UNIVERSITY OF EDINBURGH.

"STUDIES IN THE PHYSIOLOGY OF MILK BACTERIA WITH SPECIAL REFERENCE TO CONDITIONS OF GROWTH AND RESISTANCE TO DESICCATION".

by

MARION KATHLEEN STEVENS, B.Sc.

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INTRODUCTION.

The thesis has been divided into three sections. Section I describes a convenient method for estimating the viable count of a bacterial population, which was evolved during the course of the work; Section II deals with the death rates of the commoner milk micro-organisms when subjected to desiccation, and Section III with the growth of pure cultures of these organisms in milk. The work that was undertaken first was that described in Section III. It was originally intended to investigate the behaviour of bacteria in pure culture in milk and then to deal with mixed cultures and their mutual effects, under the same conditions. The results obtained indicated however, that the problem was more complex than had been anticipated and that a large amount of work with pure cultures would be necessary before the problem of mutual influences could be explored satisfactorily. On the advice of Dr. T. Gibson it was decided that this could not be overtaken in the time that was available and this problem was therefore abandoned in favour of the work described in Section II on the desiccation of micro-organisms.

My grateful thanks are offered to Professor S. J. Watson, Principal of the Edinburgh & East of Scotland College of Agriculture, and to Dr. T. Gibson, under whose supervision this work has been carried out; to Mrs. A. C. Stirling, B.Sc. for her assistance in the work described in Section I; to Dr. D. N. Lawley for advice on statistical analysis, and to Mr. J. Campbell for technical assistance.

I. A CONVENIENT METHOD FOR DETERMINING THE VIABLE COUNT OF A BACTERIAL POPULATION BY MEANS OF COLONIES DEVELOPING IN STRIPS OF AGAR IN TUBES.

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A CONVENIENT METHOD FOR DETERMINING THE VIABLE COUNT OF A BACTERIAL POPULATION BY MEANS OF COLONIES DEVELOPING IN STRIPS OF AGAR IN TUBES

Studies in the multiplication and rates of death of bacteria require a simple but accurate method for determining the viable count. The plate count is the standard method most often employed in such enumerative work, owing to the reliable results it is known to yield. When dealing with a large number of replicates it does, however, become rather cumbersome as it necessitates a large stock ofPetri dishes, much medium and considerable incubator space. The labour involved in washing and sterilization and the cost of replacements must also be considered. Modifications of the plating method have been suggested from time to time with a view to reducing the amount of materials and labour involved without impairing the accuracy of the count.

One of the earliest modifications was the roll tube method introduced by Esmarch (1) and later investigated on a quantitative basis by Wilson (2). It considerably reduces the amount of glass ware and medium required and lessens risk of contamination. In addition Wilson (2) claims that colonies developing within roll tube cultures are more easily counted than in plates.

The/

The method however, has not been used extensively probably owing to the dexterity and practice required in rolling the tubes. Inoculation and rolling of the tubes can best be carried out by separate workers, but even when this is possible considerable practice appears to be necessary to produce evenly distributed films of agar free from wrinkles. Various mechanical devices have been suggested, for instance (3) (4) (5), but it is not always convenient to introduce such specialised apparatus in all laboratories and at best, the process is slow and laborious.

Various surface counting methods are also in use at present. Those described by Snyder (6) and Crone (7) do not appear to economise in time or materials and necessitate the preparation of dried plates some time before use. Miles and Misra (8) recently described a method in which measured drops of the suspension are seeded on dried agar plates by means of dropping pipettes. This method has met with considerable favour and has been investigated in detail by Reed and Reed (9) using pure cultures of bacteria. Campbell and Konowalchuk (10) found it satisfactory for counts of raw milk. It is less laborious and a little more accurate than the plate count and can/

can be used with opaque as well as transparent media. It however, requires a certain number of Petri dishes and a constant supply of calibrated dropping pipettes. The main disadvantage appears to be in the preparation of the dried plates, which process must be carefully adjusted to ensure complete absorption of the drops in 15-20 minutes.

In an endeavour to find a simple method by which colony counts might be made, inoculation of small quantities of agar in test-tubes followed by sloping to form thin strips of agar, was tried. This gave promising results.

EXPERIMENTAL

Preliminary trials were carried out to determine the most suitable quantity and concentration of agar to use in each tube and the best methods of mixing and of forming the agar strips.

The technique finally adopted was as follows: 6 x $\frac{5}{8}$ in. test-tubes are used, each containing 1·5 - 2 ml. (estimated approximately when tubing) of 2% nutrient agar. The agar is melted and held in a water bath at 45°C for $\frac{1}{2}$-1 hr., before it is inoculated. This allows the condensation water to run down the tube and be incorporated in the agar again, thus lessening the amount of moisture appearing at the foot of the tubes during incubation. The presence of much moisture encourages the development of spreading growth, especially if the count is high.

A 1 ml. pipette graduated in 0·01 ml. is used to deliver inoculum into each/

each tube.

The agar and inoculum are mixed by rotating the tube 5 times and the tube is then laid on the bench with the stoppered end slightly raised to prevent agar from reaching the cotton wool plug. This produces a layer of agar 3-4 in. long. If necessary the tube is slightly tilted when it is being laid down to ensure that the agar flows evenly over the glass. The colonies that develop are counted with greater ease when the strip of agar is of good length. The agar solidifies rapidly and when the tubes are cool they are set upright in racks and placed in the incubator.

Statistical analysis of method.

Experiments were carried out to provide material for a statistical analysis, using a suspension of the spores of Bacillus megatherium which was stable and gave reproducible results throughout a period of 3 years.

Experiment I

This experiment was designed to compare the counts obtained in tubes with those obtained by the standard method in which 1 ml. quantities of spore suspension were mixed with approximately 10 ml. of nutrient agar in Petri dishes.

Six workers each inoculated 120 tubes and 10 plates; each worker used one pipette for the inoculation of both tubes/

tubes and plates. In order to detect any differences due to pipetting 0·1 ml. quantities, the 120 tube inoculations were divided into three groups:

(a) 40 tubes inoculated with consecutive 0·1 ml. amounts;

(b) 40 tubes inoculated with alternate 0·1 ml. amounts, i.e. 1 st., 3rd., 5th., 7th., and 9th., 0·1 ml. amounts;

(c) 40 tubes inoculated with the 1st and 6th 0·1 ml. amounts from each pipetteful.

In (b) and (c) the unused quantities were discarded into a sterile tube.

Both tubes and plates were kept in order of inoculation so that it was possible to correlate the counts with the 0·1 ml. quantities measured.

Results.

The colony counts obtained by each method are shown in table 1. The total values obtained by each worker for all tubes and plates are as follows:

Worker	Total tube counts.	Total plates counts.
1.	3,719	3,026.
2.	3,503	3,122
3.	3,539	2,955.
4.	3,602	3,028.
5.	3,501	2,964.
6.	3,491	3,127.
Total for all workers	21,355	18,222.

There appears to be good agreement in all cases.

The/

Table 1. Individual colony counts obtained by six workers after inoculation of 720 tubes and 60 plates.

Worker	Counts from tubes						Count from plates
	Group a		Group b		Group c		
	30	30	25	33	21	19	298
	29	42	23	35	21	35	317
	28	47	30	29	31	32	291
	28	27	37	23	31	24	291
	23	30	36	45	34	25	324
1.	35	32	30	32	37	24	335
	25	29	34	28	22	30	318
	31	40	27	30	24	37	314
	27	31	43	34	22	27	283
	46	30	35	36	32	37	255
	24	34	30	35	46	29	
	30	19	25	34	27	36	
	23	35	29	28	23	39	
	33	38	30	32	36	43	
	34	6	47	40	36	23	
	23	31	28	27	33	28	
	28	28	23	23	24	27	
	27	33	31	41	33	30	
	38	38	29	35	31	32	
	36	36	31	38	32	31	
	31	19	28	36	33	21	308
	32	29	24	27	27	42	293
	26	40	39	28	30	23	292
2.	22	28	25	30	33	25	268
	31	43	23	28	15	37	273
	27	30	27	0	25	12	281
	29	37	30	33	38	20	284
	33	24	29	27	31	29	523
	29	26	31	21	32	30	290
	27	36	20	34	20	38	310
	41	37	20	29	26	37	
	32	35	32	28	36	37	
	36	24	33	26	37	32	
	27	29	33	26	26	28	
	28	36	37	0	22	33	
	28	1	30	32	37	32	
	27	30	36	35	31	39	
	33	26	25	31	34	27	
	21	30	27	31	28	31	
	41	36	27	30	39	17	

Table 1 (Continued)

Worker	Count from tubes						Count from plates.
	Group a		Group b		Group c		
	27	35	34	30	29	28	358
	37	29	30	14	35	25	310
	29	27	21	28	31	22	263
	32	26	21	36	23	22	331
	26	32	25	31	33	38	
	28	28	29	31	31	34	266
	34	23	29	19	34	27	302
	29	36	35	25	25	41	289
	34	28	37	37	28	33	
	28	37	27	27	29	36	279
	32	23	31	34	24	29	283
3.	37	21	23	31	39	33	274
	27	36	28	41	30	33	
	28	0	29	38	29	21	
	27	20	33	28	33	40	
	36	33	21	34	36	31	
	34	32	27	36	25	27	
	31	25	39	28	40	25	
	23	26	24	26	30	36	
	28	33	24	31	30	25	
	49	32	29	33	26	40	328
	26	26	43	29	27	29	351
	20	25	19	27	33	37	297
	30	27	34	34	21	39	317
	29	32	38	23	32	24	301
	24	36	23	41	40	28	338
4.	16	8	17	40	26	32	311
	29	36	41	16	38	40	325
	40	26	36	26	31	32	266
	36	45	39	31	37	33	294
	33	28	1	43	43	29	
	16	34	34	32	29	36	
	23	38	27	43	0	40	
	38	35	30	40	24	27	
	25	30	43	25	29	20	
	20	24	32	39	30	26	
	32	31	2	17	34	28	
	31	35	30	41	34	28	
	27	37	34	34	31	26	
	32	35	29	32	33	17	

Table 1 (Continued)

Worker	Counts from tubes						Counts from plates
	Group a		Group b		Group c		
	41	34	36	24	34	41	
	19	31	31	32	27	20	310
	23	26	34	35	26	40	319
5	27	33	33	32	25	28	
	29	33	28	27	39	33	299
	25	34	36	42	28	27	288
	25	20	30	29	31	30	319
	18	42	35	24	32	25	271
	29	25	27	28	29	29	273
	35	36	28	31	29	25	292
							288
	17	22	45	38	31	39	305
	39	26	26	37	27	29	
	28	26	35	25	29	40	
	25	28	38	21	30	31	
	35	24	25	38	28	26	
	28	29	34	30	20	18	
	25	17	0	31	32	25	
	0	31	22	29	34	31	
	22	23	34	25	31	41	
	36	29	32	24	29	26	
	26	37	25	28	32	31	
	28	36	34	30	37	25	321
	37	30	45	34	28	26	299
6	26	34	24	38	29	29	301
	28	31	27	28	18	34	303
	24	29	31	39	18	39	307
	29	29	24	30	32	43	322
	24	22	31	22	38	34	284
	16	30	33	30	26	27	324
	27	32	32	32	24	22	335
							331
	27	41	22	27	46	36	
	21	20	26	29	33	26	
	26	35	31	23	25	29	
	29	40	27	23	36	26	
	24	30	34	30	41	27	
	19	24	27	37	21	31	
	14	13	24	33	30	37	
	28	27	32	31	29	29	
	18	25	33	33	35	28	
	31	25	24	29	27	24	

The total tube counts are slightly greater than the total plate counts which would appear to show that the cultural conditions for the development of colonies in tubes are quite as favourable as those in plates.

The following figures give the mean counts for plates and tubes (X10, since in this case the inoculum was 0·1 ml.).

Worker	Plates	Tubes.
1.	302±7·5	312·0±5·4
2.	288·8±4·7	299·2±5·7
3.	295·5±9·6	298·2±5·0
4.	302·8±8·7	309·6±6·7
5.	296·4±5·4	296·7±5·1
6.	312·7±5·2	290·9±4·8
All workers	299·8±2·9	301·1±2·2

In calculating these figures the eleven tube counts below 10 were omitted since the tubes were found on reinoculation, to contain an inhibitory substance which prevented growth.

The counts do not differ significantly.

The results of the tube counts were subjected to an analysis of variance (Table 2.) in order to ascertain the accuracy of the experimental technique. The variance ratio calculated/

TABLE 2.

Analysis of variance of Experiment I.

Method of inoculation	Factors involved	Degrees of freedom	Sum of Squares	Mean square	Variance ratio
Consecutive 0·1 ml. amounts	Position of 0·1 ml. amounts in the pipette (P)	9	880·0	97·8	2·77 ⊠ ⊠
	Intraction of workers & positions (W X P)	45	1,504·6	33·4	0·95
	Pipettefuls (P_p)	18	820·3	45·6	1·29
Alternate 0·1 ml. amounts	P	4	188·9	47·2	1·34
	W X P	20	855·3	44·3	1·25
	P_p	42	900·2	21·4	0·61
1st and 6th 0·1 ml. amounts	P	1	82·8	82·8	2·35
	W X P	5	348·0	69·6	1·97
	P_p	114	4,395·0	38·6	1·09
Combined results	Workers (W)	5	437·0	87·4	2·48 ⊠
	Methods (M)	2	137·1	68·5	1·94
	Intraction (W X M)	10	401·7	40·2	1·14
	Error	433	15,277·6	35·3	
	Total	708	26,228·5		

⊠ significant at the 5% level

⊠ ⊠ " " " 1% "

calculated from the combined results of the six workers indicated a significant difference between workers at the 5% level, which may have been due to inexperience with the method. No significant difference was found between the various pipettefuls measured or between the three methods of inoculation, but the consecutive 0·1 ml inocula showed a significant difference at the 1% level when the results for all workers were combined, though this did not show when the results were examined separately for each worker. The discrepancy with the consecutive 0·1 ml amounts appeared to be due mainly to the last 0·1 ml. delivered by each pipette, which in most cases gave a higher mean count than the remainder, as is shown by the following figures:

Consecutive 0·1 ml inocula	Mean count.
1.	31·25
2.	28·92
3.	29·79
4.	29·79
5.	29·58
6.	28·17
7.	26·46
8.	30·08
9.	27·88
10.	33·88

The/

The higher count may have been due to the slowness of delivery of the pipette which was obvious with the last 0·1 ml quantity, or to the difficulty in seeing the graduation mark which came within the test-tube. This source of error could be avoided by ignoring the last 0·1 ml quantity. The seventh 0·1 ml quantity appears to be somewhat smaller than the others though the divergence is not so marked as with the tenth. The checking of the pipettes by weighing the quantities delivered gave not explanation of any difference in the seventh position.

The value of X^2 for tube counts calculated by dividing the error sum of squaresof the analysis of variance by the mean count for all tubes was 507·4 with 433 degrees of freedom. This is significantly large at the 1% level. Further examination revealed, however, that this high value was almost entirely due to the variable counts obtained by one worker, for which there may have been some special reason. When the results for this worker were omitted the value of X^2 was 388·4 which with 363 degrees of freedom is not significant. There is thus no indication that the counts obtained from tubes deviate significantly from the expected Poisson distribution. A given number of tubes did not however, give as reliable a result as the same number of plates, the coefficient of variation for plates being 7·3. and for tubes 19·9%, This was thought to be due to the number/

number of colonies counted in plates having been larger than in tubes, so a second experiment was designed to show the influence of colony numbers.

Experiment II.

In this experiment two dilutions of spore suspension were used, one calculated to give a count ten times that of the other. Two workers each inoculated 12 plates with 1 ml quantities of the higher dilution and 120 tubes with each dilution, using consecutive 0·1 ml amounts of inoculum. The number of colonies developing on a plate should therefore be the same as the number developing in a tube inoculated with the lower dilution and thus permit a comparison of the two methods at the same level of count. The colony counts are given in table 3.

For the same mean count (Table 4) there was no significant difference between the plate method and the tube method. The means found by the two workers are in good agreement. The observed coefficients of variation agree well with their expected values. In only one case, namely that of worker 1, with low dilution tubes, is the observed coefficient of variation greater than or appreciably/

TABLE 3

Colony counts obtained from plates, and from tubes inoculated with two dilutions of the spore suspension.

Worker	Counts from tubes						Counts from plates
	High dilution			Low dilution			
	3	6	4	57	76	78	75
	11	4	10	70	65	86	65
	6	9	7	71	76	63	68
	9	10	4	79	87	81	78
	7	10	6	90	80	76	90
	3	7	9	73	91	87	69
	7	8	6	76	75	86	91
1.	7	12	8	59	77	59	67
	7	12	6	84	64	67	74
	3	7	7	66	76	72	69
							78
	9	6	9	79	85	70	79
	7	6	9	68	60	76	
	3	9	4	65	87	50	
	12	9	11	69	64	88	
	9	9	11	80	82	68	
	4	10	7	72	70	75	
	7	8	17	72	83	68	
	6	3	6	66	63	80	
	8	7	5	65	69	47	
	6	5	9	71	57	66	
	6	5	9	86	93	68	
	7	9	5	82	81	93	
	10	6	10	91	85	56	
	4	9	10	71	80	83	
	8	8	3	84	69	76	
	10	11	8	76	85	67	
	10	12	8	79	73	65	
	5	6	7	73	73	86	
	8	11	16	93	86	79	
	9	11	6	56	78	57	
	5	10	8	71	88	91	
	8	7	11	73	101	68	
	9	9	10	77	80	71	
	13	9	8	90	72	78	
	7	5	8	77	87	59	
	7	7	12	82	67	82	
	9	7	6	75	66	73	
	5	9	4	80	81	70	
	8	8	4	72	71	79	
	8	10	8	71	90	71	

TABLE 3. (Contd.)

Worker	Counts from tubes						Counts from plates
	High dilution			Low dilution			
	8	12	7	67	66	59	76
	3	3	7	82	79	80	79
	10	7	9	77	75	74	73
	5	5	6	75	72	76	69
2.	8	9	3	68	61	72	84
	14	7	8	68	75	87	75
	7	9	7	71	59	72	72
	4	5	7	70	93	60	71
	8	5	5	79	68	69	75
	5	10	8	68	67	53	79
							65
	8	8	11	61	76	54	97
	2	9	11	63	75	62	
	10	6	6	78	78	67	
	5	6	6	60	59	68	
	5	6	7	80	73	73	
	8	4	6	77	69	69	
	5	4	3	73	74	57	
	9	7	6	72	65	91	
	4	4	10	71	70	73	
	13	11	5	65	80	64	
	11	3	9	77	76	70	
	8	11	8	78	84	58	
	7	9	10	69	75	71	
	7	8	13	82	78	70	
	9	13	11	85	73	71	
	5	13	9	79	56	78	
	11	6	11	62	76	74	
	5	6	5	80	87	64	
	6	10	7	76	70	78	
	7	8	8	67	76	86	
	8	15	6	70	68	62	
	7	11	11	72	94	71	
	5	9	3	76	58	79	
	9	9	12	69	67	68	
	10	8	7	70	68	75	
	8	10	7	88	74	83	
	8	9	7	68	58	70	
	7	6	5	74	85	69	
	8	8	4	69	79	68	
	10	7	10	73	89	71	

TABLE 4.

Mean counts in plates and tubes and the observed and expected coefficients of variation.

	Mean count		Error Vari-:ance	Coefficient of variation	
				Observed	Expected
Plates (12 per worker)	Worker 1	75·25 ±2·46	72·75	11·3%	11·5%
	" 2	76·25±2·37	67·66	10·8	11·4
Tubes, low dilution (120 per worker)	" 1	74·99±0·89	94·34	13·0	11·6
	" 2	72·08±0·75	66·87	11·3	11·8
Tubes, high dilution (120 per worker)	" 1	7·77±0·24	6·69	33·3	35·9
	" 2	7·62±0·24	6·63	33·8	36·2

ably different from expectation.

The tube counts were subjected to an analysis of variance (table 5). **This shows no significant difference** between counts from different 0·1 ml. quantities or from different pipettefuls.

The value of X^2 for all the tube counts was 484·3, with 476 degrees of freedom. The counts therefore do not appear to deviate significantly from the expected Poisson distribution.

This ~~expectation~~ experiment indicates that one tube inoculated with a 0·1 ml quantity can replace one plate inoculated with a 1 ml quantity, provided the resulting mean colony counts lie at the same level.

DISCUSSION

The inoculation of agar in tubes in the manner described above is simple and enables many replicate counts to be made with little expenditure of time, labour or materials. The method is comparable with that of the roll-tube but is simpler in practice since it dispenses with the handling necessary for roll-tubes and with the use of special equipment. Colonies developing within strips of agar are counted more easily than those in roll-tubes. The method also compares favourably with surface/

TABLE 5.

Analysis of variance of Experiment II.

Dilu-:tion	Factors involved	Degrees of freedom	Worker 1.		Worker 2.	
			Sum of Squares	Mean Square	Sum of Squares	Mean Square
Low	positions	9	941·8	104·65	1,071·7	119·08
	pipettes	11	521·4	47. 40	1,561·9	141·99
	error	99	6,620·0	66·87	9,339·4	94·34
	Total	119	8,083·2		11,973·0	
High	positions	9	79·53	8·837	69·80	7·756
	pipettes	11	94·17	8·561	70·67	6·425
	error	99	656·67	6·633	663·00	6·697
	Total	119	830·37		803·47	

surface counting methods (6) (7) (8), where plates must be prepared beforehand and dried and where considerable amounts of medium often are required (6) (7). There are, however, several points which require consideration.

(a) Concentration of agar. The accuracy of the count is reduced if the agar slips within the tubes during incubation. This trouble can be avoided by using a suitable concentration of agar. With the media and incubation temperatures used throughout all my work 2% nutrient agar gave a sufficiently large margin of safety without exerting on inhibitory action on bacterial growth. Incubationtemperatures up to and including 37°C. were used. The basic medium used was 2% powdered agar, 0·5% peptone, 0·5% lemco, and 0·25% yeastrel, with the addition of 0·5% sugar for specific organsims. The method also was used successfully with the mannitol yeastrel agar and the nitogen-free mannitol agar described on p. 42 of part II. No difficulty was apparent in the counting of colonies developing in Wilson's milk agar (11), despite its opaque nature. Two percent agar is easily handled during filtration and tubing but it may/

may have to be altered to suit special conditions such as the use of richer media, different brands of agar (eg. New Zealand agar) or a warmer climate. At temperatures above 37°C. it may be feasible to incubate the tubes in a horizontal position.

(b) Glassware. The quantity of agar in each test-tube is small; consequently it becomes necessary to ensure perfect cleanliness of the tubes as any trace of a substance inhibitory to bacterial growth might still be present in sufficient concentration to reduce the count or delay the appearance of colonies (see experiment 1.) Any counts showing an unduly large variation therefore should be regarded with suspicion.

(c) Inoculation. It has generally been accepted that the sampling error is apt to become very large when small quantities (less than 1 ml.) are measured. In all measurements this error must exist but the results obtained in these experiments suggest that it need not be large. Assuming normal care, the error introduced by the measurement of 0·1 ml. using a 1 ml. pipette is cancelled out by other errors that are inherent in any plating method. Snyder (6) compared different methods of inoculation and measurement and found that 0·1 ml. delivered from a capillary/

capillary dropping pipette was more accurate than 0·1 ml from a 1 ml pipette,,but concluded that there is no appreciable contribution from the pipetting error unless the total error~~(unless the total error)~~of the method is very small. One method recommended by Snyder has since been studied in more detail by Crone (7). Counts obtained by spreading 0·1 ml inocula, measured by a capillary pipette or a 1ml. graduated pipette, over the surface of previously prepared plates, are shown to conform within the limits of experimental error to a Poisson distribution and the total count from all the plates agrees well with that obtained from a corresponding number o f roll-tube cultures. No large error therefore, appears to have been introduced in the measurement of 0·1 ml inocula. Miles and Misra (8) used a capillary pipette for the seeding of 0·02 ml drops on agar plates. Greater accuracy of measurement of the inoculum could undoubtedly be achieved by the use of calibrated dropping pipettes but for the inoculation of tubes the 1 ml. pipette is easier to handle and provided the last 0·1 ml quantity is not used, there seems little disadvantage in its use. Pipettes with good points and having a suitable rate of delivery were used in our experiments and were checked by weighing the successive 0·1 ml amounts delivered. Those not graduated to the tip were selected, but the blow-out type of pipette can also be used provided the last 0·1 ml amount is discarded.

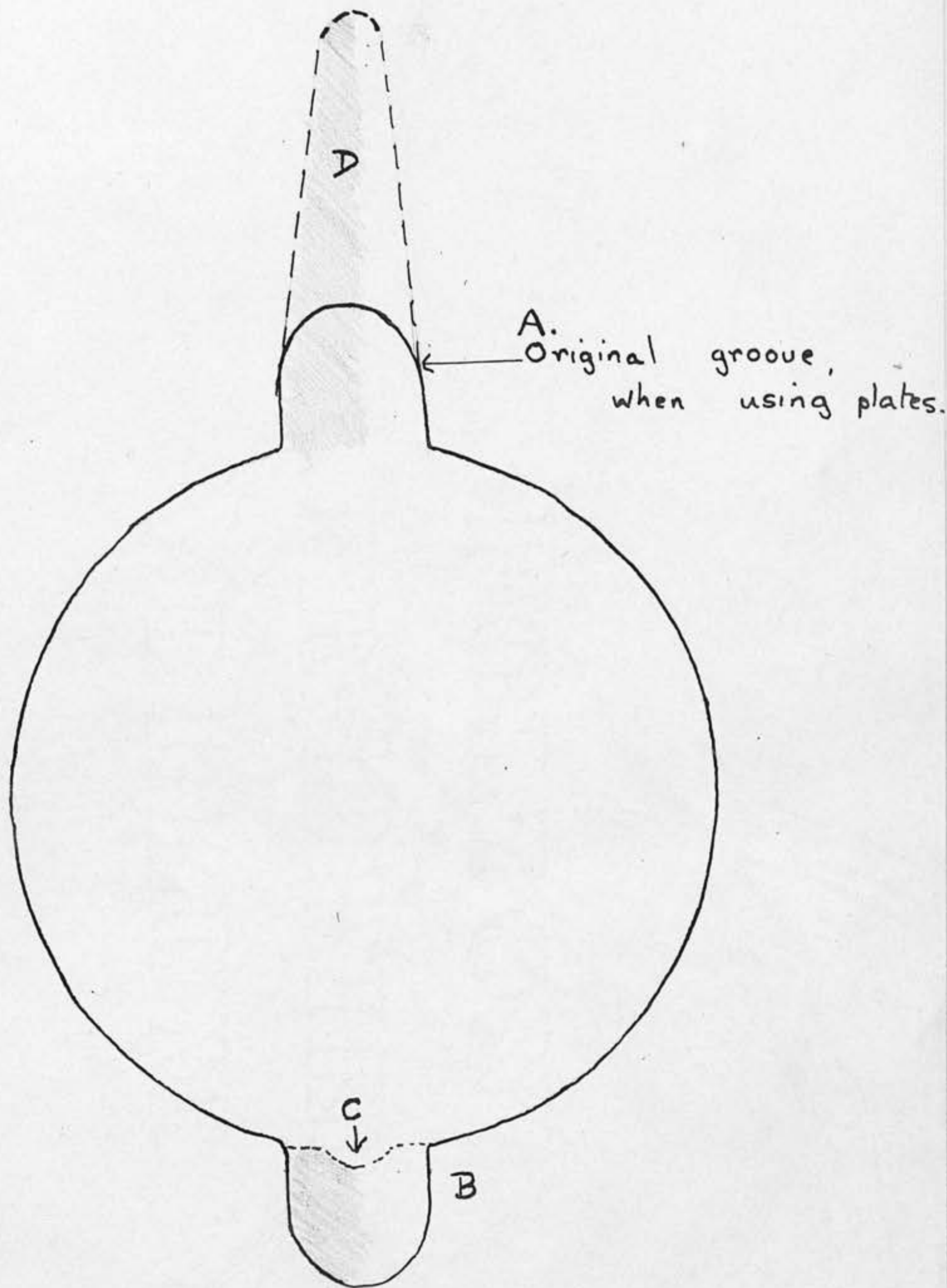
Statistical/

Statistical analysis shows that there is no great error introduced by measuring consecutive 0·1 ml. amounts and that the results obtained will follow the expected Poisson distribution.

<u>(d) Counting of colonies</u>. Colonies developing within the agar can be counted by using the lateral illumination box used for couting colonies in Petri dishes (see fig1). The lower groove B. should be slightly notched close to the glass (c). in order to support the base of the tube; the opposite groove A. should be elongated to accommodate the upper part of the tube (D). In this way the tube is held in position and can be rotated slightly if required.

As with the plate count, the coefficient of variation (table 4) rises rapidly as the mean count decreases eg. with a mean count of 72·08 the coefficient of variation is 11·3% and with a mean count of 7·62 it is 33·8%. This indicates that the number of colonies should be as large as possible without introducing any error due to overcrowding. It is suggested that the number of colonies counted in a tube should lie between 30 and 300. The maximum will depend on the type of colony that is being counted/

Fig. I. Lid of counting box, showing illuminated field and grooves for holding tubes.



counted. Counts of 250-300 have been made quite readily and as many as 400 with small streptococcal colonies. The surface seeded drop counts recommended by Miles and Misra appears to be of a more limited range, the maximum count quoted being 100. Five fold dilutions can be used if required to obtain a convenient count, but in all the work undertaken this was found to be unnecessary.

Miscellaneous Factors.

(1) Organisms splashed on to the glass of the tube during the mixing of the inoculum with the agar, and those developing at the top of the slope where the agar film is apt to be thin, were detected easily without any magnification.

(2) The agar strip does not dry up undully during normal periods of incubation.

(3) The surface area in proportion to the amount of medium is approximately the same in tubes and in plates.

Application of the method.

The agar strip method was used extensively throughout the work described in the remainder of this thesis. It has proved satisfactory with pure cultures of the following microrganisms:

Bacillus subtilis./

Bacillus subtilis.

Bacillus megatherium.

Coliform organisms.

Pseudomonas sp.

Alcaligenes viscosus.

Micrococcus sp.

Streptococcus sp.

Corynebacterium sp.

Actinomycetes sp.

Yeasts.

Azotobacter.

Rhizobium.

Oidium lactis.

With *Bacillus subtilis* the spreading growths did not interfere with counting to a greater degree than they did in the usual plating method. With bacteria such as Azotobacter and Rhizobium that form mucoid colonies which tend to flow, prolonged incubation periods should be avoided. No trouble however, was experienced with incubation periods necessary to give satisfactory counts eg. 2-3 days at 30°C. Moulds that produce rapidly spreading growths may prove troublesome. With *Oidium lactis* counts of up to 120 colonies per tube were obtained after 24 hr. at 30°C. The range of count will probably be found/

found to be lower with moulds than with bacteria and short incubation periods will probably be essential.

The suitability of the method for the examination of mixed populations remains to be tested in detail. It has been used successfully for counts of a number of milk and water samples.

SUMMARY.

An estimate of a viable population can be obtained from colonies developing in strips of agar in tubes.

A 1 ml. graduated pipette is used to deliver 0·1 ml of inoculum into tubes containing 1·5 - 2 ml of nutrient agar. The tubes are sloped to give thin strips of agar and are incubated upright in racks.

With the medium used 2% agar was found to prevent slipping of the strips within the tubes during incubation.

The results were subjected to statistical analysis which showed that the error introduced by the measurement of 0·1 ml. as opposed to 1 ml., is cancelled out by the other inaccuracies of any plating method. The accuracy of the pipettes should be checked and the last 0·1 ml should be discarded, since this tends to give a higher count than the other nine 0·1 ml. quantities.

Accurate counts may be obtained from tubes containing 30-300 colonies.

This method gives an estimate of a viable population which does not differ significantly from that/

that obtained by plates inoculated with 1 ml. quantities, provided the level of the colony count is the same.

The method evolved allows many replicate counts to be made with little expenditure of time, labour or materials. In this respect it compares favourably with any other plating techniques.

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THE BACTERICIDAL EFFECT OF DESICCATION.

It is well known that a large proportion of the bacteria found in milk are derived from dairy utensils. Contamination from this source can be reduced by the processes of sterilisation and drying. The resistance of bacteria to heat and chemical disinfectants has been extensively investigated in the past and these treatments are now recognised processes on the farm and in industry. Resistance to desiccation however, has received little attention.

Drying is known to be capable of preventing bacterial growth but to be comparatively inefficient as a means of killing bacteria. A considerable amount of information is now available concerning factors that promote the survival of bacteria when they are dried, but little is known about the death rates of different species when they are dried in air and stored at ordinary atmospheric humidities. The work to be discussed was undertaken with the object of obtaining a better understanding of the lethal effects of desiccation under natural conditions. The findings of the investigation were expected to have application in relation to dehydrated foods and to the treatment of dairy utensils and other industrial/

equipment.

LITERATURE.

Many workers have studied desiccation as a means of preserving bacterial cultures in a viable condition without frequent subculture. This work is reviewed adequately by Morton & Pulaski (1) and the more important contributions are summarised by Stamp (2). Most of the methods in use are based on either the drying of liquid bacterial suspensions over a dehydrating agent such as P_2O_5 or the freezing of suspensions immediately before and during the drying process. Throughout all this work however, there is remarkably little quantitative data provided and very few attempts to study the course of death.

The death of bacteria as the result of any unfavourable environmental condition is generally acknowledged to be proportional to the number of living cells present, in contrast to that of higher organisms which conforms to the S-shaped curve associated with a random distribution of the death rate. The logarithmic order of death of bacteria was first noted by Madsen and Nyman (3) in an experiment on the destruction of spores of B.anthracis by mercuric chloride and by Chick (4) with B.typhosum exposed to heat. Since these/

these early experiments the reality of the logarithmic course of death has gained considerable support and attempts to explain it have been based mainly on a comparison with the monomolecular type of chemical reaction. However a number of workers have obtained survivor curves of a sigmoid nature, either saggingbelow or bulging above the straight line. These have been attributed either to an inherent variation in the resistance of the cells or to the effect of cell aggregation. The opposing theories have been discussed in detail in reviews by Rahn (5), Buchanan and Fulmer (6) and Watkins and Winslow (7).

Rahn (5)summarised allknown experiments dealing with bacterial death and found that in nearly fifty percent of the experiments the intial number of bacteria was not given. The results therefore, could not be used to prove the logarithmic order of death since it is in the early part of the curve that abnormalities are likely to occur. This criticism applies to the early experiments on the drying of bacteria, by Paul (8) and Paul, Birstein and Reuss (9). These workers dried cultures of staphylococci on garnets and kept them in test tubes under varying conditions in order to determine the rate of death of the bacteria. They claimed to have observed a logarithmic/

logarithmic decrease in count.

More recently Buchbinder and Phelps (10) investigated the death of streptococci settling from the air of an experimental chamber and found it to be logarithmic. A similar type of death was noted by Campbell Renton (11) with freeze dried cultures of the cholera vibrio and by Heller (12) with freeze dried cultures of Streptoccus pyogenes and Escherichia coli. Heller examined his cultures over a period of 90 days but suggested that the logarithmic death rate may not have continued until all the organisms were dead. Ficker (13), noted a decrease in death rate over long periods of storage and attributed this to the protective action of the medium within its deeper layers. Rahn (14) revealed a similar decrease in death rate in the work of Supplee and Ashbough (15) on the survival of the microflora of milk powder after storage for several months. However, he explained the break in the curve obtained by plotting their figures against time, as due to the mixed flora of the milk powder and consequently to the presence of spores or of species showing a resistance above the average.

Factors influencing the death rate of bacteria on desiccation.

The following principles appear to influence the death rates of dry bacteria, independent of the method of desiccation employed/

employed.

1. Rate of Drying. Rapid drying generally is accepted as less destructive than a slow process. Kitasato (16) Germano (17) (18) and Ficker (19) observed that when organisms were dried rapidily in a desiccator they survived longer than when dried in ordinary room air. These results were confirmed by Rogers (20) who obtained a high recovery from milk cultures subjected to rapid spray drying and by Otten (21) who varied the rate of vacuum drying over conc. H_2SO_4. Stamp (2), in a comparison of a slow drying process over P_2O_5 in vacuo with two rapid freeze drying processes, found that the latter were more destructive. However, his observations were based on the drying of one organism only, namely Chromobacterium prodigiosum. He suggested that some particularly sensitive bacteria might only be dried satisfactorily by a lyophilization method and that the higher survival rates obtained by drying over P_2O_5 might be accounted for by growth during the drying process.

2. Nature of surface on which drying occurs. A variety of substances have been investigated such as silk threads (Kitasato (16)); garnets (Paul (8)); glass (Heim, (22, 23, 24) Brown (25)); filter paper (Hammer (26); Brown (25)); paper money (Dold and Chen Yü hsiang (27); Keifer (28)); cotton (Miller and Schad (29); Chester (30)) and wood (Miller and Schad (29)).

Schad (29)). These investigations appear to indicate that a porous surface affords a greater degree of protection than a smooth one. For instance, Pseudomonas campestris was found by Harding and Prucha (31) to live for at least a year on dried seeds of cabbage but for only 10 days when dried on glass. Miller and Schad (29) however, obtained living cells of meningococci after 10 days on glass beads in contrast to 7 to 8 days on cotton or wood.

Rahn (14) mentions that drying on metal surfaces or on glass containing free alkali is especially destructive.

3. Medium. The composition of the medium in which the bacteria are dried has an important effect on the death rate. Soil appears to possess certain protective qualities and these have been utilised in the preservation of cultures by Lipman and Burgess (32), Giltner and Langworthy (33), Barthel (34) and Greene and Fred (35). Giltner and Langworthy (33) found that bacteria could be preserved for a longer period in a rich clay soil than in sand. They claim that this is not entirely due to the retention of hygroscopic moisture by clay as the solution extracted from such a soil also exerts a protective/

protective influence.

The protective properties of soil may be due to its colloidal nature since most colloidal substances appear to increase the survival rates of bacteria suspended in them during drying. They have been found to afford considerable protection in drying from the liquid state over various dehydrating agents (Ficker (13); Otten (21, 36); Stark and Herrington (37); Stamp (2); Weiser and Hennum (38)) and in freeze drying experiments (Rogers (20); Heller (12); Naylor and Smith (39); Stamp (2); Weiser and Hennum (38)). Heller (12) determined the death rate of freeze dried cultures of <u>Streptococcus pyogenes</u> in a number of colloidal materials. The death rates decreased in the following order: aluminium hydroxide, starch, gum tragacanth, gastric mucin and peptone. Thus, the least hydrophilic of the colloids used afforded the lowest degree of protection. The results can also be correlated with Zsigmondy's gold number as decreasing gold numbers are accompanied by decreasing values for percentage redubtion per day.

Heller (12) also determined the death rate of bacteria dried in various crystalline materials. The rate of death was greater than in colloidal menstruums and varied considerably/

ably in the different crystalline materials. Thus, the death rates of <u>Strep. pyogenes</u> and <u>Esch. coli</u> were greatest when these bacteria were dried in distilled water or saline and were least in glucose or sucrose. Xylose, trytophane and salicin provided intermediate degrees of protection. Heller therefore concluded that a decrease in the death rate can be brought about by drying in crystalline menstruums of a soluble nature and in ones which the organisms are capable of dissimilating under normal growth conditions.

Naylor and Smith (39) also found that certain inorganic compounds promoted the survival of dried bacteria. In freeze drying experiments the inclusion in the medium of a commercial yeast extract "marmite" was found to be beneficial. The active constituents of the marmite appeared to be certain ammonium salts. An optimum concentration of 0·5% NH_4Cl added to the medium to be dried resulted in substantial increases in the percentage survival. Naylor and Smith also suggested that NaCl possesses protective properties but to a lesser degree than NH_4Cl.

Ascorbic acid (Naylor and Smith (39); Stamp (72)) and cysteine and thiourea (Naylor and Smith (39)) also pro-mote/

mote the survival of dried bacteria. These substances possess reducing properties and it has been suggested that they counteract the harmful process of oxidation (Stamp (2)). Proom and Hemmons (40) obtained a higher percentage survival when bacteria were dried in broth than when saline was used as the suspending medium. This was found both with cultures dried at room temperature and with freeze dried cultures.

4. Gases. Oxidation appears to be largely responsible for the death of dried bacterial cells. Paul, Birstein and Reuss (9) found that the rate of decrease in viable count of staphylococci was approximately proportional to the square root of the oxygen concentration with which they were in contact. Rogers (20), Naylor and Smith (39) and Proom and Hemmons (40) compared the death rate of bacteria when stored in various gases and found that oxygen had the greatest lethal effect.

Other gases such as nitrogen (Rogers (20); Naylor and Smith (39); Proom and Hemmons (40)), hydrogen and carbon dioxide (Rogers (20)) appear to be less destructive than oxygen but to induce a higher death rate than when storage is carried out *in vacuo*.

5. Humidity. Rogers (20) and Proom and Hemmons (40) found that the survival of dried cultures was reduced in the presence of traces/

traces of moisture.

In contrast, Stamp (2) in his comparison of drying over P_2O_5 with lyophilization, suggests that the greater percentage survival in the former case may be linked with the slightly higher moisture content of the resulting discs of dried material. Growth during the slower process or protection by the medium seem to be more likely explanations.

Dunklin and Puck (41) found that if organisms were dried in 0·5% saline the highest mortality rate on storage was obtained at 50% relative humidity. Storage humidities appeared to show no marked differences if the organisms were dried in salt-free media. Watts (42) reports the maximum survival of dried milk cultures of <u>Streptococcus agalactiae</u> at 15-25% R.H. This finding has recently been supported by Higginbottom (43) who obtained the optimal survival of the flora of spray dried milk at humidities in the range of 5-20%. Above 70% R.H. there was a rapid reduction in numbers during the first few days, followed by the growth of moulds, and bacteria, particularly the spore-forming bacilli. The microflora of the powders stored at lower humidities, however, appeared to be composed mainly of streptococci and/

and micrococci suggesting that spore-formers die out rapidly in milk powders of low moisture content. This is an unexpected observation in view of the recognised resistance of bacterial spores to unfavourable agents.

6. Temperature of storage. Dried cultures may be preserved for long periods in a refrigerator or ice box but the time of survival is reduced as the temperature of storage is raised (Ficker (13); Kirstein (44); Paul (8); Rogers (20); Buchbinder and Phelps (10); Miller and Schad (29); Weiser and Hennum (38); Proom and Hemmons (40)).

7. Light. Naylor and Smith (39) and Chester (30) suggest that dried cultures should be stored in the dark but appear to offer no decisive experimental evidence in support of the suggestion. Ørskov (45) showed that sunlight had a deleterious effect on actinomycetes dried on paper. The only other relevant investigations appear to have been carried out with meningococci. Elser and Huntoon (46) and Miller and Schad (47) both found that dried cultures of these bacteria died rapdily when exposed to sunlight or diffuse daylight, The information however, appears to be of a specialised nature and may not be applicable to all dried bacteria.

8. Age of bacterial cell. Young cells are generally believed to be more susceptible to an unfavourable environment than mature/

mature cells. The relevant literature on the problem has been reviewed by Winslow & Walker (48).

The relationship between stage of growth and resistance to desiccation has not been fully investigated and much confusion appears to have arisen owing to a failure to define accurately the stages at which young and mature cells may be obtained. Ficker (19) found that a 48-hour culture of the cholera vibrio was more resistant to drying than a 24-hour culture. Incubation for longer than two days however, again produced less resistant cells. The results of Kitasato and Berckholtz , quoted by Giltner and Langworthy (33), showed approximately the same resistance in cultures of the cholera vibrio from 1 to 5 days old. Cultures older than 5 days showed a marked decrease in resistance. Naylor and Smith (39) worked with cultures of <u>Serratia marcescens</u> at the end of the logarithmic phase of growth, in order to obtain a maximum resistance to drying. All these results indicate a relatively high degree of resistance in cells of a mature state of growth. Both younger and older cells are of a less resistant nature. The results of Proom and Hemmons (40) would appear to contradict such an observation as they suggest an increasing sensitivity with age. A 7-hour culture of <u>Shigella dysenteriae</u> was more/

more resistant than those incubated for 23 hours, 72 hours or 6 days. In this case, however, the authors may have been comparing cells in a mature state of growth (7 hours) with those in the phase of decline and not cells in a young or mature condition.

9. Bacterial mass. There appears to be little decisive evidence on the effect of the density of the cell suspension to be dried. Campbell Renton (11) noted very little difference in the proportion of cholera vibrios recovered when drying three suspensions of differing concentrations. In contrast Stamp's (2) results with *Streptococcus pyogenes*, *Pasteurella pestis* and *Salmonella typhimurium* show a general tendency for the percentage survival to rise with a diminution in cell concentration.

Variations in the rate of death of specific bacteria.

Some bacteria possess morphological characters such as spores or capsules, which render them particularly resistant to unfavourable environments. Apart from this, however, there appears to be a considerable variation in the resistance of species.

In the literature dealing with desiccation it is perhaps surprising to find little reference to comparative work on bacterial species. Some variations in resistance however/

however, have been reported in work on vacuum drying from the liquid state, freeze drying and drying in the atmosphere. Among the most resistant species are the corynebacteria (Frobisher _et al_ (49); Stamp (2)), streptococci (Stark and Herrington (37), Heller (12). Frobisher _et al_ (49), Stamp (2)) and staphylococci (Stark and Herrington (37); Wells and Stone (50); Frobisher _et al_ (49); Proom and Hemmons (40)). _Esch coli_ (Stark and Herrington (37); Wells and Stone (50); Heller (12); Proom and Hemmons (40)), other allied intestinal organisms (Wells and Stone (50); Frobisher _et al_ (49)) and Shigella (Proom and Hemmons (40)) are far more susceptible but not so sensitive as species of Neisseria and Vibrio (Stamp (2); Proom and Hemmons (40)) or Fusiformis (Stamp (2)). A variety of other species are dealt with by Frobisher _et al_ (49), Proom and Hemmons (40) and Stamp (2), but it is difficult to detect any order of resistance in their results.

Stark and Herrington (37) tested the resistance of seven organisms to rapid vacuum drying over P_2O_5 and $CaCl_2$. They were, in order of decreasing resistance, _Streptococcus paracitrovorus_ and _Str. lactis_, _Saccharomyces cerevisiae_, _Staphylococcus aureus_ and _Staph albus_, _Esch coli_ and _Lactobacillus acidophilus_.

Buchbinder/

Buchbinder & Phelps (10) compared the death rate of three streptococcal groups when allowed to settle from the air of an experimental chamber. GroupA streptococci appear to be more resistant than GroupB β-hemolytic strains and considerably more resistant than the α-hemolytic types.

Stamp (2) obtained a considerable variation in resistance with different strains of the same species, namely. Salmonella typhi, but did not investigate the matter in greater detail.

EXPERIMENTAL METHODS USED THROUGHOUT THE WORK.

Previous investigations (discussed in the foregoing review of literature) have shown that the rate at which micro-organisms die in the dry state is very largely dependent on the conditions under which desiccation is carried out. This knowledge has led to the development of freeze drying methods now commonly used in the preservation of large collections of bacterial cultures. The aim of the work, to be described, however, was to study the lethal action of drying under natural conditions and to provide information applicable in the dairying and other associated industries. Thus, it was necessary to evolve a method of laboratory drying in which the organisms would be exposed neither to destructively high temperatures nor to freezing and during which they would be in contact with atmospheric oxygen. A method based on the evaporation of moisture under reduced pressure was adopted.

The choice of experimental conditions was limited by other considerations. The bacterial suspension had to be dried rapidly in order to avoid multiplication of the cells or possible changes in their resistance during the process, but had to contain a sufficiently large number of organisms to yield accurate counts which would indicate the course of death. In order to obtain strictly comparable results, it was essential to store the dried films under constant conditions/

conditions of temperature and atmospheric humidity. The standards chosen in each case were within the range of normal atmospheric conditions.

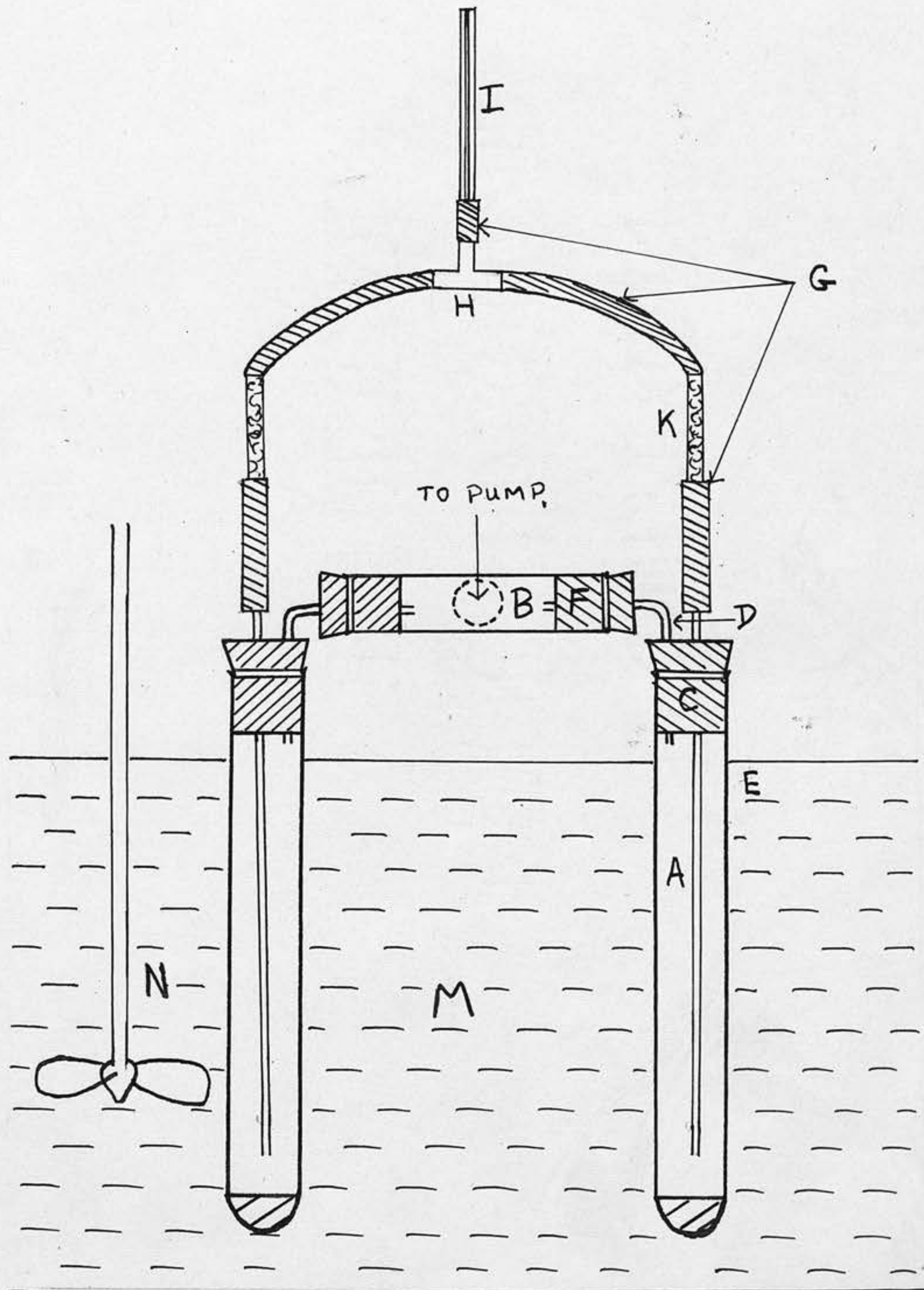
The Apparatus.

The following method was adopted after some preliminary investigations. It was chosen with the object of satisfying all the above requirements as nearly as possible.

One ml. quantities of the suspension or culture are dried in thoroughly clean 6 x $\frac{5}{8}$ in. tubes of pyrex glass. These tubes were expected to provide a relatively inert surface. Rapid drying was achieved by means of the apparatus shown in figure 1.

Two 6 x $\frac{5}{8}$ in. tubes (A) containing the suspension to be dried, are connected to a rotary vacuum pump by means of a manifold (B) of $\frac{1}{2}$ in bore. Each tube is fitted with a rubber stopper (C), through which passes two pieces of narrow bore glass tubing (D & E). The shorter length (D) is bent at right angles and connects the tube to the manifold by means of a 1-hole stopper (F). In order to promote the rapid removal of water vapour the connections from A to the pump are kept as short and wide as possible. The longer length/

FIGURE. I.



length (E) terminates $\frac{3}{4}$ in. from the base of the tube and is connected by means of pressure tubing (G) and a T-piece (H) to a piece of capillary tubing (I). When the pump is working the small stream of air admitted through I (510 ml. per min.) provides a low concentration of oxygen in A and at the same time it keeps the suspension thoroughly mixed by a slight stirring action. Drying in complete absence of air would eliminate one of the most important lethal factors involved. The incoming air is filtered through a $1\frac{1}{2}$ in. plug of cotton wool at K. The same piece of capillary tubing was used throughout all the work and it alone controls the flow of air into A. The diameter of the tubing E is therefore of no great importance. The diameter of the tubing D is also immaterial since the moisture vapour is carried off by means of the current of air created from I. In selecting the piece of capillary tubing it was important to realise that the degree of vacuum and consequently the rate of drying was dependent on its bore. The moist air pumped out of the system is drawn through silica gel impregnated with a cobalt salt and then through aluminium oxide, before reaching the pump. This prevents the oil in the pump from becoming/

becoming contaminated with moisture. The silica gel was reactivated at intervals by drying in an air oven.

The tubes were immersed in a thermostatically controlled water bath (M) kept at 40°C. and provided with a stirrer (N). This arrangement provides the energy necessary to evaporate the water quickly without exposing the organisms to a temperature that would in itself be harmful. Trials showed that the temperature of the bacterial suspension quickly comes to 27°C. when the pump is working and as a result of the cooling effect of evaporation it remains at this level until all visible moisture disappears at the end of 12 min. The pumping was continued for a further 8 min. and during this time owing to the cooling effect of the incoming air, the temperature within the tube rises only slowly to 35°C. In the preliminary investigations a manifold was not used and the tubes were then dried singly. All visible moisture evaporated in the first 9 min. and pumping was continued for a further 6 min. This had the disadvantage of greatly increasing the total amount of pumping required to dry a series of tubes in any one experiment.

The pump reduces the pressure in the evacuated system

to/

to approximately 60 min. Hg. The pressure was checked by a mercury manometer at the start of each drying in order to guard against any leakage which would affect the rate of desiccation and consequently the lethal action of the process (c.f. the review of literature).

Before the sterilisation of the 6 x $\frac{5}{8}$ in. tubes they were plugged with cotton wool. The plugs were wrapped in muslin so that fibres would not be left in the tube and thus make it difficult to obtain a good joint with the rubber stopper. After use the tubes were scrubbed with a hot solution of washing soda and rinsed thoroughly in tap water. This routine washing was supplemented at intervals with thorough cleaning in a chromic-sulphuric acid mixture. The parts E,C,D,F & K were assembled and wrapped in paper for sterilisation. The stoppers C & F were lubricated with sterile glycerol before connecting to the tubes and manifold.

Before stopping a rotary pump, air must be admitted into the evacuated system so that oil in the pump is not sucked back. In order that this might be done without drawing unsterile air into the tubes containing dried bacteria, a tap provided with a narrow constricted opening was used for slowly releasing the vacuum. This tap was situated close to the pump and connected directly to it by

a/

a short piece of glass tubing. In this way only air that had passed through the sterile cotton wool filter would enter the dried tubes. Preliminary trials were carried out to ensure that this occured in practice.

The determination of the viable count.

Throughout the work described in this section the density of micro-organisms was estimated by the agar strip method described in Part I of the thesis. The speed with which tube inoculations can be carried out made it possible to obtain triplicate counts for each dilution without extending the time required to prepare the dilutions and to mix the bacterial suspension with agar beyond the customary 15 min. All dilutions were made in 0·1% gelatin.

The preparation of cultures to be dried.

It was necessary to establish a standard technique for handling bacterial spores and vegetative cells before desiccation. The concentrated suspension of spores in distilled water, which was used throughout the work, showed no deterioration in viable count after storage for 3 years. It was prepared from 7-10 day cultures on nutrient agar by repeated centrifugation and washing, followed finally by pasteurisation at 63°C. for 30 min. The spores were finally suspended in glass distilled water and stored in 1 oz screw-top/

top vials which had been previously cleaned in a chromic-sulphuric acid mixture, to remove all traces of nutrient material. The concentration of the suspension was determined by plating and it was then stored and diluted for use as required.

Vegetative cells were grown in broth or other suitable liquid media. The degree of turbidiy was used to obtain an approximation of the cell ~~organisms~~ concentration. The composition of the basic medium used, except with Azotobacter and Rhizobium was

0·5% Evan's peptone.
0·5% Lemco.
0·25% Yeastrel.

Sugars were added when necessary to meet any specific growth requirements. Azotobacter was grown in:-

1% Mannitol.
0·01% KNO_3
0·05% K_2HPO_4
0·02% $MgSO_4$
0·01% Iron citrate.

and Rhizobium was grown in

0·5% Mannitol.
0·25% Peptone.
0·25% Yeastrel.

Aerobic and facultative anaerobes other than the streptococci, were grown in approximately 5 ml. quantities of broth in 100 ml. conical flasks. This shallow layer of broth was expected to ensure an adequate supply of oxygen for rapid growth. Streptococci were incubated in 6 x $\frac{5}{8}$ in. tubes/

tubes since they appear to prefer conditions of limited aeration. (cf. Part III of thesis). Cultures activated by frequent transfer were used as inocula and incubation was carried out at either 22°C. or 30°C. depending on the optimum temperature for growth of the organism, until the required degree of turbidity was attained. In most of the preliminary work the cultures thus obtained were centrifuged and the sediment was resuspended in an equal quantity of the medium in which drying was to be carried out. It was thought unnecessary to prolong the process by repeated washing of the sediment.

PRELIMINARY STUDY OF EXPERIMENTAL METHODS.

1. A STUDY OF A METHOD OF DRYING A NUMBER OF SAMPLES OF A BACTERIAL SUSPENSION WITH A UNIFORM RESISTANCE TO DRYING.

In order to study the decrease in viable count of dried micro-organisms over a period of time, it is necessary to dry several 1 ml. quantities of each bacterial suspension. This raised the problem of preserving the culture in a uniform condition of resistance during the drying of a number of replicate samples. Vegetative cells may undergo rapid changes in resistance, especially during the early stages of the growth cycle and an attempt to stabilise them was made by storing at a low temperature.

The cultures were centrifuged and the sediment was resuspended/

resuspended in 0·1% gelatin, a relatively inert but soluble medium, and held in a refrigerator at 5°C. Samples were withdrawn at intervals and subjected to desiccation in order to determine any variation in resistance arising out of the treatment. Recovery was carried out in 4 ml quantities of 0·1% gelatin at 40°C. (see following section).

Trials were carried out with Bacterium coli, Micrococcus albus and a corynebacterium, with the results shown in table 1.

B. coli and M. albus both decreased in ability to survive desiccation when stored at 5°C for 2 and 4 hours. This was probably caused by some physiological effect of low temperature storage on the cells, similar to that observed by Hegarty and Weeks (51) and Sherman and Naylor (52). In view of the results this procedure was abandoned.

Relatively stable cultures were obtained in all future experiments by incubating until the cells had reached the end of the logarithmic phase of growth. No great variation in resistance was found to occur if such cultures/

TABLE 1.

The influence of storage of bacteria at 5°C on their resistance to desiccation.

Species	Period of storage (hours)	Count before drying	% survival
B. coli	0	4,800,000	0·75
	2	4,200,000	0·31
	4	7,100,000	0·38
M. albus	0	10,000,000	9·96
	2	12,000,000	6·76
	4	14,000,000	5·18
Corynebacterium sp.	0	15,000,000	52·81
	2	16,000,000	42·51
	4	16,000,000	59·70

cultures were kept on the bench, samples being withdrawn centrifuged and diluted afresh for each period of drying.

II. THE RECOVERY OF VIABLE ORGANISMS DRIED ON A GLASS SURFACE.

(a) The resistance to desiccation of spores of Bacillus subtilis.

At the outset of this work it was essential to determine whether all cells surviving drying could be recovered, and counted as colonies on nutrient agar. Failure to do so would cause a considerable error in the estimation of the lethal action of the process. In all the literature on drying this source of error appears to have been overlooked and has probably led to a number of unjustified conclusions concerning the effect of the suspending medium and the drying surface. The unaccountable variation in the percentage survival throughout a series of replicate dryings obtained by Proom and Hemmons (40), may also be caused by failure to recover all the viable organisms in each case.

In order to estimate the degree of cell recovery after drying, it was decided to carry out preliminary investigations with an organism completely resistant to desiccation/

desiccation. A suspension of the spores of <u>Bacillus subtilis</u> appeared to fulfil this requirement. Before proceeding further however, it was essential to determine whether these spores actually could withstand the method of drying to be employed.

The suspension was diluted in sterile tap water to give a count of approximately 5,000,000 cells per ml. and six 1 ml. quantities were dried at the pump. The dried films were reconstitution in 4 ml. quantities of 0·5% saline solution at 40°C and shaken for 30 sec. (see p 49.-53) Viable counts of the suspension before drying and of the dried films on reconstitution were obtained, one dried sample being examined immediately on removing from the pump and the remainder at intervals throughout the following seven days. The dried samples were stored in screw-top jars, each containing a shallow layer of a saturated solution of calcium nitrate, held at 22°C. This was calculated to provide a relative humidity of 53% within the jars (53). The spores were dried in tap water in order to prevent them from germinating during the preparation of the original dilutions or the early stages of drying at the pump, when traces of moisture will still be present. It is well known that bacterial spores undergo changes in morphology within/

within a very short period of time in the presence of nutrient material as can be demonstrated by their ability to absorb aqueous dye solutions & at the same time they decrease in their resistance to desiccation.

The percentage recovery figures are plotted in fig. 2. Any deviations from a straight line are within the limits of experimental error. Spores of B. subtilis can therefore be assumed to resist the desiccation employed and can be used in experiments designed to distinguish between the recovery and survival of dried organisms.

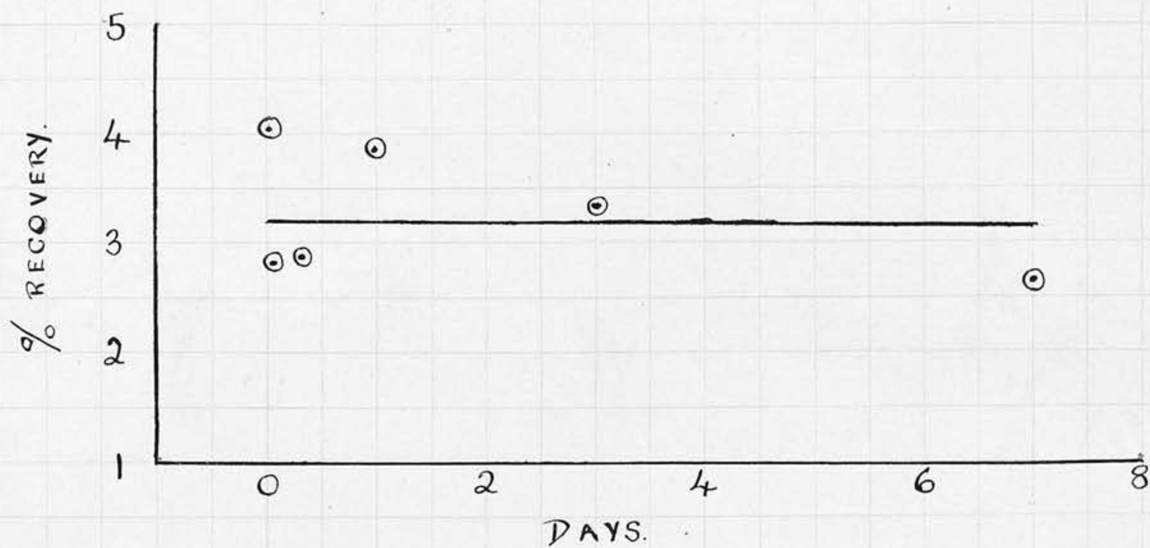
It is noticeable that the percentage recovery is extremely low but this can only be accounted for by failure to resuspend all the cells after drying. Experiments were therefore carried out in an attempt to increase this figure by varying the medium in which the spores were dried, the nature of the resuspending solution and the amount of shaking during redispersal of the cells.

(b) The recovery of spores of B.subtilis after drying.

The conditions for recovering dried bacteria were explored.

A suspension of spores of B. subtilis was diluted to a convenient concentration in various media and 1 ml. quantities were dried immediately. In this experiment/

Fig. 2. THE SURVIVAL OF SPORES OF BACILLUS SUBTILIS AFTER DRYING.



experiment the tubes were dried singly (in absence of the manifold) and thus, were only attached to the pump for a period of 15 min. each.

Media dried. The media selected for drying are listed in table 2.

(i) Distilled water was chosen as the simplest suspending fluid.

(ii) In order to simulate conditions likely to occur after centrifugation of organisms grown in liquid media, the spores were dried in both distilled water and gelatin containing traces of broth.

(iii) Naylor and Smith (39) found that the addition of ammonium chloride to the suspending medium before drying increased the percentage survival of *Serratia marcescens*. Use of this salt alone therefore seemed to merit further investigation.

(iv) Saline, was chosen in view of its frequent use in bacteriological work as a solution isotonic with protoplasm.

(v) The phosphate buffer, a M/15 solution of Na_2HPO_4 KH_2PO_4 with a pH of 7·0, was expected to provide information on the effect of a medium possessing buffering properties and to be more soluble than an organic/

organic buffer medium. Proom and Hemmons (40) found that a similar solution increased the survival rate of vegetative cells.

(vi) Of the three more complex media, 1% milk was chosen owing to its practical importance in the dairy industry .

(vii) 0·1% gelatin was selected as a colloidal substance possessing approximately the same buffering capacity as 1% milk but is more easily dissolved after drying.

(viii) Broth was included with the object of drying organisms directly in the medium in which they had been grown, thus dispensing with the use of the centrifuge. It produces dried films of considerable solubility.

<u>Recovery fluids</u>. Various resuspending fluids were examined, 4 ml quantities being used. In most cases the recovery solutions were added at 40°C. The dried milk films were reconstituted at 50°C. since several workers (54, 55, 56) have shown that milk powder gives its maximum viable count when reconstituted at this temperature. The films of dried gelatin were reconstituted at 35°C and then heated slowly to 40°C. in order to promote rapid solution. Addition of the recovery fluid at temperatures greater than 35°C. produced/

produced a glutinous mass of gelatin which was only redissolved with difficulty.

The results are presented in table 2.

Discussion. It is difficult to assess the significance of these results since insufficient data were collected on which to base accurate comparisons. Several facts may be noted, however,

Firstly, the degree of recovery is influenced largely by the medium in which the spores are dried. Drying in 0·5% saline solution has given the highest rate of recovery. It was thought possible however, that sensitive vegetative cells might be damaged if dried in a saline solution so this choice was abandoned and saline was only used in future as a resuspending fluid.

Milk, gelatin and broth all gave a higher degree of recovery than distilled water. The three methods differ considerably in their ease of solution, dried milk being far moreinsoluble than either broth or gelatin. This variation in solubility is not reflected in the results, particularly in those for gelatin and milk, so there would appear to be some other limiting factor involved in the recovery process. Drying of spores in ammonium chloride gives approximately/

TABLE 2.

The recovery of Bacillus subtilis spores after drying.

Medium dried	Recovery fluid	Period of shaking	Count before drying per ml.	% recovery
distilled water	tap water	20 sec.	760,000	18
		2 min.		32
		4 min.		28
	distilled water	1 min.	770,000	56
			760,000	47
	Na Cl	1 min.	770,000	12
distilled water + 1% broth	tap water	20 sec.	970,000	15
		2 min.		25
		4 min.		19
0·5% NH_4Cl	NH_4Cl	1 min.	840,000	60
0·5% Na Cl	Distilled water	1 min.	830,000	79
			920,000	72
	NaCl	1 min.	600,000	75
M/15 phosphate buffer	phosphate buffer	1 min.	830,000	28
1% milk	tap water	5 sec.	760,000	25
		30 sec.		61
		2 min.		51
0·1% gelatin	tap water	0 (mixed by rotation)	760,000	36
		20 sec.		50
		2 min.		58
0·1% gelatin + 1% broth	tap water	20 sec.	760,000	38
broth	NaCl	30 sec.	980,000	62
			910,000	67
			1,100,000	68

approximately the same recovery as drying in broth. The inorganic phosphate buffer solution seems to be of little value. Traces of broth in distilled water and gelatin have no decisive effect as far as can be seen from these results.

Gelatin and broth were selected for further investigation as media to be dried.

It proved difficult to make a choice among the recovery fluids but it was finally decided to carry out further investigations with gelatin and saline. Tap water was regarded as unsatisfactory since when used to reconstitute dried milk it left traces of milk on the glass. This may have been caused by the precipitation of casein by the calcium ions present in the water.

(c) The effect of shaking on the percentage recovery.

In table 2. there appears to be some correlation between the amount of shaking and percentage recovery. When spores are dried in milk, distilled water, and distilled water and broth, the percentage recovery appears to rise with an increase in the amount of shaking but finally to drop off again when this is prolonged. The decrease in recovery with long shaking suggests a clumping of the spores. A similar effect is described by Cone and Ashworth (57). They found/

found that prolonged mechanical shaking of a reconstituted solution of spray dried milk powder at room temperature resulted in a lower plate count than reconstitution at 50°C with no additional mechanical shaking. This result however, may be due to the variation in the temperature of reconstitution and not to the amount of shaking. Wilson (58) found that shaking led to the disintegration of clumps in whole milk or diluted milk and hence an increase in count.

A further experiment was designed since the data presented in table 2. are not sufficiently detailed to be conclusive. Trials were carried out using the spore suspension of Bacillus subtilis; Micrococcus albus an organism with a tendency to occur in clumps, and Bacterium coli a non-clumping organism. Twelve 1 ml. quantities of each bacterial suspension were dried in broth and reconstituted in 4 ml. quantities of distilled water, 0·5% saline or a buffered saline solution of pH 7·0 consisting of 0·5% NaCl, 0·75% K_2HPO_4 and 0·2% KH_2PO_4. Johnson and ZoBell (59) used this buffered saline solution when preparing a suspension of "individual spores of Bacillus/

Bacillus subtilis," so it was thought desirable to determine whether it had any beneficial effect on the rate of recovery in these experiments. Using any one recovery solution, duplicate tubes dried simultaneously at the pump were reconstituted with (a) 30 sec. shaking and (b) 3 min shaking, by hand. The amplitude and speed of shaking was approximately 1 ft. per sec. in all cases. The percentage recovery figures are listed in table 3.

The amount of shaking had not decisive effect although the possiblity that the optimum period lies between 30 sec. and 3 min. cannot be ignored. The magnitude of variation within this range is not likely to be large however, and provided a standard method is adopted the amount of shaking is considered to be unimportant. A period of 30 sec. was selected for use in all future work as it is less laborious than a longer period. It must be noted however, that this choice is based on the recovery of organisms dried in broth. Other media may differ in their behaviour on shaking.

There is little variation in recovery with changes in the nature of the reconstituting fluid. This substantiates earlier impressions that the decisive factor is the medium in/

TABLE 3.

The effect of shaking on the percentage recovery of dried bacteria

Organism.	Period of shaking	% recovery in		
		distilled water	0·5% saline	saline buffer
B. subtilis spore sus-:pension	30 sec.	83·7 61·12	71·68 63·30	72·85 61·31
	3 min.	69·08 72·73	59·75 80·58	67·69 71·24
Micrococcus albus	30 sec.	48·54 49·52	39·99 46·07	30·37 28·89
	3 min.	65·07 47·45	57·41 49·71	28·20 34·45
Bacterium coli	30 sec.	2·331 0·7214	4·109 0·9445	1·816 0·8037
	3 min.	1·479 0·73	5·088 0·6918	0·5535 0·8399

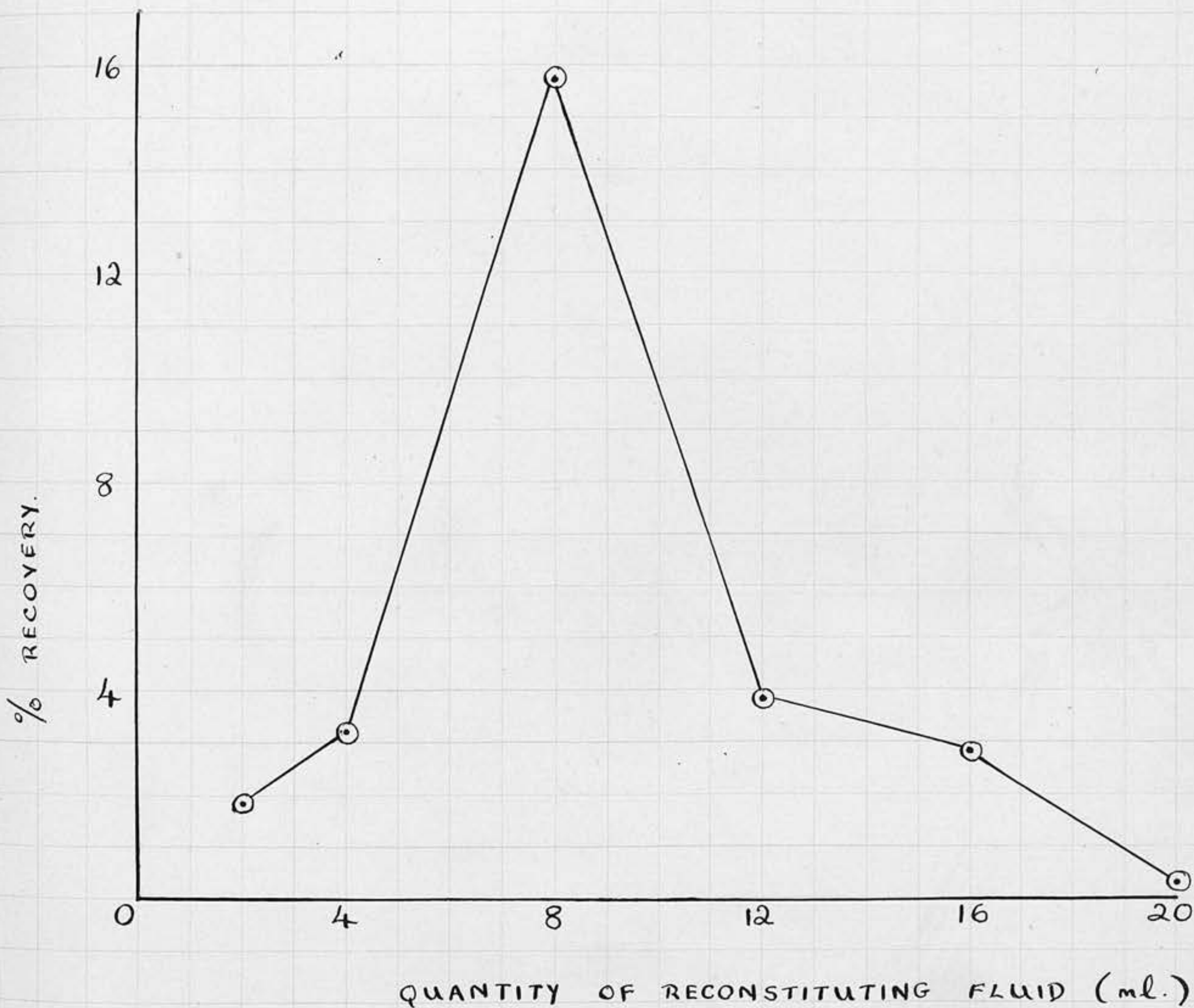
in which the organisms are suspended during drying and not the method of reconstitution.

(d) The effect of varying the quantity of recovery fluid.

In the trials so far described 4 ml. quantities of reconstituting fluid were used. The effect of varying this quantity was investigated by drying 1 ml quantities of the spore suspension in sterile tap water and reconstituting in volumes of 0·5% saline ranging from 2 to 20 ml. The percentage recovery figures are plotted in fig. 3. The average degree of recovery is low, but compares with that in fig. 2; where drying was also carried out in tap water. Repeated trials with tap water at a later date failed to give such low recovery, so it seemed probable that the sample (sterilised in one batch) used in these earlier experiments contained some harmful substance. The percentage recovery appears to increase rapidily with an increase in the amount of saline-employed for reconstitution, until a peak is reached in the region of 8 ml. Larger quantities of saline make it difficult to ensure complete mixing of the suspension during the shaking process and this factor may account for the rapid drop in the curve between 8 and 20 ml.

The/

Fig. 3. THE EFFECT OF VARYING THE QUANTITY OF SALINE USED IN THE RECOVERY OF DRIED SPORES OF BACILLUS SUBTILIS.



The results of this experiment are inconclusive owing to the inexplicably low degree of recovery and in all future work 4 ml. quantities of reconstituting fluid were used. This avoided any change in technique and gave results strictly comparable with earlier work. Larger quantities of recovery fluid (4-8 ml.) may cause the resulting suspension to be too dilute for purposesof counting when only a few viable organisms are present.

The beneficial effect of dilution during reconstitution suggested the possibility of a variation in the recovery depending on the concentration of cells in the suspended dried. The experiments described in the following section were therefore designed.

(e) <u>The influence of cell concentration on the recovery of organisms.</u>

(i) <u>Bacterial spores</u>. A preliminary experiment was carried out in which two dilutions of the spore suspension of *B. subtilis* were examined. Samples of each dilution were dried in distilled water and in broth and were recovered in 4 ml. quantities of 0·1% gelatin or 0·5% saline. The following results were obtained:

Medium dried	Recovery fluid	Count before drying	% Recovery
Distilled water	gelatin	760,000	47
		7,800	67
Broth	saline	1,080,000	68
		913,000	71

In both cases the more concentrated suspension gave a lower percentage recovery.

A second experiment was designed to investigate this question more fully. Four dilutions of the spore suspension were dried in broth and reconstituted in saline, duplicate tubes of each concentration being dried. A suspension of the spores of <u>Bacillus megatherium</u> was also examined, but owing to its dilute nature it was only practicable to dry two concentrations. The result of both trials are summarised in table 4 and fig.4.

The recovery rate appears to be largely dependent on the density of the suspension dried. This is apparent from a study of the results of <u>B. subtilis</u>. Those of <u>B. megatherium</u> are insufficient to be decisive, but show the same general trend. If a variation of over 40% is obtained when samples of a stable suspension differing only in their density, are dried under identical experimental conditions,,the possibility of a larger error in experiments of this type cannot be ignored. The adjustment of the cell density at the outset of an experiment is obviously of great importance.

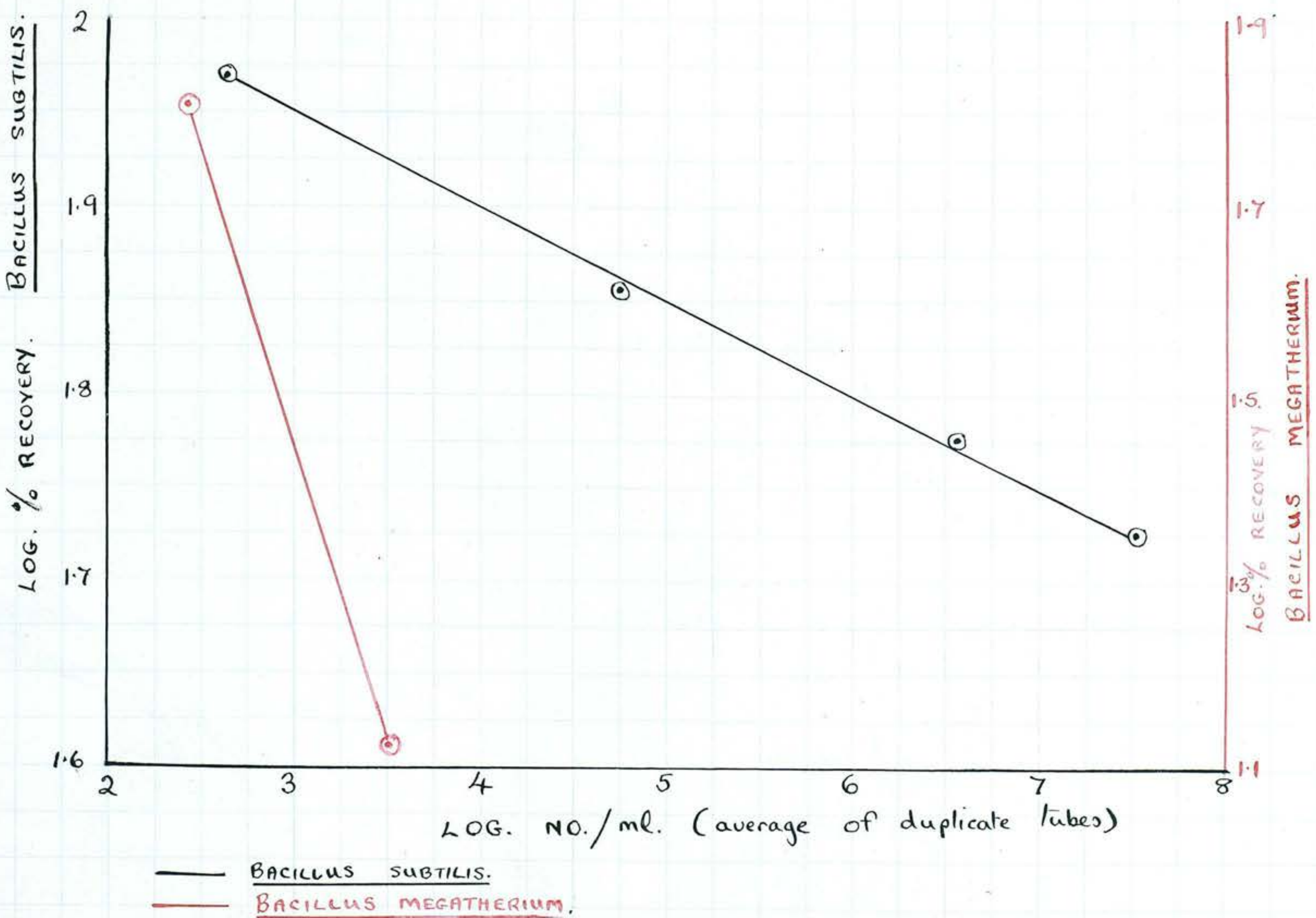
(ii) <u>Vegetative cells</u>. Cultures of <u>Micrococcus albus</u> and Bacterium/

TABLE 4.

The effect of cell concentration on the recovery of spores.

Organism	Original count	% recovery
Spores of *B, subtilis*	36,000,000	54·45 56·66
	4,930,000	63·30 63·68
	50,300	71·34 71·34
	480	94·61 92·92
Spores of *B. megatherium*	3,330	13·70
	248·3	65·55 74·09

Fig. 4. THE EFFECT OF CELL CONCENTRATION ON THE RECOVERY OF SPORES OF BACILLUS SUBTILIS AND BACILLUS MEGATHERIUM.



Bacterium coli were dried at two concentrations. Owing to the rapid destruction of organisms during the process of drying and in the early hours of the storage period, it was considered unsatisfactory to base any comparison on the recovery rate immediately after drying. A number of 1 ml. samples of each culture were therefore, dried in broth and reconstituted at intervals throughout 5 days, using 0·5% saline as recovery fluid. The dried cultures were stored at 53% relative humidity and 22°C during this period. Figs.5 and 6 show that the percentage recovery is again influenced by the cell concentration. This variation observed between the two concentrations could easily have been mistaken for a variation in resistance of cells if similar results had not been obtained previously with spores and if cultures at the same stage of growth had not been used.

The results obtained with B.coli are not so decisive as those for M. albus and there is an unexpected crossing of the lines at approximately 12 hours after drying. This can possibly be accounted for by the fact that the samples of differing concentration were dried on consecutive days and thus, were drawn from different transfers/

Fig. 5. THE EFFECT OF CELL CONCENTRATION ON THE RECOVERY OF DRIED CELLS OF MICROCOCCUS ALBUS.

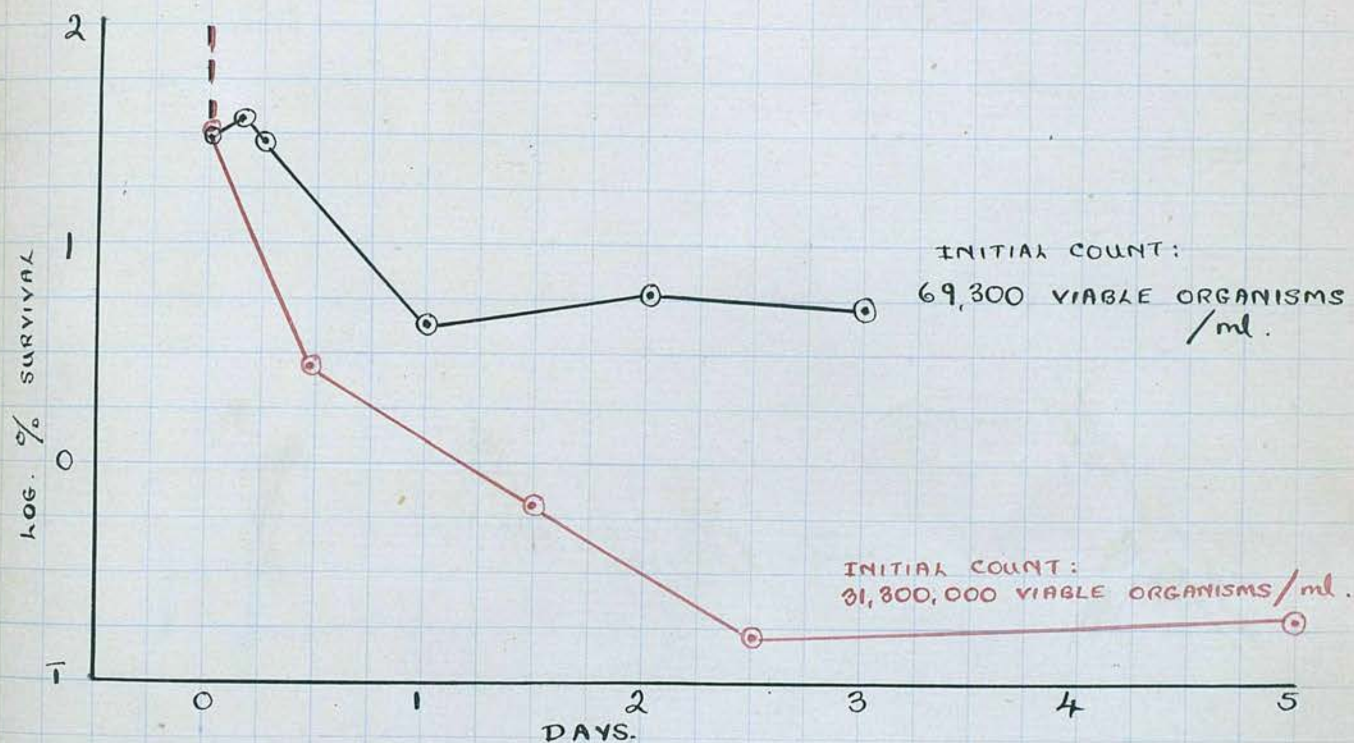
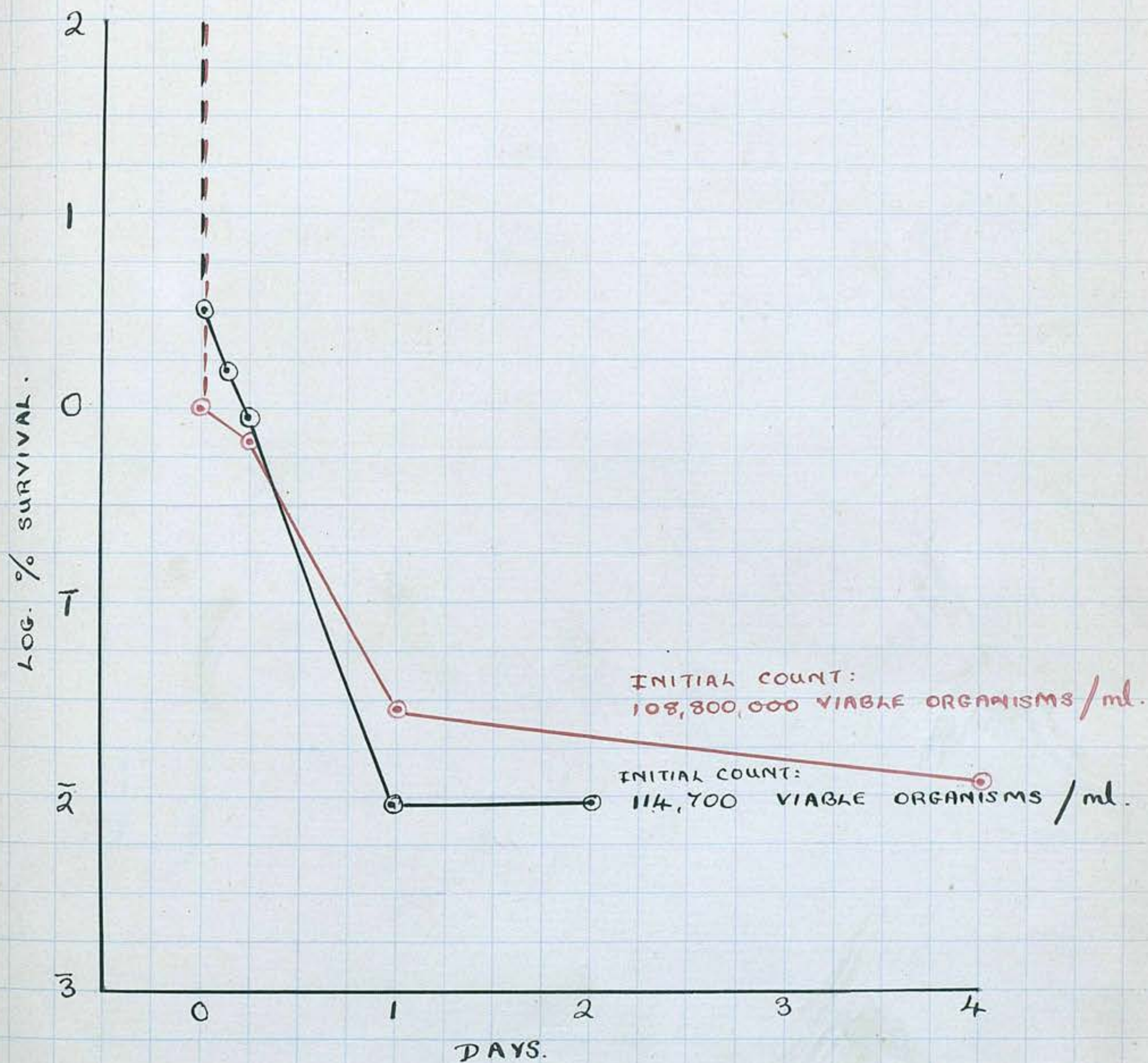


Fig. 6. THE EFFECT OF CELL CONCENTRATION ON THE RECOVERY OF DRIED CELLS OF BACTERIUM COLI.



transfers of the bacterial culture. This may have given rise to some slight variation in the resistance of the cells.

The increase in count obtained at approximately 3 hours with M. albus was probably caused by the breaking up of clumps of cells. This is not unlikely to occur with a clumping organism such as a micrococcus. Results suggesting the disintegration of clumps during drying have been obtained occasionally throughout my work. A similar effect is reported by Proom and Hemmons (40) who obtained survivals of over 100% with cultures of Streptococcus pyogenes and Staphylococcus aureus dried in broth. Naylor and Smith (39) appear to have obtained survivals in excess of 100% with Serratia marcescens, but attribute this to an inherent plating error. Heller (12) provides microscopical evidence on the effect of drying cells of Streptococcus pyogenes. He examined films in a Petroff-Hauser counting chamber before and after freezing and drying in 1% sucrose. An increase in the average total number of chains per ml. and a decrease in the average number of cocci per chain was apparent. Failure of the viable count to show a corresponding increase was probably due to the death of a considerable number of cells during the process. Heller (12)/

(12) also examined the dried cultures over a period of 20 days storage and found that the viable count increased gradually during the first 15 days. The total count remained approximately constant. He suggests that cells damaged by the drying process may be able to recover sufficiently to give rise to colonies after a period of time. It is difficult to see how such a recovery could occur.

Very few workers seem to have considered the importance of the concentration of the suspension to be dried, possibly because they have been concerned mainly with the problem of preservation. In such work it is usual to dry highly concentrated suspensions in order to obtain as large a number of viable organisms as possible in the dried material. Stamp (22) dried _Streptococcus pyogenes_ over P_2O_5 and obtained an apparent survival of 58% when the material immediately after drying contained $9{\cdot}7 \times 10^6$ viable organisms per mg of the dried film and 91% when it contained $1{\cdot}2 \times 10^5$ organisms per mg. Some variation was still obtained after storage for 2 and 4 years. He also noted a similar effect with _Pasteurella pestis_ and _Salmonella typhimurium_, but attributes all three/

three cases to an increased survival rate with a diminution in cell concentration. In view of the results obtained in my work however, it seems extremely likely that the variation is mainly due to a failure to resuspend all the cells after drying and not to the degree of survival. Campbell Renton (11) failed to obtain any decisive difference in the percentage of viable organisms recovered after 24 hours when she dried single standardised drops of a suspension of the cholera vibrio containing 9×10^6, 9×10^4 and 9×10^3 organisms per drop. It is difficult to explain this result since differences in recovery in my experience, become apparent when the concentration of the original suspension is varied by ten or hundred-fold dilution.

These investigations show that in all future work it is of utmost importance to standardise the concentration of the suspension before drying if strictly comparable results are to be obtained.

(f) The effect of a surface agent on the recovery of spores.

Trials were carried out to determine whether the addition of a surface active material to the reconstituting fluid/

fluid would increase the percentage recovery of dried spores. The material selected was the commercial detergent "Stergene" manufactured by Domestos Ltd.

First the bactericidal or bacteriostatic properties of this detergent were determined by testing its action on M. albus. Failure to decrease the count of this vegetative bacterium would ensure a wide margin of safety with spores. A 24 hour broth culture of M. albus was diluted in sterile broth to approximately 10 million cells per ml. One ml. quantities of 1/10, 1/100 and 1/1000 dilutions of Stergene in tap water were added to 4 ml. quantities of the diluted broth culture and the mixtures were shaken for 30 sec. to simulate conditions occuring in the reconstitution of dried films. The viable counts of the diluted broth cultures and of these cultures after treatment with Stergene are given in table 5. Counts after the addition of Stergene have been adjusted to compensate for the dilution factor.

Stergene does not appear to possess any strongly bactericidal or bacteriostatic properties, as only a slight decrease in the count of the micrococcus is evident with the lowest/

TABLE 5.

The lethal action of Stergene on Micococcus albus.

Dilution of Stergene	Original Count	Count after addition of Stergene	% survival
$\frac{1}{5000}$	10,350,000	11,500,000	111·1
$\frac{1}{500}$	9,550,000	11,190,000	117·1
$\frac{1}{50}$	14,150,000	13,390,000	94·64

lowest dilution. The 1/500 dilution appears to have increased the count to a slightly greater extent than the 1/5000 dilution and therefore was selected for use in the following experiment.

The effect of Stergene on the recovery of spores of B. subtilis was investigated. One ml. quantities of the spore suspension were dried in broth and each set of duplicate tubes dried simultaneously, was examined as follows:-

Tube 1. The dried suspension was reconstituted in 4 ml 0·5% saline.

Tube 2. The dried suspension was reconstituted in 3 ml 0·5% saline plus 1 ml Stergene (1/400 dilution).

Since this appeared to have little effect on the percentage recovery of dried spores, the addition of Stergene was also investigated in 1/40 and 1/4 dilution. The results are given in table 6. A 1/40 dilution of Stergene slightly increases the percentage recovery of dried spores, a 1/400 dilution has only a negligable effect and a 1/4 dilution exerts slight bactericidal or bacteriostatic properties. A surface active agent therefore appears to be of/

TABLE 6.

The effect of Stergene on the recovery of spores.

Saline % recovery	Stergene	
	Dilution	% recovery
52·86	$\frac{1}{400}$	53·13
63·68	$\frac{1}{40}$	72·57
53·18	$\frac{1}{4}$	42·85

of little benefit in promoting the recovery of dried spores.

Discussion

A survey of the results so far obtained shows a consistent failure to obtain a 100% recovery of viable cells after drying. This failure could be due either to an adherence of organisms to the glass of the tubes or to a clumping together of cells during drying. That the results obtained are caused by an adherence of organisms to the glass is unlikely since there appeared to be no correlation between recovery rate and the solubility of the medium dried, and no beneficial effect to be gained by prolonged shaking. The clumping together of cells during drying seems a more likely theory and is supported by the variation in recovery obtained with different cell concentrations; clumping might be expected to occur less readily in a dilute suspension than in a concentrated one. Failure substantially to increase the degree of recovery by prolonged shaking also points to some clumping effect.

Microscopical examination of cultures. Attempts were made to detect clumping by microscopical methods. It was considered necessary to use either hanging drop preparations in/

in which the cells were mounted in a dilute solution of crystal violet to make them more easily visble, or nigrosin films in the preparation of which the suspensions were dried at room temperature and then smeared with the background stain. The fixing and staining of ordinary smears might obscure the actual degree of aggregation of the cells.

Concentrated suspensions of the spores of _B. subtilis_ and of vegetative cells of _M. albus_ and _B coli_ were dried by the vacuum method adopted throughout all this work. The original cultures before drying and the dried films after reconstitution in 0·5% saline were then examined microscopically. Very little differences in the clumping of the spores and of _M. albus_ before and after drying could be detected but drying of _B. coli_ appeared to cause the cells to clump together slightly. However, none of the results were decisive.

The effect of the resuspending fluid was investigated by examining hanging drop preparations and nigrosin films of undried cells mounted in distilled water, tap water and saline. The amount of clumping was considerably less in saline than in distilled water and tap water. This possibly explains the relatively high degree of recovery of spores dried or recovered in saline, which was obtained earlier

in my work.

Microscopical methods however, did not appear to yield any decisive information.

III. The Resistance to Desiccation of Bacteria at Different Stages of Growth.

A detailed study of the relationship between resistance to desiccation and stage of growth was considered to be beyond the scope of this work. Nevertheless, for the purpose of comparing the effect of drying on different species it was deemed essential to use cultures in the same phase of growth and containing cells uniform in resistance. It was also necessary to detect any large degree of variation in resistance to drying associated with the phase of growth in order to qualify such general conclusions as might be based on the work carried out at a later stage. Cultures were therefore examined at two stages, of growth: near the beginning and towards the end of the logarithmic phase.

(a) Cells in the early stages of growth.

Flasks containing 5 ml. of broth were inoculated with approximately 0·2 ml. of a culture in the logarithmic phase of growth. This inoculum was large enough to provide a fairly high cell concentration in a short time, but/

but did not produce any turbidity on addition to the sterile broth. The fresh cultures were incubated at 22° or 30°C. according to the requirements of the organisms, until a faint turbidity was detected. Of the bacteria examined, cultures of *Pseudomonas fluorescens*, *Micrococcus albus* and the coliform organisms reached this stage in 1½ hours at 30°C. while those of the corynebacterium required 17 hours at 22°C. One ml. samples were then dried in broth and recovery was carried out in 4 ml. quantities of 0·1% gelatin. The quantity withdrawn from drying was replaced by an equal volume of sterile broth and the cultures were kept at either 22° or 30°C. This maintained an approximately constant cell concentration throughout the experiment.

(b) *Cells at the end of the logarithmic phase of growth*.

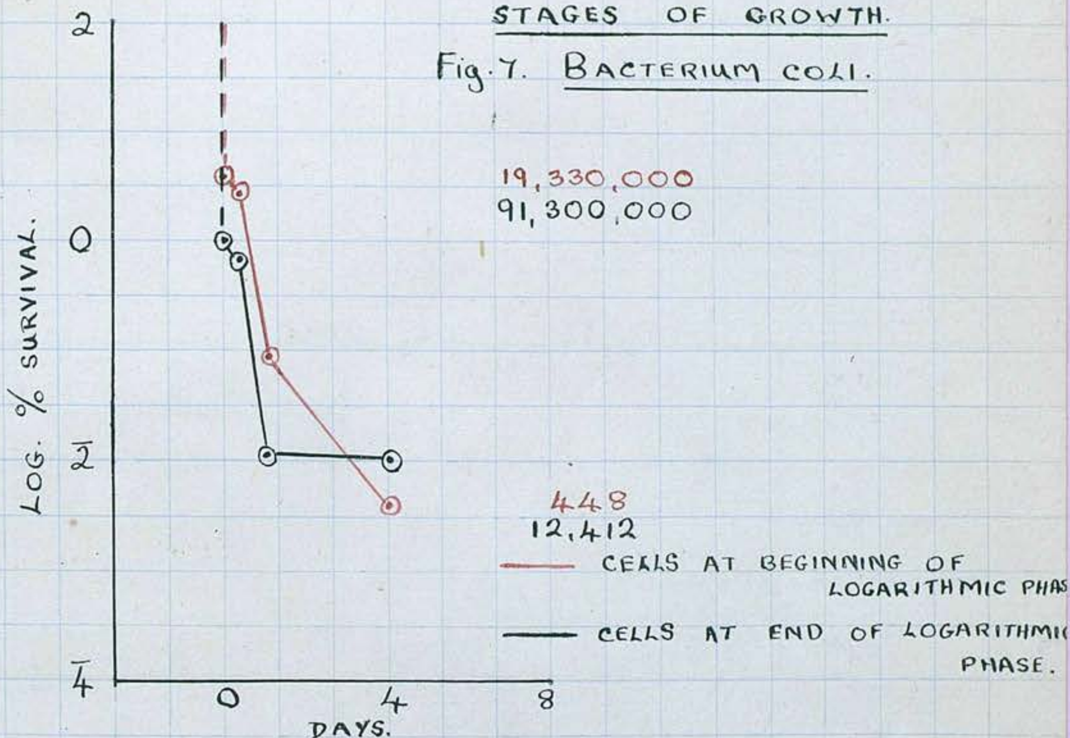
The broth cultures, inoculated as described above, were incubated until densely turbid. This stage was reached in 17 hours at 30°C. with the four more rapidly growing species and in 48 hours at 22°C with the corynebacterium. Samples from each culture were diluted in sterile broth until a turbidity matching that of the cultures in the early stage of growth was obtained. This ensured that approximately the/

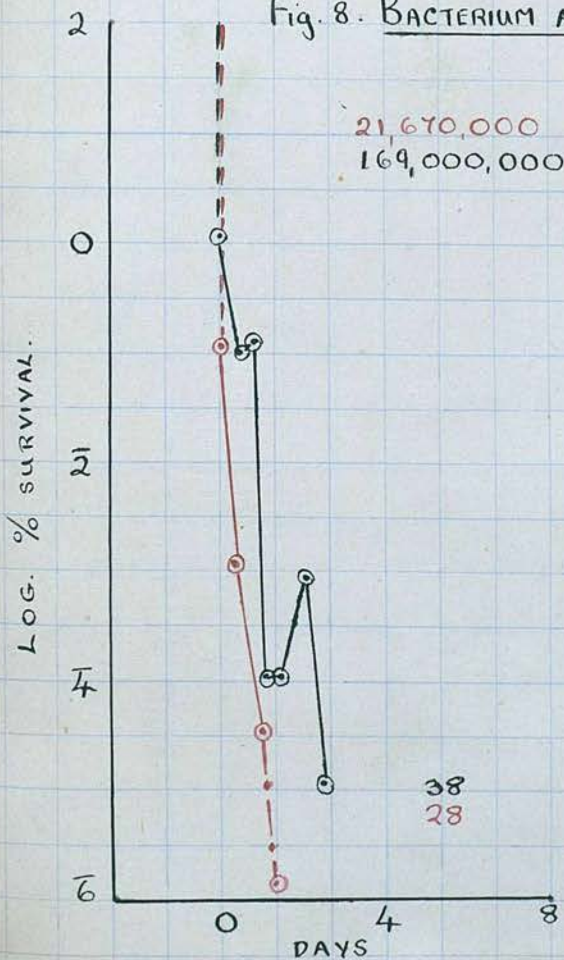
the same concentration of cells was dried in each case. Drying was carried out immediately after dilution. The original cultures were kept on the bench and diluted afresh for each drying in order to avoid variations in resistance due to renewed growth. Drying and recovery was carried out as described above.

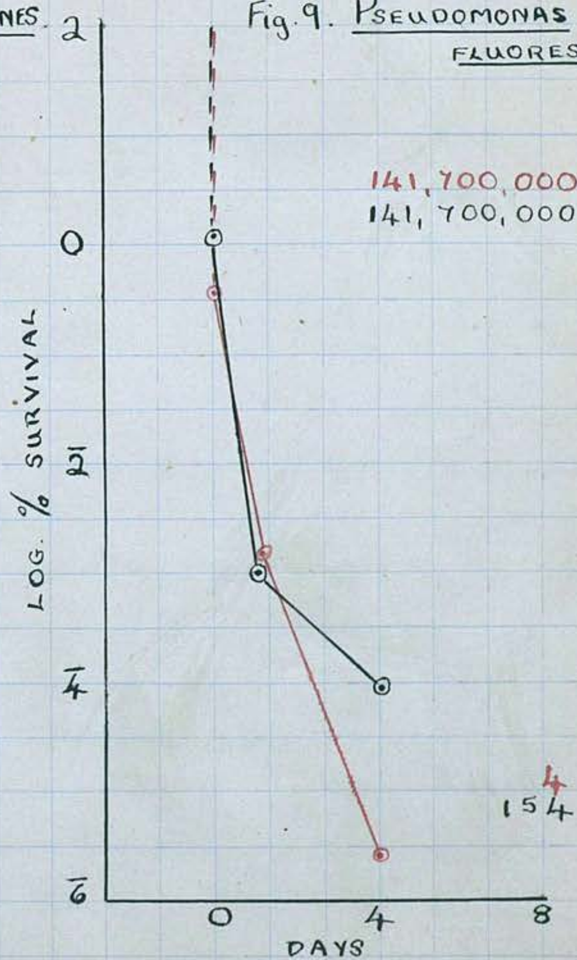
Results of these experiments presented graphically in fig. 7-11, do not appear to be decisive. In one case only, namely that of M. albus, is there a marked increase in resistance of cells at the end of the logarithmic phase. B. aerogenes and P. fluorescens show the same effect to a very slight degree.

The physiological changes which occur at various stages of the growth cycle have been studied in detail by several workers and reviewed adequately by Winslow & Walker (48). Cells appear to be most susceptible to an unfavourable environment for a short period towards the end of the lag phase when an increase in cell size is occuring prior to active cell division. In the experiments described in this section, it is possible that the cells do not pass through this period of lowered resistance owing to the use of actively growing cultures as inocula, or that it occurs/

RESISTANCE TO DESICCATION SHOWN BY ORGANISMS AT DIFFERENT STAGES OF GROWTH.







RESISTANCE TO DESICCATION SHOWN BY ORGANISMS AT DIFFERENT STAGES OF GROWTH. (CON.).

Fig. 10. MICROCOCCUS ALBUS.

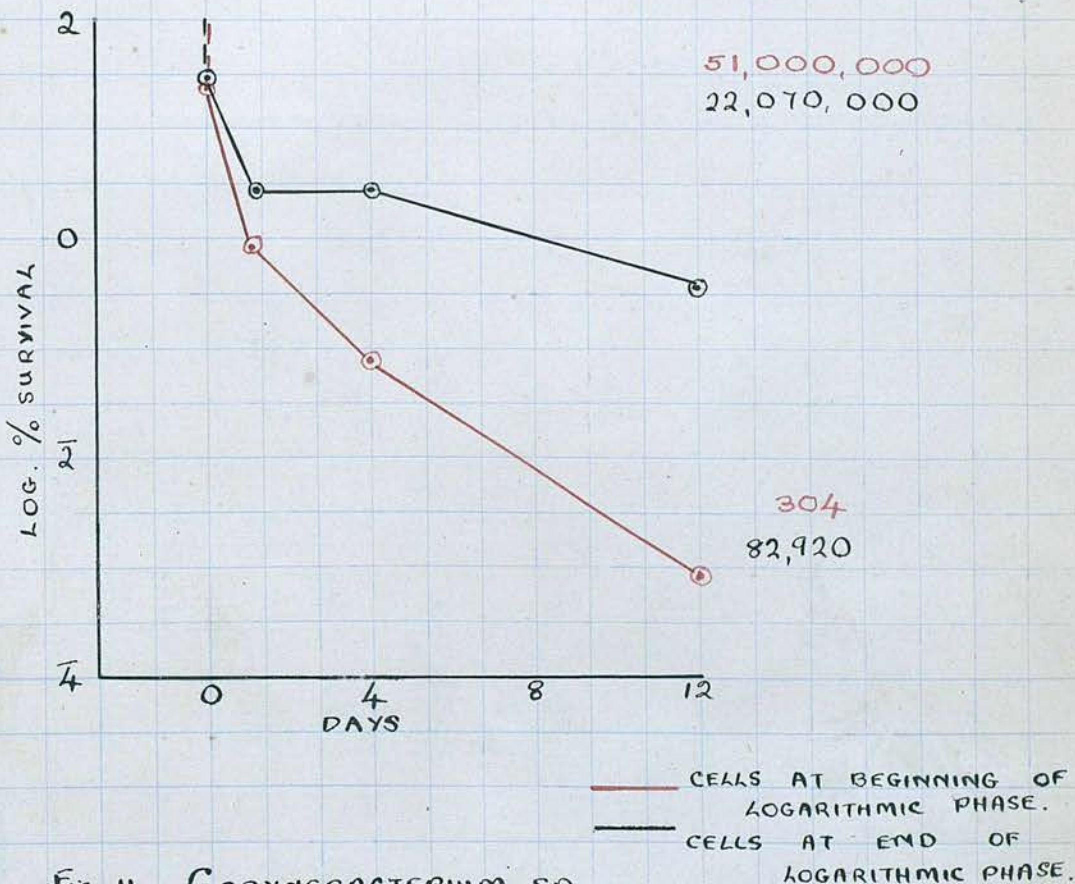
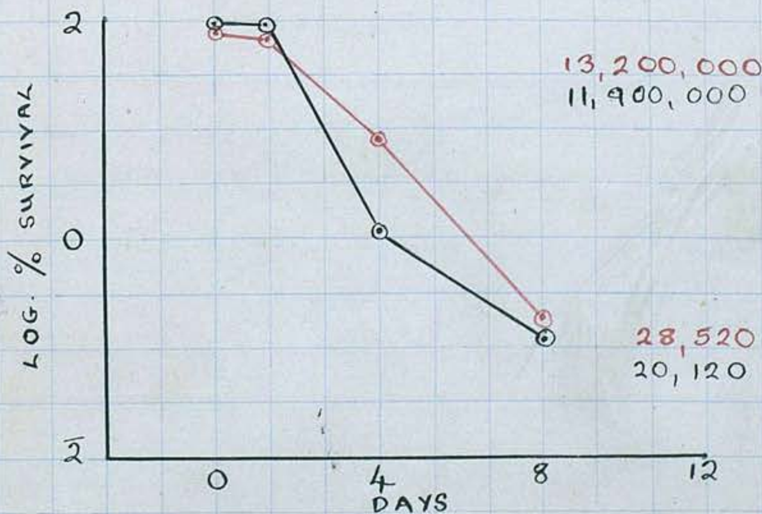


Fig. 11. CORYNEBACTERIUM. sp.



occurs only in the very early stages of incubation. The decrease in resistance to drying with ageing of cultures of *Shigella dysenteriae* reported by Proom and Hemmons (40) can probably be attributed to the long incubation period that they employed. The greatest resistance was shown after incubation for 7 hours when it is likely that the cells are well within the logarithmic phase of growth. In my experiments the older cultures had probably not reached the phase of decreased resistance reported to occur by Proom and Hemmons (40) at 23 hr - 6 days and by Ficker (19) after 2 days incubation.

Since it appears that cells towards the beginning and the end of the logarithmic phase of growth do not differ greatly in resistance to desiccation, it was considered unnecessary in future work to compare cultures in more than one phase of growth or to consider this factor in assessing the value of the results. In order to ensure ~~appropriate~~ approximate uniformity it was decided to work with cells approaching the end of the logarithmic phase. Such cultures are convenient to handle in practice since they are unlikely to change rapidly in resistance.

IV The Storage of Dried Organisms

The/

The rate of death of dried bacteria is known to vary considerably with changes in environment (see the review of literature). In this work it was necessary to select for the storage of dried organisms a set of conditions that could be applied uniformily throughout. The choice was limited to conditions that may occur in practice on the farm. The dried films were held in the presence of air and at a temperature of 22°C. It was desirable to store the dried cultures in air of a constant humidity since any dry material will readily establish an equilibrum between itself and the surrounding air. Dried feeding stuffs and plant materials in equilibrium with air of 75 - 100% R.H. have been shown (60) to absorb sufficient moisture to support microbial growth.

Normal atmospheric humidity is subject to considerable variation, as is indicated by the daily weather reports issued by the Meteorological Office, London. For instance the figures registered at Renfrew throughout the year 1948 vary between 20 and 100% R.H. The most common values however, occur between 50 and 90% R.H. For the purposes of the present work, relative humidities of 53%, 75% and 94% were selected. These values lie within the normal atmospheric range and can be obtained easily at 22°C. by means of saturated salt solutions. (53). The following/

following solutions in the presence of an excess of the solid phase, were used at 22°C:

$Ca(NO_3)_2$ = 53% R.H.

$NaNO_3$ = 75% R.H.

KNO_3 = 94% R.H.

Tubes containing the dried cultures were stored in screw top jars containing a small quantity of the requisite salt solution. The cotton wool plug of each tube was cut off level with the glass flange in order to encourage free circulation of air and an equating of the moisture content of the atmosphere in the jars with that in the tubes.

Preliminary trials were carried out using young actively growing broth cultures of M. albus and a coryne-bacterium. These cultures were centrifuged and the cells were resuspended in 0·1% gelatin prior to drying. Three dried samples of each bacterium were stored at each relative humidity and were reconstituted at intervals in 0·1% gelatin. The results given in table 7 show a peculiar irregularity when cultures of M. albus are stored at 75% and 94% R.H. Within the first 24 hours the percentage survival decreases in normal manner, probably because at this stage an equilibrium/

TABLE 7.

The effect on dried bacteria of storage at different relative humidities.

Period of Storage	Count before drying	% survival		
		53% R.H.	75% R.H.	94% R.H.
Micrococcus albus				
0 hr.	10,500,000) 12,000,000) 13,600,000)	7·961	6·761	5·184
24 hr.	10,500,000	0·2151	3·744	0·3629
5 days	12,000,000	0·01629	0·08058	148·0
10 days	13,600,000	0·00305	2·591	> 100
Corynebacterium sp.				
0 hr.	15,300,000) 16,000,000) 16,500,000)	52·81	42·51	59·7
24 hr.	15,300,000	12·28	6·301	3·417
5 days	16,000,000	0·3756	5·484	0
10 days	16,500,000	0·00002	0·01975	0

equilibrium has not become established between the dried cultures and the humid atmosphere in the jars. Bartholomew and Norman (60) found that such a process was apt to be slow when exposing dried plant material to a stream of moist air. The minimum time involved in their work was 24 hours. It is perhaps surprising to note that the death rate at 75% R.H. is considerably less than at 53 or 94% R.H., during the first 24 hours. Following the period of adjustment the micrococcus showed a considerable increase in numbers in 5 days at 94% R.H., and 10 days at 75% R.H. The increase in count obtained at 94% R.H. is too great to be accounted for by disintegration of clumps and can only be reasonably explained by assuming that multiplication of the surviving cells has occured. No such evidence of growth is shown by the corynebacterium.

Moisture absorbed from the atmosphere under these conditions would only be sufficient to give rise to a relatively concentrated solution of gelatin in which bacterial growth would not be expected to occur. Micrococci are able to grow in solutions of high osmotic tension (61, 62, 63, 64, 65, 66). This possibly is linked with their ability to multiply in gelatin films of a low moisture content.

In order to investigate this problem more fully cultures/

cultures at the end of the logarithmic phase of growth were dried in broth and stored for varying periods at each humidity. The following organisms were examined:

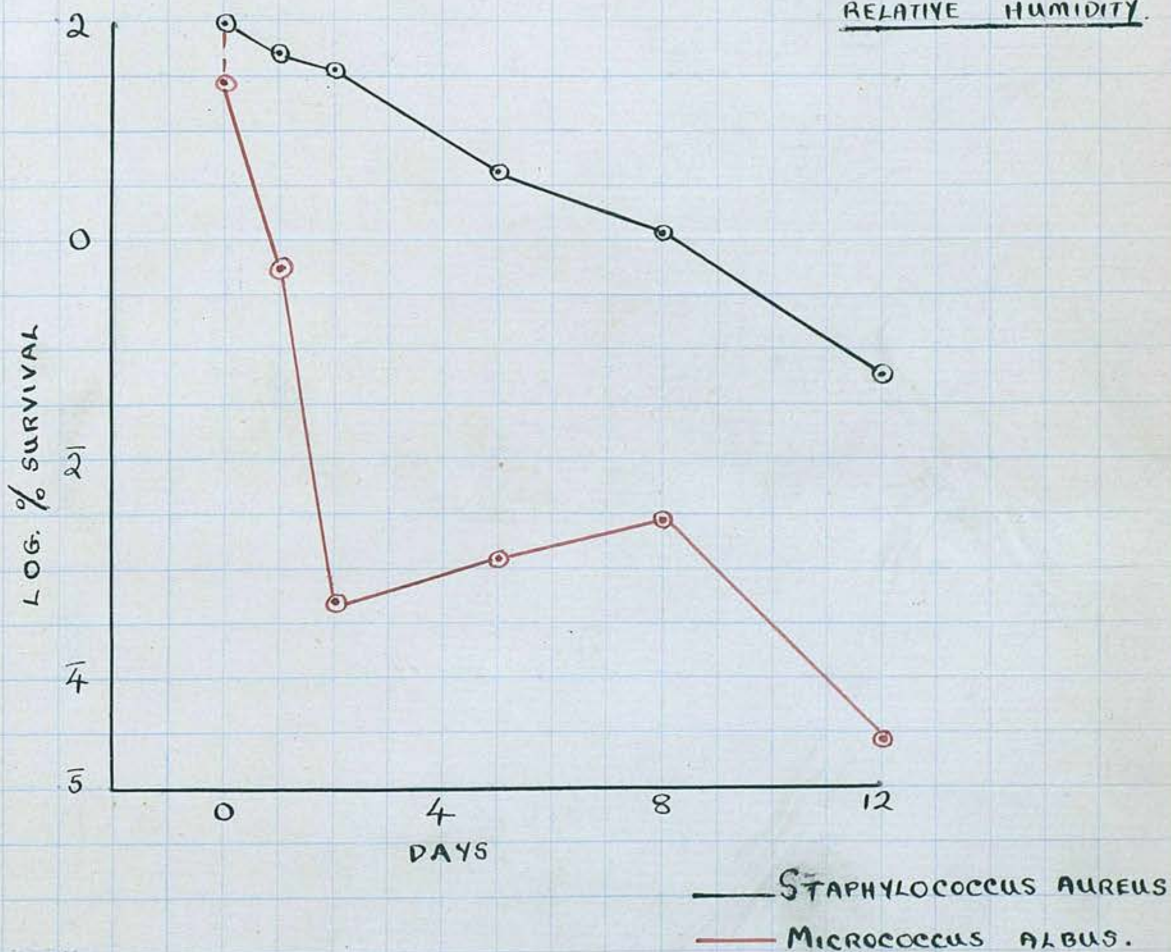
Organism	R.H.
Mic. albus	94% R.H.
Staph. aureus (Coagulase +)	
Staph. albus (Coagulase +)	53%. 75% & 94% R.H.
Mic. luteus	
Str. Liquefaciens	

The results are given in figs. 12-15. The films stored at the two higher relative humidities appeared to have absorbed a considerable amount of moisture after 5 days storage, giving rise to a drop of liquid broth at the base of each tube. There was however, no decisive evidence of an increase in count after storage in any of these experiments. That shown by M. albus was only very slight and was probably due to the disintegration of clumps.

The ability of M. albus. to multiply in a concentrated solution of gelatin but not in broth may be due to a difference in the hygroscopic capacity of the two materials. Failure to grow in broth may also be due to its greater content of osmotically active substances.

The corynebacterium used in the preliminary trial and/

Fig. 12. THE DEATH RATES OF STAPHYLOCOCCUS AUREUS AND MICROCOCCUS ALBUS STORED AT 94% RELATIVE HUMIDITY.



THE DEATH RATES OF BACTERIA STORED AT 53%, 75% AND 94% RELATIVE HUMIDITY.

Fig. 13. STAPHYLOCOCCUS ALBUS.

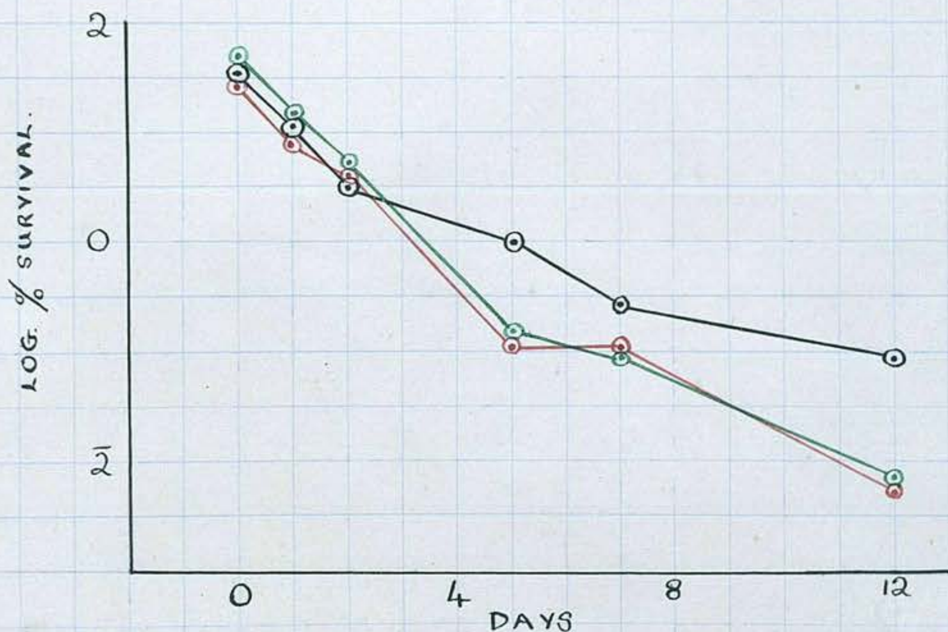
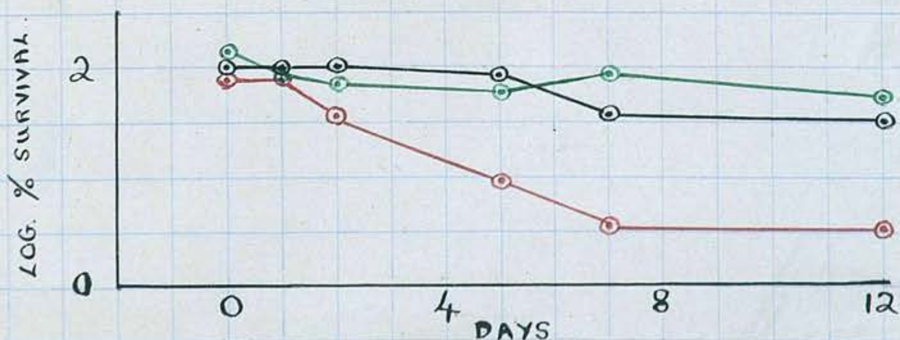
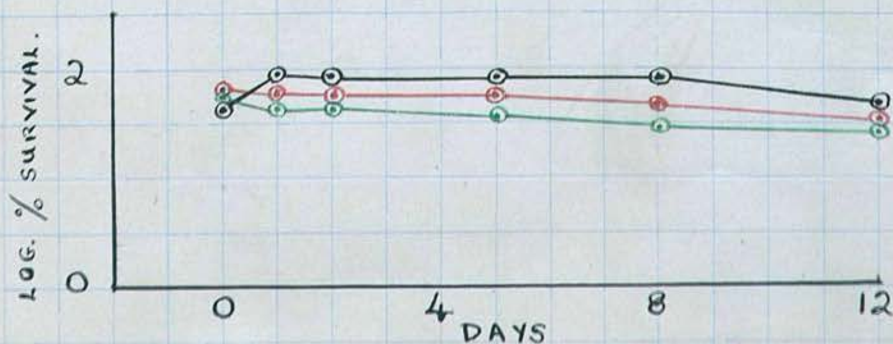


Fig. 14. MICROCOCCUS LUTEUS.



53% R.H.
75% R.H.
94% R.H.

Fig. 15. STREPTOCOCCUS LIQUEFACIENS.



and the cultures of <u>Staph. albus</u> and <u>Str. liquefaciens</u> appear to survive longest when stored at 53% R.H. The variation between humidities is not very large or decisive in these experiments but it can be correlated with the results of Rogers (20) and Proom and Hemmons (40). Rogers obtained a maximum survival of the flora of dried milk powder when it was stored at a low moisture content and Proom and Hemmons found that freeze dried cultures survived for a longer period if subjected to secondary drying over P_2O_5 *in vacuo*. Higginbottom (43) working with the natural microflora of milk powder and Watts (42) with dried milk cultures of <u>Str. agalactiae</u> noted an increased survival at low relative humidities with the exception of 0% R.H. which appeared to initiate a higher death rate than the slightly higher humidities but their results are not sufficiently detailed to be conclusive. It therefore, seems possible to presume that in the absence of sufficient moisture to support growth, slightly damp cultures die off more rapidly than drier ones. The susceptibility of bacteria to disinfectants also appears to vary with the amount of moisture present. For instance, Wolf and Batchelor (67) and Cousins (68) both found that a greater percentage of bacteria were killed by/

by hypochlorite aerosols in air of a high relative humidity than in air of a lower moisture content.

A relative humidity of 53% was adopted in all future work in order to avoid any multiplication of cells such as might occur when cultures are stored under more humid conditions. Such multiplication is unlikely to occur when bacteria are dried in broth, but a relatively low humidity provides a wider margin of safety and therefore is more satisfactory in practice.

THE RESISTANCE TO DESICCATION OF SPECIFIC MICROORGANISMS.

Experimental Method.

The microorganisms to be investigated were grown in broth and dried in this medium when they had reached approximately the end of the logarithmic phase of growth, as described in the previous section. Samples from each culture were diluted in sterile broth, to give an approximately standard cell concentration and six 1 ml. quantities were dried, two samples being dried simultaneously for a period of 20 min. Any changes in the composition of the original broth cultures due to the accumulation of metabolic products was considered unimportant since each sample was diluted in a relatively large volume of sterile broth of constant composition. Viable counts were obtained from the undried culture and from one sample immediately on removing from the pump. The remaining five dried tubes were stored at 22°C. in a closed jar containing saturated Ca $(NO_3)_2$ giving 53% R.H. Tubes were removed at intervals and the dried cells were recovered in 0·5% saline. All dilutions were made in 0·1% gelatin.

In the early stages of the work organisms commonly/

commonly occuring in milk were selected for examination in order to provide information applicable in the dairy industry. Later additional species were included to investigate more fully the apparent correlation between resistance to desiccation and Gram reaction and to complete as comprehensive a study of species variation as time would permit. Whenever possible, two or more species of each type were examined.

Presentation of results.

The results are presented mainly in the form of graphs in order to demonstrate the course of death over a period of time. As approximately seven hundred colony counts, each an average of triplicate counts, were made in this part of the work, alone, it seemed impracticable and unnecessary in view of the aims of the work, to list these figures in detail. Throughout the work good agreement was obtained between parallel counts and only eleven tubes were discarded owing to counts showing an undully large deviation from the average. These variations were due to the inhibition of bacterial growth caused by the use of improperly washed glass ware.

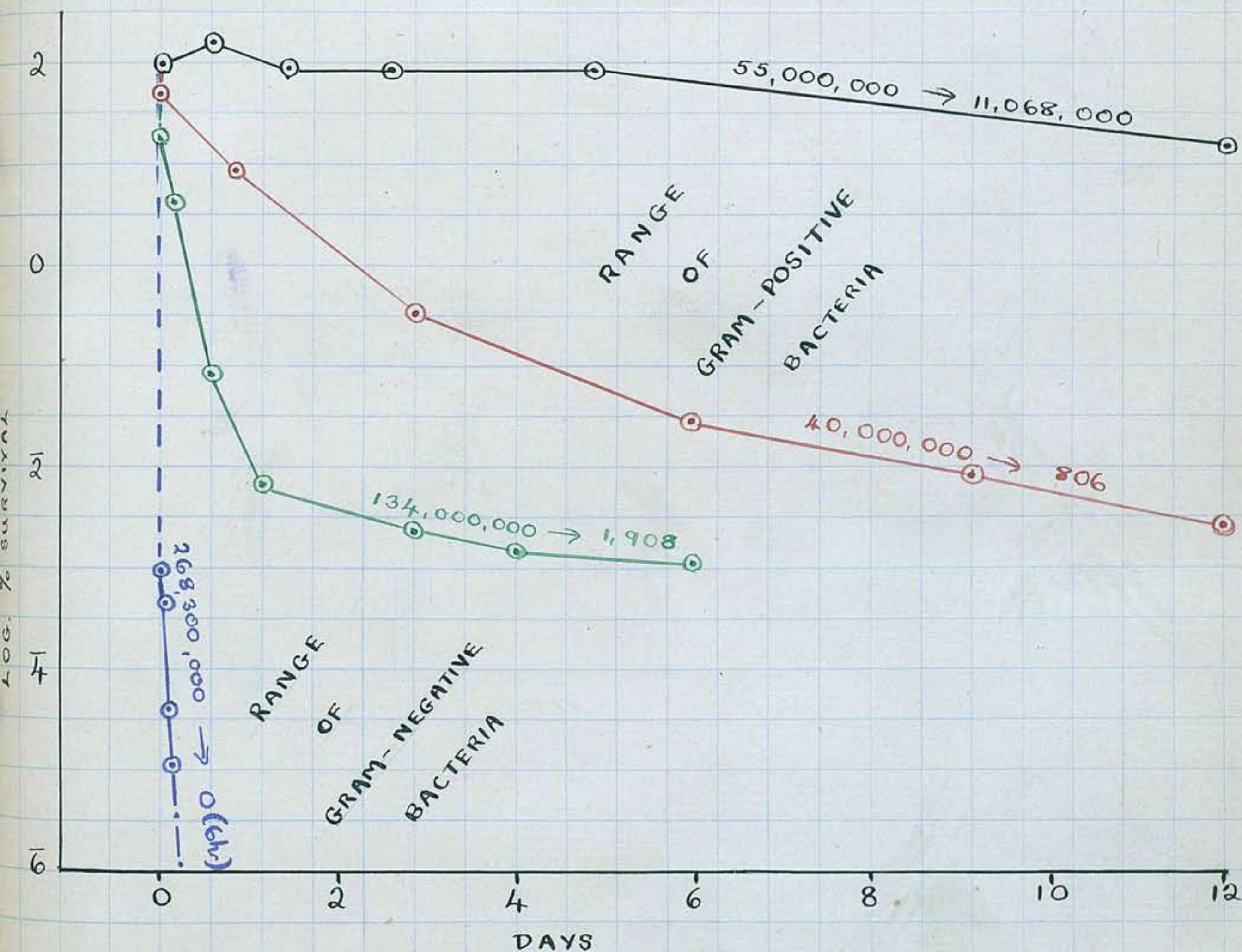
In/

In the graphs (figs 16-34) as in those in the previous section (figs. 5-15), the vertical drop (indicated by a broken line) from 100% (log. 2) at 0 hours represents the death of microorganisms during the 20 min period of rapid drying at the pump. In some cases the graphs are continued by a second type of broken line towards the end of the process. This is done where the recovery of viable organisms was less than thirty per ml. In such cases the reliability of the count is uncertain since the sampling error would be great.

The number of viable organisms in the original suspension before drying and in the last dry culture to be examined are shown in each figure.

The counts in table 8 were obtained at a later stage in an attempt to determine the total period throughout which the more resistant species would survive. In most cases a few viable cells were still present after 6 weeks storage but time did not permit any further extension of the period. Some variation will be noted between these and earlier results. This variation may be/

Fig. 16. THE EXTREME TYPES OF RESISTANCE TO DESICCATION SHOWN BY GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA.



CORYNEBACTERIUM LACTICUM (1).
MICROCOCCUS VARIANS (2).
RHIZOBIUM sp. (LUCERNE STRAIN).
AZOTOBACTER sp.

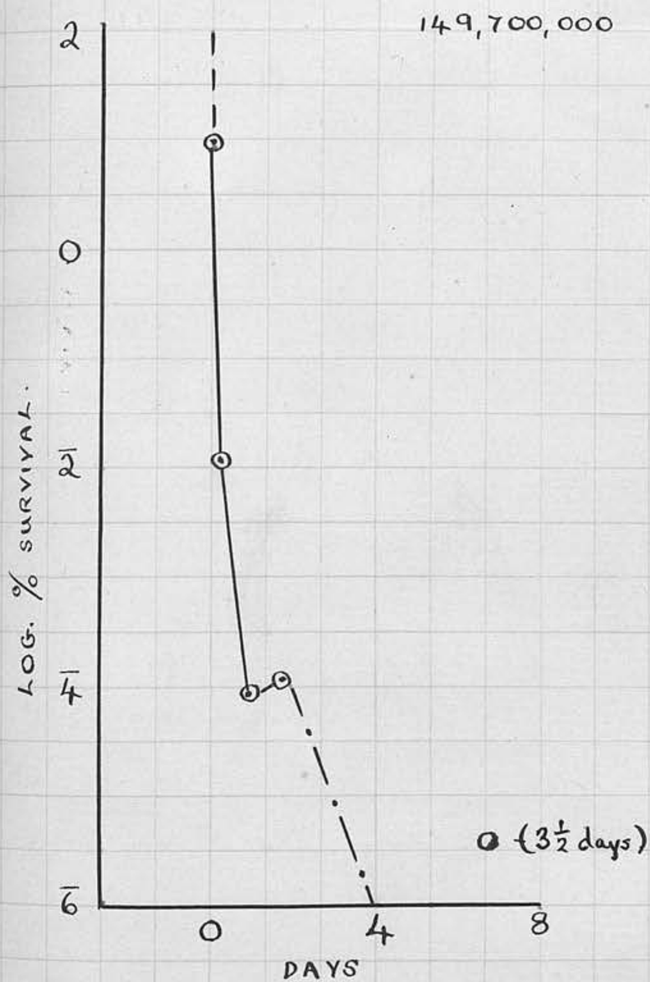


Fig. 17. Pseudomonas sp.
149,700,000
2
0
$\bar{2}$
$\bar{4}$
$\bar{6}$
LOG. % SURVIVAL.
0 (3½ days)
0
4
8
DAYS

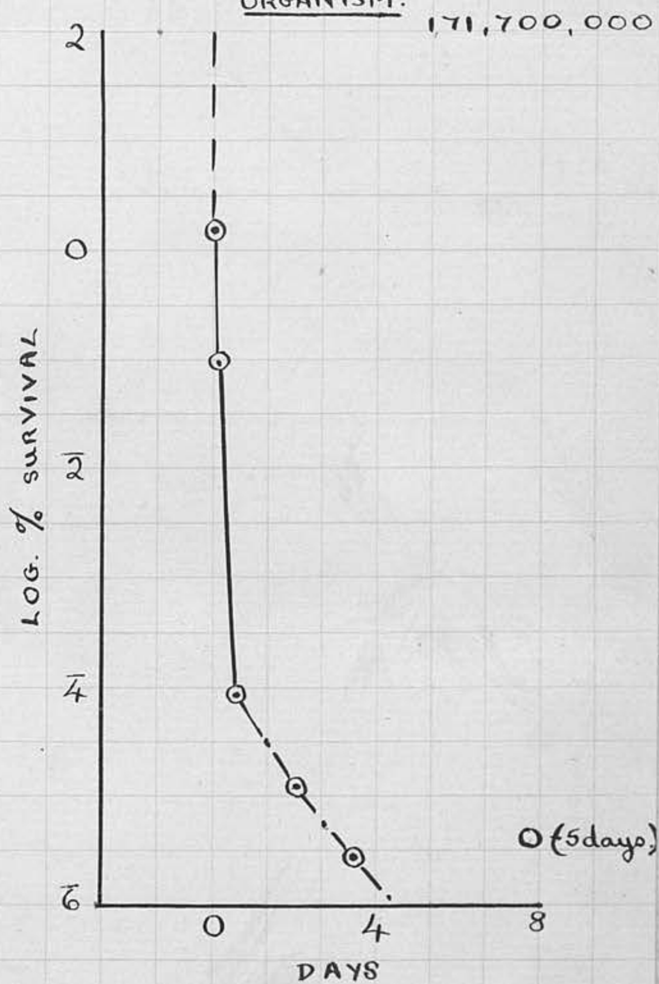


Fig. 18. Intermediate coliform organism.
171,700,000
2
0
$\bar{2}$
$\bar{4}$
$\bar{6}$
LOG. % SURVIVAL
0 (5 days)
0
4
8
DAYS

Fig. 19. ALCALIGENES VISCOSUS (1).

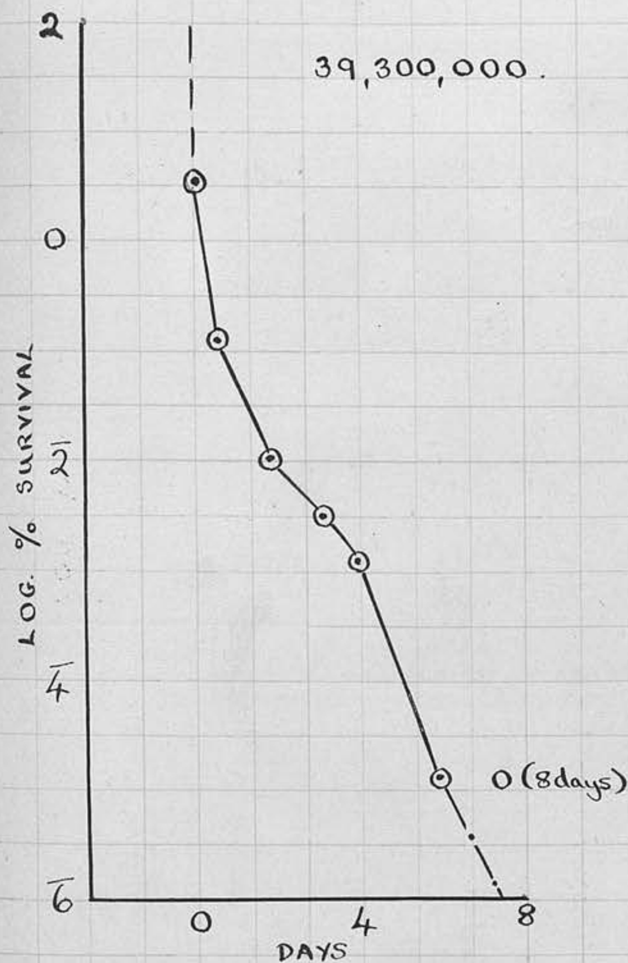


Fig. 20. ALCALIGENES VISCOSUS (2).

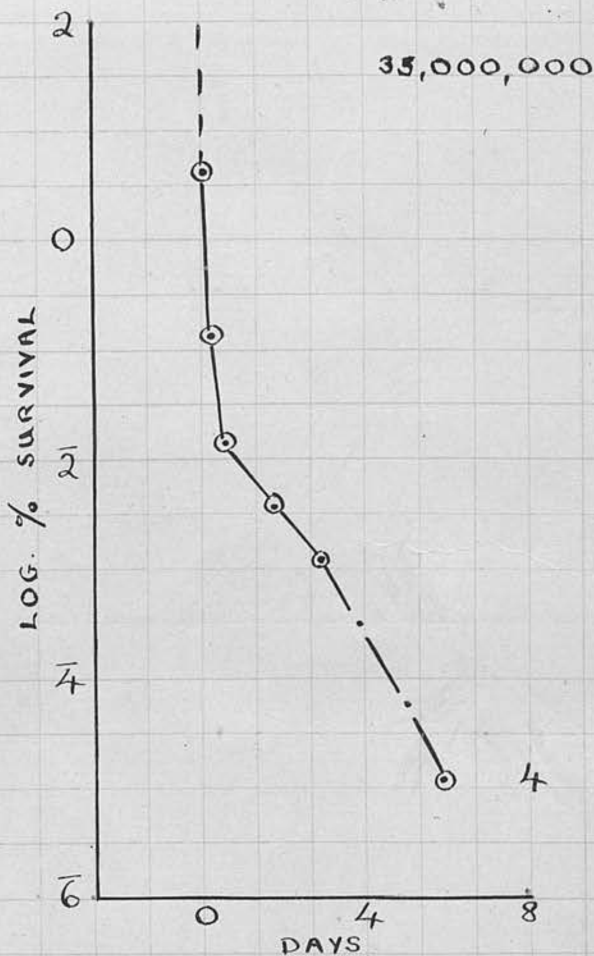


Fig. 21. RHIZOBIUM sp. (SOYA STRAIN).

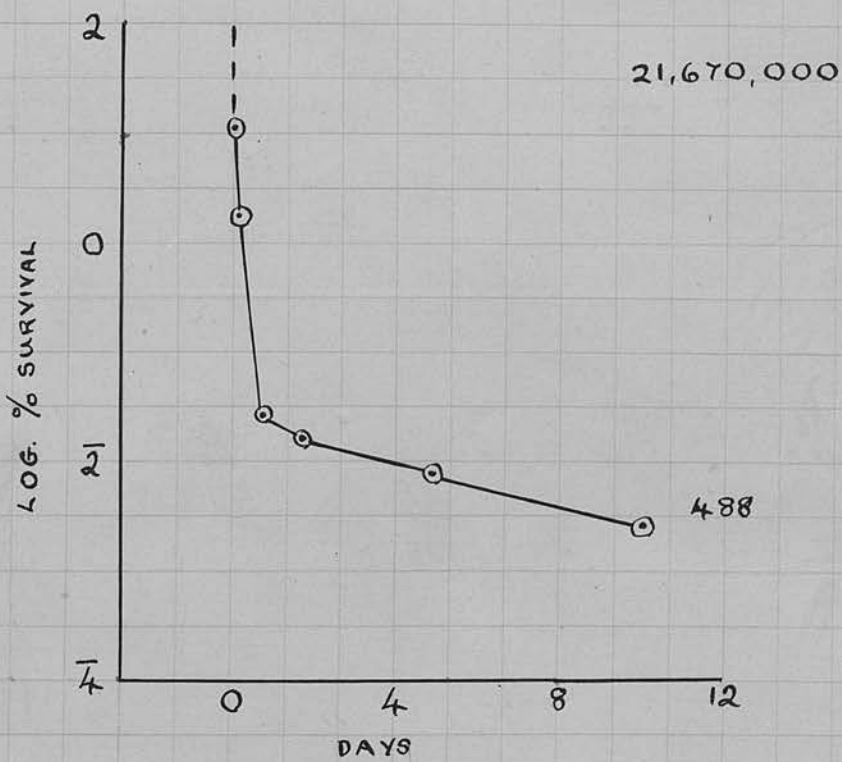


Fig. 22. Oidium lactis (oidia).

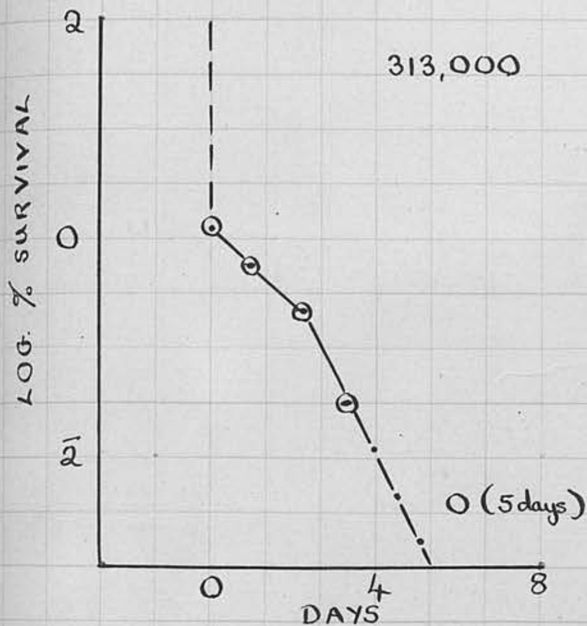


Fig. 23. Pink yeast.

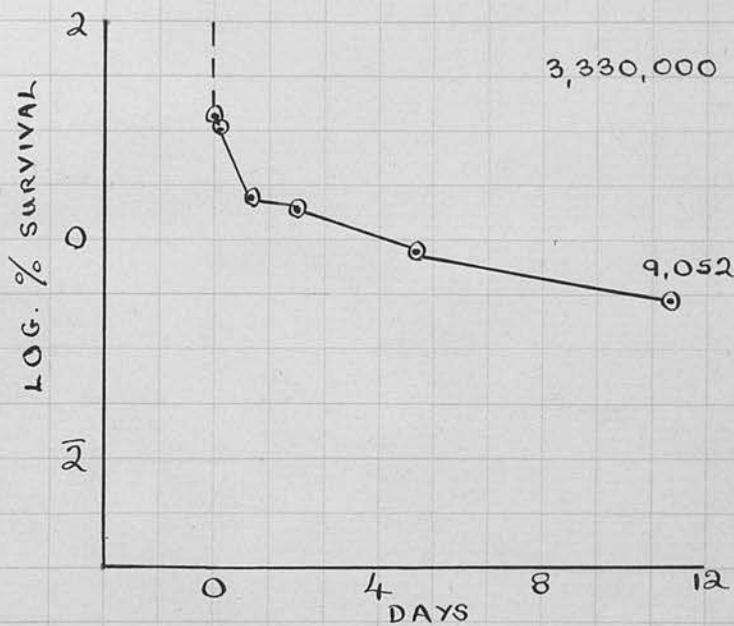


Fig. 24. Torulopsis kefyr.

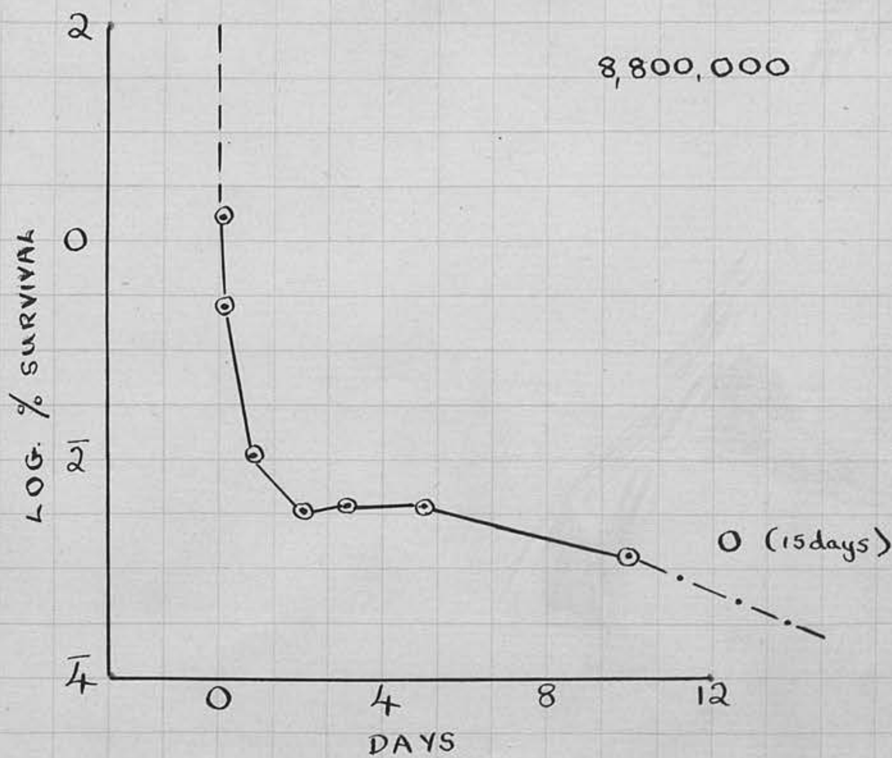


Fig. 25. STREPTOMYCES SP. (SPORES).

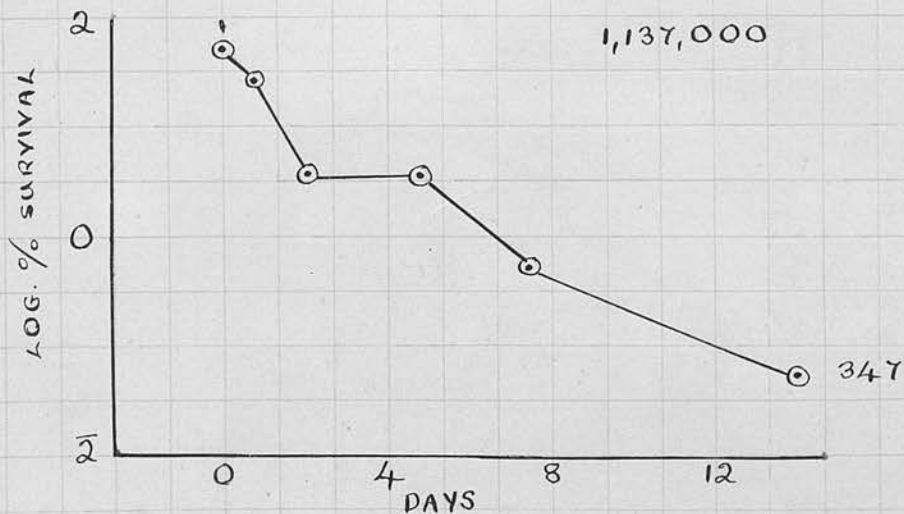


Fig. 26. NOCARDIA SP.

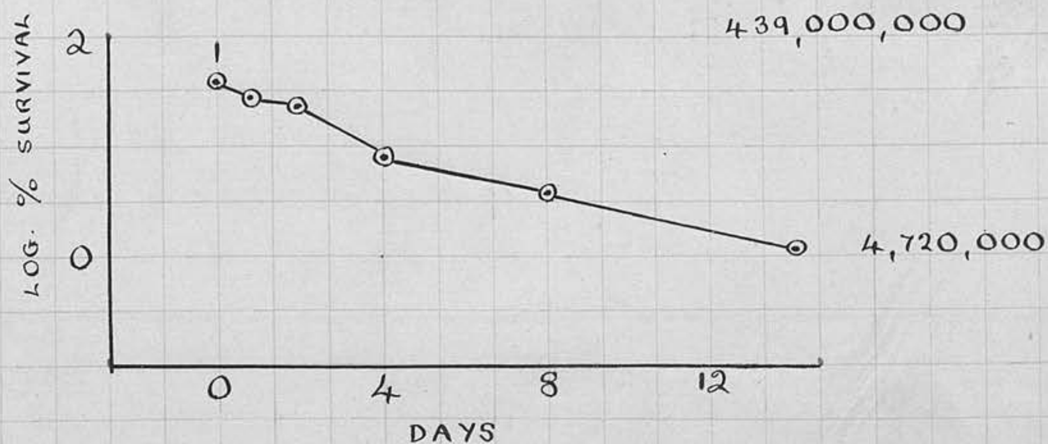


Fig. 27. MICROCOCCUS LUTEUS.

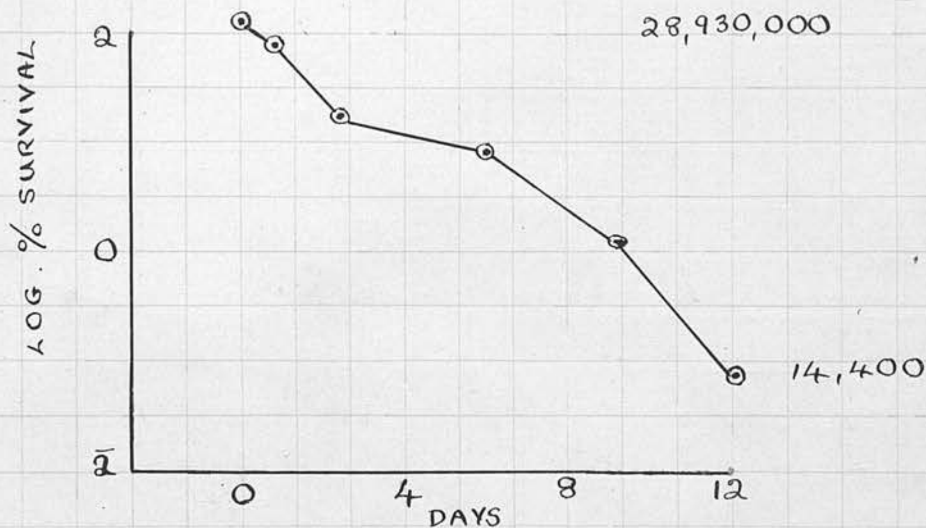


Fig. 28. MICROCOCCUS VARIANS (1).

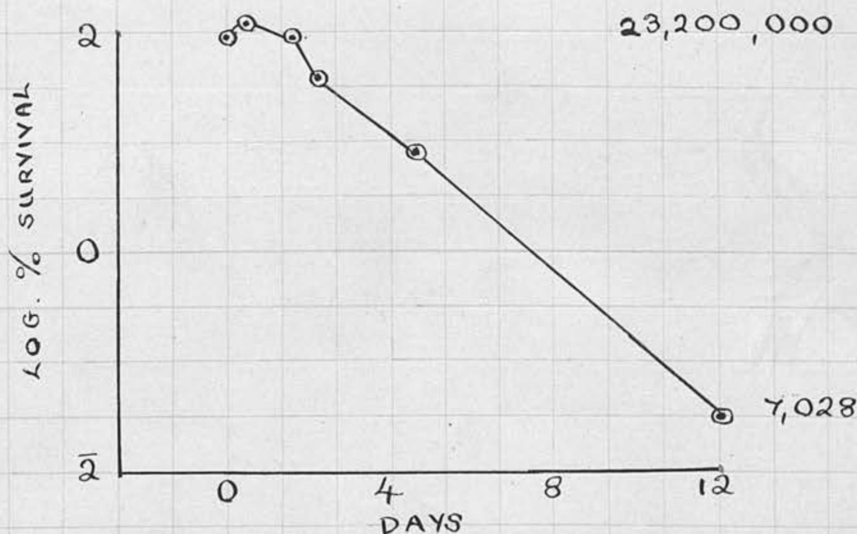
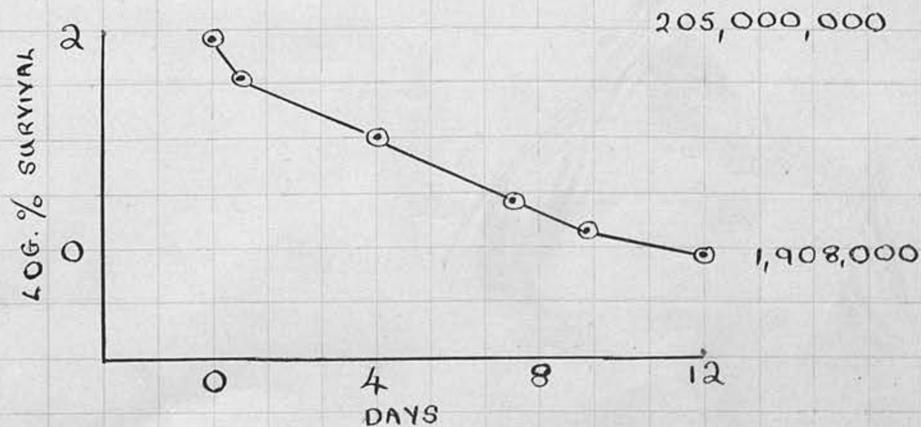


Fig. 29. CORYNEBACTERIUM LACTICUM (2).



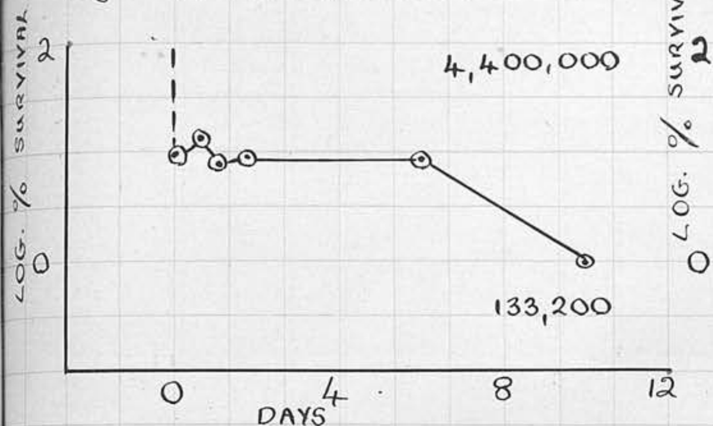


Fig. 30. STREPTOCOCCUS LACTIS.
LOG. % SURVIVAL
2
0
4,400,000
133,200
0
4
8
12
DAYS

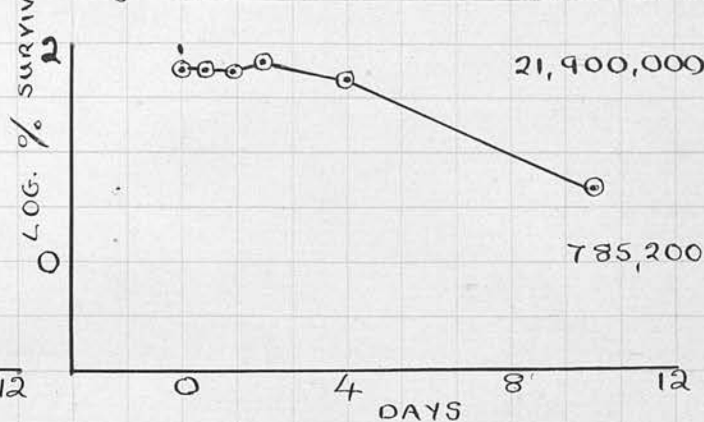


Fig. 31. STREPTOCOCCUS LIQUEFACIENS.
LOG. % SURVIVAL
2
0
21,900,000
785,200
0
4
8
12
DAYS

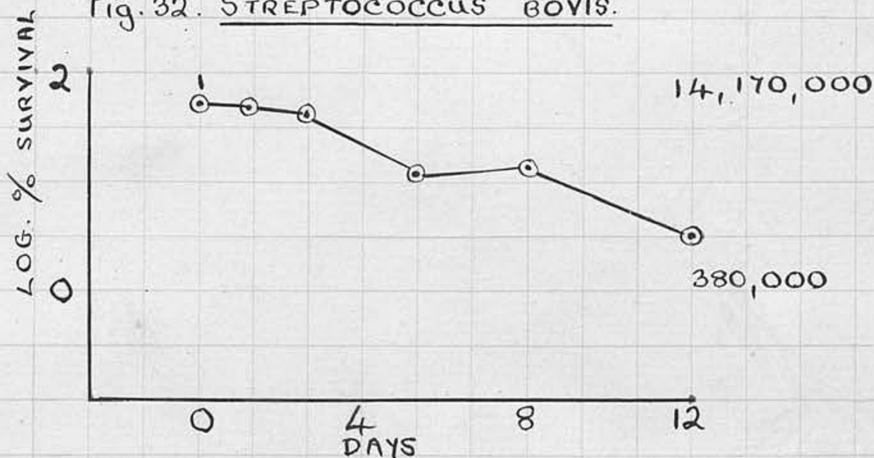


Fig. 32. STREPTOCOCCUS BOVIS.
LOG. % SURVIVAL
2
0
14,170,000
380,000
0
4
8
12
DAYS

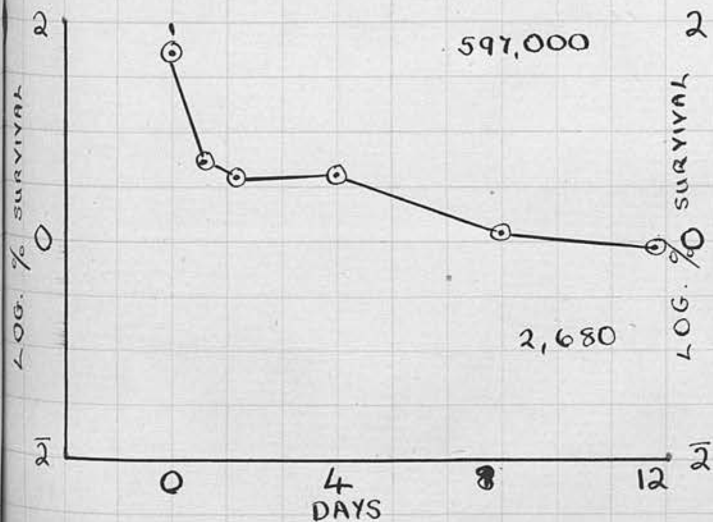


Fig. 33. STREPTOCOCCUS THERMOPHILUS.
LOG. % SURVIVAL
2
0
$\bar{2}$
597,000
2,680
0
4
8
12
DAYS

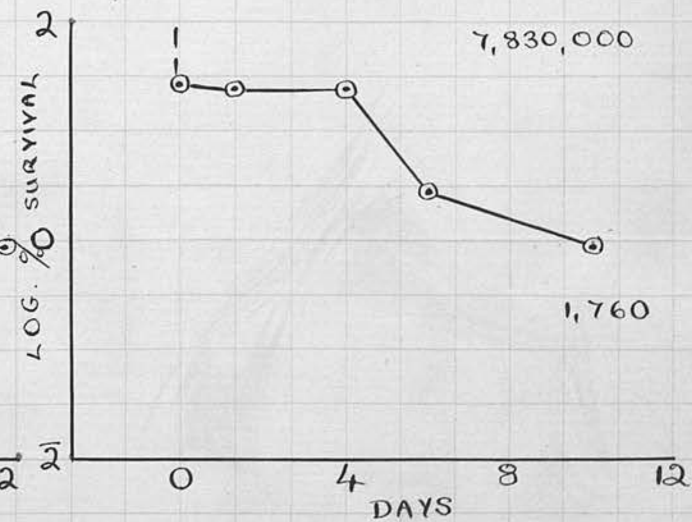


Fig. 34. STREPTOCOCCUS KEFIR.
LOG. % SURVIVAL
2
0
$\bar{2}$
7,830,000
1,760
0
4
8
12
DAYS

TABLE 8.

The survival of species relatively resistant to desiccation during a prolonged storage period.

Species	% survival after storage for		
	2 weeks	4 weeks	6 weeks
Streptococcus liquefaciens	43,5000	5·2160	0·2786
Str. bovis	38·3600	11·5300	0·6940
Str. thermophilus	18·6000	2·0130	0·0883
Str. lactis	9·4060	0·1142	0·0020
Str. kefir	7·6580	0·0230	< 0·0001
Corynebacterium lacticum (1)	1·5960	0·1714	0·0830
C. lacticum (2)	0·2647	0·0081	0·0018
Corynebacterium sp.	0·1065	0·0073	0·0057
Micrococcus varians (2)	1·0960	┼	0·0424
M. varians (1)	0·9541	0·0287	┼
M. luteus	0·0884	1·3580	0·0527
M. albus	0·1465	0·0003	< 0·0001
Streptomyces sp.	0·0305	0·0020	0
Nocardia sp.	1·0750	┼	0·0367
Pink Yeast	0·0358	< 0·0001	0

┼ Dilution plated was too low to yield accurate counts.

< 0·0001 Less than 30 colonies per ml. and consequently a large sampling error.

be due to a failure to recover the total number of viable cells in all cases.

Discussion.

The limitations of the experimental method must be taken into account when drawing any general conclusions from the results. The preliminary work already described indicates that there are two main sources of error:

(1) a disintegration of cell groups as a result of drying and

(2) a failure to recover all the viable cells after drying.

The following bacteria gave apparent survivals of over 100%:

Expt (a) Corynebacterium lacticum (1)

10 hr. storage 256·6%

Expt (b) Corynebacterium lacticum (1)

0 hr. storage 111·5%

Corynebacterium sp.

0 hr. storage 194·9%

Expt (a) Micrococcus varians (1)

10 hr. storage 101·7%

Expt (b) Micrococcus varians (1)

0 hr. storage 104·1%

M. varians (2)

0 hr. storage 149·6%

M. luteus (as defined by Abd-El-Malek &

<u>M. luteus</u> (as defined by Abd-El-Malek & Gibson (69)

0 hr. storage   114·3%

Both micrococci and corynebacteria are relatively resistant to desiccation. The individual cells of these organisms tend to remain attached after division and these clumps may become broken up during drying. A similar tendency with sensitive organisms would be indistinguishable from the lethal action of desiccation. The results obtained with the spores of <u>B. subtilis</u> indicate that the observed percentage survival is probably in ~~all~~ some cases lower than the actual figure, due to a failure to recover all the viable cells. To avoid these difficulties as far as possible, the death of the organisms was followed over a period of time. The results so obtained are probably comparable and they indicate a large variation in resistance to drying among different organisms. Strains of the same species usually exhibit approximately the same order of resistance.

(a) <u>Logarithmic order of death</u>. The theoretical curve of logarithmic decrease is not shown by all the organisms examined in this work. In a number of cases (figs. 7-10, 16 (Azotobacter and Rhizobium), 17-24, 33) a large reduction in the count occurs during the 20 min period of drying at the/

the pump. (see dotted lines parallel to vertical axes). This apparently high death rate persists for approximately 24 hours. A change in the direction of the curve then becomes apparent and the organisms die off at a considerably reduced rate. This type of curve is confined mainly to organisms that are readily destroyed by drying but no clear demarkation can be made on this basis since it is also evident to some extent in the results with M. albus and Str. thermophilus. In general, the more resistant species are not appreciably affected during the period in which water is rapidly removed and during storage they show a decrease in count which is approximately logarithmic.

Other workers investigating the destruction of micro-organisms by a variety of physical and chemical agents, have noted deviations from the logarithmic order of death (see review of literature). The finding of Reichenbach (70) on the effect of heat and of Chick (71) on the action of phenol, appear to be comparable with the results obtained in this study of desiccation. These workers obtained sigmoid curves of decreasing death rate when Bacterium/

Bacterium paratyphosum was exposed to temperatures of approximately 50°C. (70) and to 0·6% phenol (71). They explain their results by assuming that the cultures contained a mixture of young and mature cells, since much of the variation in death rate was eliminated by repeated transfer of the cultures or by incubation until all cells had reached maturity. In my work sigmoid curves were obtained with certain organisms despite the routine treatment in which cultures prior to drying, were transferred frequently and were incubated until they had reached the end of the logarithmic phase of growth. The cells thus obtained should have been relatively uniform.

If a variation in the resistance of individual cells is responsible for the decreasing death rate, it might be expected that the sigmoid type of curve would have been obtained in all the trials. The more resistant species however, appeared to show approximately the logarithmic order of death during the period of the experiments. Detailed examinations ~~contained~~ continued over a longer period of time may perhaps reveal a decrease in the death rate of these organisms but this does not seem likely as counts at 2,4, and 6 weeks do not indicate any such tendency.

Another/

Another possible explanation of the break in the curves may be protection of residual cells by accumulated debris and a subsequent reduction in their rate of death. In such an event the protective effect would be expected to appear gradually and to give rise to more gently sloping curves. It should occur in all experiments and should be less obvious with dilute than with concentrated suspensions. This was not the case (figs. 5 and 6). King and Alexander (72) in experiments on the mechanical destruction of bacteria by shaking with glass beads, obtained a similar break in the logarithmic death rate. They also rejected the theory of protection by accumulated debris as a possible explanation.

A third possible explanation of my results is that two processes are involved in the destruction by drying of sensitive micro-organisms. The rapid decrease in count during the early stages may be due to some irreversible change in the bacterial protoplasm caused solely by the removal of moisture. The surviving cells may be killed only by some slower process, perhaps an oxidation of certain cell constituents. The presence of oxygen has been shown to increase greatly the death rate of dried bacteria (9, 20, 39, 40). The more resistant species may be capable of withstanding/

withstanding the first type of change and in these, oxidation may be the major factor responsible for cell death.

(b) Gram-reaction of organisms and their resistance to desiccation.

The ability to survive desiccation appears to be linked with the Gram-reaction of the organisms. This can be seen in fig. 16 in which are plotted the results obtained with the most resistant and the least resistant of the Gram-positive and the Gram-negative organisms. Gram-positive species such as corynebacteria (figs. 11, 16, 29) and streptococci (figs. 31-34), with the exception of Str. lactis (fig. 30), did not decrease appreciably in numbers during the removal of water and were still viable after storage for 6 weeks in the dried state. The Gram-negative species on the other hand, were reduced considerably in numbers by the removal of water and only survived in the dried state for a few days (figs 7-9 16-21). The extreme case is that of Azotobacter (fig 16) which was completely nonviable 6 hours after drying. Alcaligenes viscosus (figs. 19. 20) was more resistant than/

than most other bacteria in this group, probably on account of its Gram-variable nature. It was however, more readily killed than <u>Rhizobium</u>. The resistance of the latter species perhaps is influenced by the size of its cells (see p 88). The yeasts (figs. 23-24) and <u>Oidium lactis</u> (fig 22) were intermediate in resistance between the Gram-positive and the Gram- negative bacteria, probably again on account of their cell size (p.88).

Gram-positive and Gram-negative species differ in their reaction to many harmful agents:

1. Ultraviolet light (73).
2. Salts and their constituent anions and cations (74).
3. Synthetic detergents (75, 76).
4. Aniline dyes (77, 78).
5. Oxidizing and perhaps reducing agents (79)
6. Antibiotics (80).
7. Solution in alkalies (81)
8. Digestion by enzymes (81).
9. Lysis by an immune serum in presence of complement. (81).

No. 1-6 Gram-positive organisms more susceptible than Gram-negative organisms.

No. 7-9/

No. 7-9 Gram-negative organisms more susceptible than Gram-positive organisms.

To the second group of agencies may now be added desiccation.

It is obvious that the Gram-staining mechanism is based on some important property of the cells, but the basis is still not fully understood. Detailed references to early theories are given by Knaysi (82). Recently Henry & Stacey (83) and Stacey (84) obtained eveidence that the Gram reaction depends on the presence in the surface layers of the cells, of a magnesium salt of ribonucleic acid in combination with inert polysaccharides and traces of protein. However, it does not seem possible at present to suggest that the magnesium ribonucleate content of Gram-positive bacteria endows them with their ability to resist desiccation.

Taylor (85) has shown that Gram-positive and Gram-negative species differ in their ability to concentrate free aminoacids, particularly lysine and glutamic acid, in the internal cell environment and Gale (86) has found a similar/

similar variation in cells of Str. faecalis at different stages of growth. A relatively high concentration of these acids appears to occur in the cells of Gram-positive species and in cells at the end of the period of active cell division. These findings do not seem to be connected in any obvious way with resistance to desiccation.

Other writers do not appear to have mentioned any correlation between Gram-reaction and resistance to drying buta survey of their results has revealed the following relevant points. Stamp (2) dried cultures over P_2O_5 in a partial vacuum for several days and found that Corynebacterium diphtheriae gave a survival of at least 44% in contrast to Fusiformis fusiformis and Neisseria meningitidis which failed to grow on subculture. Stark and Herrington (37) found that rapid vacuum drying over P_2O_5 and $CaCl_2$ killed Esch. coli more readily than streptococci, a yeast and staphylococci. Another Gram-positive species, Lactobacillus acidophilus was however, sensitive to drying. Frobisher et al (49) preserved some Gram-positive species such as streptococci and staphylococci for as long as 19 years on grains of sand/

sand dried under vacuum, while the least resistant species which were non-viable within a few months were those of Neisseria, Brucella and Hemophilus. Proom and Hemmons (40) found that freeze dried cultures of Staph. aureus were more resistant than Esch. coli and Shigella dysenteriae and considerably more resistant than Neisseria intracellularis, N. gonorrhae and Vibrio comma... Apart from these results however, Stamp (2), Frobisher et al (49) and Proom and Hemmons (40) all obtained intermediate degrees of resistance of a variable nature, with certain Gram-positive and Gram-negative species and this may account for their failure to correlate Gram-reaction with resistance to desiccation.

The relationship of the two properties is marked in the results of Wells and Stone (50) who determined the survival of bacteria in the air of an experimental chamber. The organisms investigated were recovered after the following periods of time:

B. subtilis 96 hours.

Staph. aureus 72 hours.

Pneumococcus
Diphtheria bacillus
Str. hemolyticus. } 48 hours.

Various Gram-negative intestinal bacteria 4-8 hours.

Pfeiffer's/

Pfeiffer's bacillus<1 hour.

These results clearly indicate the ability of Gram-positive bacteria to survive desiccation in the air for longer periods than Gram-negative species.

(c) *Cell size*. It may be possible to correlate resistance to desiccation will cell size, provided the observations conform to the major classification based on Gram-reaction as previously discussed. Throughout my results it is apparent that large cells are more easily killed than smaller ones. Within the Gram-negative group *Azotobacter* was the least resistant of the organisms examined and *Rhizobium* the most resistant. The line representing the rate of death of *Azotobacter* (fig 16) approaches the vertical and suggests that the removal of moisture is by far the most important cause of death. Any other process, such as an oxidation, probably plays a relatively insignificant part. The small cells of *Rhizobium* (figs. 16, 21) are able to withstand desiccation to a considerable degree and are even more resistant than the Gram- variable bacterium, *Alcaligenes viscosus* (figs 19. 20.)

Within the Gram-positive group the least resistant organisms are the yeasts (figs 23 24) and *Oidium lactis*

(fig/

(fig 22) and the most resistant species are the streptococci and corynebacteria. The micrococci occupy an intermediate position in both cell size and resistance to desiccation. Bacterial cells about to enter the logarithmic phase of growth are characterised by their large size and by their susceptibility to unfavourable environments (48). In view of the evidence obtained in my work on the relative inability of large cells to withstand desiccation it would be anticipated that in every bacterium whose cells enlarge during the lag phase, susceptibility would reach a maximum immediately before the start of cell proliferation. That variations in resistance to other harmful agencies during the earlier part of the growth cycle are attributed to changes in size, appears to be less probable. The mechanical strength of the cell may be an important factor in its resistance to drying and to unfavourably high or low temperatures, but there seems to be no reason to suspect that large fragile cells would be more susceptible than smaller ones to agencies such as concentrated salt solutions or chemical disinfectants.

The slime formed by certain bacterial species does not appear to afford them any degree of protection against drying. Three bacteria which form large amounts of slime, were examined. Two, namely Rhizobium and Alcaligenes viscosus/

viscosus showed a comparatively high degree of resistance to desiccation, while the third one, *Azotobacter*, was extremely susceptible.

(d) *The microflora of air and dry soils.* It seems probable that the bacterial populations of air are determined to a large extent by the ability of the microorganisms to survive desiccation. Proctor (87) and Proctor and Parker (88) collected samples of air at altitudes ranging from 1,500 to 16,500 ft, and found them to contain a number of moulds and the following bacteria:

Bacillus sp.
Achromobacter sp.
Staphylococcus sp.
Micrococcus sp.
Sarcina
Kurthia Zopfii
Actinomyces sp.

These organisms, with the exception of the Achromobacter sp. would be expected on the evidence of my work to resist desiccation. The moulds probably occured in the form of spores.

As previously mentioned, Wells and Stone (50) found that Gram-positive bacteria survived longer in the air of an experimental/

experimental chamber than did Gram-negative bacteria.

Lochhead (89) isolated a number of bacteria from the snow in the Ottawa district and found them to be mainly spore formers and a micrococcus. These organisms were derived from dust originating in the Western States and carried for a considerable distance across the continent before being precipated in the snow. They therefore must have been capable of withstanding considerable desiccation.

In bacteriological work cultures may become contaminated from the air. In my experience the microorganisms most commonly derived from this source are moulds, micrococci and sarcinae, and bacteria that form spores. In contrast Gram-negative bacteria are rarely obtained from the air.

The microflora of desert soils may also be determined by the resistance to desiccation of microorganisms. Feher (90) found a number of fungi, algae and bacteria, including actinomycetes, in desert soils of the Sahara. The species of actinomyces used in my work showed a considerable degree of resistance to desiccation. Ørskov (45) also preserved actinomycetes on dry filter paper for 8 months. Barthel (34) preserved "lactic acid bacteria of the Str. lactis group" in dry soil for 5 years. Other literature on/

on the survival of microorganisms in soil is cited by Giltner and Langworthy (33). It includes references to experiments in which _Azotobacter_ was found to remain alive in dry soil for a considerable time. This perhaps is surprising in view of the susceptibility of this bacterium to drying shown in my work. However, soil possesses certain protective properties which have been attributed (Giltner & Langworthy (33) and references cited in their paper) to its content of colloidal matter and its ability to retain hygroscopic moisture.

(e) _The microflora of dairy utensils_. Microorganisms derived from improperly treated utensils are a common source of contamination in the dairy industry. To date, much stress has been laid on the sterilisation of dairy utensils by steam or chemical disinfectants, but their subsequent storage has received less attention. Drying is known to prevent the proliferation of bacteria which survive sterilisation or are introduced afterwards, but little information is available concerning its bactericidal powers.

Thomas, Jones, Beaven and Thomas (91) found that dry milk churns had a lower count of thermoduric organisms than wet ones, but no attempt was made to show whether the difference was due to bacterial proliferation or to the effects/

effects of desiccation, Hughes and Ellison (92) investigated the microflora of clean, dry churns one hour after washing and found it to be composed mainly of micrococci, corynebacteria and other Gram-positive non-spore-forming rods. Gram-negative non-sporing rods were found only occasionally and none of these produced gas in MacConkey's broth at 37°C. Streptococci were uncommon. This is somewhat surprising in view of their ability to resist desiccation.

The results of my work show that some of the more common contaminants of dairy utensils, namely coliform organisms and pseudomonads, are unable to survive for long in the dry state. On the other hand *Corynebacterium lacticum*, the streptococci and micrococci might be expected to suffer little reduction in numbers on dry surfaces. This may well account in part, for the frequency with which these bacteria occur in milk. It is noteworthy that the organisms that withstand pasteurisation are relatively resistant to desiccation while the non-thermoduric types are relatively susceptible.

Where milk soon after production gives a bacterial count of several thousands per ml., the sterility of the utensils is at once suspected. If in such circumstances Gram-negative rods/

rods are numerous it is probably a fair assumption that some item or part of the equipment has been left in a moist condition. Unsterile utensils which have been dried would more probably yield a Gram-positive population.

The known facts concerning the bacteriostatic or bactericidal effects of desiccation suggest that in relation to the bacteriological condition of dairy equipment, drying may at times have a more potent influence than sterilisation. This at once raises the practical problem of how to dry certain items such as rubber tubes and metal pipes or of finding a suitable alternative method of storing them.

SUMMARY.

1. A method was devised for drying micro-organisms at reduced pressure in the presence of oxygen. The process was designed to resemble as nearly as possible, drying under natural conditions.
2. When spores were dried in broth, on a glass surface, only about 60% could be recovered on resuspending in saline. This did not seem to be due to any lethal action of the process. The percentage recovery with vegetative cells of different species was not determined, but it may be subject to considerable variation.
3. The recovery of dried cells was found to vary inversely with the concentration/

concentration of the suspension. It therefore was necessary to use suspensions of an approximately constant density in all comparitive work.

4. In order to avoid any confusion due to poor recovery, the death rates of organisms were examined throughout a period of storage at 53% R.H.

5. A slight variation in resistance to desiccation is shown by organisms at different stages of growth, cells at the beginning of the logarithmic phase being more susceptible than those towards the end of the logarithmic phase.

6. The organisms selected for examination were:

Gram-negative species.

Bacterium coli

Bacterium aerogenes

Intermediate coliform organism.

Pseudomonas fluorescens

Pseudomonas sp.

Alcaligenes viscosus (2 strains)

Azotobacter.

Rhizobium (2 strains)

Gram-positive species.

Micrococcus albus.

Staphylococcus albus.

Staphylococcus aureus.

Micrococcus lutéus.

Micrococcus varians (2 strains)

(According to Abd-El-Malek and Gibson (69)).

Pink Yeast.

Torulopsis kefyr

Oidium lactis

Streptoccus lactis

Streptococcus liquefaciens.

Streptococcus bovis

Streptococcus thermophilus

Streptococcus kefir

Corynebacterium sp.

Corynebacterium lacticum.
(2 strains)

Streptomyces sp.

Nocardia sp.

The Gram-positive species have been found to be more resistant to desiccation than the Gram-negative organisms.

7. Large cells were found to be more susceptible to desiccation than smaller ones.

8. The rate of death of resistant species is approximately logarithmic but the death of species sensitive to desiccation appears to be more complex. These sensitive species show a period of rapid decline in numbers at the commencement of drying, which is followed later by a period of death at a much reduced rate.

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III. A QUANTITATIVE STUDY OF THE GROWTH OF PURE CULTURES OF BACTERIA IN MILK.

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III. A QUANTITATIVE STUDY OF THE GROWTH OF PURE CULTURES OF BACTERIA IN MILK.

A large amount of quantitative work has been carried out on the normal microflora of market milk but there appears to be little precise information available on the rates of growth in milk, of specific microorganisms. During distribution milk is subjected to widely varying conditions of temperature and aeration. The effect of temperature on the entire microflora of milk has been investigated in some detail, but little attention has been given to the effects of aeration. During production and transportation milk is subjected to considerable agitation and aeration but at the distribution centres it is frequently stored in large quiescent volumes where gaseous exchange is limited to the surface layers. It might be expected that under such widely differing conditions of aeration the original microflora of the milk would become modified considerably and than in particular, the growth of strict aerobes would be restricted. In order to investigate this problem the growth of pure cultures of some of the commoner milk bacteria was studied in raw and in sterilised milk, incubated under aerobic and anaerobic conditions.

In a mixed microflora such as that of market milk the/

the different species might be expected to have a considerable influence on each other. It was intended originally to investigate the interaction of the bacteria, but for reasons stated later, this section of the work was abandoned.

LITERATURE.

Much of the work on the growth of microorganisms and on the various phases of the bacterial growth cycle has been reviewed by Winslow and Walker (1) and more recently by Monod (2) and Hinshelwood (3). Detailed quantitative data on the growth of pure cultures of bacteria in milk can be found in papers by Luxwolda (4), Foter and Rahn (5), Dorn and Rahn (6) and Rahn (7). These workers were concerned mainly with the effect of temperature on the rates of growth and fermentation and each of them investigated only a limited number of species.

Wilson (8) compared the growth of Bacterium coli and Bacterium aerogenes at 37°C. in sterile raw milk with that in broth. In the milk cultures the lag phase was prolonged for varying periods up to 6 hours and the growth curves flattened out prematurely when counts of approximately 100,000 viable organisms per ml. were attained. In broth the logarithmic phase of growth was extended considerably until counts of at least 100-200 million viable organisms per ml. were obtained. Microscopical examination of the milk cultures showed/

showed that the cells tended to clump together after passing the 100,000 ml. level, but no attempt appears to have been made to compare the number of individual cells present with the viable count. It, therefore, is impossible to determine whether the short logarithmic phase in milk was due to the aggregation of cells or to a reduction in the total amount of growth.

It is well known that a long lag phase may occur when microorganisms are grown in raw milk. This is attributed to some bacteriostatic or bactericidal property of the milk which is destroyed only by heating to temperatures of 70-80°C. (9,10,11,12.). The effect of the factor responsible for the inhibition varies with the season of the year (10) and in milk drawn from different cows (9, 11, 13, 14) or from different quarters of the udder (12). It also differs in its effect on specific bacteria (9,.10). The nature of the milk constituent which is responsible for delayed multiplication has not been investigated fully. Jones and Simms (15. 16) isolated "lactenin" in a practically pure form. This substance was composed mainly of protein and appeared to cause the bacteriostatic effect of the milk. Curran (17) obtained evidence that the inhibitory material probably was derived from the blood. Hobbs (18) investigated the growth of a number of bacteria in raw and heat treated/

treated milk and in whey. She obtained evidence that the prolonged lag period in raw milk was due in part, to a growth inhibitory substance, but also to the agglutination of cells, which was detected by examination of Breed smears at half hourly intervals. Both properties were destroyed by heating to 70-75°C for 1 hour. The factor responsible for agglutination was found to be present in whole milk and in whey, but the growth inhibitory substance was found only in whole milk.

Morris and Edwards (19) investigated the changes in the morphology of Streptococcus lactis which occur when this bacterium is grown in raw and in heated milk. In the heat treated milk the bacterium produced normal short-chains and diplococci, but in the raw milk it tended to form longer chains which appeared to result from the proliferation of single cells and not from any agglutination process. The chain length appeared to be maximal after 3-4 hours incubation at 37°C. After 5-6 hours incubation a decrease in chain length became apparent which continued until finally after 24 hours there was again a predominance of short chains and diplococci.

Hobbs, and Morris and Edwards appear to have based their observations on the examination of Breed smears, during the preparation of which the degree of clumping might have been modified/

modified. It is an interesting possiblity however, that actual changes in chain length as well as the aggregation of single cells may be responsible in part, for the apparent differences in the growth of bacteria in raw and in heat treated milk.

<u>The effect of aeration on bacterial growth in milk.</u>

In 1902 Marshall (20) carried out a gas analysis of milk and attempted to link bacterial growth with the quantities of oxygen, carbon dioxide, hydrogen & nitrogen present in this medium. He compared the multiplication of bacteria in raw milk incubated in completely filled test-tubes stoppered to exclude air, with that obtained in shallow layers of milk in flasks and found that in the latter case a considerably higher count was reached. However, he failed to take account of the variation in oxygen requirements of different species.

Wilson (8) noted that the amount of bacterial growth in sample bottles completely filled with milk was less than in partly filled bottles. He suggests that this may have been due to either the degree of aeration or the relatively greater amount of shaking which was possible/

possible in the partly filled bottles.

Winslow, Walker and Sutermeister (81) studied the growth of Escherichia coli in broth and in peptone water. They found that aeration of bacterial cultures caused a period of lag, which was followed by rapid growth at a later stage, giving a higher final population than that obtained in quiet cultures. This finding was confirmed later by Rahn and Richardson (22) working with pure cultures of Pseudomonas fluorescens in sterile milk. It did not however, apply to cultures of Streptococcus lactis. In an earlier paper (23) Rahn and Richardson give the results of an experiment in which the multiplication of bacteria in methylene blue milk under a seal of oil is compared with that obtained in milk exposed to the air. Samples were examined after the reduction of the dye had occured and it was found that Streptococcus lactis multiplied as rapidly in confined milk as in freely aerated milk. The rate of growth Escherichia coli however, was considerably reduced under anaerobic conditions and Pseudomonas fluorescens and Bacillus mesentericus failed to multiply at all. In later work Bacillus mesentericus/

mesentericus, *B. subtilis* and *B. cereus* were grown in 1% Bacto-peptone under seals of oil or vaspar. *B. mesentericus* and *B. subtilis* died off rapidly, but *B. cereus* was capable of multiplication for one or two generations. In the same medium *Pseudomonas fluorescens* was found capable of very slow multiplication under a vaspar seal. It therefore, was suggested that aerobic species may vary in their tolerance to anaerobic conditions. In view of these results and in particular, of the growth of a strict aerobe such as *P. fluorescens* under a vaspar seal, it seems likely that some air was capable of diffusing through the seals of oil or vaspar employed to protect the cultures from oxygen. The results therefore, probably do not indicate the growth of bacteria under completely anaerobic conditions.

METHODS

Samples of Milk

The organisms under consideration were grown in skim milk sterilised by intermittent steaming and in raw whole milk. The raw milk was drawn aseptically from a cow in a herd free from streptococcal mastitis. Milk from the same cow and when possible from the same quarter of the udder, was used in order to obtain samples of a uniform/

uniform nature, particularly in respect of their bacteriostatic properties. Each sample was examined for its bacterial content before it was used, The counts per ml. of most samples were 200-300 Corynebacterium lipolyticum and 3-4 udder micrococci. In a few cases samples contained up to 100 micrococci per ml. which multiplied during incubation and made it impossible to obtain accurate counts of the bacteria under investigation. C. lipolyticum does not multiply appreciably at the temperature used for incubation (22°C) and therefore was unlikely to have contributed to the counts of the organisms inoculated into the milk. The leucocyte count immediately after milking was approximately 200,000 per ml. The samples were aged at 4-5°C. for approximately 24 hours before use so that their bacteriostatic or bactericidal action might be reduced to a level more typical of average market milk.

Incubation of cultures.

Litmus milk cultures actively growing at 22°C were used as inocula. These cultures were diluted in sterile water and finally in sterile milk in order to obtain a convenient cell concentration. The required volumes of sterile and raw milk were inoculated in bulk with the final/

final dilution of the culture, at the rate of 1 ml. of inoculum in 5 ml. of milk. The freshly inoculated milk then was measured out aseptically in 5 ml. quantities into 100 ml. conical flasks and into 6 x $\frac{5}{8}$ in. test-tubes. The flasks provided a shallow layer of milk with an ample supply of oxygen. The tubes were incubated in anaerobic jars.

Method of incubation.

A modification of the McIntosh and Fildes' anaerobic jar was used. These jars were evacuated by means of a water pump and then were filled with gaseous hydrogen. In addition to the cultures under observation, each jar contained:

(a) A tube containing an oxygen indicator. In the earlier experiments a three-solution indicator (24) was used, but this was later replaced by the single-solution indicator recommended by Ulrich and Larsen (25). A slight blue colour returns to the surface of the reduced indicator during the evacuation of the jars. Owing to the low temperature in use, further reduction of the methylene blue during incubation was slow and with the three-solution indicator it often did not occur until after 66 hours. The single/

single-solution indicator was completely reduced at 24 hours.

(b) Freshly chopped potato tissue to assist in the removal of oxygen in the early stages of incubation.

(c) Marble chips + HCl, giving a concentration of approximately 5% CO_2. This was included as a precautionary measure to ensure that there was a plentiful supply of CO_2 for bacterial growth. The marble chips were dislodged into the acid at the base of the jar after evacuation at the pump.

In the later experiments (b) and (c) were replaced by a pyrogallol - Na_2CO_3 mixture. This helped to absorb oxygen in the early stages of incubation and also provided CO_2.

4. <u>Temperature of incubation</u>.

A temperature of 22°C. was selected as it promotes the growth of all the common milk bacteria in a reasonably short period of time. The jars and flasks were incubated in a water bath. Preliminary trials indicated that the rate of growth of the organisms is influenced easily by slight changes in temperature and an anhydric incubator was found to be subject to too great a fluctuation.

5. <u>Determination of the viable count</u>.

The/

The cultures were examined at intervals to determine the rate of growth. The viable count was estimated in the earlier experiments by the usual plating technique, but this was replaced later by the tube method of counting (see Section I).

Bacteria commonly occuring in milk were selected for examination.

6. Determination of the degree of acidity developed by three streptococci in milk

The rate of acid formation in the cultures of Streptococcus bovis, Str. thermophilus and Str. Kefir was determined at intervals throughout the incubation periods by titration with standard NaOH, using phenolphthalein as the indicator. The percentage of lactic acid is shown in figs. 10,11 & 12. Cultures incubated in the anaerobic jars were selected for titration since their growth appeared to be slightly in advance of that in the freely aerated cultures. In each case the values plotted are those obtained after subtraction of the titration figures given by an uninoculated control.

PRESENTATION OF THE RESULTS.

Preliminary trials were carried out in order to determine the approximate rate of growth of each organism/

organism. These trials were followed by more detailed experiments carried out over a longer period of time, the results of which are given in figs. 1-12. It seemed unnecessary to present in tabular form the large amount of data amassed in the course of the work, since all the essential points are illustrated in the graphs. The viable counts were plotted logarithmically. The results obtained in the later stages of the experiments with Corynebacterium lacticum, with Micrococcus varians (strain 1) and the final count of Streptococcus thermophilus in raw milk were obscured by the multiplication of udder micrococci.

DISCUSSION.

1. The phases of the bacterial growth cycle in milk

(a) Lag phase. In all cases the intial period of lag was longer in raw than in sterile milk, indicating that the bacteriostatic property of freshly drawn milk is not overcome by ageing at 5°C. for 24 hours. Hanssen (10) also found that raw milk was capable of delaying multiplication after holding in an ice box for 24 hours. Microscopicalexamination of my cultures showed that both the chain length and the degree of clumping was approximately the same in raw and sterile milk.

The growth of all bacteria was not delayed to the same extent, for instance, the coliform organisms (figs. 1 & 2) Alcaligenes viscosus (fig 3), Pseudomonas fluerescens (fig. 4)/

Fig. 1. Bacterium coli

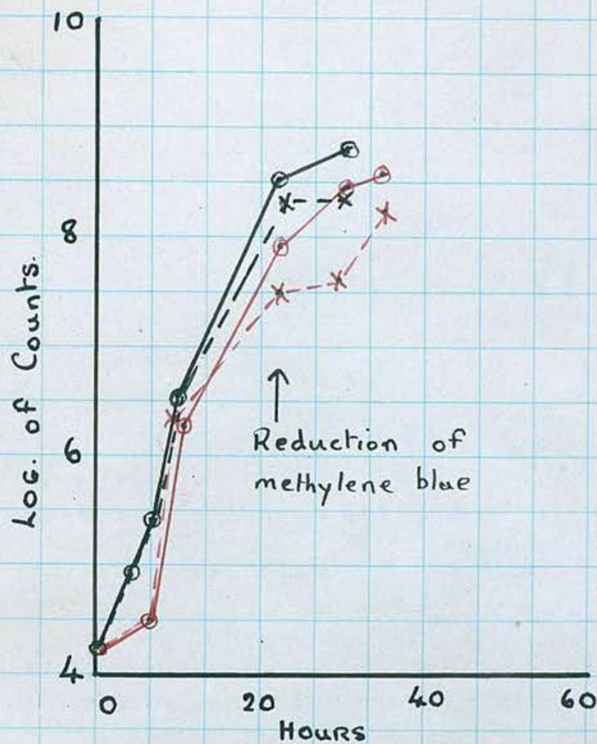
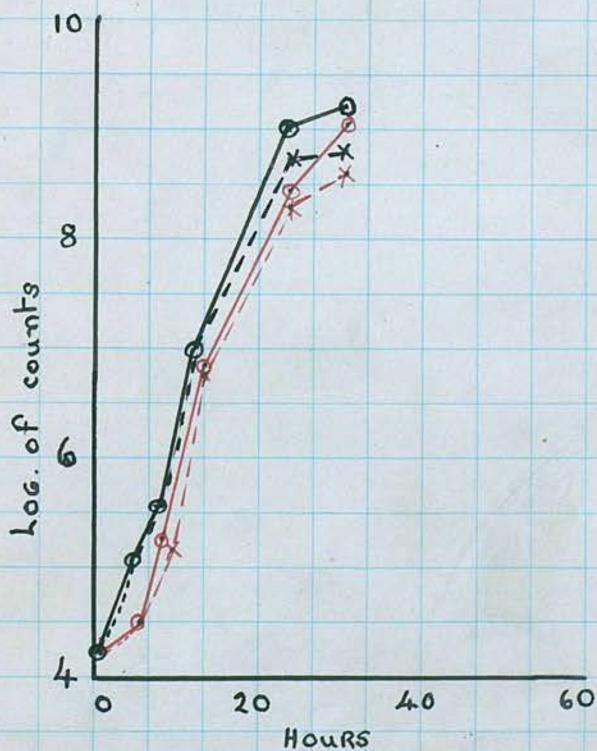


Fig. 2. Bacterium aerogenes.



Sterile milk
aerobic. ———
anaerobic. - - -
Raw milk
aerobic ———
anaerobic - - -

Fig. 3. Alcaligenes viscosus.

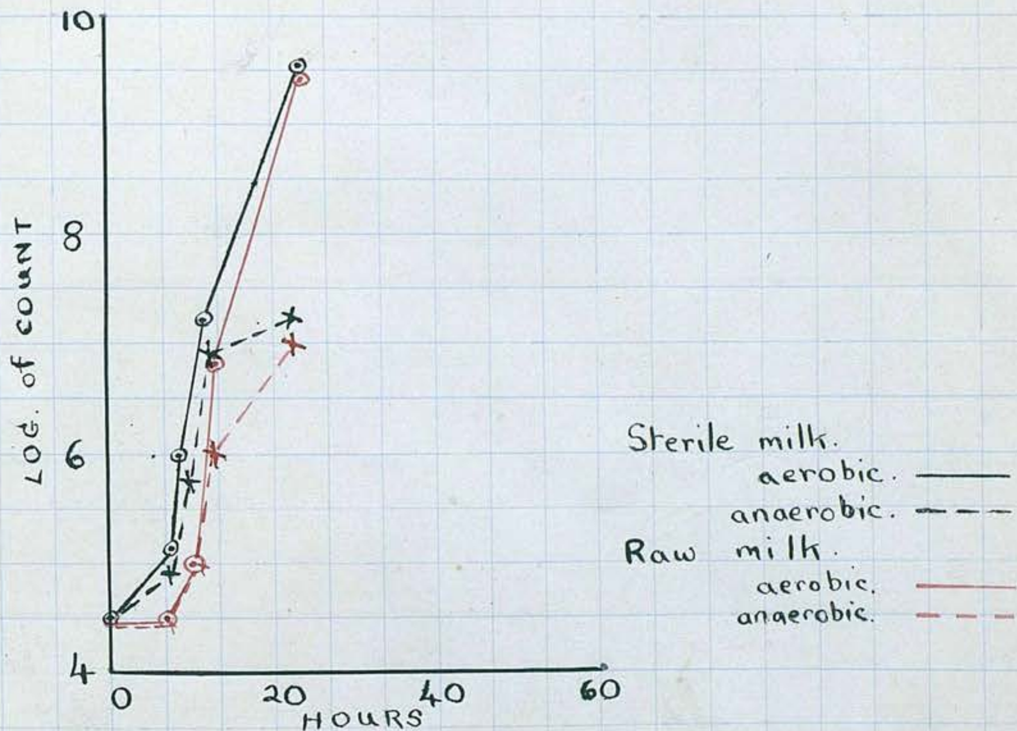


Fig. 4. Pseudomonas fluorescens.

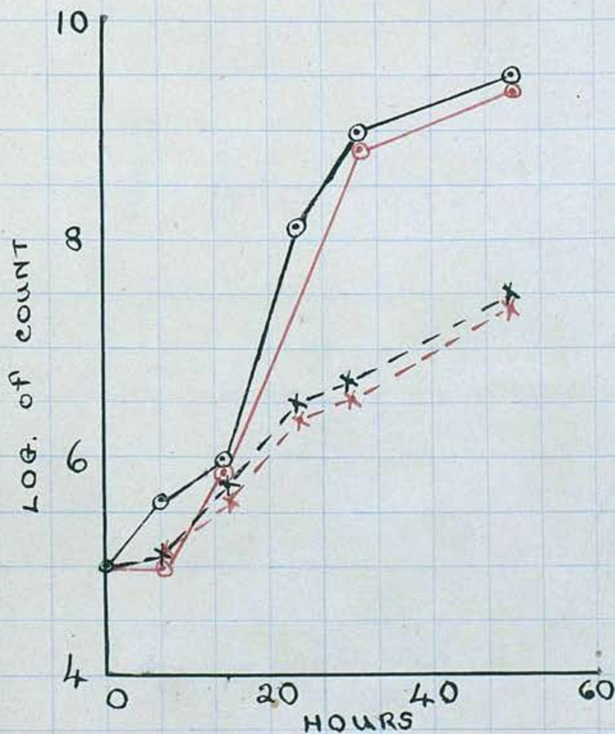
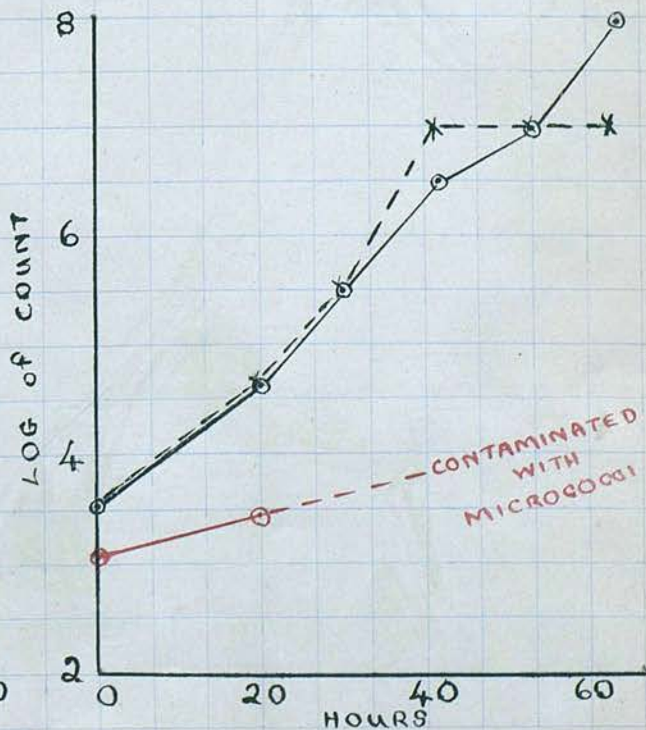


Fig. 5. Corynebacterium lacticum.



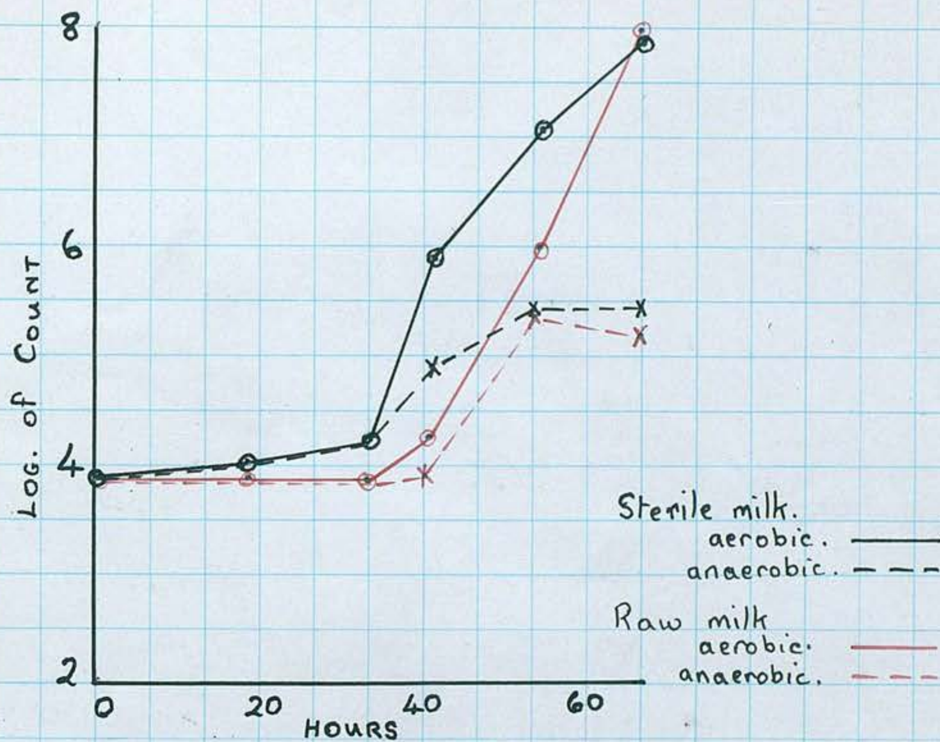


Fig. 6. Micrococcus luteus.

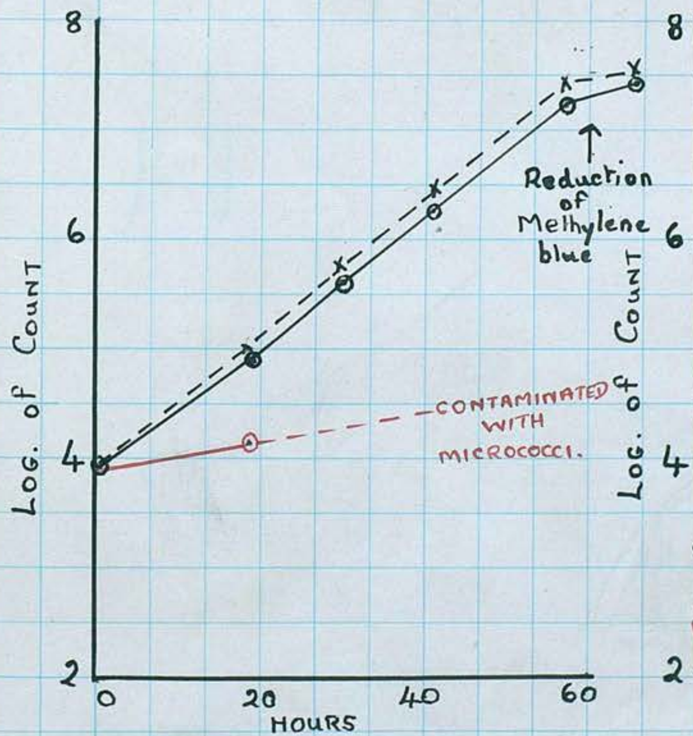


Fig. 7. Micrococcus varians (1)

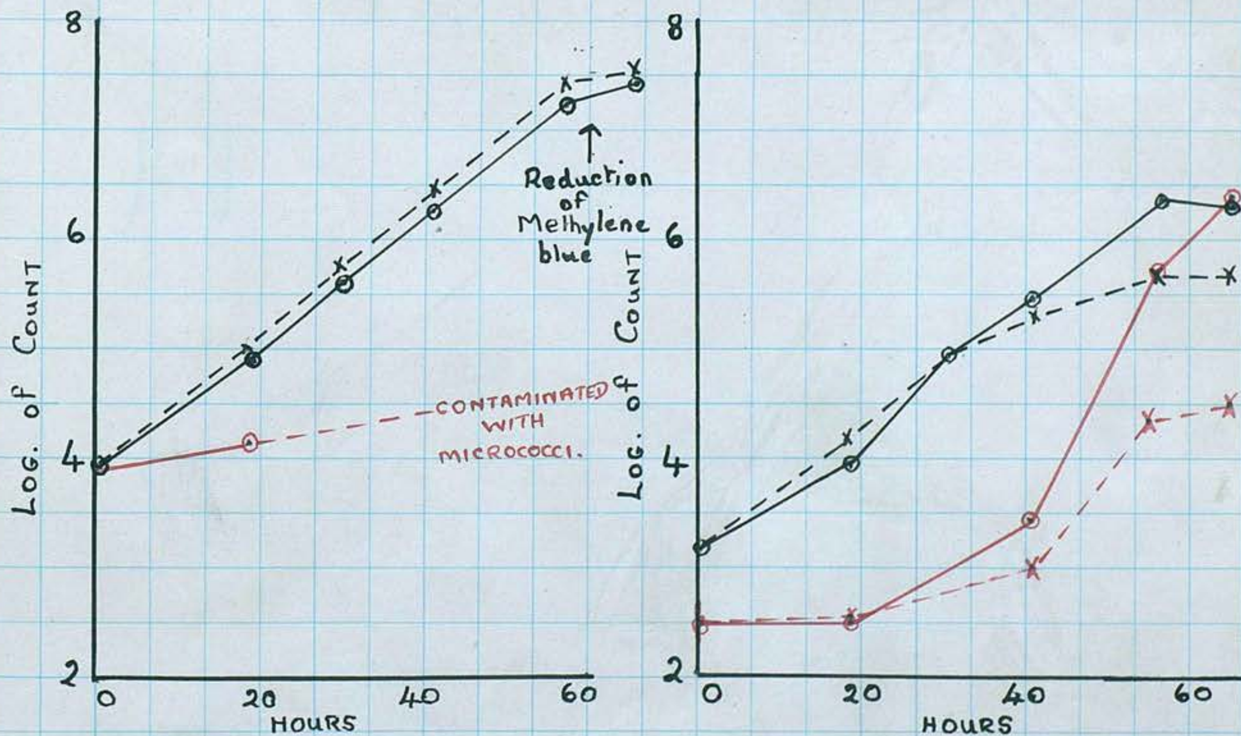


Fig. 8. Micrococcus varians (2)

Fig. 9. Streptococcus lactis.

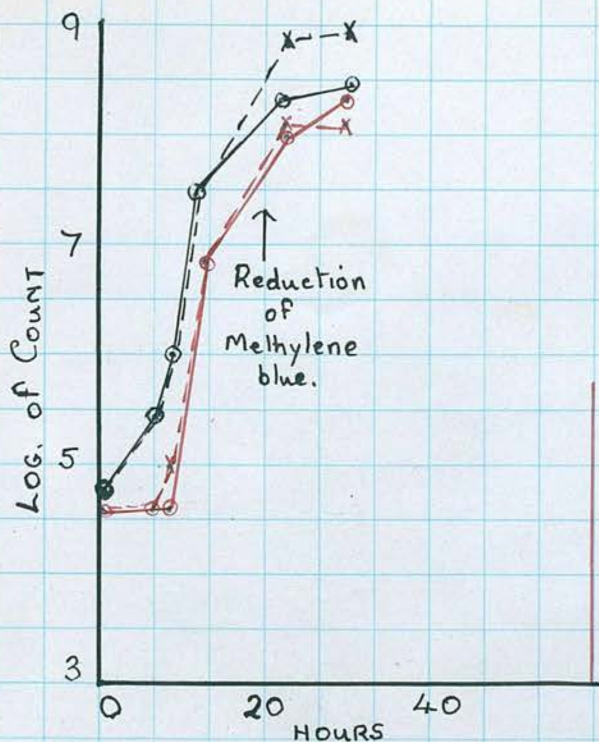


Fig. 10. Streptococcus kefir

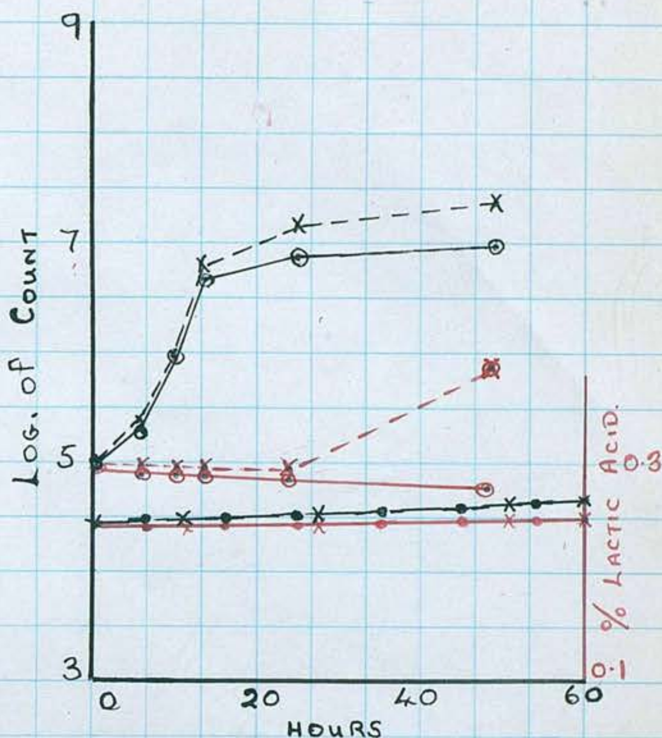


Fig. 11. Streptococcus bovis.

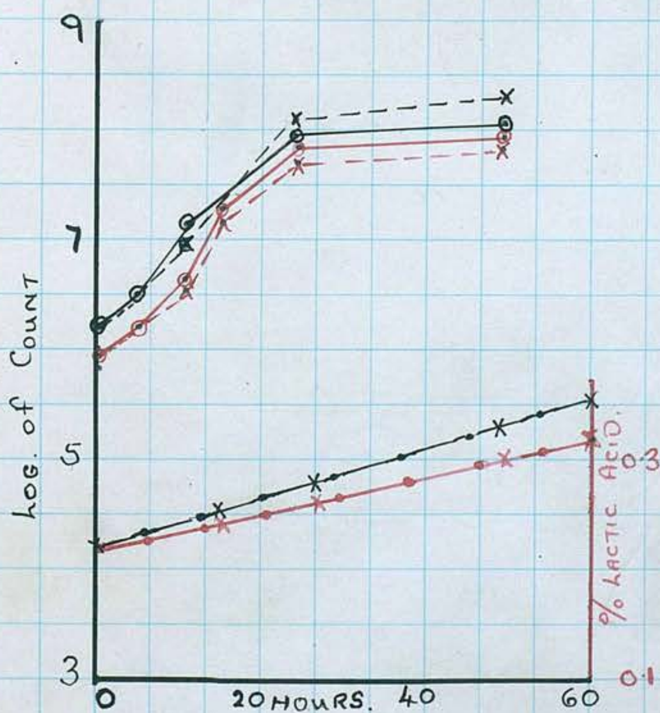
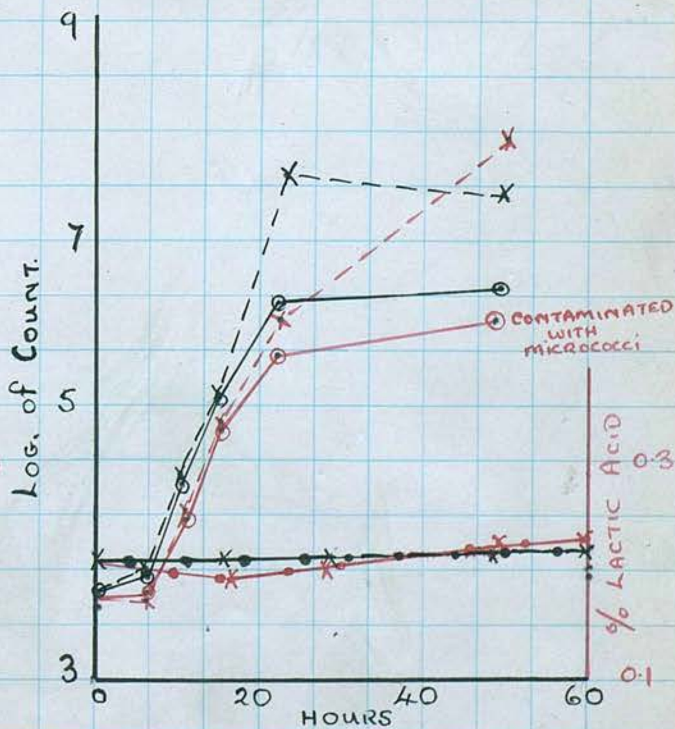


Fig. 12. Streptococcus thermophilus.



Sterile milk
aerobic. ——————
anaerobic. — — —

Raw milk.
aerobic. —————— (red)
anaerobic. — — — (red)

% Lactic Acid.
Sterile milk •—•
Raw milk •—• (red)

(4) Streptococcus lactis (fig 9), Str. bovis (fig 11) and Str. thermophilus (fig 12) showed only a short lag period in raw milk. In contrast, Str. kefir (fig 10) showed no appreciable increase in numbers in raw milk in the 50 hours following inoculation, while Micrococcus varians (2) (fig 8) was intermediate in its reaction. These differences may have been due either to some specific property of the organisms or to variations in the bacteriostatic properties of the raw milk samples. Other workers have noted a variation in the length of the initial lag period in milk, with different bacteria. For instance Hanssen(10) found that Bacillus typhosus was more sensitive than ordinary milk bacteria to the bactericidal effect of raw milk and Chambers (9) demonstrated that Bacterium lactis-acidi was less easily inhibited than B. coli but more sensitive than the organisms originating in the udder of the cow, from which the milk was drawn. All the species investigated in my work occur naturally in milk, and might not be expected to be specially sensitive to any inhibition. However, they had been maintained for a number of years on sterile media (agar or litmus milk) and their reaction to the properties of raw milk might have changed during that period.

The/

The variation in the length of the lag period might ~~have~~ also have been due to variations in the bacteriostatic property of the milk. This property has been shown by Hanssen (10) to be subject to a seasoned fluctuation and Wilson (8) noted an apparent variation from day to day when attempting to determine the rates of growth of B. coli and B. aerogenes in raw milk. The experiments in my work were carried out on different days, often separated by a considerable period of time and it therefore is possible that the variations in the length of the lag period were due mainly to changes in the bacteriostatic effect of the milk.

A period of lag was not completely absent when the bacteria were grown in sterile milk. This is noticeable particularly with Micrococcus luteus (fig. 6)and probably is comparable with the lag phase which occurs in most bacteriological media. Recent investigations (2.3.) suggest that the lag phase is a period during which intermediate metabolic products necessary for growth are accumulated. Its length appears to be determined by a number of factors, but it is difficult to ascertain accurately the stage of development/

development of these cultures. Hinshelwood (3) discusses the relationship between the length of the lag phase and the size of the inoculum, a factor which varied considerably in my work as can be seen in table 1. This influence is demonstrated most clearly by the results obtained with the streptococci. The largest inoculum was that of Str. bovis which contained 1-2 million cells per ml. and in this case the growth curve (fig 11) shows very little evidence of lag. The rate of inoculation of the other streptococci was considerably less and their growth curves (figs 9, 10, 12) do not show any appreciable lag period.

(b) Logarithmic phase. The minimum generation time for each organism durin g the period of most rapid multiplication is given in table 2 and those calculated from growth under aerobic conditions are plotted in fig. 13, to give hypothetical growth curves with 10^2 as their origin. The generation times were calculated by means of the formula:

$$G = \frac{T . \log 2}{\log b - \log a}$$

where G = generation time.
T - time interval
a = initial number of cells.
b = final number of cells.

This formula is based on the somewhat controversial assumption that all the bacteria survive in each generation. It/

Table 1 The initial and highest viable counts of pure cultures of bacteria in milk at 22°C.

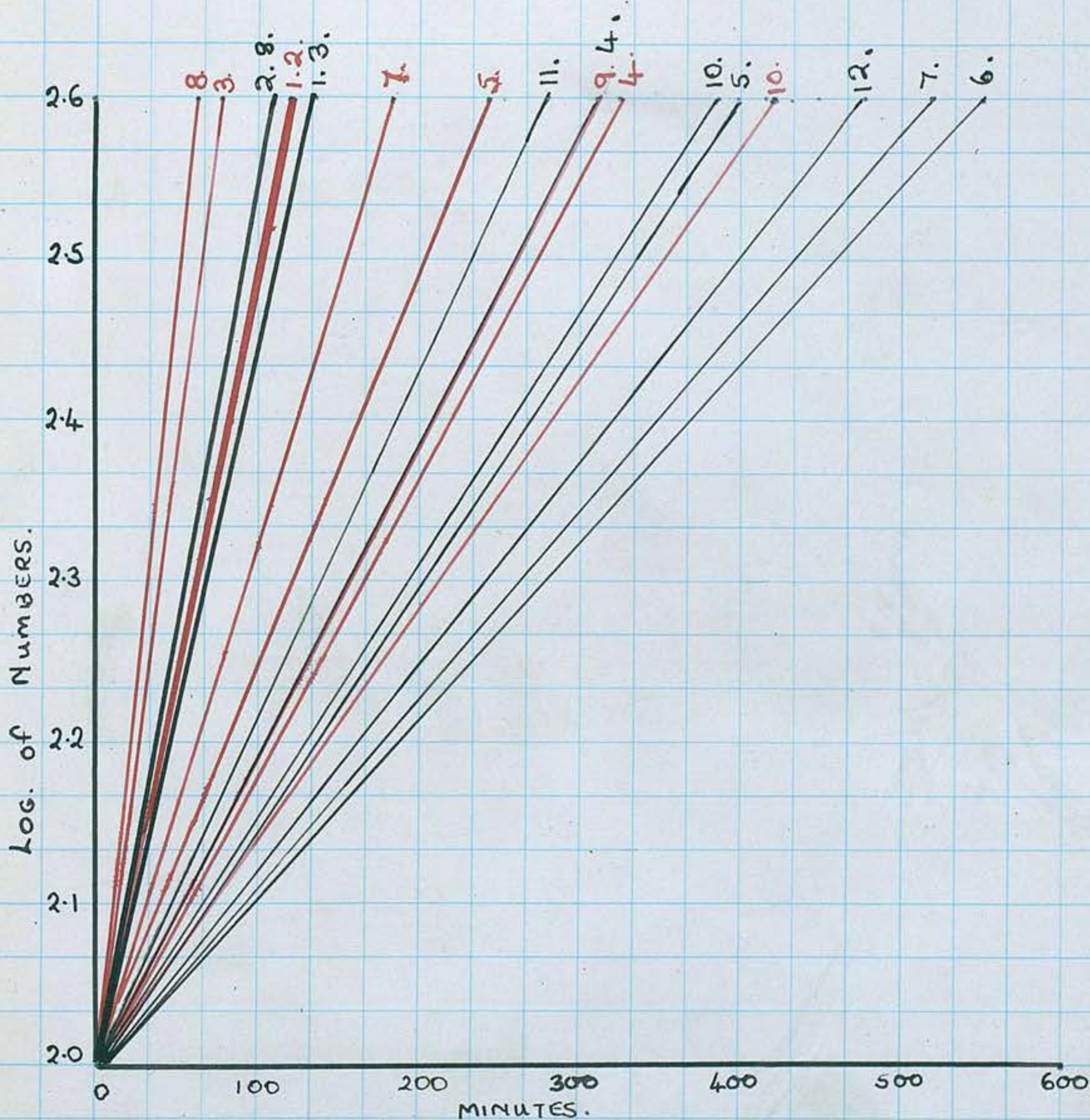
Species	Initial count per ml. (in thousands)		Final count per ml. (in thousands)			
	Sterile Milk	Raw Milk	Sterile Milk		Raw Milk	
			Aerobic	Anaerobic	Aerobic	Anaerobic
Bacterium coli	14.1	20.8	2,050,000	730,000	1,500,000	680,000
Bacterium aerogenes	25.1	24.9	1,100,000	343,000	1,000,000	269,000
Alcaligenes viscosus	39	27.3	+ 2,700,000	24,300	+ 1,850,000	3,370
Pseudomonas fluorescens	93.6	97	4,000,000	39,300	3,730,000	33,000
Micrococcus luteus	7.8	7	+ 64,000	430	+ 99,000	410
Micrococcus ~~varionoli~~ varians (1)	5.3	5.1	34,100	43,400	contaminated with micrococci	
Micrococcus varians (2)	2.2	0.4	2,190	530	+ 2.130	28
Streptococcus lactis	47.5 ~~4.75~~	29.8	227,000	947,000	203,000	134,000
Streptococcus bovis	2,810	1,503	135,000	217,000	103,000	70,000
Streptococcus thermophilus	4.3	4.5	4,010	27,500	contaminated with micrococci	+ 85,000
Streptococcus kefir	80.3	75.3	8,200	20,800	+ 48	+ 333
Corynebacterium lacticum	3.7	0.6	+ 61,800	12,600	contaminated with micrococci	

+ Cultures apparently had not reached their maximum population.

Table 2. The approximate minimum generation times of Bacteria grown in milk at 22°C.

Species	Generation time (min.)			
	Cultures in sterile milk		Cultures in raw milk	
	Aerobic	Anaerobic	Aerobic	Anaerobic
Bacterium coli	61.5	62.9	60.4	51.0
Bacterium aerogenes	58.5	57.7	59.5	58.1
~~Bacterium viscosum~~ Alcaligenes viscosus.	61.2	56.7	38.2	62.0
Pseudomonas fluorescens	143.2	224.7	152.2	263.1
Micrococcus luteus	169.0	456.9	116.9	108.4
Micrococcus varians (1)	258.4	243.6	contaminated with micrococci	
Micrococcus varians (2)	241.0	418.3	90.2	154.6
Streptococcus lactis	57.5	54.6	32.7	40.1
Streptococcus bovis	too crowded to give accurate count		143.6	172.5
Streptococcus thermophilus	154.8	96.1	176.0	151.2
Streptococcus kefir	124.6	110.9	cultures not yet in logarithmic phase	
Corynebacterim lacticum	218.8	210.5	contaminated with micrococci	

Fig. 13. Hypothetical growth curves obtained by plotting generation times (aerobic)



—— Sterile milk.
—— Raw milk.

1. Bacterium coli.
2. Bacterium aerogenes.
3. Alcaligenes viscosus.
4. Pseudomonas fluorescens.
5. Micrococcus luteus.
6. Micrococcus varians (1)
7. Micrococcus varians (2)
8. Streptococcus lactis.
9. Streptococcus bovis.
10. Streptococcus thermophilus.
11. Streptococcus kefir.
12. Corynebacterium lacticum.

It was considered sufficiently accurate however, in this work since the generation times could only be on approximate estimate of the maximum rate of multiplication owing to the limited number of counts obtained in each experiment.

These results show that when the bacteriostatic property has been overcome the rate of growth in raw milk tends either to be equal to or in some cases, greater than that in sterile milk. This difference is not sufficiently large however, to indicate that either medium is more favourable to bacterial growth.

(c) Phase of deacceleration. On leaving the logarithmic phase of growth most of the cultures appear to enter a comparitively long period of slower multiplication instead of changing abruptly to a stationary phase.

The factors which bring about a decrease in the growth rate have been discussed in detail by Monod (2) and Hinshelwood (3). A great deal of the work carried out by Monod appears to have been concerned with the relationship between bacterial growth and the concentration of nutrients in the medium. In connection with this effect, Monod discusses the existence of complex growth cycles containing several consecutive logarithmic phases, each representing a distinct/

distinct rate of growth. The possible existence of such complex cycles in milk might account for the variability of the curves shown during the logarithmic phase, in my work, but insufficient counts were obtained to prove the existence of a number of successive rates of growth during this period. The type of curve obtained in my work might perhaps have resulted from the presence in milk, of a limited quantity of some growth factor, using this term in its widest sense. When this supply is exhausted the bacteria might still be capable of obtaining all their nutrient requirements, but at a reduced rate which would become reflected in a slower multiplication of the cells.

The highest count reached by each bacterium is given in table 1. In some cases the graphs indicate that the final population probably had not been reached and this may account to some extent for the variation between different organisms. It can be seen that the coliform organisms, Alcaligenes viscosus and Pseudomonas fluorescens are capable of reaching higher counts per ml. than the micrococci and streptococci. This does not appear to be linked with the optimal growth temperatures of the bacteria or with their/

their oxygen requirements. Rahn (7) showed that milk does not contain sufficient nitrogenous material to produce the maximal growth of streptococci, since he was able to increase their counts per ml. by the addition of peptone. The deficiency of some nutrient material might account for the low maximum populations of the streptococci and micrococci in my experiments.

Apart from the exhaustion of nutrient materials, the termination of growth in a bacterial population might be due to the accumulation of toxic metabolic products or to adverse changes in ion equilibrium, especially pH (2.3.) With the data available it is impossible to ascertain the relative importance of these effects in milk since each may operate singly, or in combination and each may become the limiting factor, under slightly different conditions. It is interesting to note that the size of the original inoculum has no significant effect on the maximum population (table 1).

The influence of aeration on the size of the final population is discussed later in relation to the oxygen requirements of the organisms.

2. Acid production.

Figs. 10, 11, and 12, indicate that the three streptococci examined did not form any large quantity of acid in the early stages of growth. The cultures of

Str. kefir.

Str. kefir and Str. thermophilus showed no appreciable increase in the percentage of lactic acid present at the end of a 60 hour incubation period, although the bacteria had reached the stationary phase of growth some hours previously. Acid production with Str. bovis began at a slightly earlier stage but the degree of acidity developed at the end of 60 hours incubation was again very small. In no case did clotting of the milk occur during the period of the experiment. Cultures containing sterile bromo-cresol purple remained practically unchanged during the period of incubation indicating that no appreciable change in pH. had occured.

Foter and Rahn (5), Dorn and Rahn (6) and Ritter (26) obtained a good agreement between the growth and fermentation rates of bacteria in milk at varying temperatures, except below 10°C (5). Rahn (7) however, appears to have obtained results indicating that acid formation does not commence at as early a stage as does growth in cultures of Str. lactis in sterile milk at 20°C. It seems unlikely that the lag in acid production obtained in these results and in my work was due entirely to the incubation of the cultures at a suboptimal temperature, since the streptococci examined multiplied/

multiplied readily in milk at 20 and 22°C and reached the stationary phase of growth. In Rahn's experiments the percentage of lactic acid continued to rise for some time after the viable count had become static.

3. Oxygen requirements.

In the work undertaken, the degree of aeration appeared to influence the size of the maximum bacterial population but the rate of growth was affected to only a very slight degree. Walker, Winslow, Huntington and Mooney (27) found that the aeration of a peptone medium prolonged the logarithmic phase of growth and increased the final bacterial population. Similar effects were noted by Winslow et al (21) in broth and peptone water, and by Rahn and Richardson (22) in milk (see review of literature).

Among the facultative anaerobes, the coliform organisms (figs 1. 2.) *Micrococcus luteus* (fig 6), *Mic. varians* (2) (fig 8) and *Corynebacterium lactisum* (Fig 5) reached slightly higher counts under aerobic conditions. The reverse was true of the streptococci (figs 9-12) which attained higher maximal populations in the cultures incubated in an atmosphere free from oxygen. *C. lactisum* and *Mic. luteus* both reached higher counts when incubated in the presence of oxygen. The anaerobic culture of *C. lactisum*/

lactic<u>um</u> (fig 5) reached the stationary phase at the end of the experiment, but the aerobic culture showed every indication of still being in the logarithmic phase of growth. The difference between the aerobic cultures of M. luteus is even more clearly defined.

Jones-Evans and Thomas (28) compared the growth of a species of Microbacterium at 18°C in sterile and in raw milk, but obtained no appreciable increase in count in raw milk after incubation for 72 hours. This seems likely to have been caused by the bacteriostatic property of the raw milk.

It perhaps is surprising to note the behaviour of the two obligate aerobes: Alcaligenes viscosus (fig 3) and Pseudomonas fluorescens (fig 4). Both these bacteria multiplied rapidly in milk incubated in an oxygen-free atmosphere and the final count reached several millions per ml. before the rate of growth decreased. The most likely explanation of this phenomenon is that oxygen was available to the organisms. In view of the fact that in establishing anaerobic conditions the jars were evacuated at the pump, the oxygen must have been held firmly by the milk. Support for this suggestion has been provided by the work of Noll/

Noll and Supplee (29), who had great difficulty in removing all traces of oxygen from milk. They found that this could only be accomplished by severe means such as flushing of the milk with nitrogen when under a vacuum of 25 inches; heating to 185°F. cooling to 100°F in an atmosphere of nitrogen and subjecting to a vacuum of 24-29 inches; or treatment with ascorbic acid.

In order to investigate this question, cultures containing sufficient 0·5% sterile aqueous methylene blue to give a faint colour, were incubated in the anaerobic jars. Such cultures were not used for estimating the viable count in case the dye exerted an inhibitory influence on bacterial growth, but they were expected to give an indication of the oxygen content of the plain milk cultures. While the mechanism of dye reduction in milk is a complex phenomenon it may be assumed that methylene blue would not remain for long in its oxidized form in a culture entirely free from oxygen. Reduction of the dye was found to be very slow as is shown in figs 1,7 & 9. In all other cases it did not occur until some time after the completion of examinations.

The results from this study of bacterial growth ~~obtained~~/showed

~~obtained~~ show that even the obligate aerobes are capable of reaching a count of at least several million per ml. in milk stored in an oxygen-free atmosphere. It, therefore, seems unlikely that any variation in aeration of market milk during distribution will have an important influence on the growth of its bacterial flora and consequently on its keeping quality.

The results obtained indicate that a number of factors were not under control in the experimental method employed and that the problem was of an extremely complex nature. Moreover, variations of the magnitude obtained in these experiments in which the milk of one cow only was used, might be expected to be increased considerably in ordinary bulked milk. Investigations therefore were discontinued and the work originally planned on the mutual effects of bacteria in mixed culture was abandoned.

The application of the findings to dairying practice.

In the application of these findings in the practical field certain limitations must be recognised. All the work was carried out with pure cultures of bacteria and with milk from only one cow. The behaviour of bacteria in pure culture, particularly after they have been maintained on/

on sterile media for a considerable time, may not be an accurate indication of their behaviour in a mixed population under natural conditions, while milk from one cow might not provide conditions of growth comparable with those occurring in other samples or in bulked milk.

In the experiments described above marked differences in the rate of growth of specific bacteria were shown. The following bacteria are listed in order of decreasing growth rates. Those within each group showed little individual variation.

Streptococcus lactis.
Bacterium aerogenes.
Alcaligenes viscosus.
Bacterium coli.

Streptococcus kefir
Streptococcus bovis
Pseudomonas fluorescens
Streptococcus thermophilus
Micrococcus luteus.

Corynebacterium lacticum
Micrococcus varians (2)
Micrococcus varians. (1).

Still/

Still greater differences were detected however, in the size of the maximum population reached. Milk was shown to be capable of supporting relatively large numbers of coliform organisms and other Gram-negative species, but to impose a much lower limit on the maximum population of streptococci, micrococci and probably corynebacteria. This finding was unexpected since it is well known that in most bulked raw milk streptococci normally became the dominant bacteria and since Streptococcus lactis was shown in my work to grow rapidly during the logarithmic phase. The limitations of work with pure cultures are here obvious. A complex bacterial population may alter milk in a way that promotes the growth of organisms such as streptococci. In a mixed culture inhibitory effects are also likely to operate. The experiments do indicate, however, that in milk containing few types of bacteria or held at selectively low temperatures a relatively rapid and extensive growth of coliform organisms , Alcaligenes viscosus and possibly Pseudomonas fluorescens might be expected. Samples of milk for example, frequently appear to show this result during the winter season.

The degree of aeration appears to be of little importance/

importance in relation to microbial growth in milk. There appears to be sufficient oxygen possibly adsorbed to some of the milk constituents to support the growth of strict aerobes for a considerable period of time. An oxygen supply sufficient to support the growth of pure cultures of bacteria may not however, be adequate when the organisms are present in mixed culture and in particular when milk of a high bacterial count is encountered. In such low grade milks methylene blue may be reduced in an extremely short period of time and under these conditions it seems likely that oxygen would not be available. This would impose a restriction on the growth of strict aerobes.

SUMMARY

1. The growth rates of pure cultures of the common milk bacteria were determined in sterile skim milk and in raw whole milk at 22°C. The cultures were incubated aerobically in flasks and in an oxygen-free atmosphere obtained by means of a modification of the McIntosh and Fildes anaerobic jar.

2. The species selected for examination were:

Bacterium coli	*Corynebacterium lacticum*
Bacterium aerogenes	*Micrococcus luteus*
Alcaligenes viscosus	*Micrococcus varians* (2 strains

Pseudomonas fluorescens

Streptococcus lactis
Streptococcus bovis
Streptococcus thermophilus
Streptococcus kefir.

3. Raw milk aged for approximately 24 hours, exerts a bacteriostatic influence on bacterial growth during the early stages of incubation. When this has been overcome the rate of growth in raw milk is equal to or greater than that obtained in sterile milk. A short period of lag also occurs in sterile milk.

4. Bacteria grown in milk possibly enter a prolonged phase of de-acceleration before reaching the stationary phase of growth.

5. The rate of acid production in milk by certain streptococci does not necessarily indicate their rate of growth.

6. Incubation in an oxygen-free atmosphere has little effect on the growth of facultative anaerobes. It may influence the size of the final population, but does not appreciably alter the rate of multiplication.

7. Obligate aerobes are capable of reaching counts of several millions per ml. in milk incubated in an oxygen free atmosphere.

8. Under experimental conditions using pure cultures, the bacterial content/

content of milk does not appear to be affected appreciably by the degree of aeration. Aeration may however, have a more pungent effect in milk with a mixed microflora of high bacterial count.

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