BACTERIAL SURVIVAL IN SYSTEMS OF LOW MOISTURE CONTENT

PART I. ENUMERATION AND DISTRIBUTION OF ORGANISMS IN SPRAY-DRIED POWDERS

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INTRODUCTION

THE growth and metabolism of bacteria and their response to altered environmental conditions when growing in liquid media, or such media solidified by addition of a small percentage of agar, have been extensively studied both quantitatively and qualitatively. Much has been written about the enumeration of bacteria suspended in liquids. On the contrary, the behaviour and enumeration of bacteria in powders, pastes, and oils, that is to say in systems of relatively low moisture content, have been studied to a much lesser extent. This situation is probably due to the difficulty, experienced in the past, of obtaining powders of predetermined composition, containing an even distribution of suitable numbers of a known organism, with which the experiments could be carried out.

As long ago as 1909 Shackell[1] called attention to the possibilities and advantages of freeze-drying for the purpose of obtaining bacterial cultures in a form in which they would retain their viability, cultural characteristics and state of virulence. Swift[2], and Elser, Thomas and Steffan[3], showed that hæmolytic streptococci and meningococci were resistant to freeze-drying and subsequently remained viable over a long period of time. Heller[4] used this method of drying for the quantitative investigation of environmental factors affecting dried samples of *Streptococcus pyogenes* and *Escherichia coli.* He did not, however, specify his limits of error. The products of freeze-drying tend to occur as flakes, and an examination of the literature has not revealed any report establishing that viable micro-organisms are uniformly distributed in the powders formed from such products.

The work described in the present communication was foreshadowed in a previous paper from this Department[5]. It was there suggested that a spray-dried powder containing a known species of micro-organism in a medium of known composition might prove suitable for the study of the effects of environmental conditions on the organism in such a powder, or such a powder suspended in oil.

Before enquiry into the effects of environmental conditions can be made, certain facts must be established. Organisms suitable for the investigations on hand must be chosen. It must be shown that these organisms can be counted satisfactorily, or at any rate that reasonably concordant replicate viable counts can be obtained and the errors of such counts must be statistically evaluated. It must be shown that the viable organisms counted are uniformly distributed in the powder or that with suitable treatment such uniformity can be attained. The organisms used should remain viable for considerable lengths of time in the powder,

but if they cease to be able to reproduce during the course of the experiment then it must be possible to estimate the extent of such degeneration over the periods of time involved in the experiment. It should clearly be understood that the fact that a particular bacterium does not produce a colony when the medium is plated out may not mean that the organism is dead as an individual, but it does mean that it is incapable of reproduction. In the following work such organisms will, as is usual, be regarded as non-viable.

EXPERIMENTAL TECHNIQUES

Choice of Test Organisms. Considerable thought has been given to the question of the choice of suitable organisms. For the early work on spray-drying it was essential that the test organisms should be non-pathogenic since some of the dried culture might be inhaled. Preliminary experiments with yeasts and moulds did not appear promising, largely on account of difficulties of culture and enumeration. Bacteria, although smaller in size and so more difficult to examine microscopically and distinguish from unorganised matter in total counts, yielded more repeatable figures.

It was considered that both spores and vegetative forms should be examined in this work, for the following reasons. (i) To be satisfactory, suggested processes for sterilisation must destroy the viability of spores, but (ii) a substance which will kill, or even profoundly inhibit vegetative organisms, even if it does not affect spores, may be considered to be satisfactory for the maintenance of sterility in, for example, multiple dose containers. (iii) For the understanding of the life processes of bacteria the behaviour of both spores and vegetative forms is of interest.

Bacillus subtilis appeared to be the most obviously satisfactory non-pathogenic sporing organism. To perform "useful" viable counts on *B. subtilis* in the vegetative form is probably an impossibility owing to the tendency of the cells to form chains and the tendency of these chains to form a matted growth or pellicle on the surface of liquid media. It is difficult, not only to break down the chains into individual cells, but the pellicle is very difficult to wet so that it has proved impossible to obtain the even suspension of organisms necessary for concordant replicate counts. When, however, *B. subtilis* is allowed to spore on the surface of agar each spore is formed individually in a separate bacterial cell. This process is followed by the autolysis of the original vegetative cells; the whole surface becomes moist and the spores can easily be mixed with a suspending fluid. Microscopical examination of the resultant suspension shows the spores to be separate with no tendency to clump. Such a suspension has proved very satisfactory in this work. The details of preparation are as follows.

The organism used was *Bacillus subtilis* (Marburg, No. 3610) obtained from the National Collection of Type Cultures. The surface growth on 10-day agar slopes was washed off with 20 ml. of sterile water, and the resulting suspension was suitably diluted and distributed in glass

ampoules. These were heated at 80°C. for 3 minutes, to destroy any vegetative organisms, and were then stored in a refrigerator. Such suspensions showed no significant decrease in viable count after 6 months' storage.

To find a suitable non-sporing organism has proved to be much more difficult. *Bacterium lactis aerogenes*, which was used in the work described in the previous paper, is non-pathogenic, evidences no marked tendency to chain formation and gives rise to colonies which can easily be counted, but over 99 per cent. of the bacteria are usually destroyed on spray-drying and the survivors fairly rapidly die off in the resultant powder under ordinary conditions of storage. It is possible that some of the difficulties are inherent in the nature of vegetative bacteria. Recently it has been found that *Streptococcus fæcalis* appears to be more resistant to drying and storage in the resultant powder, and provided that difficulties involved in obtaining a viable count of a *Streptococcus* can be overcome, this organism may prove very satisfactory. For the present, however, the use of *Bact. lactis aerogenes* has been continued, the strain used being No. 418, obtained from the National Collection of Type Cultures. The details of its use are as follows. For each spray-drying 10 ml. of peptone water was inoculated with the organism and incubated at 30°C. for 24 hours. The suspension so obtained was thoroughly mixed by means of a sterile pipette and added to the solution to be dried.

Choice of Method for Viable Counts. Wilson[6] advocated a method for counting viable organisms, using roll-tubes which were inoculated by means of dropping pipettes, and the accuracy of the technique has been established by Withell[7] and others. Anderson and Stuart[8] and Miles and Misra[9] used dropping pipettes to perform "surface-viable" counts. By this method surface growths of *B. subtilis* tend to spread, and since only a relatively small number of colonies are counted errors due to interference from spreading are magnified by the large multiplication factor involved. To minimise the difficulty Davis[10], using this method, reduced the colony size by incorporating sodium taurocholate in the medium, but, while he obtained a satisfactory statistical uniformity, he found the salt to have an inhibitory effect. This might operate unequally in the presence of other chemical agents. Such inhibition would be detrimental to the objects of the present investigation. With dropping pipettes as used by the above workers, the size of drop delivered is not only governed by the external diameter of the tip, but is also influenced by the temperature and viscosity of the liquid dropped. They are not, therefore, particularly suitable for measuring samples of liquids where the viscosity may differ, as is the case with original and reconstituted samples before and after drying. Snyder[11] compared the use of dropping pipettes with that of graduated pipettes in combination with both roll-tubes and plates and with surface counts. He found graduated pipettes to be more accurate, but the significance of the difference was lost when estimating viable counts because the pipetting

errors contributed only a small proportion of the total error. It was decided, therefore, to use pipettes marked with two graduations for the delivery of 1 ml. for dilutions and inoculations: roll-tubes were used for the counts, which were carried out in the following manner. 9 ml. of diluent were placed in each of a number of plugged test-tubes. 1 ml. of the suspension to be counted was pipetted into the first of these tubes. A second pipette was used to mix the contents of the tube and to transfer 1 ml. of the mixed suspension to the second tube. A suitable number of such serial dilutions was made, a fresh pipette being used for each transference. 1 ml. portions of the final dilution were inoculated into roll-tubes containing 5 ml. of melted agar medium which had previously been maintained at a temperature of 46°C. The roll-tubes, after inoculation, were held horizontally under a stream of cold water and rotated until the agar set. They were then placed, plug-downward in the incubator. The accuracy of the technique has been assessed statistically according to the method used by Withell[7], Davis[10], Berry and Michaels[12] and others. In the immediately following paragraphs the results refer to experiments with *B. subtilis* spores. A summary of the corresponding results with *Bact. lactis aerogenes* is given on pages 891 *et seq.*

The Accuracy of the Graduated Pipettes. The pipettes used were made of fine bore glass tubing tapered at one end to a stout point. A ring was marked round the tube about 2 cm. from this end, and a second mark made at a distance above this corresponding to a delivery of 1 ml. The pipettes were calibrated gravimetrically. The weights of water delivered by the pipettes were all greater than 0·9873 and less than 1·0073 g. and the mean deviation from the theoretical values (0·9973 g.) was 0·0043 g., giving a mean percentage deviation of 0·431 per cent. Jennison, Marshall and Wadsworth[13] stated that pipettes suitable for viable counts should have an accuracy within ± 1 per cent.

In a viable count, however, the delivery of liquid is not controlled as carefully as in the above calibration. Twenty pipettes were taken at random and fitted with rubber teats by means of which water could be drawn up to the upper mark and ejected until the meniscus reached the lower mark. The water so discharged was weighed, and the process repeated 3 times for each pipette. In order to make the conditions as severe as possible no specific time was allowed for drainage, and the various pipettes when being filled were inserted at different depths into the water. The results are given in Table I.

From the weights of water discharged from the same pipette the variance was calculated and from the mean of these the co-efficient of variation was found to be 0·77 per cent. From the same figures three variances of the weights of water discharged from different pipettes were also calculated and from the mean of these the co-efficient of variation was found to be 1·32 per cent. These figures compare with 1·03 per cent. and 1·83 per cent. obtained by Withell[7], using dropping pipettes.

The Accuracy of the Dilution Technique. In most "viable counts," 10^{-1}, 10^{-2}, and 10^{-3}, dilutions have been prepared, the last dilution being tubed. Each dilution was prepared by pipetting 1 ml. of suspension into 9 ml. of diluent. As diluting agent quarter-strength Ringer's solution was used with *Bact. lactis aerogenes* while distilled water was used with *B. subtilis* since it was found to have no destructive effect on spores.

TABLE I

ERRORS INVOLVED IN MEASURING 1 ML. OF WATER WITH PIPETTES, FILLING AND EMPTYING BEING EFFECTED BY A RUBBER TEAT

	Weights			Mean ($\bar{x}$)	$S(x-\bar{x})^2$	Variance
	0·9830	0·9825	0·9800	0·9818	0·00000517	0·000002585
	0·9950	0·9920	0·9750	0·9873	0·00023267	0·000116335
	0·9793	0·9687	0·9620	0·9700	0·00015218	0·000076090
	0·9730	0·9809	0·9651	0·9730	0·00012482	0·000062410
	0·9945	1·0055	0·9972	0·9991	0·00006573	0·000032865
	0·9865	0·9700	0·9820	0·9795	0·00014550	0·000072750
	0·9785	0·9560	0·9793	0·9713	0·00034993	0·000174965
	0·9845	0·9822	0·9864	0·9843	0·00000886	0·000004430
	0·9785	0·9934	0·9841	0·9853	0·00011329	0·000056645
	0·9718	0·9867	0·9753	0·9779	0·00012141	0·000060705
	0·9994	0·9831	0·9834	0·9886	0·00017393	0·000086965
	1·0000	1·0100	0·9916	1·0005	0·00016971	0·000084855
	1·0020	1·0032	0·9928	0·9993	0·00006475	0·000032375
	0·9792	0·9960	0·9920	0·9891	0·00015403	0·000077015
	1·0086	0·9925	0·9945	0·9985	0·00015401	0·000077005
	0·9731	0·9807	0·9910	0·9816	0·00016142	0·000080710
	1·0050	1·0058	1·0000	1·0036	0·00001976	0·000009880
	0·9873	0·9841	0·9959	0·9891	0·00007448	0·000037240
	0·9807	0·9800	0·9759	0·9789	0·00001345	0·000006725
	1·0069	1·0131	1·0117	1·0106	0·00002115	0·000010575
Mean ($\bar{x}$) ...	0·9883,	0·9888,	0·9858	Total		0·001163125
$S(x-\bar{x})^2$ ...	0·00272404,	0·00419382,	0·00274324	Mean Variance ...		0·000058156
Variance ...	0·00014337,	0·00022073,	0·00014438	S. Deviation... ...		0·007626
M. Variance	0·00016949			Co-efficient of Variation		0·77%
S. Deviation	0·01302					
Co-efficient of Variation ...	1·32%					

Wilson[14] showed that distilled water was lethal to vegetative organisms and found quarter-strength Ringer's solution to be satisfactory for suspending such an organism. He also showed that distilled water had a dispersive effect on clumps of bacteria occurring in milk. The 9-ml. quantities of diluent were delivered into the test-tubes from a burette consisting of a graduated 10-ml. pipette, plugged at the upper end with cotton wool, and connected at the lower end by means of a two-way tap, either to a flask containing the diluent, or to a hooded nozzle from which the diluent could be measured into a test-tube. The co-efficient of variation of ten 9-ml. samples delivered from the apparatus was found to be 0·287 per cent.

In order to estimate the overall error of diluting and pipetting, 20 serial dilutions were carried out. From each of 20 spore suspensions, 10^{-1}, 10^{-2}, and 10^{-3} dilutions were prepared in duplicate. Five roll-tubes were then inoculated from each of the 10^{-3}, dilutions giving 2 sets of 5 tubes from each spore suspension. The results are given in Table II.

The variance of the mean counts obtained from each pair of dilutions was calculated and from these the mean co-efficient of variation was found to be 3·06 per cent. Another similar experiment by a different

worker gave a mean co-efficient of 3·37 per cent. These figures may be compared with 4·95 per cent. obtained by Withell[7] and 3·79 per cent. obtained by Berry and Michaels[12].

The Error of Counting Colonies of B. subtilis. If a roll-tube be counted on successive occasions counts will be obtained which differ slightly from one another. The differences may be attributed to:—(a) Inability to recognise small colonies; (b) The appearance of "double" colonies where two colonies lie one above the other in the medium; (c)

TABLE II

ERRORS OF DILUTING AND PIPETTING

Experiment	1st 10^{-3} dilution (mean count of 5 tubes)	2nd 10^{-3} dilution (mean count of 5 tubes)	Variance
1	168	163	12·5
2	218	219	0·5
3	199	197	2·0
4	186	195	40·5
5	187	195	32·0
6	206	203	4·5
7	208	201	24·5
8	212	219	24·5
9	196	204	32·0
10	212	226	98·0
11	237	253	128·0
12	131	124	24·5
13	119	128	40·5
14	124	120	8·0
15	159	160	0·5
16	134	131	4·5
17	132	137	12·5
18	135	129	18·0
19	78	72	18·0
20	79	75	8·0

The suppression of some colonies by local crowding; (d) The occurrence of spreading surface colonies both at the medium-air interface and the medium-glass interface; (e) The development of daughter colonies arising from the surface colonies; (f) Parallax error. Colonies which have been counted are identified by marks made on the surface of the glass. As the tubes are rotated, the relative positions of the marks and of the colonies lying deeper in the agar may alter so that colonies which have been marked appear to be unmarked and vice versa.

Wilson[14] investigated the first three of these sources of error. He concluded that the use of a small hand lens magnifying 2 or 3 diameters greatly aided the recognition of small colonies. He also examined the structure of double colonies appearing as circular surface colonies upon which smaller, lenticular colonies, lying deeper in the agar, were superimposed, and suggested that where the lenticular colony was placed centrally with respect to the circular colony these should be regarded as one, whilst when the lenticular colony was eccentrically placed the two colonies should be regarded as separate. Wilson also showed that if the tubes are overcrowded suppression of some colonies may occur and recommended that the count should lie between 30 and 300. All these recommendations have been followed in the work described in this paper.

The presence of spreading surface growth has been generally held to

cause low values for the viable count and various attempts have been made to reduce the tendency of such colonies to form and to reduce their size when they do occur. Thornton[15] endeavoured to control them by reducing the nutrient content of the agar medium but the period of incubation had to be extended to 10 days which is unsuitable for roll-tubes because they dry out unless the incubator atmosphere is kept saturated with water, in which case the surface growth is greatly increased. Various methods of drying the surface of the agar before incubation were tried but all proved unsatisfactory.

The surface growth may affect the count in two ways. Firstly, it may become confluent with other colonies growing on the surface. The proportion of these, however, is small and the surface growth occupies usually only one-third or less of the total surface. The number of colonies affected in relation to the total number occurring in the tube is therefore not likely to be large. Moreover, discrete colonies are often observed lying on the surface in the middle of the spreading growth and surrounded by a clear ring, which suggests that during the period of incubation they have not lost their identity. Secondly a more serious difficulty is caused by the presence at the edge of the spreading growth of numbers of small discrete daughter colonies, produced on the surface from it. These colonies differ in appearance from those submerged colonies normally seen in tubes where no spreading growth occurs; differentiation was based on the following characteristics.

Submerged Colonies are small, irregular, or "woolly" colonies, ½ to 1 mm. in diameter, opaque, white or pale cream by reflected light, brown by transmitted light.

Surface Colonies may be produced from the submerged colonies. When these have grown to reach the upper surface they produce a spreading colony thereon. The size of this depends on the amount of moisture present at the surface of the medium. During incubation this moisture film gradually dries up, so that the depth at which an organism is implanted in the medium and the speed with which it grows govern the size of surface colony produced. Thus many colonies produce no surface growth. Others produce circular surface colonies; these are thin, or slightly raised, the surface being smooth or showing radiate ridges or wrinkles; the submerged colony can be seen as a denser spot lying below the centre of the circular colony. More advanced surface colonies show transitions from the circular type through lobate or pinnatifid forms to much-branched, somewhat radiate, moderately thin, colonies, the ends of the branches and their subdivisions being club-shaped. A small submerged colony can be seen at the centre of radiation. In the largest "spreaders" the centre is occupied by a continuous film of growth. Since these have probably been produced from organisms implanted on or very near the surface of the medium there is no submerged colony apparent. Surface colonies are occasionally produced at the medium-glass interface. These are very thin with irregular edges and are of an even, granular texture.

Daughter Colonies are sometimes produced from the edges of extensive

surface " spreaders." They are circular or scaly, effuse and much thinner than the " spreader " or the circular colonies described above. Since they are not produced from submerged colonies they show no central spot. They occur in groups the members of which are similar in size and texture and the presence of submerged colonies growing below them can usually be easily recognised. Daughter colonies may also be formed from colonies at the medium-glass interface. They resemble their parent colonies but occur in well-marked groups.

It appeared that it should be possible to perform a " viable count " satisfactorily even in the presence of the surface growths. Counting tests were therefore performed to determine the effect of these growths and of the parallax error.

Twenty tubes were taken and each was counted three times. Three methods of performing the count were used, as follows:—

Method (1). 8 longitudinal lines and 3 transverse rings were marked on each tube with a wax pencil. The tube was thus divided into small areas within which the colonies were counted. Colonies touching the line were counted only on the upper and right hand margins of each area. All colonies including indistinct and daughter colonies were counted.

Method (2). As above, but any daughter colonies or colonies rendered indistinct by the surface growth were ignored. A new set of tubes was used, half the tubes having a high count and half a lower count.

Method (3). As in (2) but marking the tubes with 1 longitudinal line and 6 transverse rings.

The results are set out in Table III.

TABLE III

THE ERRORS OF COUNTING TUBES ON SUCCESSIVE OCCASIONS BY DIFFERENT METHODS

Method (1		Method (2)		Method (3)	
Counts	Variance	Counts	Variance	Counts	Variance
347 350 346	4·5	436 431 432	7·0	433 426 430	12·5
610 599 617	81·5	463 465 475	41·5	463 453 465	41·5
325 324 322	3·5	404 389 419	226·5	409 407 409	1·5
563 566 561	6·5	448 422 437	170·5	393 394 398	7·0
546 549 527	142·5	408 384 394	145·0	443 459 443	85·5
598 590 596	17·5	464 448 453	65·0	400 400 408	21·5
537 522 486	687·0	420 385 399	310·5	408 395 394	61·0
400 402 398	4·0	431 395 402	364·5	394 399 401	13·0
457 434 432	176·5	421 393 391	281·5	431 429 432	2·5
369 364 374	25·0	194 189 191	6·5	193 194 193	0·5
344 356 356	48·0	167 170 165	6·5	172 173 171	1·0
339 334 331	18·0	196 197 201	7·0	189 199 192	26·5
339 322 334	76·5	186 192 188	9·5	197 187 182	58·5
273 275 290	86·5	159 160 158	1·0	177 160 161	91·0
305 277 278	253·0	202 198 191	31·0	215 225 223	28·0
277 265 273	37·5	193 196 188	16·5	194 188 188	12·0
249 282 287	426·5	177 177 181	5·5	178 178 178	0·0
281 271 276	25·0	186 184 189	16·5	193 184 186	22·5
272 257 272	75·0	206 209 207	2·5	196 201 195	11·5
275 273 277	4·0	211 210 209	1·0	205 214 210	20·5
	2198·5		1708·0		518·0
Overall variance ...	=109·9	Overall variance	= 85·4	Overall variance	= 25·9
Standard deviation	=10·48	Standard deviation	= 9·24	Standard deviation	= 5·09
S.E. of mean of three counts	=6·098	S.E. of mean of three counts ...	= 5·34	S.E. of mean of three counts ...	= 2·94

The Standard Error of the Mean of Three Counts using Method (1) is 6·098, and the majority of the variances are excessive. The effect of the surface growth, either by virtue of obliteration of other colonies or by masking of them at its fringes, would be greatest on the lower counts. The use of Method (2) should minimise the effect. On the other hand, the parallax error should not be considerable on tubes of low count, for the distance separating the colonies is greater and the number of colonies bordering the demarkation lines fewer than in tubes of high count. The experimental results of Method (2) are in agreement with this, the variances of the lower counts being significantly improved, while those of the higher counts show no such improvement. Using Method (3) the parallax error has been minimised, for this occurs almost entirely along the longitudinal markings, since a slight rotation of the tube easily brings a colony from one side of the line to the other. Using this method the variances of both high and low counts were satisfactory and the Standard Error of the Mean of Three Counts was found to be 2·94, comparing favourably with that of 2·44 obtained by Berry and Michaels[12] using *E. coli.* It may be mentioned that a different worker using Method (2) obtained a Standard Error of 4·73. The same worker using Petri dishes instead of roll-tubes obtained the figure of 7·26 for the Standard Error.

A direct estimate of the effect of the surface growth on the viable count was also afforded by a series of tubes inoculated from the same suspension, in which a considerable variation occurred in the size of surface colonies present. The results are given in Table IV.

TABLE IV

THE EFFECT OF SURFACE GROWTH ON THE VIABLE COUNT OF *B. SUBTILIS*

Count	Area of Surface Growth	Count	Area of Surface Growth
146	Extensive	143	Very slightly
137	Nil	111	Slight
133	Moderate	143	Nil
123	Slight	146	Nil

The highest count was obtained in two tubes, one of which bore an extensive surface growth, while the other showed none at all, and the tube with the lowest count had only a small " spreader." There is, in fact, no significant difference between the counts which can be attributed to the effect of the surface growth, and it appears that viable counts may be satisfactorily performed even in the presence of extensive spreading colonies.

The Suitability of the Agar Medium. As pointed out above, an organism is regarded as viable if it produces a colony in the roll-tube, and as non-viable if it fails to produce such a colony. The composition of the medium used in the roll-tubes is to some extent responsible for the rate of growth of the colonies and the ultimate size which they attain. It is even more important that certain, possibly damaged or weakened, individual cells may fail to grow in one medium, while they produce a

colony and so give rise to a higher count in a more favourable substrate. The medium is, therefore, very important. For work such as that described in this paper it should (i) be accurately reproducible, (ii) be uniform throughout, all the ingredients preferably having been in solution, (iii) give concordant replicate counts from time to time and from batch to batch, (iv) give maximal counts for any given bacterial suspension.

Broadly speaking, three types of media are available: (i) Synthetic media prepared entirely from pure chemicals. Different batches may be identical, but they are difficult to prepare and opinion is by no means fixed as to the proportions of ingredients which they ought to contain. Their sensitivity is likely to vary greatly with the addition or omission of trace substances or vitamins. (ii) At the other extreme are the media prepared by direct extraction of meat tissues, with or without addition of serum. Such media are very sensitive, often giving the highest counts, but there is no guarantee of their constancy of composition from batch to batch. (iii) In between the above classes are the media prepared from peptone, with or without addition of commercial meat extracts. These have the advantages of being moderately sensitive and yet reproducible, for relatively large samples of peptone and meat extract can be purchased so that the batches of media prepared from them during the course of a lengthy set of experiments will not vary detectably. For these reasons this type of medium was chosen and three examples were examined. They consisted of:—

A. 3 per cent. of agar with 2·0 per cent. of peptone and 0·5 per cent. of sodium chloride.

B. The same with the addition of 0·5 per cent. of proteolysed liver extract.

C. The same as A with the addition of 0·5 per cent. of Lab-Lemco.

To prepare the media the ingredients were dissolved in distilled water, adjusted to *p*H 7·6 with N caustic soda, solution being effected by heating in an autoclave at 10 lb. pressure. The solution was filtered through washed sand and filter-paper pulp. The medium was then sterilised by autoclaving at 10 lb. pressure. The final *p*H was 7·2.

To compare the media, 25 roll-tubes of each were inoculated with 1 ml. from the same suspension of the test organism. Each of these batches was divided into 5 groups of 5 tubes which were incubated at different temperatures, the same 5 temperatures being applied to all three batches. The results are given in Table V.

The mean counts obtained with medium C are lower than those obtained with the other media. The counts on Medium A at temperatures between 26°C. and 42°C. do not differ significantly from those on Medium B, but the surface growth was thicker on the latter. Medium A was, therefore, chosen for use with *B. subtilis*.

Thornton[15] suggested that batches of media should comply with two requirements: (i) Different batches should give reproducible results. (ii) Parallel platings from the same batch should develop the same number of colonies within the limits of sampling variance.

TABLE V

THE EFFECT OF TEMPERATURE AND COMPOSITION OF MEDIUM ON THE VIABLE COUNT OF *B. SUBTILIS*

	Medium A					Medium B					Medium C				
	Incubation Temperature					Incubation Temperature					Incubation Temperature				
	20°C	26°C	32°C	37°C	42°C	20°C	26°C	32°C	37°C	42°C	20°C	26°C	32°C	37°C	42°C
	90	131	155	127	120	79	112	136	198	75	53	72	77	82	121
	109	136	135	133	122	36	110	126	139	94	56	95	88	89	126
	86	118	120	120	127	7	131	116	107	115	57	73	77	83	138
	103	111	122	142	138	10	149	125	112	85	57	95	127	97	103
	86	141	145	106	108	22	124	137	124	97	44	80	114	93	117
Mean ...	95	127	135	126	123	17	125	128	116	93	53	83	97	89	121

To test requirement (i), 5 tubes were rolled from each, using the same suspension of the test organism in each case. The results for successive batches are given in Table VI.

TABLE VI

THE REPRODUCIBILITY OF VIABLE COUNTS ON SUCCESSIVE BATCHES OF MEDIA

Batch I		Batch II			
Count	Mean	Count	Mean	T	P
82 90 76 86	84	118 71 92 85	91	0·462	0·6 to 0·7
97 146 112 111 116	116	115 123 123 119 116	119	0·363	0·7 to 0·8
126 140 127 130 110	126	125 113 120 133 110	120	0·942	0·3 to 0·4

The probability is in each case satisfactory and it may be concluded that different batches of the medium can be prepared having the same sensitivity to the test organism.

Requirement (ii) can be tested by the use of the statistic χ^2 calculated in the form

$$\chi^2 = \frac{S(x-\bar{x})^2}{\bar{x}}$$

Berry and Michaels[12] tested each batch of medium by counting 20 replicate tubes and comparing the value of χ^2 obtained from them with that to be expected if the variation involved only the normal sampling variance. For comparison, results of similar tests are included here. Table VII shows a typical result for one batch and Table VIII shows the summarised results for all the batches used in the present experiments. In all cases P was found to be satisfactory.

This test, however, is really a test of all the errors involved in carrying out the count and while, assuming all other errors to be small, the agar may be assumed to be satisfactory, the test is not sufficiently comprehensive to assess the overall error. Moreover, such a test would conceivably

be of use in testing media used with mixed bacterial cultures where, for instance, the encouragement of certain fast-growing organisms might militate against the development of slower growing colonies, but it is difficult to see how a homogeneous medium could increase the variance of counts on a pure culture.

The Duration and Temperature of Incubation. The optimum temperature for growth of *B. subtilis* is given by Bergey[16] as 30° to 37°C. and by Topley and Wilson[17] as 37°C. It is possible also that it may vary to some extent with the medium employed and a test was therefore carried out to examine this. Table V, to which reference has already been made, shows the counts resulting from incubation at various temperatures and on various media. In these experiments the temperature for maximal counts for *B. subtilis* appears to lie betwen 26°C. and 42°C. and incubation temperatures within this range were therefore used for this organism.

TABLE VII

GOODNESS OF FIT OF χ^2 OBTAINED FROM 20 REPLICATE ROLL-TUBES OF *B. SUBTILIS*

Count (x)	Mean ($\bar{x}$)	$(x-\bar{x})$	$(x-\bar{x})^2$	$x^2 = \frac{S(x-\bar{x})^2}{\bar{x}}$
180		13	169	
181		12	144	
206		13	169	
192		1	1	
195		2	4	
180		13	169	
206		13	169	
181		12	144	
189		4	16	
207	193	14	196	$\frac{2782}{193}$
203		10	100	=14·42
183		10	100	
180		13	169	
202		9	81	
211		18	324	
203		10	100	
190		3	9	
180		13	169	
178		15	225	
211		18	324	

N = 19 x^2 = 14·42 P = 0·8 — 0·7

TABLE VIII

SUMMARY OF VALUES OF χ^2 OBTAINED FROM SETS OF 20 ROLL-TUBES OF *B. SUBTILIS*

Batch No.	No. of Tubes	N	X^2	P
1	20	19	14·42	0·8—0·7
2	20	19	17·98	0·7—0·5
3	20	19	14·13	0·8—0·7
4	20	19	13·17	0·9—0·8
5	20	19	15·60	0·7—0·5
6	20	19	18·59	0·5—0·3
7	20	19	11·83	0·9—0·8
8	20	19	14·81	0·8—0·7
9	19	18	11·83	0·9—0·8

In order to determine the incubation period for *B. subtilis* likely to produce the most reliable counts 5 tubes were incubated at 32°C. and counted at suitable intervals. The results are given in Table IX.

TABLE IX

EFFECT OF DURATION OF INCUBATION ON THE VIABLE COUNT OF *B. SUBTILIS*

Period of Incubation	Count	Mean	Variance
24 hours	160, 165, 143, 161, 115, 148	149	343
40 hours	168, 163, 145, 160, 150, 146	155	93
48 hours	171, 166, 146, 154, 150, 151	156	98
60 hours	175, 155, 146, 152, 148, 143	153	133

The mean counts show no significant differences but at 24 hours the variance of the counts appears excessive, probably because many of the colonies are too small to be seen satisfactorily. While the experiment is too small to permit of accurate conclusions, the mean count at 24 hours would appear to be unreliable. After 60 hours' incubation the surface growth is thicker and, while this has produced no significant diminution in count, it renders the process of counting more difficult. The most suitable incubation period for *B. subtilis* would therefore appear to be about 48 hours. The fact that the count has not diminished after 60 hours tends to confirm the findings discussed above that the surface growth has little effect upon the viable count.

Table X shows the result of another experiment in which temperature and duration of incubation were both varied. It suggests that a temperature between 32°C. and 40°C. should be used with an incubation period of about 48 hours.

TABLE X

EFFECT OF TEMPERATURE AND DURATION OF INCUBATION ON THE VIABLE COUNT of *B. SUBTILIS*

	Temperature							
	26° C.		32° C.		37° C.		40° C.	
Period of Incubation	Count	Mean	Count	Mean	Count	Mean	Count	Mean
24 hours ...	65, 58, 50, 55, 45	65	188, 192, 206, 220, 185	196	336, 246, 307, 245, 410	309	367, 414, 314, 429, 416	382
48 hours ...	89, 119, 105, 108, 111	106	222, 205, 223, 221, 242	223	442, 301, 369, 356, 420	374	401, 470, 345, 446, 405	393
120 hours ...	107, 154, 142, 130, 140	135	235, 226, 242, 217, 210	226	385, 330, 333, 346, 410	361	411, 378, 383, 392, 405	394
168 hours ...	105, 151, 141, 118, 131	129	240, 227, 235, 226, 221	230	390, 315, 326, 350, 411	357	397, 457, 383, 385, 407	386

The Normal Sampling Variance. Fisher, Thornton and Mackenzie[18] showed that plate counts approximated to small samples of a Poisson series and deduced from this that χ^2, the Index of Dispersion, should exhibit a characteristic distribution which was tabulated by Elderton[19]. The distribution of the values of χ^2 determined experimentally could then be compared with the hypothetical distribution, and the Goodness

of Fit determined. Such a comparison can be used to examine the overall errors of performing viable counts and provides a more critical test than the use of a single x^2. The values of x^2 obtained with 100 samples of spore suspensions of *Bacillus subtilis* have been tabulated in Table X and their Goodness of Fit tested.

The observed distribution of x^2 shows no significant departure from the theoretical distribution and the Probability obtained is satisfactory. It may be concluded, therefore, that the technique used is reliable and that accurate and reproducible results can be obtained with it.

Results of Test of Counting Technique using Bact. lactis aerogenes. Tables XI, XII, XIII, XIV and XV relating to *Bact. lactis aerogenes* correspond with Tables III, V, VII, IX and X respectively for *B. subtilis.* Table XVI shows that quarter-strength Ringer's solution is a suitable diluent for use with *Bact. lactis aerogenes.* These tables establish the fact that reliable and reproducible values for the viable count of this organism can be obtained using Medium A and incubating at 20°C. to 40°C. for 24 hours.

TABLE X

GOODNESS OF FIT OF VALUES OF χ^2 OBTAINED FROM COUNTS ON SETS OF FIVE ROLL-TUBES, USING *B. SUBTILIS*

Value of x^2	Expected Frequency (m)	Observed Frequency (m+x)	Difference (x)	$\frac{x^2}{m}$
Under 1	9·02	13	3·98	1·756
Between 1 and 2	17·40	11	−6·40	2·355
Between 2 and 3	17·79	19	1·21	0·082
Between 3 and 4	15·18	20	4·82	1·530
Between 4 and 5	11·87	9	−2·87	0·695
Between 5 and 6	8·82	8	−0·82	0·076
Between 6 and 7	6·33	6	−0·33	0·017
Between 7 and 9	7·48	8	0·52	0·036
Over 9	6·11	6	−0·11	0·002

$\chi^2 = 6{\cdot}549$ N = 8 P = 0·5—0·7

This result was confirmed by another worker, who obtained the following results:—$x^2 = 6{\cdot}571$, N=7, P=0·3 – 0·5.

TABLE XI

THE ERRORS OF COUNTING TUBES ON SUCCESSIVE OCCASIONS BY METHOD (III)

Counts	Variance	Counts	Variance
183 190 187	12·5	238 236 240	4·0
208 206 211	6·5	218 214 219	7·0
201 205 193	37·5	216 211 215	7·0
209 207 207	1·5	237 234 244	26·5
210 206 208	4·0	224 224 218	12·0
182 175 180	13·0	230 233 228	7·0
209 209 210	0·5	200 202 201	1·0
206 205 202	4·5	188 189 188	0·5
203 203 203	0·0	205 200 207	13·0
221 218 218	3·0		
232 231 229	2·5		

Overall Variance = 8·175
Standard Deviation = 2·86
S.E. of Mean of Three Counts = 1·65

TABLE XII

THE EFFECT OF TEMPERATURE AND COMPOSITION OF MEDIUM ON THE VIABLE COUNT

	Medium A					Medium B					Medium C				
	Incubation Temperature					Incubation Temperature					Incubation Temperature				
	20°C	26°C	32°C	37°C	42°C	20°C	26°C	32°C	37°C	42°C	20°C	26°C	32°C	37°C	42°C
	595	575	659	609	591	544	553	620	601	614	416	531	403	499	306
	644	610	679	608	603	596	566	706	616	596	325	507	403	504	319
	621	550	660	567	603	555	574	616	657	650	308	498	452	429	391
	636	583	658	657	681	591	586	572	564	653	321	413	414	501	398
	658	541	693	574	591	644	564	659	663	—	365	549	433	481	396
Mean ...	631	572	670	603	614	586	569	635	620	628	347	499	421	483	362

At 18 hours many colonies are too small easily to be seen. At 48 hours large colonies show "tailing." At 24 hours colonies are all discrete and can be distinguished with ease.

TABLE XIII

GOODNESS OF FIT OF χ^2 OBTAINED FROM 20 REPLICATE ROLL-TUBES OF *BACT. LACTIS AEROGENES*

Count (x)	Mean ($\bar{x}$)	$(x-\bar{x})$	$(x-\bar{x})^2$	$\chi^2 = \frac{S(x-\bar{x})^2}{\bar{x}}$
220		9	81	
230		19	361	
224		13	169	
197		14	196	
203		8	64	
208		3	9	
191		20	400	
177		34	1156	
244		33	1089	
209		2	4	
201		10	200	
228	211	17	289	$\frac{4705}{211} = 22{\cdot}3$
205		6	36	
235		24	576	
207		4	16	
207		4	16	
209		2	4	
202		9	81	
218		7	49	
208		3	9	

$\chi^2 = 22{\cdot}3$ N = 19 P = 0·2–0·3

TABLE XIV

EFFECT OF DURATION OF INCUBATION ON THE VIABLE COUNT OF *BACT. LACTIS AEROGENES*

Period of Incubation	Count	Mean	Variance
18 hours	34 43 37 40 36	38	50
24 hours	32 41 37 40 36	37	51
48 hours	32 42 38 38 36	37	53

TABLE XV

GOODNESS OF FIT OF VALUES OF χ^2 OBTAINED FROM COUNTS ON SETS OF FIVE ROLL-TUBES USING *BACT. LACTIS AEROGENES*

Value of χ^2	Expected frequency (m)	Observed frequency (m+χ)	Difference (χ)	$\frac{\chi^2}{m}$
Under 1	8·48	11	2·52	0·75
Between 1 and 2	16·36	20	3·64	0·81
„ 2 and 3	16·72	17	0·28	0·01
„ 3 and 4	14·26	13	−1·26	0·11
„ 4 and 5	11·15	10	−1·15	0·12
„ 5 and 6	8·26	4	−4·26	2·20
„ 6 and 7	5·94	5	−0·94	0·15
„ 7 and 9	7·03	9	1·97	0·55
Over 9	5·74	5	−0·74	0·10

$\chi^2 = 4·80$. N = 8. P = 0·7—0·8.

TABLE XVI

THE NON-DESTRUCTIVE ACTION OF QUARTER-STRENGTH RINGER'S SOLUTION ON *BACT. LACTIS AEROGENES*

	Period of Exposure (Minutes)				
	0	30	60	120	180
Replicate Counts	436	398	405	398	398
	403	331	365	402	418
	422	359	383	363	357
	407	406	402	431	364
	400	390	388	367	408
Mean...	416	377	389	392	389

RESULTS WITH DRIED POWDERS

The Preparation of the Spray-Dried Powders. The technique of spray-drying and its use for the preparation of powders containing viable organisms have been described by Bullock and Lightbown[5]. In the present work the substrate used was 4 per cent. peptone water. This was adjusted to *p*H 7·6, filtered distributed in bottles of 1-1. capacity and sterilised by autoclaving. The contents of the bottles were inoculated with a quantity of the suspension of the test organism (spore suspension in the case of *B. subtilis*) calculated to give rise to a count of approximately 2×10^5 per ml. of peptone water. The resultant suspension, cooled in ice, was then spray-dried using an air inlet temperature of 180° to 190°C. for *B. subtilis* and of 70° to 80°C. for *Bact. lactis aerogenes.* Free-flowing powders were obtained in each case. These were stored over phosphorus pentoxide in a desiccator.

Reconstitution of the Bacterial Suspension. A weighed quantity of the powder obtained was dissolved in about 9 ml. of diluent (glass-distilled water in the case of *B. subtilis* and quarter strength Ringer's solution for *Bact. lactis aerogenes*). Bullock and Lightbown[5] compared the strength of this reconstituted solution with that of the original by estimating the chloride content of each. In the present case, however, the substrate was

required to be of minimal chloride content for use in subsequent experiments. The two solutions were, therefore, compared by a colorimetric method. The powder was dissolved in such a quantity of diluent as would produce a solution stronger than the original solution from which the powder was prepared. 4 ml. of this reconstituted suspension was transferred by means of a sterile pipette to one cup of a Spekker photoelectric absorptiometer; 6 ml. of the original suspension was placed in the other cup of the absorptiometer. The two solutions were compared using a dark-blue filter and water was measured into the reconstituted suspension until it matched the original. Mixing of the diluted suspension was effected by means of a platinum wire and the extent of the dilution was noted. The remainder of the reconstituted suspension was diluted proportionately with the sterile diluent. During the process of spray-drying some darkening of the peptone may occur. This would affect the colorimetric reconstitution, increasing the dilution of the reconstituted suspension and causing it to have a lower count. This effect was investigated by spray-drying a 4 per cent. solution of peptone containing 0·5 per cent. of sodium chloride. The relative strengths of the original solution and a reconstituted sample were then determined both by the colorimetric method and by estimation of the chloride content. The results are given in Table XVII.

It was considered established that the colorimetric method was sufficiently accurate.

Percentage mortality of the organism on drying. To determine the effects of spray-drying on the organism in suspension viable counts were carried out on the suspensions fed to the dryer and the material reconstituted as described in the previous paragraph. As will be seen from Tables XVIII and XIX, *B. subtilis* spores suffer up to 10 or 12 per cent. mortality, while only 0·05 to 1·6 per cent. of the *Bact. lactis aerogenes* survive.

TABLE XVII

	Weight Taken (g.)	Volume of Water (ml.)	Colorimetric Factor	Back Titre of Ammonium Thiocyanate Solution	Titre of Sample	Chemical Factor	Error of Colorimetric Method
Original ...	—	—	1·000	5·5 ml.	15·55 ml.	1·000	—
Sample 1 ...	0·7777	15·0 ml.	1·000	3·2 ml.	14·65 ml.	0·945	−5·5 per cent.
Sample 2 ...	0·7563	14·0 ml.	1·175	1·8 ml.	17·45 ml.	1·123	−5·4 per cent.

20 ml. of Silver Nitrate Solution ≡ 21·05 ml. of Ammonium Thiocyanate Solution

TABLE XVIII

PERCENTAGE OF *B. SUBTILIS* SPORES (SUSPENDED IN 4 PER CENT. PEPTONE WATER) KILLED BY SPRAY-DRYING AT VARIOUS TEMPERATURES

Temperature of Inlet Air	Percentage Mortality	Temperature of Inlet Air	Percentage Mortality
134°C.	12·2	165°C.	0·0
150°C.	0·76	181°C.	3·6
150°C.	11·7	205°C.	0·0

TABLE XIX

MORTALITY OF *B. LACTIS AEROGENES* (SUSPENDED IN 4 PER CENT. PEPTONE WATER) DURING SPRAY-DRYING AT VARIOUS TEMPERATURES

Temperature of Inlet Air	Percentage Mortality	Temperature of Inlet Air	Percentage Mortality
70°C	98·40	120°C.	99·95
75°C	99·38	150°C.	99·68
75°C	99·37	180°C.	99·61
80°C	99·90		

The Distribution of Organisms in the Powder.—To test the distribution of the organisms in the powders obtained by spray-drying, 10 samples of powder were weighed out and dissolved in quantities of diluent proportional to their weights, so that the resultant solutions contained equal concentrations of peptone. The weights of powder taken were such that the solutions obtained from them gave a count of about 200,000 per ml. The 10^{-3} dilution from each sample was then plated out in quintuplicate and the counts determined, as described in the earlier part of this paper. These were compared by means of the Analysis of Variance. The counts obtained with *B. subtilis* are recorded in Table XX and the Analysis of Variance of these in Table XXI.

The results for a similar experiment using *Bact. lactis aerogenes* are given in Table XXII and the corresponding Analysis of Variance in Table XXIII.

TABLE XX

QUINTUPLICATE PLATING OF 10 SAMPLES OF THE SAME SPRAY-DRIED POWDER (*B. SUBTILIS*)

Sample	I	II	III	IV	V	VI	VII	VIII	IX	X
Weight (g.) ...	·5198	·5615	·5311	·4954	·5131	·6555	·5295	·4860	·6582	·5095
Volume (ml.) ...	8·8	9·5	9·0	8·4	8·7	11·1	8·95	8·2	11·2	8·65
Counts	249	224	232	210	228	233	252	230	247	247
	230	238	236	247	241	256	264	253	226	231
	260	227	211	244	233	205	227	232	230	241
	234	227	223	245	220	246	248	255	223	224
	238	262	233	207	278	216	239	233	236	214
Total Counts ...	1211	1178	1135	1153	1200	1156	1230	1203	1162	1157

TABLE XXI

ANALYSIS OF VARIANCE OF QUINTUPLICATE PLATING OF 10 SAMPLES OF THE SAME SPRAY-DRIED POWDER (*B. SUBTILIS*)

Source of Variation	Sum of Squares	N	Mean Square	Variance Ratio	P
Difference between samples	1710·9	9	190·1	1·296	>0·2
Difference between individuals (error) ...	9859·6	40	246·5		
Total	11570·5	49			

Table XXIII suggests that the organisms are not very evenly distributed in the powder. A further spray-drying was performed and samples taken and counted as before. The results are given in Table XXIV and the Analysis of Variance in Table XXV.

TABLE XXII

QUINTUPLICATE PLATING OF 8 SAMPLES OF THE SAME SPRAY-DRIED POWDER (*BACT. LACTIS AEROGENES*)

Sample	I	II	III	IV	V	VI	VII	VIII
Weight (g.) ...	·5284	·4680	·5044	·4991	·5061	·4331	·4037	·5702
Volume (ml.) ...	13·21	11·7	12·61	12·48	12·4	10·83	10·09	13·01
Counts	160 181 163 180 180	191 163 173 158 163	151 149 152 140 154	183 187 177 152 188	142 154 146 148 158	171 166 141 150 163	198 138 167 189 184	178 149 149 134 159
Total Counts	864	848	746	887	748	791	876	759

TABLE XXIII

ANALYSIS OF VARIANCE OF QUINTUPLICATE PLATING OF 8 SAMPLES OF THE SAME SPRAY-DRIED POWDER (*BACT. LACTIS AEROGENES*)

Source of Variation	Sum of Squares	N	Mean Square	Variance Ratio	P
Difference between samples	4100	7	585·7	2·505	0·01—0·05
Difference between individuals	7482	32	233·8		
Total	11582	49			

Table XXV shows that the organisms in the powder obtained from the drying of a suspension of *Bact. lactis aerogenes* were distributed very unevenly. The remainder of the powder was therefore placed in a sterile vaccine bottle together with some sterile glass beads. The bottle was closed by means of a rubber cap and fixed to a revolving wheel by means of which it was slowly rotated for a period of 24 hours. 10 further samples were then taken and viable counts performed as before. These are given in Table XXVI and the Analysis of Variance in Table XXVII.

TABLE XXIV

QUINTUPLICATE PLATING OF 10 SAMPLES OF THE SAME SPRAY-DRIED POWDER (*BACT. LACTIS AEROGENES*)

ample	I	II	III	IV	V	VI	VII	VIII	IX	X
Weight (g.) ...	·3170	·2600	·2345	·2555	·2075	·2160	·2355	·1830	·2215	·3305
Volume (ml.) ...	9·0	7·4	6·65	7·25	5·9	6·15	6·7	5·2	6·3	9·4
Counts	53 59 36 52 53	54 86 71 77 71	55 47 54 53 60	40 38 48 49 31	46 51 43 56 53	58 53 49 44 74	53 45 55 49 61	35 39 34 45 51	42 41 49 44 64	35 45 46 46 44
Total Counts ...	253	359	269	206	249	278	263	204	240	207

TABLE XXV

ANALYSIS OF VARIANCE OF QUINTUPLICATE PLATING OF 10 SAMPLES OF THE SAME SPRAY-DRIED POWDER (*BACT. LACTIS AEROGENES*)

Source of Variation	Sum of Squares	N	Mean Square	Variance Ratio	P
Difference between samples	3825·5	9	425·05	4·71	<0·01
Difference between samples	3604·8	40	90·12		
Total	7430·3	49			

TABLE XXVI

QUINTUPLICATE PLATING OF 10 SAMPLES OF THE SAME SPRAY-DRIED AND MIXED POWDER (*BACT. LACTIS AEROGENES*)

Sample	I	II	III	IV	V	VI	VII	VIII	IX	X
Weight (g.) ...	·2385	·2640	·2570	·2605	·3000	·2895	·2870	·3850	·4230	·3250
Volume (ml.) ...	7·0	7·75	7·55	7·65	8·8	8·5	8·4	11·3	12·4	9·55
Counts	76 80 95 80 85	91 86 89 71 94	91 106 93 110 112	109 89 119 76 94	56 92 95 88 98	82 93 100 91 72	81 103 106 112 97	112 114 72 97 86	91 102 104 89 105	88 88 95 118 71
Total Counts ...	416	431	512	487	429	438	499	481	491	460

TABLE XXVII

ANALYSIS OF VARIANCE OF QUINTUPLICATE PLATING OF 10 SAMPLES OF THE SAME SPRAY-DRIED POWDER (*BACT. LACTIS AEROGENES*)

Source of Variation	Sum of Squares	N	Mean Square	Variance Ratio	P
Difference between samples	2097·9	9	233·10	1·184	>0·2
Difference between individuals (error)...	7877·4	40	196·93		
Total	9975·3	49			

Tables XXVI and XXVII show that after thorough mixing by the above method the organisms have become evenly distributed in the powder.

The Viability of Organisms in Stored Powders.—The powders obtained by spray-drying were stored over phosphorus pentoxide in desiccators at room temperature. Samples were weighed out at intervals and dissolved in quantities of diluent proportional to their weight. The resultant suspensions were suitably diluted and the final dilutions were plated out in quintuplicate (10^{-3} for *B. subtilis*, 10^{-2} for *Bact. lactis aerogenes*). The mean count was determined for each set of 5 tubes and these means are set out in Table XXVIII.

TABLE XXVIII

EFFECT OF STORAGE ON COUNT OF ORGANISMS CONTAINED IN SPRAY-DRIED POWDERS

B. subtilis							
Period of Storage (Days)	0	12	22	48	80	124	157
Mean Count of 5 Tubes	129	124	126	132	131	132	128
Bact. lactis aerogenes							
Period of Storage (Days)	0	6	7	9	13	21	37
Mean Count of 5 Tubes	5426	743	513	309	301	200	64

DISCUSSION

Viable counts have always been subject to much criticism. In the last few decades some of the objections have been overcome. As a result of carrying out a large number of counts and submitting the results to statistical analysis it has been shown that, with certain organisms and using particular techniques, counts may be performed so as to give results reproducible within certain ascertained limits of error. Methods have also been elaborated for establishing the suitability or otherwise of particular media. In the present work *B. subtilis* spores and *Bact. lactis aerogenes* non-sporing organisms have been submitted to this type of examination.

Tables V to X show that the medium used (Medium A, Table V), is suitable for *B. subtilis* and that the spores can be counted accurately, the errors involved being no greater than the normal errors of random sampling. Having shown that satisfactory viable counts of *B. subtilis* spores could be performed if the spores are in the form of an even suspension, the next task was to examine the spray-dried powder containing these spores. Table XVIII shows two things. In the first place the mortality on drying is low and in the second place variations in the conditions of drying, e.g., in the temperature of the inlet air or the rate of flow of the liquid, have comparatively little effect on the percentage of organisms surviving. Thus one might expect to obtain an even distribution of spores in the resultant powder since local conditions at the jet, or variations in the length of time the powder is lodged in the machine, would not be expected greatly to alter the count of different portions of the powder. Further, in a previous paper it was shown that spores in dry powders were resistant to comparatively high temperatures. It has also been shown that the spores remain viable in the resultant powder over considerable periods of time and that there is little, if any, diminution in the viable count of the powder (Table XXVIII). That the expected even distribution of spores in the powder is in fact obtained is proved in Table XXI. The count variation from sample to sample of powder is shown statistically to be accounted for by the normal errors of random sampling. It is clear from the conclusions established that spray-dried powders containing *B. subtilis* spores are suitable for use

in further experiments. Such experiments involving the exposure to antiseptics and heat of both the powder itself and the powder suspended in oil, are in progress in this department.

In the case of *Bact. lactis aerogenes* the circumstances are not so simple. Table XIX shows that between 98·4 and 99·9 per cent. of the organisms are killed, i.e., that 0·1 to 1·6 per cent. survive, a very great variation. Furthermore, Table XXVIII shows that the organisms die off rapidly in the dry powder, a result previously reported by Bullock and Lightbown[5], who also showed that *Bact. lactis aerogenes* even in powder form is considerably more sensitive to heat than the spores of *B. subtilis*. Thus we should expect that slight variations in drying conditions around the spray jet and in the time during which the various portions of powder are in contact with the metallic surfaces of the drying chambers would have a considerable effect on the number of organisms surviving. It is not therefore surprising to find that as shown in Tables XXIII and XXV there is evidence of uneven distribution of the organisms in the dried powder as discharged from the dryer. However, the powder is light and easily mixed and this treatment is shown in Table XXVII to result in a powder in which the organisms are evenly distributed.

It would appear, therefore, that the satisfactory nature or otherwise of a spray-dried powder probably depends upon the resistance of the organism to the drying process. If the organism is resistant, as in the case of *B. subtilis* spores, the powder is eminently satisfactory for further work. If the organism is susceptible as in the case of *Bact. lactis aerogenes,* then the powder is not so suitable. It requires careful mixing and in examining the effects of heat or antiseptics on the powder, and powder suspended in oil, the high death rate normally associated with the organisms must be taken into account. Experiments are therefore in progress to see if a more suitable organism than *Bact. lactis aerogenes* can be found and some, more promising, results have been obtained with *Streptococcus fæcalis.* Meanwhile it can be said that a powder containing *Bact. lactis aerogenes* in even distribution can be obtained and is suitable for use in experiments concerned with the study of environmental conditions on a relatively sensitive organism.

Summary

1. The technique of performing viable counts using graduated pipettes with roll-tubes has been examined and the accuracy of the method has been assessed by statistical analysis.

2. It has been shown that satisfactory viable counts can be obtained of spores of *B. subtilis* in suspension or in powders. The spreading surface growth of the organism has been shown to have no significant effect upon the count and roll-tube counts may be performed satisfactorily in its presence.

3. It has been shown that if even suspensions of *B. subtilis* spores are spray-dried the viable organisms are evenly distributed in the resultant powder.

4. It has been shown that if even suspensions of *Bact. lactis aerogenes* are spray-dried the viable organisms are not evenly distributed in the powder as taken from the spray-drier, but that an even distribution can be obtained if the powder is thoroughly mixed by mechanical means.

5. The effect of storage on the viability of the organisms in peptone powders has been examined. The viable count of *B. subtilis* was found to undergo no significant diminution after 6 months' storage, whereas the viable count of *Bact. lactis aerogenes* fell rapidly, most of the organisms dying within the first few days.

6. It is concluded that spray-dried powders containing spores of *B. subtilis* are very suitable for examining the effects of environmental conditions on the spores. On the other hand, the use of powders containing organisms, such as *Bact. lactis aerogenes,* which show a high mortality on spray-drying, involves greater difficulties, which may be inherent in the problem.

It is a pleasure to express our thanks to Professor M. S. Bartlett for suggestions and advice concerning the statistical treatment of the results reported in this paper.

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DISCUSSION

Miss Winifred Keepe presented the paper.

MR. B. A. BULL (Nottingham), deputising for the Chairman, said that the work described was an extension of the valuable work of Dr. Bullock and his colleagues on spray drying. The authors seemed to have evolved a suitable technique for a complex subject.

DR. K. R. CAPPER (London) said that bacteria in dust were in an environment of low moisture content, and dust was a very probable