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Some Factors influencing the Survival of *Bacterium coli* on Freeze-drying

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SUMMARY: Quantitative studies have been carried out on the survival of bacteria on drying from the frozen state. Attention has been confined mainly to one species of vegetative bacteria, viz. *Bacterium coli*, of which several different strains were examined. When suspended in a phosphate buffer (pH 7.6) and dried from the frozen state (-85°), it was found that the percentage of organisms surviving varied widely with the strain used. With a given strain, it was found that a well-defined relationship existed between the concentration of organisms in the suspension before drying and the percentage of viable survivors recovered after reconstituting the dried product; the more dilute the suspension the lower the percentage of organisms surviving. When a low concentration of organisms was suspended in the cell-free filtrate obtained from a higher concentration of the same organisms, the percentage of viable organisms recovered approximated to the figure to be expected for the higher concentration of organisms. The dependence of percentage survival on the concentration of organisms in the suspension being dried was shown to be due to soluble material derived from the organisms themselves. On freezing alone, percentage survival was independent of the concentration of organisms in the suspension; the material affords protection during the drying stage of the process only. The protective effect of the material during freeze-drying was enhanced by the addition of glucose, especially during prolonged secondary drying.

The widespread development of freeze-drying apparatus and techniques in recent years has focused increasing attention on the preservation of bacteria by this means. The first real quantitative data on survival rates are to be found in the work of Stamp (1947), and this has since been followed by papers by Proom & Hemmons (1949) and Fry & Greaves (1951). The subject was briefly reviewed in Flösdorff's *Freeze-drying* (1949); various aspects are dealt with in the *Institute of Biology's Symposium on Freezing and Drying* (1951). Interest has so far centred on the practical problem of the preservation of organisms in a viable state in good yield and during storage for long periods, and work has been mainly directed to developing media and studying the conditions best fitted to achieve this goal. With the introduction of their 'mist. desiccans', a serum broth glucose mixture, Fry & Greaves (1951) appear to have made a notable advance in this direction.

The present work represents a departure from this general approach in that studies of survival on freeze-drying have also been carried out in the absence of any protective substances other than any that may be supplied by the organisms themselves. Under these conditions, it was found that the percentage of organisms surviving drying increased with the concentration of organisms in the suspension being dried. It is further shown that the phenomenon is the result of the presence in the suspension of soluble material derived from the organisms themselves.

METHODS

Roux bottles containing tryptic meat broth were inoculated with a pure culture and incubated for 18 hr. at 37°. The broth was then transferred to 1 oz. screw-cap bottles and centrifuged at 1600 g. for 15 min. The supernatant was poured off and the residue of bacteria drained, after which, having been re-suspended in phosphate buffer, it was shaken in a small machine for 10 min. This washing process was repeated three times, further washings having been found to have no further effect on the results presented. The aim was to obtain a suspension entirely free from the growth medium, and this consideration dictated the choice of broth instead of nutrient agar for this purpose. After the third washing, the bacterial deposit was finally re-suspended either in water or phosphate buffer, as required, with 20 min. shaking (4.5 cm. stroke, 450 r.p.m.) to ensure the dispersal of any clumps, and to give a bacterial concentration in the range 10^9 – 10^{10} organisms/ml. The suspension was placed in the cold room (+2°) for 2 hr. before use. The phosphate buffer used throughout this work was the standard sterile buffer developed in this department for general bacteriological use, as a dilution fluid for plate counts, etc. It was made up (in %, w/v) from KH_2PO_4 , 0.45; $(\text{NH}_4)_2\text{SO}_4$, 0.05; NH_4Cl , 0.05; adjusted to pH 7.6 by the addition of NaOH.

Serial dilutions were made in phosphate buffer with, usually, a tenfold dilution at each stage, except in the region where the final counts were made, when closer ratios were often used. Nine ml. volumes of phosphate buffer were placed in a set of 1 oz. screw-cap bottles, and 1 ml. of the suspension delivered into the first bottle from an accurate mark to mark pipette. After thorough mixing, 1 ml. of this diluted suspension was transferred (fresh sterile pipette each time) to the next 9 ml. volume of phosphate buffer, and so on until a suitable dilution for making the final counts was reached.

Counts were made by a modified Miles & Misra (1938) surface-culture method, 12 drops from each dilution being delivered from a calibrated dropping pipette (36 drops/ml.) on to each of six Petri dishes containing 30 ml. tryptic meat agar. The surface agar had been suitably dehydrated by pre-treatment in an incubator at 50° (1 hr. in the inverted position) such that the test 1/3 ml. delivered on the agar surface was absorbed into the agar in about 5 min. The colonies were counted after a suitable period of incubation at 37°.

The freeze-drying of suspensions

The Cryostat. This apparatus shown diagrammatically in Fig. 1 was designed primarily for the preparation by freeze-drying of histological specimens for microscopic examination. It was well adapted for the freeze-drying of small quantities (0.2 ml.) of bacterial suspensions, since the temperature throughout the drying process, a matter of considerable doubt with the usual types of freeze-drying equipment, could be controlled precisely. The part of the apparatus containing the ampoules was immersed in a bath of acetone contained in a 1 gal. thermos jar, and refrigerated by a copper coil connected via an automatic regulator to a refrigerator compressor. The bath temperature

was maintained within a degree or so of any desired temperature by a Sunvic thermostat which switched the compressor on and off via an inductive hot-wire relay. By using two domestic-type refrigerator units in cascade, bath temperatures down to -65° could be obtained using Freon 12 as a refrigerant. The 0.2 ml. samples contained in small tubes, and held in a rack, were rapidly frozen by dipping in the acetone bath for a few minutes. They were then transferred to the Cryostat, which had already been cooled to the desired drying temperature. The apparatus was evacuated to about $1\ \mu$. Hg pressure by means of an Edwards two-stage rotary oil pump, pressure being read on a sensitive McLeod gauge. Moisture was absorbed from the system by phosphorus pentoxide contained in a tray; such an absorbent is satisfactory for the small

Fig. 1

quantities of material being handled. At no time during the drying process can the temperature of the product exceed that of the bath, which therefore represents the maximum temperature of drying and, in fact, the temperature at which the final stages of the drying process are carried out. For very small amounts of material, an apparatus employing a refrigerated chamber is probably essential if true freeze-drying is to be achieved. Stamp (1947) described freeze-drying at a controlled temperature (-23°) by means of the McFarlane process.

It was found that the survival rates obtained under these conditions at temperatures down to -50° were not significantly different from those obtained using the department's standard equipment in which the low temperature of the drying mass is maintained by virtue of its own evaporation. Most of the present work has been carried out on this standard equipment, where larger volumes could be handled more conveniently and quickly.

The M.R.D. freeze-drying apparatus. This equipment, designed by one of us (B. R. R.) and built in the department's workshops, has not before been described in the literature, so a brief account will be given here.

The design has been based on the vacuum spin-freeze principle introduced by Greaves (1944), and was adopted in the first place to deal with the problem of freeze-drying from 8 lb. Kilner bottling jars (250 ml./jar) without the necessity for pre-freezing. The first machine accommodated eighteen of these jars, with the provision for vertical spinning of each jar about its own axis in the vacuum chamber, by means of a train of gears. The satisfactory results obtained with this machine led us to construct a second machine to accommodate sixty of the 1 oz. screw-cap type of bottle, each bottle being rotated at 2000 r.p.m. about its own vertical axis, by gear drive from a central shaft, driven from a motor external to the vacuum chamber.

This departure from the usual practice of placing the ampoules or bottles in inclined holes drilled in a large rotor, while no longer as essential as with the larger size of bottle, has the merit of distributing the contents of each bottle in a thin film of approximately uniform thickness round the entire wall of the bottle, instead of in the form of a wedge at a lower corner. Much more rapid and uniform drying is thereby achieved, 10 ml. quantities being dry in about 4 hr. without any heat being applied.

Pl. 1, fig. 1, shows the external view of the vacuum chamber. The refrigerated coil occupying the lower part of the chamber is maintained at -45° by a 1 h.p. compressor, located behind the panel in a sound-proof compartment. This also contains the vacuum pump (Edwards, 2S 150) of the two-stage rotary oil type. Pl. 1, fig. 2, shows an interior view of the upper half of the chamber, with the sixty gear-driven containers. The temperature of a representative sample is recorded during the drying process by means of a fine wire thermocouple connected to a copper:constantan slip ring assembly fixed to one of the containers, as seen in the illustration.

The bottles are spun for the first 20 min. or so only, by which time the vacuum has reached about 0.1 mm. Hg and the product temperature -25° , after which the spinner motor can be stopped and the drying allowed to proceed in the usual manner. With a coil temperature of -45° , the product assumes a temperature of about -40° with uncapped bottles, or about -35° when aseptic precautions are taken by the fitting of sterile cotton-wool and gauze caps, as in the present work. From the chart recording product temperature, it appears that freezing is practically instantaneous, there being no evident break in the curve at any point. An appreciable part of the water in the solution is evaporated in the initial stage of the process in withdrawing heat to freeze and to cool the remainder down to -35° . A further quantity is lost in cooling the walls of the bottle down to this temperature and for this reason it is not advisable to use too small a volume of solution per bottle if a true freeze-dry is to be ensured. Most of this work was carried out with 5 ml./bottle which provides an ample margin. If the volume greatly exceeds 15 ml. loss of liquid occurs from the neck of the bottle during the spinning stage.

While primary drying is complete in about 4 hr., we have, as a routine in this work, continued the process overnight so that all samples have had a period of 24 hr. in the primary chamber. The results obtained after this treatment are referred to as the survival on primary drying only. In a number of cases we have continued the drying of the primary dried product by storing in a vacuum of <0.01 mm. Hg at room temperature over phosphorus pentoxide.

In quantitative work involving freeze-drying it is necessary to be on guard in case material should be ejected from the bottle during the drying process—the so-called ‘spill’ (see, for example, S. T. Cowan, p. 127, and Wilson Smith, p. 139, of the *Inst. Biol. Symp.* 1951). This is especially likely to occur with dilute salt solutions, or with particle suspensions in water or dilute salt, where the finely divided dried layer on the inside appears to be carried away by the high-velocity vapour stream emerging from beneath it. Sodium chloride is notorious in this respect, nearly half being ejected during the freeze-drying of a 1% solution from an open-necked bottle. The phosphate buffer used for the present work is by no means exempt, losses of 10–20% being found with open bottles. With the gauze/cotton-wool drying caps on the bottles, however, gravimetric determinations showed no significant loss of phosphate buffer from the bottle under the conditions used. (With 1% NaCl a heavy deposit piles up on the underside of the cap.)

RESULTS

Comparison of six different strains of coliform bacteria

Suspensions containing approximately 10^6 orgs./ml. in phosphate buffer were freeze-dried. The survival figures after primary drying are given in Table 1.

Table 1. *Survival of various strains (approx. 10^6 orgs./ml. in phosphate buffer) after freeze-drying*

Strain	Viable survivors (%)
<i>Bact. coli communis</i>	1.4
<i>Bact. coli</i> ‘Jepp’	0.2
<i>Bact. aerogenes</i> (NCTC 418)	0.17
<i>Bact. coli</i> ‘1100’	0.06
<i>Bact. coli</i> (NCTC 4450)	0.01
<i>Bact. coli</i> American Type B	0.006

It is seen that the survival under given conditions depended to a great extent on the strain of organism used. The most sensitive ‘American type B’ and a fairly robust strain ‘Jepp’ were selected for more detailed study.

The effect of cell concentration on survival

The question of cell concentration has been largely ignored in studies on the effect of freezing, and freeze-drying on viability. Stamp (1947) asserted that in the drying of *Streptococcus pyogenes*, *Pasteurella pestis* and *Salmonella typhimurium* in nutrient gelatin + ascorbic acid, the percentage survival rate rose with a diminution in cell concentration. The opposite effect was reported by

Otten (1930) who dried various pathogenic bacteria in saline at reduced pressure in a desiccator over H_2SO_4 . Fry & Greaves (1951) were unable to demonstrate any significant difference in the survival rate of paracolon D20 IM over the range 4.7×10^9 to 4.7×10^4 orgs./ml. when freeze-dried in their 'mist. desiccans'. In the absence of any protective medium, we found that the concentration of organisms in the suspension being dried had a very marked effect on the percentage surviving freeze-drying, the survival rate falling with diminishing cell concentration. The experimental points in Fig. 2 were obtained from three independent experiments, with *Bact. coli* 'Jepp' freeze-dried in phosphate buffer. The curve is drawn to indicate the general trend.

Fig. 3 shows a similar series of results obtained with the fragile 'American B' strain of *Bact. coli*. In this case there were no survivors immediately after freeze-drying a 10^4 orgs./ml. suspension.

Fig. 2. Freeze-drying of *Bact. coli* Jepp in phosphate buffer. (24 hours drying).

Fig. 3. Freeze-drying of the American 'B' strain of *Bact. coli* in phosphate buffer.

It appears at first sight that the surviving organisms are protected by the proximity of their moribund neighbours, and indeed, Otten (1930) had already shown that a killed suspension of the same or even of a different organism had a protective action on living organisms dried in saline under the conditions used by him (reduced pressure over H_2SO_4). In the absence of clumping, however, such a hypothesis is untenable in the case of true freeze-drying since in the completely frozen condition, below the eutectic point of the suspending medium, each organism is fixed firmly in position out of range of its neighbours. It becomes necessary to postulate, therefore, the presence of some material derived from the organisms themselves, and already in solution before freeze-drying commenced, and it seemed reasonable to suppose that the concentration of this material present in solution would depend on the concentration of organisms in suspension, thus explaining the concentration/survival relationship observed.

The effect of the cell-free supernatant on survival

The presence of a soluble protective substance was readily demonstrated by taking the cell-free supernatant (obtained by centrifuging and filtering through a sintered glass filter) from a 10^{10} orgs./ml. suspension of *Bact. coli* 'Jepp' in

phosphate buffer, the suspension having been made up 2 hr. before. A 10^4 orgs./ml. suspension of the same organism when freeze-dried from the supernatant showed a survival of 5.7 % as against a value of about 0.2 % for a 10^4 orgs./ml. suspension in phosphate buffer only. In fact, the 10^4 orgs./ml. suspension now showed the same survival as that for a 10^{10} orgs./ml. suspension.

It is evident, therefore, that the organisms release, either by secretion or by the lysis of a small fraction of their number, a substance which, even in very low concentrations, has a remarkable effect in protecting viable cells against damage during freeze-drying.

Preparation of the bacterial exudate from Bact. coli 'Jepp'. By freeze-drying an aqueous suspension of *Bact. coli*, reconstituting with water, and freeze-drying a second time, a product was obtained practically devoid of living cells (0.00001 % survivors). This contained a large proportion of the bacteria in solution, e.g. a 10^{10} orgs./ml. suspension of well-washed *Bact. coli* 'Jepp' treated in this manner, gave after centrifuging down the cell debris (and Seitz filtration), a solution containing 0.15 % soluble material. This was dispensed in screw-capped bottles and stored in the freeze-dried state.

The effect of the bacterial exudate on survival. The freeze-dried bacterial exudate thus obtained was taken up in phosphate buffer and serially diluted in tenfold steps. A freshly washed suspension of *Bact. coli* 'Jepp' was added to each tube to give a final concentration of approximately 10^4 viable orgs./ml. This is a convenient concentration of organisms for exploring the effect of any additive on survival, there being usually sufficient survivors to count with fair accuracy without at the same time sufficient organisms present to influence the result to any extent. The results are given in Table 2.

Table 2. *The survival of Bact. coli (Jepp) (10^4 orgs./ml.) after freeze-drying in varying concentrations of Bact. coli exudate*

Concn. exudate (g./100 ml.)	Survival (%)
0.80	12.6
0.80×10^{-1}	8.7
0.80×10^{-2}	4.0
0.80×10^{-3}	1.4
0.80×10^{-4}	0.66
0.80×10^{-5}	0.34
0.80×10^{-6}	0.24
Control: phosphate buffer only	0.21

The effect of prolonged secondary drying

When the freeze-dried suspensions of *Bact. coli* 'Jepp' in phosphate buffer were subjected to extended and vigorous drying over P_2O_5 under a vacuum of 0.01 mm. Hg or less, the fall in viability was rather rapid. Fig. 4 shows the general trend; after 21 days secondary drying a 4×10^8 suspension contained only 0.1 % survivors, while a 4×10^7 suspension had no survivors.

When dried in the presence of the prepared *Bact. coli* exudate (approx. 0.05 %) more stable conditions obtain, e.g. a 10^8 orgs./ml. suspension of *Bact.*

coli 'Jepp' which had a survival after primary drying of 20% showed a viability of 4% after 7 days drying, 2.8% after 21 days and 2.1% after 58 days. It was not, however, until the addition of glucose (Fry & Greaves, 1951) that any real stability was obtained. Thus, in the presence of *Bact. coli* exudate (0.25%) and 5% glucose the figures shown in Table 3 were found for *Bact. coli* 'Jepp'. The sensitive 'American B' strain of *Bact. coli* dried under the same conditions showed some fall in viable count in the initial stages, but settled down to a figure of about 10% survival.

Fig. 4. Freeze-drying of *Bact. coli* Jepp in phosphate buffer. Prolonged secondary drying.

Table 3. *Survival of Bact. coli* 'Jepp' after secondary drying of suspensions containing *Bact. coli* exudate (0.25%) + glucose (5%)

Concn. of organisms before drying (orgs./ml.)	Survival after secondary drying for (days)					
	1	16	30	59	188	330
	Survival (%)					
1×10^8	51	44	41	37	41	38
1×10^4	42	39	38	36	34	35

DISCUSSION

The role played by the concentration of organisms in a suspension in determining the resulting survival after freeze-drying has been investigated in detail so far only in the coliform group of bacteria, though we have found a similar

though less pronounced effect in the case of *Chromobacterium prodigiosum* (*Serratia marcescens*), and the absence of a gradient with *Corynebacterium xerosis*. Independently, Hutton & Shirey (1951) observed a similar effect with *Brucella abortus*, though only over a very limited range of concentration. It would appear that the phenomenon may be expected to occur with any species of organisms which are easily disrupted and release the protoplasmic contents of some of their cells into solution. The phenomenon is of course masked in the presence of a medium with very strong protective properties, and it is not therefore surprising that Fry & Greaves (1951) failed to find any concentration effect in the case of bacteria dried in their 'mist. desiccans'. Even cultures of *Bact. coli* grown on nutrient agar tend to give variable results owing to the presence of free particles of agar in the washed suspensions.

The effect of the age of a suspension on the percentage survival has not been investigated, but it appears probable that the concentration of material in solution would increase with the duration of storage and so result in higher survival rates, always supposing that the viable cells surviving still retain their original robustness. The initial shaking of the suspension, an essential step towards securing a dispersion of single organisms free from clumping, doubtless has a similar effect in breaking down the weaker cells and in raising the concentration of material in solution. The effect of shaking on the destruction of *Bact. coli* is dealt with in detail in a recent paper by Furness (1952).

Since freezing is an inseparable preliminary part of the freeze-drying process, it was of interest to examine the effect of bacterial concentration and of added bacterial exudate in protecting organisms against death on freezing alone. A single rapid freeze (-78°) and thaw of a *Bact. coli* 'Jepp' suspension in phosphate buffer resulted in a 40 % loss of viable organisms regardless of the concentration of organisms in the suspension over the range from 10^{10} to 10^4 orgs./ml. and was not appreciably affected by the addition of bacterial exudate. It therefore appears that the exudate offers no protection against damage by ice crystal formation, concentration of salts, etc., but is effective only during the drying stage of the freeze-drying process.

Whether the protective action during the drying stage is due to the formation of a layer of colloidal material which reinforces the fragile cell envelope, to the retention of a certain minimal amount of moisture in the cell or to other causes is not at present understood. That free moisture is in fact essential to survival would appear open to question in view of the results obtained in the presence of coli 'extract' with the addition of 5 % glucose, where negligible loss in viability occurred after nearly one year of continuous rigorous drying (see Table 3). If moisture is essential, clearly the amount required is infinitesimal.

The physico-chemical properties of the bacterial exudate have only been briefly investigated. It passes very slowly, if at all, through a cellophane membrane; after 4 days dialysis at 2° , the presence of protective material in the dialysate was still in doubt; from its behaviour in the ultracentrifuge it appears to be very polydisperse. Its protective effect is not specific for the organism from which it is prepared, e.g. the material obtained from *Bact. coli* offered substantial protection to *Chr. prodigiosum*. Whether the material, or

some component of it, has any property which makes it unique in the field of bacterial preservation remains still to be assessed.

Of chief importance perhaps is the realization of the existence of the phenomenon described, namely that under adverse conditions, such as the freeze-drying of aqueous suspensions of organisms, survival may depend to a large extent on their concentration, the survivors deriving protection from a material supplied by the organisms themselves, and a degree of protection proportional to the concentration of this material.

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EXPLANATION OF PLATE

- Fig. 1. External view of vacuum chamber. The compressor for the -45° refrigerated coil situated in the lower part of the chamber, and the Edwards two-stage rotary oil type vacuum pump are located behind the panel on the left in a soundproof compartment.
- Fig. 2. A view of the interior of the upper half of the chamber showing the sixty gear-driven containers. The fine-wire thermocouple for temperature recording during the drying process is shown fixed in one of the containers.

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