would be of interest, therefore, to make some observations with this organism on the same lines as those made with *Lact. bifidus*, but with slight modifications in the drying media and the heat treatment. After freeze-drying ampoules were heated at 100° C. for 60 and 120 min. and also at 120° C. for 10 and 30 min. Table 7 shows that with sodium glutamate alone as the drying medium the survival rates after

heating at 100° C. were reasonably good, but with combinations of sodium glutamate and PVP, polyvinyl alcohol (PVA), or precipitated calcium carbonate in the experiments with heating at 120° C. the survival rates were significantly better.

Table 7. *Survival of dried* Streptococcus faecalis *after heat application*

Type and concentration of drying medium	Log_{10} no. organisms: Before drying	After drying	Time and temperature of heat application: 100° C. 60 min.	100° C. 120 min.	120° C. 10 min.	120° C. 30 min.
Sodium glutamate 3 %	10·5	9·9	9·0	8·9	6·5	0
PVP 5 %, sodium glutamate 3 %	10·0	10·0	8·8	8·3	7·3	3·8
SCMC 0·3 %, sodium glutamate 3 %	10·0	10·0	8·8	8·0	0	0
Sodium alginate 0·3 %, sodium glutamate 3 %	10·1	10·0	8·6	7·4	1·0	0
Soluble starch 3 %, sodium glutamate 3 %	10·1	9·8	8·6	8·0	6·1	0
Calcium carbonate, sodium glutamate 3 %	10·5	9·5	9·1	8·7	6·5	3·1
Lactose 12 %	—	10·8	8·9	3·6	0	0
Polyvinyl alcohol 1·5 %, sodium glutamate 3 %	—	10·5	8·4	7·5	7·3	4·0

Comparison of viability of various strains of lactobacilli sealed in air and in vacuo *after freeze-drying*

For preserving the stability of freeze-dried organisms vacuum-sealing of the ampoules is generally considered to be more satisfactory than sealing in air. We had, however, observed that dried *Lact. bifidus* exposed to atmospheric conditions in the ampoules before sealing had a high degree of stability and therefore thought it would be of interest to examine this point in more detail. Four cultures were used: *Lact. bifidus*, *Lact. bulgaricus*, *Lact. acidophilus* and *Strep. faecalis*. *Lact. bifidus* is a strict anaerobe and the other organisms are classified as micro-anaerophilic (Bergey, 1957), but *Lact. bulgaricus* is rather more anaerobic than the others. Using sodium glutamate as the drying medium the organisms were all freeze-dried in the same chamber desiccator. After the completion of the drying process, the air, dried by passing through a filter containing silica gel, was let into the chamber to release the vacuum. One half of the ampoules were then sealed without delay and the other half were sealed after being connected with a manifold and evacuated at $\frac{1}{10}$ mm. Hg. As will be seen from Table 8 there was no significant difference in preservability between the products sealed *in vacuo* and those sealed in air when the ampoules were stored at 5° C. Stored at 37° C. *Lact. bifidus* in the ampoules sealed in air showed a somewhat better survival rate than those sealed *in vacuo*.

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The survival of *Lact. acidophilus* appeared to be better in the ampoules sealed *in vacuo*, but with *Lact. bulgaricus* and *Strep. faecalis* there was no observable difference in the viability of the dried organisms whether sealed in air or *in vacuo*.

Table 8. *Effect of storage on viability of dried organisms in ampoules sealed under vacuum and at atmospheric pressure*

Kind of organisms and drying medium	Storage condition (° C.)		Log_{10} no. organisms: Before drying	After drying	Preservation period (months): 1	2	3	5	6
Lact. bifidus (2% S.G.)	37	Air	10·0	—	8·5	7·4	6·8	—	6·3
		Vacuum	10·0	—	8·2	6·9	0 (10^6)	—	5·4
	5	Air	10·0	9·0	9·0	—	8·8	—	8·7
		Vacuum	10·0	9·0	9·0	—	8·8	—	8·2
Lact. bifidus (3% S.G.)	37	Air	9·9	—	6·8	6·4	6·1	4·2	—
		Vacuum	9·9	—	6·3	6·2	6·1	0 (10^3)	—
	5	Air	9·9	9·3	—	—	9·0	8·6	9·1
		Vacuum	9·9	9·4	—	—	9·0	8·2	8·8
Lact. bulgaricus (2% S.G.)	37	Air	10·2	—	8·8	8·0	8·1	7·3	—
		Vacuum	10·2	—	8·6	7·9	6·5	—	—
	5	Air	10·2	9·2	9·2	—	9·2	8·9	8·9
		Vacuum	10·2	9·2	9·2	—	9·2	8·8	8·8
Lact. acidophilus (3% S.G.)	37	Air	10·3	—	9·6	9·0	8·5	—	0
		Vacuum	10·3	—	9·9	9·3	8·6	—	0
	5	Air	10·3	10·2	—	10·3	10·3	—	—
		Vacuum	10·3	10·3	—	10·3	10·2	—	—
Lact. acidophilus (3% S.G.)	37	Air	10·3	—	9·2	9·1	—	6·0	—
		Vacuum	10·3	—	9·4	9·5	—	6·0	—
	5	Air	10·3	10·3	10·3	10·3	—	—	10·2
		Vacuum	10·3	10·3	10·2	10·3	—	—	10·3
Strep. faecalis (2% S.G.)	37	Air	10·7	—	10·3	—	10·1	—	9·7
		Vacuum	10·7	—	10·3	—	10·0	—	9·6*
	5	Air	10·7	10·4	—	—	10·5	—	10·5
		Vacuum	10·7	10·4	—	—	10·5	—	10·4

Note. Figures in parentheses represent the degree of dilution in respective case.
* Products preserved *in vacuo* during the first 3 months, thereafter in atmospheric pressure.

DISCUSSION

The role of the drying medium in the freeze-drying of bacteria is unquestionably a very important one and variations in the type of drying medium used may affect differently their survival during and after the process of freeze-drying and also their viability on storage and reconstitution for use. In most of the early work on this subject various mixtures of substances were used for the drying media, and it was difficult to clarify the role played by the individual constituents. In our study of this problem we have examined first the relevant properties of relatively pure substances as drying media and later on examined the effect of combining substances, which have been previously investigated separately, in

various concentrations. In any such investigation it is important that the main study should be on one bacterial strain and that in preparing such cultures for freeze-drying they should be well washed to remove all traces of the culture medium in which they were grown. Residual culture medium might well seriously affect the proper assessment of a particular drying medium.

Previous studies had already indicated the value of sodium glutamate as a drying medium for the preservation of dried bacteria. Many of these studies were in connexion with the freeze-drying of BCG vaccine (Miller & Goodner, 1953; Cho & Obayashi, 1956; Obayashi & Cho, 1957; Obayashi, 1960; Muggleton, 1960). Greaves (1960) has also drawn attention to this drying medium for the gonococcus and some paracolon bacilli and spoken of its effectiveness in preserving viability, and Ota (1959) has already reported good results with sodium glutamate for freeze-drying *Lact. bifidus* which are fully confirmed by the results of the present studies.

Scott (1960) presented a thesis to indicate that a fundamental cause of death of dried organisms is the alternation in cell protein brought about by the reaction between compounds containing carbonyl groups and amino side chains of some essential constituents of the micro-organism. Substances containing carbonyl groups, added to the suspending fluids, will increase death in the dried state, whereas the addition of amino acids to the drying medium will improve the viability on storage. Although this hypothesis may be an interesting explanation of the mechanism of drying media, the supporting evidence is only circumstantial and not decisive.

While sodium glutamate may be an effective drying medium for the organisms mentioned above, it is not necessarily the most suitable for all micro-organisms. Yanagisawa, Kitaoka, Tagaya, Soekawa, Yamanouchi, Sawada & Suzuki (1960) found it effective for drying vaccinia virus but we (unpublished report) have not found it suitable as a drying medium for some types of yeasts. It is clear that other substances may prove to be more suitable drying agents for some organisms, and further investigations are obviously indicated.

The differing affinities of drying media for water during the drying process is also an important problem. Estimations of the sublimation rates of bacterial suspensions dried under exactly the same conditions but with different types and different concentrations of drying media show that they can be classified in two groups: those such as sodium glutamate and glucose where the rate of sublimation decreases as the concentration of the medium increases and the second group, which includes PVP, soluble starch and dextran, in which the rate of sublimation does not decrease with the increasing concentration of the medium. Combination with media from different groups gave intermediate values and combinations of media from the first group gave lower sublimation rates than when the media were used singly. Since media belonging to the second group when used alone failed to show any protective action, either during the actual drying process or during the subsequent storage of the dried product, their effectiveness when used in combination with sodium glutamate may be attributable solely to their adjuvant effect on the drying, and this may mean that the second medium is not indispensable

so long as the necessary degree of desiccation can be attained without its aid. In most cases, however, and especially when the concentration of the medium in the first group is relatively high, drying will be more readily achieved by the use of combined media, and moreover the stability of the dried organism will be increased.

In our general experience soluble starch, as a member of the second group for combination with sodium glutamate, was effective over a relatively wide range of bacteria, but in the present study on *Lact. bifidus* the most effective member of the second group for combination was PVP.

For reasons already indicated we do not think it appropriate to use either glucose or sucrose in combination with sodium glutamate. Unless glucose or sucrose is expected by itself to give protection to the bacteria during drying, their use for the purpose of retaining water during the drying process could be equally well met by increasing the concentration of the sodium glutamate. Glucose is rather unstable at high temperatures and freeze-dried preparations of glucose without bacteria tend to become discoloured by boiling, and unless there is any special reason for employing this substance its use in combination with sodium glutamate is not recommended.

The question of the optimal concentration of the drying medium can only be considered in conjunction with the concentration of the bacterial suspension and the method of lyophilization. The optimal concentration of the drying medium increases with the increasing concentration of bacilli in the suspension and, in the case of sodium glutamate, the residual moisture in the dried product will increase with the increasing concentration of the drying medium. If the residual moisture rises above a critical level a drop in the viability curve of the dried product will follow; this can, however, be overcome by additional drying. This has already been discussed in a previous paper by Obayashi & Cho (1957), but the present studies fully confirm the earlier observations. For the drying of BCG, Cho & Obayashi (1956) recommended 1% sodium glutamate and for the freeze-drying of gonococci and paracolon bacilli, Greaves (1960) recommended 5–10% sodium glutamate as the optimal concentration, but such differences must be considered in relation to the concentration of the bacilli and the drying technique employed. In the routine production of freeze-dried BCG vaccine in Japan, 1·5% sodium glutamate is commonly used for a bacillary concentration of 10 mg./ml., but when the latter is as high as 80 mg./ml., about 5% sodium glutamate is used. Similarly, when dextran is used as the medium of the second group for a combined drying medium, a relatively high concentration of the first group medium should be added.

We have confirmed the value of the Greaves (1960) boiling test for assessing the stability of dried bacteria and found good correlation between the results of boiling the dried product at 100° C. and the viability tests after storage at 45° C. for long periods. It seems probable that we shall be able to rely on the boiling test for predicting the useful life of the dried product, and certainly the method is very convenient for the comparison of the different effects of various drying media and also of different techniques of lyophilization.

Although we found that *Lact. bifidus*, which was the most anaerobic of the organisms studied, showed a somewhat better survival rate when stored in air

than when stored *in vacuo*, there was no significant difference between the two methods in the survival rates of the other anaerobic bacilli tested. At the present time there are insufficient grounds for connecting these facts with the anaerobic properties of the organisms, but there is, at least, some reason for reconsidering the customary belief that the viability of freeze-dried organisms sealed *in vacuo* is always better than that obtained when they are sealed in air.

SUMMARY

1. The use of sodium glutamate as a medium for freeze-drying *Lact. bifidus* significantly enhanced the stability of the dried product, especially the heat stability.

2. The optimal concentration of sodium glutamate, as a drying medium, was found to depend on the bacillary concentration; the higher the concentration of the bacillary suspension used the higher was found to be the optimal concentration of the sodium glutamate.

3. It was possible to classify various drying media according to the degree of sublimation of the product during the process of freeze-drying. Two types were observed; in the first, which included sodium glutamate and glucose, the sublimation rate diminished with the increasing concentration of the medium, whereas in the second group, which included polyvinyl pyrrolidon K 90 (PVP), soluble starch and dextran, no such diminution was observed when the concentration of the medium was increased.

4. By the combination of sodium glutamate or glucose with a medium belonging to the second group sublimation was found to be promoted.

5. The stability-conferring effect of sodium glutamate on dried *Lact. bifidus* was markedly enhanced when the glutamate was combined with a drying medium belonging to the second group. This effect is attributed to a desiccation-promoting property of the latter medium.

6. *Lact. bifidus* as a freeze-dried preparation showed a somewhat better survival rate when sealed in air than when sealed *in vacuo*. In the case of other anaerobic organisms tested there was no significant difference in the survival rates under the two methods of storage.

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