THE SURVIVAL OF BACTERIA DURING AND AFTER DRYING

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(With 9 Figures in the Text)

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I. INTRODUCTION

In the past 40 years numerous authors have reported survival of micro-organisms after drying by various methods, usually *in vacuo* and often at low temperatures. Two recent papers on this subject are those by Stamp (1947) and Proom & Hemmons (1949), and since both these authors give full references to most of the earlier work of importance we do not propose to review this. It may be noted, however, that very few authors have produced quantitative results, being content for the most part with demonstrating the survival of *some* organisms. A notable exception is Stamp, who gives a very complete quantitative record of survival rates of his cultures, not only immediately after drying, but after storage for several years. Proom & Hemmons also give a few tables showing survival rates, but in only one table are these carried beyond the end of the drying process, and the survival after long storage is for the most part estimated by the possibility of getting some growth from a tube. By the time the cultures have reached the stage when any individual tube fails to show any survivors (see Proom & Hemmons, 1949, Table 2), it is clear that the survival rate in other tubes which are

not completely sterilized must be extremely low, and there is no certainty that the remaining few organisms are a representative sample of the original culture before drying.

Our efforts have therefore been directed to devising methods by which we could get survival rates of at least 1 % over a period of years. With organisms which are not very sensitive to drying this figure has been greatly exceeded, and though with more sensitive organisms we have had to be content sometimes with survival rates of the order of 0·1 %, even this may represent an actual viable count of between 10^5 and 10^6 organisms/ml., so that further survival of the culture is assured for a long period.

Most of the previous workers have devised a technique and employed it on a large number of different organisms. It appeared to us that there was a great dearth of information about what actually happens during the drying process, and the following points in particular needed clarification:

(1) The effect of changes in the suspending fluid.

(2) The effect of changes in the age of the culture, and in the cell concentration of the suspension.

(3) The time and conditions under which death occurs during the drying process.

To investigate these points it was clearly undesirable to start with a great variety of organisms, and we decided to work first with but one strain which should be capable of giving a suspension free from large clumps, and colonies which should be easy to count in pour-plates. It was also desirable that the selected strain should be moderately sensitive to drying by ordinary methods in order that improvements in results might be demonstrated. All these conditions suggested a member of the genus *Bacterium* as a suitable organism, and we started work with a paracolon bacillus, 'D. 201 H', which had been sent to us for preservation by Dr Joan Taylor. Later, we applied the results obtained with this to other more delicate organisms.

II. TECHNIQUE

(*a*) *Preparation of suspensions*

In order to avoid chance errors due to the presence of products of metabolism or to changes in pH, organisms were not dried in the medium in which they had been grown. They were grown on a solid medium (nutrient agar in the case of 'D. 201 H'), and some of the growth was scraped off and emulsified in the suspending fluid. Occasionally, when two or more fluids were being compared in one experiment, a thick suspension was made in N/4 Ringer, and equal small amounts of this were added to the different fluids. In most of our earlier experiments we used young cultures (4–6 hr. old) on nutrient agar, believing that these would show the best survival. As is shown later (p. 229) we were mistaken.

(*b*) *Methods used for viable counts*

Whenever possible, pour-plates were used, and three separate plates were poured for each dilution of the bacterial suspension. When dealing with organisms

that would not produce easily countable deep colonies, the surface method of Miles & Misra (1938) was used. Full details of the methods used for diluting and counting the suspensions are given in Appendix 2.

(c) *Drying technique*

Several different methods have been used, and are described in detail in Appendix 1. They may be summarized as follows:

Method A. Drying *in vacuo* at $-180°$ C., using a liquid air condenser.

Method B. Drying *in vacuo* from the frozen state at relatively high temperatures by the 'spin-freeze' method, using either a refrigerated condenser or P_2O_5 to absorb water.

Method C. Drying at reduced pressure at room temperature by the method of Stamp (1947).

III. THE EFFECT OF THE SUSPENDING FLUID ON THE SURVIVAL RATE

Before this work started, we had already laid down a stock of dried cultures of *Streptococcus pyogenes*, suspended for drying in Hiss's serum water. This medium appeared to give good survival with this and similar organisms, but our obser-

Table 1. *Showing the percentage survival of* 'D. 201 H' *dried in broth and nutrient gelatin in a number of different experiments.* (*Drying technique* '*B*')

Drying medium	Exp. no.	Drying time (hr.)	Age of culture (hr.)	Percentage survival after 1 day	1–3 months	4–7 months	9–14 months	18–22 months	36–42 months
Broth	28.*b*1	18	4	9	—	—	12	9	—
	28.*b*2	336	4	—	—	—	6·5	1·8	4
	37.*s*	18	5	1	—	2	—	1	—
	43.*a*	18	4	0·2	—	0·07	—	—	—
	56.*a*	24	6	6	—	—	—	—	—
	58	24	5	10	—	—	—	—	—
	59	24	5	4	—	—	—	—	—
	62.*a*	24	4½	3·4	—	—	—	—	—
	63.*a*	72	16	24	19	—	—	—	—
	64.*a*	72	4½	7	6	—	—	—	—
	107	48	20	10	6	6	—	—	—
Nutrient gelatin	6.*b*	168	6	25	4	3	—	—	4·5

vations were not based on quantitative work. When we used this medium for drying other organisms the results were very different, *Staphylococcus aureus* being reduced to less than 1 % after 24 hr. drying, and to 0·003 % after 12 months storage. Our paracolon bacillus, 'D. 201 H', and a strain of *Salmonella typhimurium* showed even lower survival rates, being reduced to about 0·0001 % in 1–2 months. It was quite clear that serum water was an unsuitable medium, and other suspending fluids must be tried.

Table 1 shows the survival rates per cent of 'D. 201 H' in a number of different experiments after drying in tryptic digest broth and nutrient gelatin, both immediately after drying and after storage for periods up to 3½ years. These were

all dried by method B, using either P_2O_5 or a refrigerated condenser. In Table 2 are shown the results of a number of similar experiments, in which the same organism was dried in five different solutions not containing broth. From these two tables it is seen that, with the exception of the first experiment in serum, broth or nutrient gelatin give much higher long-term survival rates than the broth-free media. The 24 hr. survival rates in broth and nutrient gelatin show considerable variation, presumably depending largely on the constitution of the different batches of broth, but most lie between 5 and 10 % and one is even higher. The two which have been followed for 3–3½ years show final figures of

Table 2. *Showing the percentage survival of* 'D. 201 H' *in different drying media not containing broth.* (*Drying technique* '*B*')

Drying medium		Exp. no.	Drying time (hr.)	Age of culture (hr.)	Percentage survival after: 1 day	3–9 days	1 month	7–14 months	22–27 months	36–42 months
Serum		24.*a*	18	6	—	—	8	10	10	—
		24.*b*	336	6	—	—	0·2	0·6	0·1	10^{-5}
Haemo-	5 %	73	18	4	6	—	—	0·03	—	—
globin	10 %	102.*a*	2½	17	2·2	0·6	0·1	8×10^{-5}	—	—
	10 %	102.*b*	24	17	1·4	0·2	0·01	8×10^{-6}	—	—
Albumin	6 %	15	120	4½	0·03	5×10^{-4}	0	—	—	—
	5 %	99	24	18	0·4	—	—	—	—	—
	5 %	100.*a*	2½	18	6·5	1·4	0·9	4×10^{-5}	—	—
	5 %	100.*b*	4½	18	3	0·6	0·1	2×10^{-5}	—	—
Gum acacia	2·5 %	19	72	5	11	0·2	0	—	—	—
	5 %	19	72	5	15	0·1	0	—	—	—
	10 %	19	72	5	14	0·2	0	—	—	—
	20 %	19	72	5	12	0·1	0	—	—	—
Aqueous	10 %	27.*a*	5	17	2·1	—	1·2	—	—	0·03
gelatin	10 %	27.*b*	336	17	—	—	0·4	—	—	10^{-4}
	10 %	89.*a*	18	5	2	—	—	$<10^{-3}$	0	—
	10 %	92.*a*	—	4	0·2	—	—	$<6\times10^{-4}$	0	—
	10 %	93.*a*	—	4½	0·3	—	—	$<2\times10^{-3}$	0	—
	5 %	101.*a*	2½	20	5·5	3	2·2	0·06	—	—
	5 %	101.*b*	24	20	0·9	0·1	7×10^{-3}	4×10^{-5}	—	—

about 4 %, and most of those which have been counted at from 1 to 2½ years show figures comparable with this. In Table 2, on the other hand, the five broth-free media show for the most part a moderately good survival rate at 24 hr., but all the survival rates, with one exception, fall off rapidly, being below 1 % at a month and sterile or nearly sterile at 1–3 years. It seems clear that the nature of the protective colloid may have a considerable effect on the survival rate, and we cannot agree with Proom & Hemmons (1949) in their statement that it is not very important.

The single exception, shown in the first line of Table 2, illustrates what we believe to be a fundamental point. The tubes in this line had received 18 hr. preliminary drying only, while those in the second line of the table were part of the same experiment, done under identical conditions, except that they were dried for a further 14 days on the secondary manifold. The survival rates are consistently much higher in the tubes which had not been completely dried. The same effect is seen with albumin, aqueous gelatin and haemoglobin. The last two

lines in each of these sections of Table 2 represent experiments in which half the tubes were sealed after only 2½ hr. drying, the remainder being dried for a longer period, 4½ hr. in the albumin experiment and 24 hr. with gelatin and haemoglobin. In all three experiments the tubes dried for the shorter time show a much higher survival rate, not only at 24 hr., but after storage for periods up to 1 year. This point is discussed more fully later (p. 234), but it appears that, when using simple protein solutions, it is essential that the water should not be completely removed.

(a) The effect of glucose added to the suspending fluid

In 1944 Leshchinskaya claimed that B.C.G. could be preserved best by drying in a 50 % aqueous solution of glucose. Because of the difficulty of making accurate measurements with such a viscous fluid we decided to do a trial experiment with 20 % glucose, and Table 3 shows the results of drying 'D. 201 H' in this solution.

Table 3. *Showing the percentage survival of* 'D. 201 H' *after drying in* 20 % *aqueous glucose.* (*Exp.* 26, *drying technique* '*B*')

	Percentage survival after					
Drying time	5 hr.	24 hr.	6 days	15 days	31 days	3 years
5 hr.	24	7	1	0·8	0·1	5×10^{-5}
5 days	—	—	0·8	1·1	1·7	0·4

Table 4. *Showing the percentage survival of* 'D. 201 H' *after drying in different concentrations of aqueous glucose.* (*Exp.* 69, *drying technique* '*B*')

	Dried 20 hr. Percentage survival after			Dried 48 hr. Percentage survival after	
Concentration of glucose (%)	24 hr.	2½ months	28 months	2½ months	28 months
2·5	13	1	$<10^{-2}$	0·9	$<10^{-3}$
5	27	4	$<10^{-3}$	6	1
10	27	$<10^{-1}$	—	6	1
20	30	$<10^{-3}$	—	4	$<2 \times 10^{-3}$
40	13	$<10^{-3}$	—	$<10^{-3}$	$<2 \times 10^{-5}$

The tubes were again divided, half being taken off and sealed after 5 hr. drying and the remainder being dried for 5 days. Here the effect of short drying is reversed, the higher survival rates being in the tubes dried for the longer period. The survival in the 5-day tubes was encouraging, though not as good as that in broth or nutrient gelatin.

Table 4 shows a similar experiment, using five different concentrations of glucose. After 24 hr. all show a fairly good survival rate, but after storage for 28 months the only tubes showing a reasonable survival are those dried in 5 and 10 % glucose which had received the longer drying, the others being practically sterile.

The addition of glucose to nutrient broth gives a somewhat similar result, with a survival rate higher than that given by either broth or glucose alone. Fig. 1 shows in graphical form the survival rate immediately after drying for 24 hr. in

broth containing different concentrations of glucose, in four similar experiments done at different times with different batches of broth. The curves are all of approximately the same shape, all showing a steep rise up to 5 or 10 % of glucose. The top curve, showing 70 % survival in 10 % glucose broth, was obtained with a batch of broth that had been deliberately overheated during sterilization. The very high survivals in this curve were not maintained on storage, and after about a month they had fallen to about the same level as in the other three experiments.

These curves show that, with the particular test organism in use, the addition of between 5 and 10 % glucose to broth produced a large increase in the survival

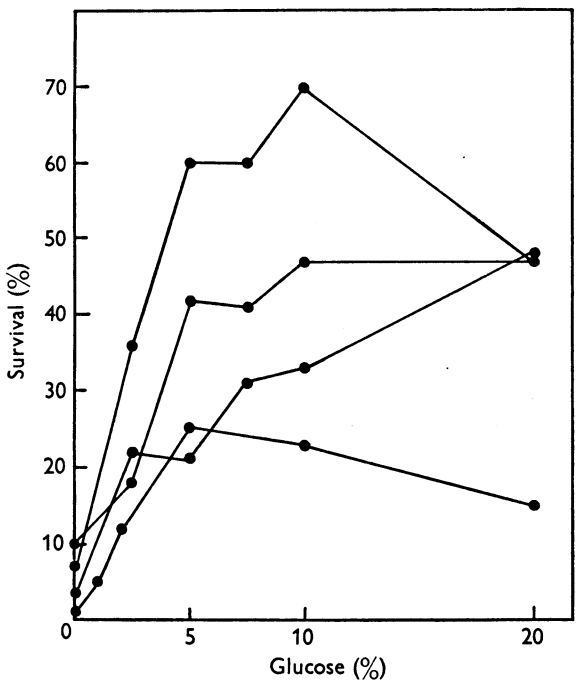

Fig. 1. Showing the percentage survival of 'D. 201H' immediately after drying in nutrient broth with varying concentrations of glucose, in four different experiments. (Drying method 'B'.)

rate. Table 5 shows the results of all our experiments with 'D. 201H' in broth containing from 5 to 10 % of glucose, giving the survival immediately after drying and after various periods of storage, up to 3 years. It is seen that the initial survival rate is generally well maintained, the fall after 1–6 months being very slight.

(*b*) *The effect of carbohydrates other than glucose*

It was clearly important to find out if this protective effect was peculiar to glucose, and four other carbohydrates, lactose, sucrose, raffinose and xylose, were therefore tested in the same way, using several different concentrations in each case. Fig. 2 shows the survival rates of 'D. 201H' immediately after drying in these sugar-broth solutions. With the exception of sucrose they all show curves somewhat similar to the glucose-broth curves, with maximum survival in the

Table 5. *Showing the percentage survival of* 'D. 201H' *after drying in broth containing from* 5 *to* 10% *glucose.* (*Drying technique* '*B*')

Exp. no.	Glucose (%)	Age of culture (hr.)	Drying time (hr.)	Percentage survival after 1 day	1 month	4½–6 month	8–13 months	19 months	22–31 months	36 months
37.*b*1	5	5	18	18	17	14	—	17	10	—
37.*b*2	5	5	192	—	10	13	—	6	—	11
37.*c*1	5	5	18	17	—	—	—	17	17	13
37.*c*2	5	5	192	—	—	—	—	7	—	7
37.*l*1	5	5	18	25	—	—	—	10	—	—
37.*l*2	5	5	192	—	—	15	—	—	—	—
37.*m*1	5	5	18	25	—	20	—	—	—	—
37.*m*2	5	5	192	—	—	34	—	—	—	—
37.*h*1	10	5	18	23	—	—	—	13	—	—
37.*h*2	10	5	192	—	—	21	—	—	—	—
37.*k*1	10	5	18	25	25	—	—	—	—	—
37.*k*2	10	5	192	—	25	—	—	—	—	—
38.*k*	7·5	6½	192	13	—	—	—	—	—	14
64.*c*	5	4½	72	60	24	—	—	—	—	—
64.*d*	7·5	4½	72	60	16	—	—	—	—	—
64.*e*	10	4½	72	70	31	—	—	—	—	—
67.*c*	7·5	4	18	8	—	—	1	—	—	—
67.*d*	7·5	4	18	8	—	—	2	—	—	—
95	7·5	15	24	50	—	—	44	47	55	—
96	7·5	18	24	54	—	65	—	—	—	—

In addition, nine other experiments were put up in 5, 7·5 or 10% glucose broth, which were only counted after 24 hr. These showed survival rates ranging from 16 to 47%, with a mean survival of 32%.

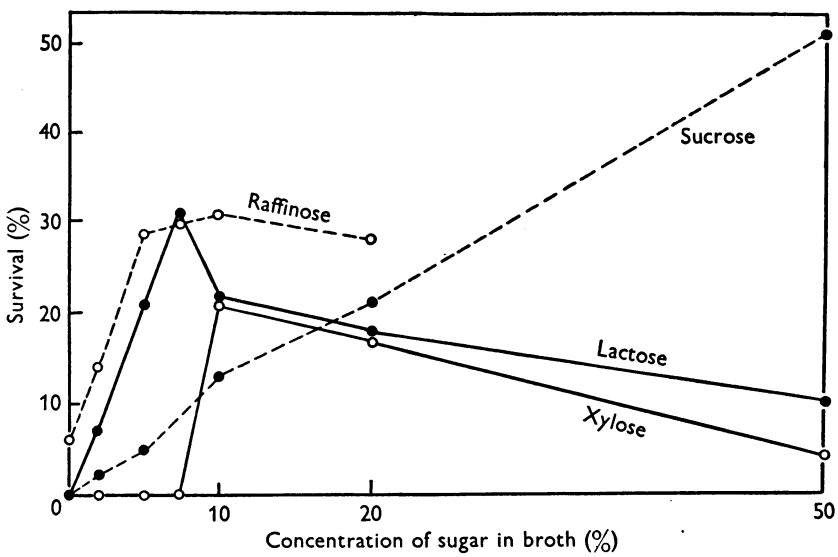

Fig. 2. Showing the percentage survival of 'D. 201H' immediately after drying in nutrient broth containing varying concentrations of four different carbohydrates. (Drying method 'B'.)

region of 5–10 % of carbohydrate. Xylose differs slightly from the other three in that there is practically no survival below 10 % concentrations, but this is not supported by one later experiment in which 5 % xylose broth gave a 24 hr. survival rate of 10 %. Sucrose is quite different from the other carbohydrates, giving a steady increase in survival as the concentration is raised up to 50 %. A further experiment with still higher concentrations suggested that the maximum survival is at about 50 % sucrose, and falls off as the concentration is further increased. It was, however, impossible to get these strong solutions of sucrose even approximately dry, so that these results have little significance.

These curves suggest that the action of glucose may be shared by many other sugars, and it is of some interest to see the long-term survival in these sugar-broth mixtures. Fig. 3 shows the survival in different strengths of lactose broth after

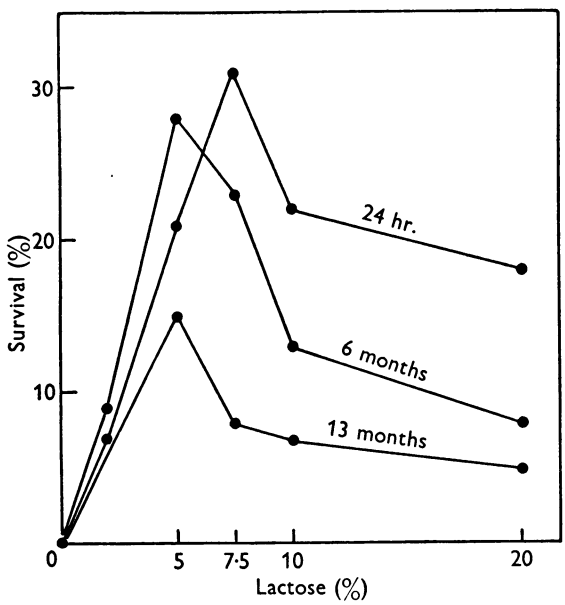

Fig. 3. Showing the percentage survival of 'D. 201H' 24 hr., 6 months and 13 months after drying in nutrient broth containing varying concentrations of lactose. (Drying method 'B'.)

24 hr., 6 months and 13 months. There is a gradual drop in survival rate with length of storage, but the shape of the survival/concentration curves remains much the same. These results with lactose show that, like glucose, it gives a good long-term survival rate. Sucrose broth showed only a moderate drop in survival after 3 years' storage in the range from 5 to 20 % sucrose, but the survival of cultures dried in 50 % sucrose broth, the optimum concentration for immediate survival, had dropped to about 1 % in 6 months. Xylose and raffinose have not yet been fully studied over long periods, but a 21 % survival at 24 hr. in 10 % xylose broth was reduced to less than 0·002 % in 3 years. This result illustrates clearly the danger of relying merely on a good survival rate immediately after drying.

(c) *The effect of changes in the protective colloid*

Table 6 shows the results obtained after drying 'D. 201 H' in most of the protein solutions shown in Table 2 and a few others not in that table, 7·5 % glucose being added to each. A comparison between Tables 2 and 6 shows that in every case the addition of glucose produces a big increase in the initial survival rate, after 24 hr. drying, but this is not always maintained after storage for long periods. This is especially noticeable with aqueous gelatin, where the cultures are almost sterile after 1 year and sterile at about 2 years. The weaker haemoglobin and 5 % albumin also show very little survival after 1 year in the tubes which

Table 6. *Showing the percentage survival of* 'D. 201 H' *in different drying media, each containing* 7·5 % *glucose.* (*Drying technique* '*B*')

Suspending medium with 7·5 % glucose		Exp. no.	Drying time (hr.)	Age of culture (hr.)	Percentage survival after: 1 day	2–3 weeks	2–3 months	4–7 months	8–14 months	24–30 months
Serum		82	18	6	42	—	—	25	12	14
Haemoglobin	5 %	73.*b*1	18	4	64	—	4	0·04	—	3×10^{-5}
	5 %	73.*b*2	48	4	—	—	10	2	—	0·02
	10 %	78	18	5	43	—	—	—	7	3·5
	10 %	105	18	24	22	—	—	—	—	—
Albumin	5 %	72.*a*	20	5	87	—	31	—	0·1	—
	5 %	72.*b*	48	5	—	—	35	—	8	—
	25 %	88	18	4½	47	—	—	—	16	6·5
Aqueous gelatin	10 %	89	18	5	38	—	—	—	$< 10^{-3}$	0
		92.*b*	18	4	11	—	—	—	$< 10^{-3}$	0
		92.*d*	18	4	5	—	—	—	$< 10^{-3}$	0
		93.*b*	18	4½	21	—	—	—	10^{-3}	0
Reduced haemoglobin		86	18	4	54	—	—	—	1	0·07
γ-Globulin		84	48	4	40	—	—	—	20	12
Milk		106	18	18	67	—	—	43	28	—
'Mist. desiccans'		53	48	4	34	25	14	—	4	1·8
		57.*b*	48	18	49	—	37	—	13	—
		60.*a*	48	4	63	—	35	—	20	—
		60.*b*	48	4	58	—	26	—	28	—
		60.*c*	48	4	85	—	42	—	21	—

had only had preliminary drying for 24 hr., but those which received a further 24 hr. secondary drying show a much higher survival rate.

The last few lines of Table 6 show results with proteins which do not appear in Table 2. They formed part of an investigation into the most suitable medium for preserving more delicate organisms, dealt with in a later section (p. 232). With the exception of reduced haemoglobin (reduced by the addition of sodium hydrosulphite) these results are quite satisfactory. 'Mist. desiccans', a mixture of one part broth with three parts serum, with 7·5 % glucose added, also gives satisfactory results up to a year, except for the first experiment (no. 53), in which the drop at 1 and 2 years is rather large. We have no explanation for this result.

IV. THE EFFECT OF AGE OF CULTURE ON SURVIVAL

During all our earlier work we were using agar-slope cultures of 'D. 201 H' which had been grown only for a short time, usually between 4½ and 6 hr., because of a feeling, for which we had no evidence, that young cultures would be more resistant to drying. One experiment, in which a 16 hr. culture was used because a younger one was not available, gave such a surprisingly high survival rate in broth (see Table 1, Exp. 63*a*, under 'Broth') that we felt that we might have been mistaken in our assumption. An experiment was therefore set up, with two agar cultures of 'D. 201 H' 4½ and 20 hr. old respectively, which were dried in the same batch of broth under identical conditions. Table 7 shows that the survival rate up to 2½ years was from five to six times higher with the older culture. A similar effect is seen in Table 5 with cultures dried in glucose broth. Exps. 95 and 96 in this table were performed with cultures 15 and 18 hr. old, and their long-term survival rates are much higher than those of any other cultures in the table.

Table 7. *Showing the percentage survival of young* (4½ *hr.*) *and old* (20 hr.) *cultures of* 'D. 201 H' *after drying in tryptic digest broth.* (*Exp.* 65, *drying technique* '*B*')

	Percentage survival after		
	24 hr.	13 months	29 months
Young culture	3·5	1	1
Old culture	15	6	6

Table 8. *Showing the percentage survival from different cell concentrations of* 'D. 201 H' *dried in* '*Mist. desiccans*'. (*Exp.* 60, *drying technique* '*B*')

Original count per ml.	Percentage survival after		
	24 hr.	3 months	8 months
47×10^8	63	35	20
47×10^6	58	26	28
47×10^3	85	42	21

V. THE EFFECT OF CELL CONCENTRATION ON SURVIVAL

Stamp (1947) says 'with a diminution in cell concentration, the percentage survival rate rises'. In most of our experiments the viable count before drying was between 100 million and 1000 million/ml., but we had taken no precautions to keep in any particular range. One experiment was therefore put up to check this point. A very thick suspension of 'D. 201 H' was made in 'Mist. desiccans', and diluted 1 in 100 and 1 in 100,000. Counts were put up, and four tubes of each suspension were dried together *in vacuo* over P_2O_5. Table 8 shows the survival rates at 24 hr., 3 months and 8 months. At 24 hr. the survival rate is rather higher in the weakest suspension than in the others, but in the later counts there is no significant difference between the three suspensions. A possible explanation of this discrepancy between our results and Stamp's is given later (p. 236).

VI. THE TIME OF MAXIMUM KILLING DURING DRYING

In most of this work the first viable count was done after drying for 24 hr., by which time from 50 to 95 % of the organisms may have been killed. It is a matter of some interest to see at what stage in the drying process this killing occurs. The introduction of the small Edwards L. 5 model desiccator made this possible, since it is easy with this machine to break the vacuum, take out one tube and evacuate again so quickly that the remaining tubes are not seriously affected.

Five experiments of this kind have been done with 'D. 201H' as the test organism, using 7·5 % glucose broth (twice), haemoglobin, 5 % albumin, and 5 % aqueous gelatin. The results are shown in Table 9. The figures after 24 hr. in three of the columns are survivals in tubes which were sealed *in vacuo* after 24 hr.

Table 9. *Showing the rate of death of* 'D. 201H' *in the early stages of drying in four different media.* (*Drying technique* '*B*')

	Percentage survival after drying in				
Time after start of drying	Glucose broth 7·5 %	Glucose broth 7·5 %	Albumin 5 %	Aqueous gelatin 5 %	Haemoglobin 10 %
30 min.	85	—	69	87	61
1 hr.	61	78	56	85	25
2 hr.	50	72	19	—	8·5
3 hr.	—	—	16	35	—
4 hr.	53	65	9	9	3·5
6 hr.	68	72	—	—	3
8 hr.	—	—	3	—	—
10 hr.	—	60	—	—	—
12 hr.	—	60	—	—	—
24 hr.	50	—	0·4	0·9	1·4
30 hr.	—	54	—	—	—
76 hr.	53	60	—	—	—
1 month	—	—	—	—	0·01
4–8 months	44	65	—	—	—
13–19 months	47	—	—	—	8×10^{-6}
22½ months	55	—	—	—	—
Exp. no.	95	96	99	101	102

drying and stored for long-term counts. In 7·5 % glucose broth the greater part of the killing has occurred in the first 2 hr., and there is very little further drop during months and, in one experiment, nearly two years. With the three protein solutions with no added glucose there is a fairly steady fall through the first 24 hr., and with haemoglobin this goes on to near sterility in 13 months. Both albumin and haemoglobin show a much steeper fall in the first 2 hr. than later, but in gelatin the reverse effect is seen, only a slight drop occurring in the first hour. This is probably due to the early formation of a thin scale on the surface of the gelatin, which hinders loss of water from the deeper layers to some extent.

One experiment was done to see the effect of very rapid drying in thin films, and for this quite different methods had to be used. A very thick suspension of 'D. 201H' was made in haemoglobin with 7·5 % glucose, and a measured volume

of 0·005 ml. was dropped on to a sterile glass slide and immediately spread in a thin film with the edge of a sterile cover-slip. Drying was complete in about 30 sec., when the slide and cover-slip were dropped into a tube containing 25 ml. of N/4 Ringer which was well shaken. Dilutions were then made, and counts put up in the usual way, a similar drop of the suspension being counted before drying. Three counts done in this way gave survival rates of 20, 14 and 13 %. The scatter in these figures is not unduly large considering the crude methods used, and they suggest that very rapid drying at room temperature is at least as lethal as the rather slower process used in our standard methods.

VII. THE EFFECT OF SUCCESSIVE DRYINGS

Drying the same organism by the same technique, it is possible to get fairly comparable survival rates in each experiment. The question naturally arises whether these survivors are more resistant to desiccation than those which have been killed. In an attempt to answer this question, 0·25 ml. of a suspension of 'D. 201 H'

Table 10. *Showing the effect of successive dryings without intermediate growth. 24 hr. culture of* 'D. 201 H' *dried in* 10 % *haemoglobin with* 7·5 % *glucose. (Exp.* 105, *drying technique* '*B*')

No. of times dried	Viable count (millions per ml.)	Percentage of original count	Percentage of previous day
0	193	—	—
1	42	22	22
2	12	6	29
3	3·5	1·8	29
4	1·3	0·7	37

in haemoglobin with 7·5 % glucose was placed in each of eighteen tubes. Two tubes, the first and the last to be filled, were counted immediately, and the other sixteen were dried together *in vacuo* over P_2O_5. After 24 hr. drying, all the tubes were reconstituted with 0·25 ml. of water, viable counts were done on three selected at random and the other thirteen were immediately redried. This procedure was repeated on the two succeeding days, three tubes being counted on each occasion. Table 10 shows the results obtained. The last column shows the survival rate each day calculated as a percentage of the previous day's count. Apart from a slight rise in the survival rate after the fourth drying, there is no evidence that the survivors show any increased resistance.

VIII. THE EFFECT OF CHANGING THE TEST ORGANISM

All the results discussed so far have been obtained with one strain of paracolon bacillus, 'D. 201 H'. Although, as stated in the introduction, most of our work has been with this strain, we have done a few scattered experiments with some other organisms which are resistant or moderately resistant to drying. Table 11 shows the survival rates of these after drying in several different suspending fluids. Several strains of *Salmonella typhi-murium* behave similarly to 'D. 201 H', dying rapidly in serum water, showing a moderate survival in broth or nutrient gelatin

and a high survival rate up to nearly two years in 7·5 % glucose broth. One strain of *Bacterium coli* type I shows a high survival rate in 'Mist. desiccans' beyond 2 years. *Staphylococcus aureus* and a haemolytic streptococcus of group E behave rather like 'D. 201 H' in 'Mist. desiccans' except that their survival up to one month is very high. The haemolytic streptococcus group B is very resistant to drying, and this strain (Cole), which shows a survival rate of 100 % even in serum water, was, in another experiment, not entirely killed 18 months after drying in distilled water. It seems impossible to kill this strain by drying.

A number of experiments have also been done with more delicate test organisms, the two chiefly used being *Neisseria gonorrhoeae* and *Vibrio cholerae* (Ogawa). These proved very sensitive to drying, and all our attempts to dry them without

Table 11. *Showing the percentage survival of a number of fairly resistant organisms after drying in various media.* (*Drying technique 'B'*)

Drying medium	Culture	Exp. no.	Percentage survival after: 24 hr.	2–9 days	2–3 weeks	1 month	2–3 months	4–7 months	8–14 months	18–22 months	24–30 months	36–42 months
Serum water	*Salm. typhi-murium*	13.*c*	0·05	—	—	2×10^{-4}	—	—	—	—	—	—
Serum water	*Staph. aureus*	4	0·8	0·2	—	—	—	—	3×10^{-3}	—	—	—
Serum water	*Haem. strep.*, group B	2	—	100	100	—	—	100	100	—	—	—
Broth	*Salm. typhi-murium*	50.*a*	7	—	—	—	—	4	1	2	—	—
Broth + 7·5 % glucose	*Salm. typhi-murium*	50.*b*	31	—	—	—	—	35	34	19	—	—
Broth + 7·5 % lactose	*Salm. typhi-murium*	50.*c*	21	—	—	—	—	21	10	26	—	—
Nutrient gelatin	*Salm. typhi-murium*	13.*b*	8	—	—	8	—	2	—	—	—	2
'Mist. desiccans'	*Haem. strep.*, group E	52	100+	100+	—	100	—	—	72	—	20	—
'Mist. desiccans'	*Staph. aureus*	54	100	100+	90	74	68	—	—	—	36	—
'Mist. desiccans'	*Bact. coli* type I	55	55	—	—	—	37	—	14	—	7	—

the addition of glucose failed. A preliminary experiment suggested that between 5 and 10 % of glucose in broth was the best concentration for these organisms as well as for the Gram-negative bacilli, but it also appeared that glucose broth alone was not very satisfactory, at least for the gonococcus, and an experiment was done to investigate the effect of adding serum to the glucose broth. Because of the uncertainty of getting any appreciable survival by our ordinary technique, this drying was done with a liquid-air condenser by method A (see Appendix 1). Fig. 4 shows the survival rate of the gonococcus immediately after drying in broth containing various concentrations of human serum, with a constant addition of 7·5 % of glucose to the mixture in each case. There is a well-marked maximum survival with 75 % of serum, and as a result of this experiment the mixture previously referred to as 'Mist. desiccans' was devised. Attempts were also made to dry the gonococcus and the cholera vibrio in various other proteins containing 7·5 % glucose, and the results of these experiments are shown in Table 12. The results in 'Mist. desiccans' are the best, but haemoglobin and egg yolk, each with added glucose, also give satisfactory results, good enough at least to ensure

survival of a culture for many years. It is strange that γ-globulin, which gave the highest survival with *Vibrio cholerae* immediately after drying, produced a sterile result in just over 2 years. *Haemophilus pertussis* has also been dried in 'Mist. desiccans' with satisfactory survival over a period of years, but we have no accurate counts of this organism.

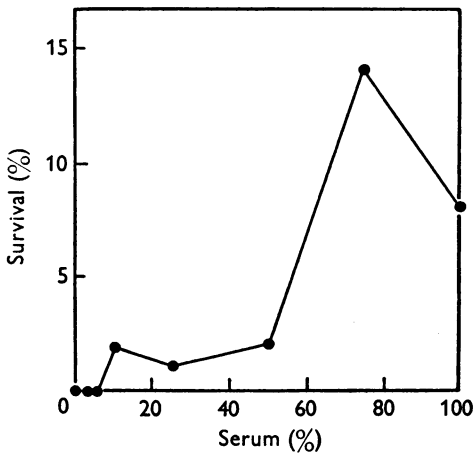

Fig. 4. Showing the percentage survival rate of *Neisseria gonorrhoeae* immediately after drying in nutrient broth containing varying concentrations of human serum, with 7·5% glucose added in each case. (Drying method 'A. 2'.)

Table 12. *Showing the percentage survival of* Neisseria gonorrhoeae *and* Vibrio cholerae *after drying in various drying media containing glucose. (Drying technique 'B')*

Drying medium		Exp. no.	Percentage survival after: *Neisseria gonorrhoeae* 24 hr.	3–4 months	12 months	*Vibrio cholerae* 24 hr.	1 month	26–28 months
'Mist. desiccans'		68	—	1	—	5	5	3
Albumin 5%	With 7·5% glucose	74	1·3	1	0·02	0·3	—	0·04
Albumin 25%		87	—	—	—	7	—	0·4
α+β-Globulin 20%		79.*b*	—	—	—	2	—	0·03
γ-Globulin 25%		84	—	—	—	12	—	0
Serum		81	1·7	—	0·002	3	—	0·08
Haemoglobin 10%		77	9	—	0·25	5·5	—	0·5
Reduced haemoglobin		85	—	0·5	0·025	2	—	0·01
Egg yolk		83	1·7	0·3	0·6	10	—	0·9
Glucose 7·5%		76	—	—	—	0·2	—	0·002

IX. DISCUSSION

Throughout this work we have been impressed with the paramount importance of the suspending fluid in which the organisms are dried, and with the fact that, whatever fluid is used, the addition to it of from 5 to 10% of glucose, or possibly lactose, will greatly increase the survival rate.

Since we were working with an organism which could utilize glucose it seemed important to rule out the possibility, however improbable, that the effect of the

added glucose was merely metabolic. For this purpose we used a non-lactose-fermenting organism, *Salmonella typhi-murium*, and dried it in (*a*) broth with no added sugar, (*b*) broth with 7·5% lactose and (*c*) broth with 7·5% glucose. Viable counts were done at 24 hr., 5 months and 18 months, and on each occasion there was no significant difference between the survival rates in the glucose and lactose broth, and each was from five to ten times as high as in broth with no added sugar.

Another much more probable explanation of the sugar effect is that the presence of glucose ensures the retention of a certain amount of moisture, which is necessary for survival. This is in direct opposition to the generally accepted view that the cultures must be made as dry as possible (see Proom & Hemmons, 1949, p. 17; Wilson & Miles, 1946, p. 111), but it is supported by a number of experiments in which we have shown that, with no added glucose in the suspending fluid, prolonged drying gives a consistently lower survival rate than drying for a short period (see p. 223 and Table 2). As a result of the experiments shown in Table 2 we are convinced that a certain minimal amount of water must be left for satis-

Table 13. *Showing the effect of different concentrations of sodium citrate in broth on the survival rate of* 'D. 201H' *after drying.* (*Exp.* 107, *drying technique* '*B*')

Concentration of citrate (%)	Percentage survival after			
	24 hr.	6 weeks	2½ months	4 months
0	10	6	5	6
2·5	16	15	15	6
5	35	24	19	8
10	36	14	24	0·4
20	25	16	0·3	0·1

factory survival, the only practical difficulty being to ensure that the right amount is left. Glucose probably acts by automatically regulating the amount of residual moisture in the cultures, and by trial and error we have arrived at 7·5% as the concentration which best achieves this result.

If this explanation is correct, it should be possible to find other substances which would act in the same way, and it was suggested to us that sodium citrate should also retain some moisture during the drying process. We accordingly set up an experiment, drying 'D. 201H' in broth containing various concentrations of sodium citrate from 0 to 20%. Table 13 shows the survival rates after 24 hr., 6 weeks and 2½ and 4 months. The results up to 2½ months are almost the same as with glucose, with a maximum survival at about 5–10% of citrate. Even 2·5% improves the survival rate considerably. In the early stages 20% citrate was also good, but by 2½ months the counts from these tubes, which look too wet for a satisfactory result, have fallen to a very low level. At 4 months the survival in all citrate concentrations has fallen badly, and a good long-term survival cannot be expected.

If, as we believe, some residual moisture is essential for survival, some explanation is necessary for the discrepancy between our results and those of other workers. In most of the published results there has been no detailed record of the

survival rates except immediately after drying, the long-term results being judged merely by the presence or absence of *some* viable organisms in the culture. Proom & Hemmons (1949, p. 14), however, give one table showing survival rates up to 6 months for two different organisms, but as these cultures had no secondary drying it is probable that they were not 'as dry as possible'. Further, the standard method used by these authors involved secondary drying in a desiccator, which was finally filled with dry nitrogen. The tubes were removed from the desiccator for sealing, and during this process there is ample opportunity for a well-dried culture to absorb a small amount of water from the atmosphere.

Stamp (1947) gives full details of survival rates in cultures dried by his method after storage for periods up to 4 years in a desiccator over P_2O_5, so that hydration of the cultures could not occur. Comparison with his figures is, however, difficult, because of the particular method of drying which he used. At the time when we were investigating killing during the early stages of drying we tried a similar experiment using Stamp's technique, drying 'D. 201 H' in nutrient gelatin at

Table 14. *Showing the 'survival' rate during drying by Stamp's technique. 17 hr. culture of* 'D. 201 H' *dried in nutrient gelatin. (Exp. 97, drying technique 'C')*

Drying time	Viable count (millions per ml.)	Survival (%)
0	162	—
2½ hr.	197	120
5 hr.	640	400
9 hr.	1340	830
24 hr.	730	450
30 hr.	585	360
2 days	382	236
3 days	560	350
5 days	444	275
6 days	385	240
9 days	365	230

room temperature over P_2O_5 at a pressure of 200 mm. Hg. The only difference was that, instead of putting gelatin drops on waxed paper, we put 0·05 ml. drops into $4 \times \frac{1}{2}$ in. tubes, which were sloped nearly horizontally and left with open ends. Table 14 shows the viable counts at intervals from 2½ hr. to 9 days, and it is seen that during the first 9 hr. at this relatively high pressure there is sufficient moisture remaining for active growth to occur. At 9 hr. the 'survival' was over 800%, and up to the 9th day it had not fallen below 200%. A last count done on three remaining tubes at 54 days gave widely scattered survival rates of 36, 73 and 160%, with a mean of 90%. It is unlikely that quite such a dramatic rise occurred in Stamp's cultures, since his initial viable counts were mostly much higher than ours, and his survival rates 2–3 days after drying, though high, were never above 100%. It is probable, however, that some growth took place in the early stages to balance any possible killing, at least with organisms that could multiply freely in nutrient gelatin at 20–22° C. Because of this possible fallacy we do not feel that any direct comparison between Stamp's figures and ours can be usefully made.

This possibility of growth during the slow-drying process at room temperature may explain Stamp's finding that lower cell concentrations gave a higher percentage survival rate, a finding which we were unable to confirm by our methods. With his technique the lower cell concentrations would clearly have the greater chance of growing during the first few hours of the drying process, and would therefore have apparently higher survival rates.

Some workers have noted difficulty in recovering cultures from the dried state. Wilson & Miles (1946) say: 'To recover the organisms, an optimal medium is desirable for primary cultivation.' Proom & Hemmons (1949) noted that dried

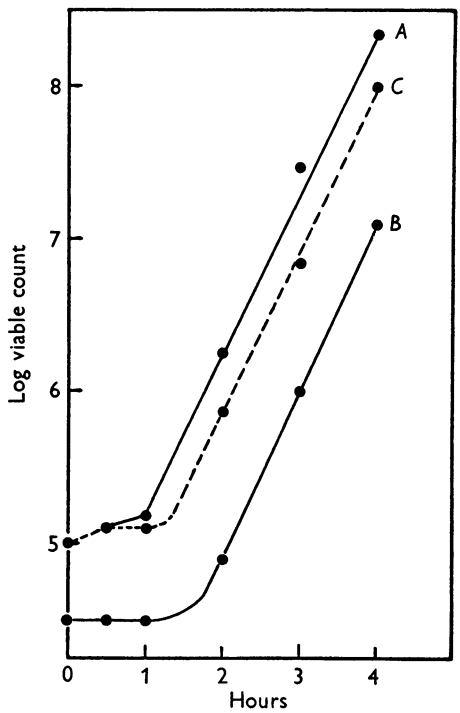

Fig. 5. Showing the growth curve of 'D. 201H' in nutrient broth (*A*) before drying, (*B*) immediately after drying for 24 hr., and (*C*) 15 days after drying. In (*C*) the amount of broth has been reduced to bring the initial viable count to the same level as in (A). (Drying method 'B'.)

cultures have an unusually long lag phase when subcultivated. These effects would of course be expected if the number of surviving organisms is very small. That they are not due to any serious lowering of the vitality of the survivors is shown by an experiment on the rate of growth of 'D. 201H' before and after drying in 'Mist. desiccans'. In Fig. 5, curve *A* shows the normal growth curve obtained by adding 0·25 ml. of suspension to 10 ml. of broth at 37° C. Curve *B* shows the growth curve after 24 hr. drying, the contents of one tube being added to 10 ml. of warm broth as before. The lag phase here is a little longer because, on account of the death of 75% of the organisms, the initial cell concentration is lower than in curve *A*. Curve *C* shows the growth curve when, 15 days later, the

contents of another tube of dried culture were dissolved in only 2·5 ml. of broth, to bring the initial cell concentration to about the same level as in curve *A*. Any lengthening of the lag phase is very slight, and curves *A* and *C* lie close and parallel to each other.

The temperature at which dried cultures should be stored is a point which we have not investigated, and all our cultures have, with one exception, been stored in cardboard boxes, protected from light, at normal room temperature. Judging by the results of Proom & Hemmons (1949), it is probable that our results might have been much better with storage in the refrigerator. The one exception mentioned is shown in Fig. 3, where the lowest curve (13 months after drying) shows the survival in a series of tubes which had been stored in a box for 7 months and then mounted on a card for demonstration. They were left on this card standing in a sunny window from January to July before the counts were done, but strong sunlight does not appear to have harmed the cultures greatly.

All our dried cultures have been sealed *in vacuo* as described in Appendix 1. We feel that this is by far the safest way of ensuring that there is no addition of an unknown amount of moisture during the sealing process. Proom & Hemmons's (1949) objection that the sudden inrush of air on opening the tube is likely to cause contamination or to push the plug down to the bottom of the tube is easily overcome by making a nick in the glass above the level of the plug and pressing a hot bead of glass into it. A minute crack develops through which the air enters gently, and in about 30 sec. the top of the tube can be removed with no inrush of air at all. The sudden popping open of a tube sealed *in vacuo* is a most unnecessary and dangerous proceeding, and, if there is no plug in the tube, it may well scatter some of the dried contents into the surrounding air.

The nature of the protective colloid used for suspending the organisms seems to be of considerable importance, secondary only to the glucose. Most of those we have tried, with the exception of nutrient broth and nutrient gelatin, seem useless for long-term survival without added glucose, and even with glucose different colloids show very different results. Aqueous gelatin with glucose, for example, failed completely judged by long-term survival, even though the immediate survival was fairly good (see Table 6). Nutrient gelatin, on the other hand, gave results very similar to those obtained with broth without the addition of gelatin. This does not agree with Stamp's statement, referring to his medium, that 'there is some evidence that the beneficial effect is mainly due to the gelatin'. Amongst the other colloids we have tried the choice is more difficult. We have always had the best results with 'Mist. desiccans', but since this is more trouble to prepare than some, and cannot be sterilized by heat, we have generally reserved it for the more sensitive organisms. For routine use with fairly resistant organisms, such as the Gram-positive cocci, salmonella group organisms and coliforms, 7·5% glucose broth is all that is needed, and is a convenient medium that can be heat-sterilized. We have had some encouraging results with filtered milk with added glucose, but further work is necessary to confirm these results over a longer period. For the numerous organisms with which we have not worked we hesitate to make any recommendations, except that we should expect them to survive

fairly well in 'Mist. desiccans', and would hope to get good survival in 7·5% glucose broth with most organisms that grow well on ordinary media.

Finally, while it is clearly impossible for any one group of workers to investigate the long-term survival of all organisms dried in many different media, we feel that, with our limited programme, we may have indicated lines for future methods of investigation.

X. SUMMARY

An investigation of the survival rate of bacteria after drying *in vacuo* by the 'spin-freeze' method showed that one of the most important factors was the fluid in which the bacteria were suspended. Broth or nutrient gelatin gave a moderate survival rate, but in various other protein solutions, even though there might be a fair survival immediately after drying, a large fall occurred in the viable count after storage. The addition of glucose to the suspending fluid in concentrations of between 5 and 10% greatly increased the survival rate both immediately and after storage. Some experiments showed that, without added glucose, the longer the drying process was continued the lower was the survival rate, and it is thought that glucose acts by automatically retaining a small amount of water which is necessary for survival. Lactose appears to produce the same effect.

It is shown that the highest death-rate generally occurs during the early stages of drying, the further drop after 24 hr. being small if glucose is used in the suspending fluid. Very young cultures ($4\frac{1}{2}$–6 hr.) seem to be far more sensitive to drying than older ones (18–24 hr.). The bacteria that survive drying are not more resistant to subsequent drying.

Most of these results have been obtained with one strain of paracolon bacillus, but a limited number of other experiments show that the same principles hold good for other organisms such as *Salmonella typhi-murium*, staphylococci, streptococci, *Neisseria gonorrhoeae* and *Vibrio cholerae*. In the case of the last two species the percentage survival is much lower than with the others, but survivals up to 1% have been obtained after storage for from 1 to 3 years.

APPENDIX 1

Techniques for freeze-drying bacterial cultures

Previous experience with the 'freeze-drying' of protein solutions had led to the conclusion that (*a*) they must be frozen rapidly in order to obtain a fine crystal structure, (*b*) they must be dried below their eutectic temperature so that no liquid phase is present during the drying cycle, and (*c*) they must be rendered as dry as possible if the minimum of deterioration is to result after long periods of storage at high temperatures. Scale preparations were regarded as evidence of poor technique and invariably led to some denaturation of the proteins.

Apparently these criteria have to be abandoned if the drying of bacteria is to be successful. Stamp (1947) has shown that, except for a few of the more delicate organisms, drying below the freezing-point is not essential. In this paper we have

shown that if cultures are rendered too dry they are killed. We consider that the beneficial effect of glucose may be due to the buffering action it exerts over the speed of loss of the last traces of moisture, so that in effect it controls the final moisture content. The addition of glucose to the drying medium leads to a dried product which tends to be frothy and scaly, a product ethically unpleasing to those who have worked with 'freeze-dried' proteins. Fortunately, these glucose scale preparations are very rapidly soluble.

In order to investigate the effect of the drying temperature on the survival rate, it was necessary to devise a technique for drying at as low a temperature as

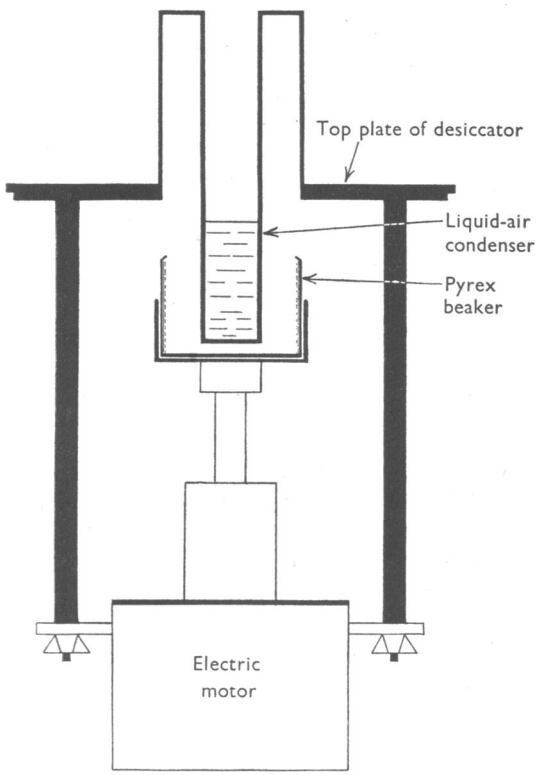

Fig. 6. Diagram of the apparatus used for drying with a liquid air condenser. (Drying method 'A. 1'.)

possible. Applying the theory as expounded by Greaves (1946, pp. 19–21), it will be appreciated that the very small volumes in which it was desired to dry the cultures rendered the task difficult. It was essential to keep the vapour path as short and unobstructed as possible, since this was easier than trying to limit the uptake of heat by the material being dried and it also led to quicker drying.

Method A. Drying at very low temperatures ($-180°$ C.)

(1) *For single experiments.* A small Pyrex beaker 2 in. in diameter and 2 in. long had its top edge rolled in to form a small internal lip. It was then spun on

its vertical axis round a brass cylinder 1 in. in diameter fitted to the top plate of a desiccator. This latter cylinder could be filled with liquid air to act as the cold condenser (see Fig. 6).

10 ml. of a bacterial suspension was placed in the beaker which was rotated at 1000 r.p.m. by a small electric motor which was also in the vacuum chamber. The rotation distributed the liquid as a very thin film on the inside periphery of the beaker so that frothing was prevented while the desiccator was being evacuated, and when the critical vacuum of about 1 mm. Hg had been reached 'snap-freezing' of the liquid occurred in this thin film (see Greaves, 1944). Since the obstruction to the flow of vapour from the frozen culture medium to the condenser is so small, the medium rapidly reaches a temperature very near to that of the liquid-air condenser, and rapid drying at a very low temperature occurs.

In order that the beaker may attain the temperature of the room, thus avoiding excessive uptake of moisture on removal from the desiccator after drying, P_2O_5 is placed in the bottom of the desiccator. After about 1 hr. when drying is complete, no further liquid air is placed in the cylinder, and the ice now on the cylinder is allowed to sublime over into the phosphorus pentoxide. After about 6 hr. the beaker can be removed warm and dry.

Though useful for preliminary experiments this apparatus could not be used for storage experiments. When storage experiments were required we therefore modified the technique to method A (2).

(2) *For storage experiments.* A metal Petri dish was fitted with a central boss, over which was placed a cap to which an $\frac{1}{8}$ in. wide edge of a coloured waterproof Cellophane square was stuck with Durofix. The Cellophane squares were about $1\frac{1}{2}$ in. square. When stuck to the metal cap their free edge was cut into radial strips $\frac{1}{8}$ in. wide; the terminal $\frac{1}{8}$ in. of the Cellophane strip was then folded upwards to facilitate picking up with forceps at the end of drying.

When the cap with the Cellophane strips attached was placed on the locating boss of the Petri dish, the Cellophane strips took up a position on the bottom of the Petri dish radiating out from the centre (see Fig. 7). The dish and its contents, covered with a glass Petri dish top, were then sterilized in the hot-air oven.

Four drops of 0·005 ml. of culture were then measured on to each strip with a graduated Wright pipette, and since the Cellophane used was of the waterproof variety these drops remained discrete. By using four different colours of Cellophane for identification purposes, four different cultures could be dried at the same time.

The Petri dish was then floated on to a larger dish filled with liquid air so that very rapid freezing occurred. Still floating on liquid air the dish was then placed in the desiccator, the condenser was filled with liquid air and the vacuum pump started. As soon as the liquid air inside the desiccator had boiled away, the chamber became evacuated rapidly to less than 10^{-4} mm. Hg and drying by sublimation started.

As in the previous method P_2O_5 was placed in the bottom of the chamber so that the dried product could be removed warm from the desiccator, but it was found that this beautifully dried product was so hygroscopic that the transfer of the Cellophane strips to their glass tubes for sealing had to be carried out in

a room at $-25°$ C. This rendered an otherwise ideal technique somewhat cumbersome, but no doubt simpler methods could be evolved to avoid this.

In the $-25°$ C. room the turned-up edge of a Cellophane strip was seized with a pair of sterile forceps, torn from the retaining cap and then placed in a small sterile glass tube which was sealed with a paraffined cork. When all the Cellophane strips had been transferred to corked glass tubes they were brought back to room

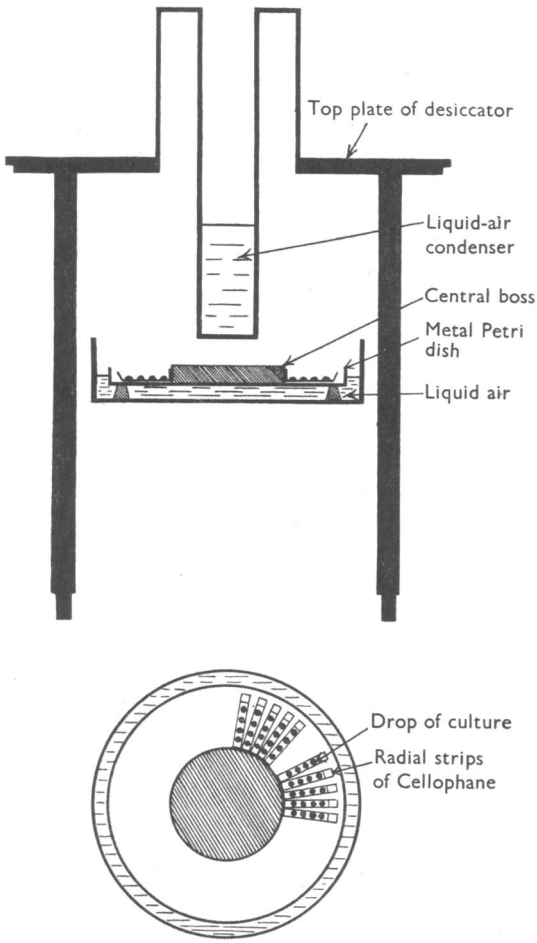

Fig. 7. Diagram of the apparatus used for drying with a liquid-air condenser for subsequent storage of the cultures. (Drying method 'A. 2'.)

temperature and the tubes were drawn out to a narrow neck about $\frac{1}{2}$ in. below their corked ends in a gas flame. The tubes were then placed on a vacuum manifold, evacuated over phosphorus pentoxide, and finally sealed off in high vacuum.

With delicate organisms such as *Neisseria gonorrhoeae* and *Vibrio cholerae* this technique proved to be remarkably good, but as has been shown elsewhere in this paper, the drying medium is of far greater importance than the temperature of drying. Thus this technique has been largely abandoned in favour of technique B, but is well worth using if the best possible results are required.

Method B. Standard technique using phosphorus pentoxide or a mechanically refrigerated condenser

Since the introduction of 'Mist. desiccans' as a drying medium even *Neisseria gonorrhoeae* can be dried reasonably successfully by this technique, which, being simple and easy in operation, has been adopted by us as our standard technique.

Fig. 8 shows the arrangement in schematic form. Basically it consists of a vacuum chamber in which there is a small electric motor fitted with a centrifuge head. The head is drilled to accommodate up to thirty tubes, 4 in. long and $\frac{5}{16}$ in. internal diameter, with their open ends inclined inwards at an angle of 10° from the vertical. For drying, 0·25 ml. of bacterial suspension is put into each tube, so

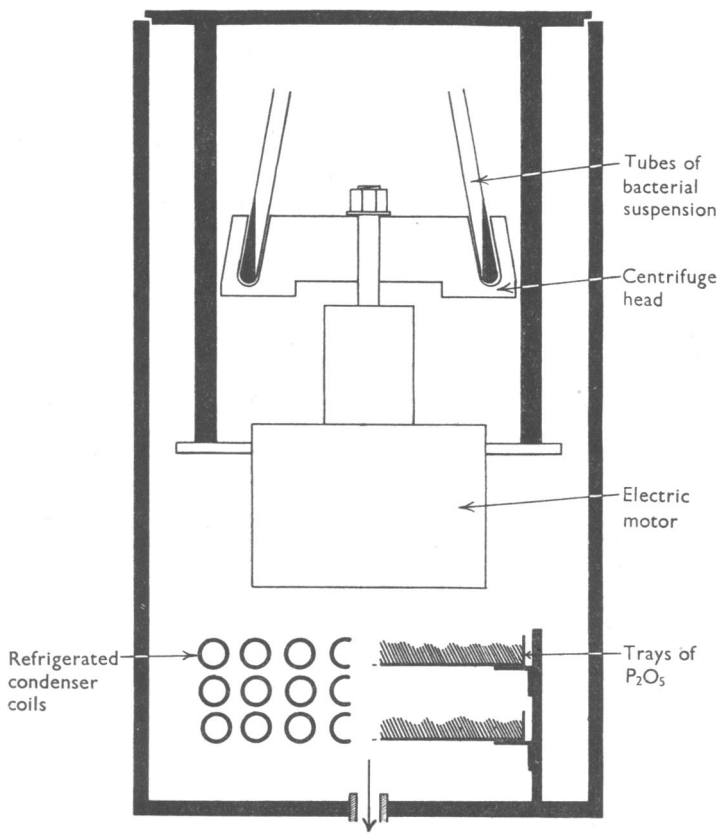

Fig. 8. Diagram of the apparatus used for drying multiple tubes by the 'spin-freeze' method, using either a refrigerated coil condenser or P_2O_5. (Drying method 'B'.)

that a full load on this machine is only of the order of 7·5 ml. Two trays filled with P_2O_5 are placed below the motor for absorption of water vapour. In a rather larger machine which takes up to sixty tubes, the chamber is fitted at the bottom with a small refrigerator coil which is maintained at −40° C. by a small domestic-type methyl-chloride compressor. This apparatus, which gives results identical with those obtained with P_2O_5, avoids the use of the large quantities of desiccant which would be necessary to deal with the heavier load.

The drying procedure is as follows. The tubes containing a measured amount of bacterial suspension are placed in the centrifuge head in the desiccator which has already been charged with P_2O_5. The centrifuge is started, and when it has attained its maximum speed (700 r.p.m. is sufficient) the vacuum pump is started and the chamber is rapidly evacuated. It should not take more than 4 min. for the vacuum to reach 1 mm. Hg, at which pressure 'snap-freezing' will occur, all frothing being suppressed by the centrifugal force (see Greaves, 1944). After freezing, the centrifuge is left running for a further 5 min. and then stopped, when it will be found that the liquid in the tubes is now frozen as a wedge on the side of each tube. By now the vacuum should be 0·01 mm. Hg or better, and drying will be complete in about 2 hr. (see Fig. 9), when the tubes may be removed and vacuum-sealed.

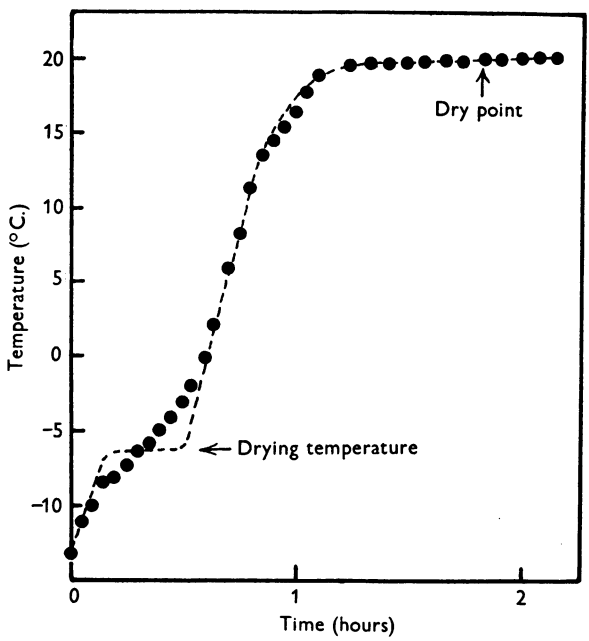

Fig. 9. Temperature curve recorded during the drying of 0·25 ml. of 'Mist. desiccans' by method 'B'. The black dots show the actual temperatures recorded. The broken line shows the assumed temperature curve, with a drying temperature at about $-7°$ C.

The recording of drying temperatures from 0·25 ml. quantities in small tubes offers considerable practical difficulties, particularly with centrifugal methods when thermocouples have to be led out through slip rings. Fig. 9 was obtained using a small thermocouple at the bottom of a wedge of material pre-frozen at $-20°$ C. It will be seen that the temperature rose rapidly to $-10°$ C., flattened slightly till $-2°$ C. was reached, and then rose more rapidly, eventually flattening out at 20° C., when dryness was reached in 2 hr. This was of course only a mean temperature but would seem to indicate a drying temperature of between -6 and $-7°$ C. (as shown by the broken line). This drying temperature was much higher than had been expected, due presumably to the very large heat uptake

for the small surface area of this 0·25 ml. amount. This temperature must be well above the eutectic of the drying medium, and with the added glucose leads to a foamy scale preparation. Nevertheless, satisfactory survival rates of delicate organisms are obtained, and further elaboration to reduce the drying temperature does not seem to be indicated.

At the end of the 2 hr. drying cycle, the vacuum in the drying chamber is let down *slowly*; the habit of letting the chamber down rapidly is greatly to be condemned (see section on sterility). The tubes are now taken out of the chamber, the drying caps are removed and replaced by $\frac{1}{2}$ in. square plugs of gamgee tissue which are pushed half-way down the tubes. The middle of the tube distal to the plug is then constricted down to a capillary, care being taken to melt plenty of glass into this capillary so as to give strength to the final vacuum seal. After constriction the tubes are placed on rubber nipples on a vacuum manifold, evacuated for 1 hr. over P_2O_5, and sealed off in high vacuum, using a small cross-fire burner, the jets of which are made from two hypodermic needles cut to a length of about $\frac{1}{2}$ in.

Method C. Drying from the liquid phase

An apparatus was set up for drying at room temperature from the liquid phase in order to repeat Stamp's (1947) experiments. It consisted simply of a vacuum bell-jar connected to an Edwards type IV rotary vacuum pump for evacuation and a mercury gauge for measuring pressures between 100 and 300 mm. Hg.

Sterility

During drying there is a rapid current of water vapour from the inside of the tube outwards, which will tend to prevent any organisms falling into the tubes. Theoretically, therefore, there should be no danger of contamination if drying is carried out using open tubes, and in practice we have found this to be so. But there is great danger that, if the vacuum is released rapidly at the end of drying, the light friable material may be blown all over the chamber and cross-infection of tubes may occur.

When drying 'Mist. desiccans' a scale-like preparation results, and there is little chance of organisms being swept out of the tube on the vapour stream. This is not so when drying organisms in distilled water, and when we tried this experimentally it was found that the contents of the tube tended to be discharged on the vapour stream and the dried organisms were scattered all over the desiccator. Some sort of capping of the tubes would therefore seem to be desirable; such a cap must offer a minimal obstruction to the vapour flow with a maximal obstruction to particles. We have used for this purpose gamgee caps which fit over the end of the tubes. They are made by taking lengths of gamgee tissue 3 in. long by 1 in. wide. A staple from a paper stapling machine is inserted at each end, and then the gamgee is folded in half and two staples placed on each side. The end staples are then curved with pliers so that they offer an opened orifice into which the tubes may be rapidly inserted.

The gamgee filters may be sterilized by autoclaving in tins and reused many times.

APPENDIX 2

Technique for viable counts

(1) *Making dilutions*

Through the whole of this work we have used an automatic delivery pipette of the mercury piston type, slightly modified from that described by Wright & Colebrook (1921, pp. 40–2). The original model of this pipette, devised by D. Parry Morgan, was adjustable for delivery of different volumes. In our modification the pipette is not adjustable, the permanent position of the wool plugs being found by trial and error. Detachable nozzles are slipped into a short rubber connexion, so that they may be changed between each diluting stage. We have tested the accuracy of the pipette which has been in constant use, which is adjusted to deliver a nominal 0·1 ml. volume. A series of weighed volumes of distilled water delivered by this pipette gave a mean weight per volume of 101·7 mg., with maximum deviations of $-1{\cdot}3$ and $+1{\cdot}2$ mg. Possible errors are therefore of the order of 1 %.

To make dilutions of a suspension for counting before drying, 9·9 ml. volumes of N/4 Ringer solution were delivered into 1 oz. screw-capped bottles (Universal containers), and 0·9 ml. volumes into small sterile tubes ($4 \times \frac{1}{2}$ in.). By delivering 0·1 ml. with the automatic pipette into these, dilutions of 1 in 100 or 1 in 10 could be made. Screw-capped bottles were used for the 1 in 100 dilutions, as they could be shaken vigorously to ensure adequate mixing. Our object in using this method was to dilute as far as possible in 100-fold steps to reduce the number of stages of dilution. When a likely range for counting was reached further dilutions were made in 10-fold steps.

When counting cultures after drying, the whole of the contents of the tube (originally 0·25 ml.) was dissolved in 25 ml. of Ringer, to give a 1 in 100 dilution. Further dilutions were then made as outlined above.

(2) *Pouring the plates*

Using the same automatic pipette, 0·1 ml. volumes of suitable dilutions were expelled into sterile Petri dishes containing a small amount of Ringer, so that this small volume could be easily spread over the bottom of the dish. Melted nutrient agar at 45–50° C. was then poured into the plate and thoroughly mixed with the culture dilution by rocking the plate. Plates from three consecutive 10-fold dilutions were usually prepared, three plates being used for each dilution.

(3) *Surface counts*

With organisms that either would not grow or were difficult to count in deep colonies, the surface method of Miles & Misra (1938) was used. Dilutions were made as described, but the final volumes discharged on to the plates were 0·02 ml., delivered either with a dropping pipette or with a fine-pointed capillary pipette graduated to contain the required volume. This latter introduced a small error due to the difference between a delivery and a container pipette. Ten such volumes were put on one plate, at least two plates, and sometimes three, being used for

each dilution. With the surface method it was found better to use a somewhat closer range of dilutions to get countable plates from more than one dilution. A threefold stage of dilution was therefore often introduced into the series.

One difficulty was encountered when counting *Vibrio cholerae* by this method. Using nutrient agar plates we found that, with old dried cultures, the counts were often very irregular, one dilution giving a confluent growth and the next showing a sterile plate or at most only a few colonies. A similar difficulty is mentioned by Miles & Misra (1938, p. 734) in their paper describing this method, but it is dismissed briefly and with no attempt at explanation. This trouble was later overcome entirely by using blood-agar plates for these counts, and it thus appears to be related to the irregularity described by Herbert (1949), who showed that small inocula of *Pasteurella pestis* would not grow on plain nutrient agar, but that growth occurred normally if whole blood or peptic digest of blood was added to the medium.

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(*MS. received for publication* 19. III. 51.)