BACTERIAL SURVIVAL IN SYSTEMS OF LOW MOISTURE CONTENT

PART III. BACTERIA IN FIXED OILS AND FATS

SECTION II. THE EFFECT OF STORAGE AND BACTERICIDES ON VIABILITY

BY KENNETH BULLOCK AND WINIFRED G. KEEPE

From the Department of Pharmacy of the University of Manchester

Received June 18, 1951

INTRODUCTION

FIXED oils may become infected with at least two types of material, moist or dry. When the oil-bearing fruits are originally pressed the oils may be contaminated with moist pulpy material or with a fine emulsion of, for example, in the case of olive oil, the olive fruit juice. Such contamination separates out as the " foots " on allowing the oils to stand and the remainder is mainly removed when the oils are clarified by filtration. The other principal source of infection by moist material, of interest in pharmacy, is the introduction of infected syringe needles into multiple dose containers. The fate of bacteria in such aqueous droplets has been studied recently by Sykes and Royce[1] and Coulthard, Chantrill and Croshaw[2] to which papers reference may be made. These are interesting papers dealing with a topic of great importance in pharmacy, but it is questionable if such a title as " The Survival of streptococci and tubercle bacilli in oils " is justified. What is being studied is the survival of the organisms in an aqueous nutritive medium suspended as droplets in oil. Substances such as phenols dissolved in the oil will presumably act, not in the oil, but after solution in the aqueous droplet. Considerable work has recently been done in this Department emphasising the importance of even small amounts of moisture in determining the death or survival of micro-organisms.

Oils on storage, or at any time during their treatment to form oily injections, may become contaminated with more or less dry bacteria from the air, the hands, clothing, unsterile apparatus, etc., as well as from unsterile, but dry, syringe needles. Concerning this aspect of the subject much less exact work has been done. Several difficulties at once become apparent. No reliable methods have been available up to the present for performing viable bacterial counts on oils. There is not even a sterility test for oils which has been proved to be reliable. This means that it is difficult to decide whether the organisms present are decreasing in number or not. The tests used by Coulthard, Chantrill and Croshaw[2] are to some extent quantitative, the magnitude involved being the unknown minimum which will cause infection in an animal after injection. Another difficulty is to obtain an evenly infected oil on which to attempt to carry out viable counts. Clarified oils of commerce are, by the tests at present in use, usually found to be sterile. The use of dried

soil or dust as infective material has many drawbacks. There is no guarantee that the organisms are distributed evenly in the powder, and unknown mineral and/or organic matter is introduced along with the bacteria. Further, nothing is known of the types of organisms introduced, or the medium most suitable for their culture. Dried cultures of bacteria have, in the past, proved difficult to handle. When powdered, with or without a diluent, the organisms tend to cohere in clumps so that viable counts are unreliable. The organisms are not evenly distributed either in the powder or subsequently in the oil. In the absence of a knowledge of how many organisms were originally added to the oil, and in the absence of reliable methods for counting bacteria in oils and of sterility tests of established sensitivity, it is not surprising that the results of experiments with oils have been of a qualitative rather than a quantitative nature.

There are a number of useful papers concerning the action of fixed oils and fats on bacteria living in an adjacent aqueous phase. Hammer and Long[3] investigating the growth of organisms in butter reported that growth takes place only in the aqueous phase, the fat being relatively resistant to infection. Williams and Feiger[4] found that oleic acid stimulated the growth of *Lactobacillus casei* while lauric, myristic and linoleic acids were inhibitory. Foster and Wynne[5] found that linoleic and oleic acids strongly inhibited the germination of spores of *C. botulinum*, while stearic acid had no effect. Oleic acid had no effect on the spores of *Bacillus brevis, B. megatherium* and *B. subtilis*. Pea-nut and cod-liver oils were found by Crimm and Martos[6] to inhibit the growth of *Mycobacterium tuberculosis*. Several papers are available relating to the ability of micro-organisms to attack the constituents of oils; summarising the position Tausson[7] stated that the following conditions are necessary:—(i) the presence of water and mineral salts, (ii) a source of nitrogen, (iii) free access to oxygen, (iv) a medium buffered at neutrality. MacMaster[8] has reported that agar slopes of *B. typhosus* can be sterilised by pouring over the surface of the culture either 0·4 per cent. of phenol or 0.3 per cent. of parachlorometacresol dissolved in liquid paraffin. The effectiveness of phenol in oil to sterilise an adjacent aqueous culture has been confirmed by Coulthard, Chantrill, and Croshaw[2]. Similar investigations have been carried out with irradiated oils. With the exception of a paper by Fairhall and Bates[9], who used oils previously dried over anhydrous sodium sulphate and " dried " spores (the method of obtaining these was not stated), all the above work refers to the effects of oils and oily solutions on organisms growing in a separate but contiguous aqueous phase.

Previous work in this Department[10,11] had established the following facts:—

1. That *S. fæcalis* and the spores of *B. subtilis* survive the spray-drying process well, and are suitable representatives of vegetative cells and spores respectively, for use in experiments concerned with the effects of conditions of low moisture content on viability.

2. That peptone powders containing *S. fæcalis* or spores of *B. subtilis*, and stearin powders containing the spores of *B. subtilis* can be obtained

by spray-drying. In each case the organisms are in even distribution throughout the powder and survive storage in sufficient numbers and for sufficient lengths of time for storage experiments to be successfully undertaken.

3. That the above powders can be mixed with oils and fats so as to give an even suspension of the infecting organism.

4. That viable counts can be carried out on such oily suspensions with an ascertainable degree of accuracy.

It might be argued that in the suspensions made from peptone powders the cells of the infecting organism are still separated from the oil by a thin film of nutritive media, but in the suspensions prepared from the stearin powder the spore surface must be in direct contact with the lipid media.

In view of the above facts, experiments were undertaken to test the effects of storage upon the viability of the organism in suspensions in oils, fats, and oily solutions of substances commonly regarded as bactericides. In the experimental work which follows, all counts of spores or bacteria should be understood to be viable counts and the number of the count refers to the number of colonies produced in a roll tube. It follows that any organism not capable of giving rise to such a colony has been regarded as non-viable.

Experimental

Methods and materials. Most of the methods and materials used in the work described in this paper have been dealt with at length in Section I (page 701). The ethyl oleate was of commercial quality supplied as being suitable for injection. The chlorophyll used was described as oil-soluble, but otherwise was of ordinary commercial quality.

Design of the storage experiments. A quantity of spray-dried powder containing a suitable concentration of either cells of *S. fæcalis* or spores of *B. subtilis* in even distribution at a particular moisture content (e.g., stored to equilibrium in air over phosphorus pentoxide or hydrated calcium chloride, etc.) was divided into 2 parts. One part continued to be stored as before and served as control. The other part was evenly suspended by light trituration in the particular oil or melted fat under investigation. The oil or fat had, of course, previously been sterilised by heating for 1 hour at 150°C. and cooled. 1 part of powder was mixed with approximately 20 parts by weight of oil or fat. Immediately after mixing, and subsequently at predetermined intervals of time, viable counts were performed simultaneously on the control powder and on the oil, by the technique described in Section I (page 701). Between counts the oils were stored in glass-stoppered bottles sealed by dipping the stopper and neck into molten hard paraffin. The bottles containing the oils were continuously inverted by mechanical means so as to prevent the powders or organisms from settling out.

All results shown in the graphs are expressed as the number of

organisms per g. of powder used to infect the oil. This figure was easily obtained in the case of the control powder. A weighed quantity was submitted to the viable count technique and the results expressed as organisms per g. of powder. The viable count technique on the oil gave the result in the first place as organisms per g. of oil, but from the known proportions in which the powder had been added to the oil this was recalculated as organisms per g. of powder. It is obvious that if the oil has had no effect on the suspended organisms the two results should be identical. The pairs of graphs shown below were obtained by plotting against time these two sets of results, one for the powder stored as such, the other for the powder stored in the oil. The divergence of the two graphs for any one experiment should give a true indication of the effect of the oil on the viability of the vegetative cells or spores suspended on it.

Results of the storage experiments. In the first group of experiments a peptone powder containing spores of *B. subtilis* was used as the source of infection. Three factors were investigated, the moisture content of the powder, the type of oil or fat and the addition of substances normally regarded as bactericides. The pairs of graphs in Figures 1A and 1B were obtained by varying the moisture content of the peptone powder by exposing it to equilibrium over phosphorous pentoxide, anhydrous calcium chloride and hydrated calcium chloride respectively before admixture with arachis oil.

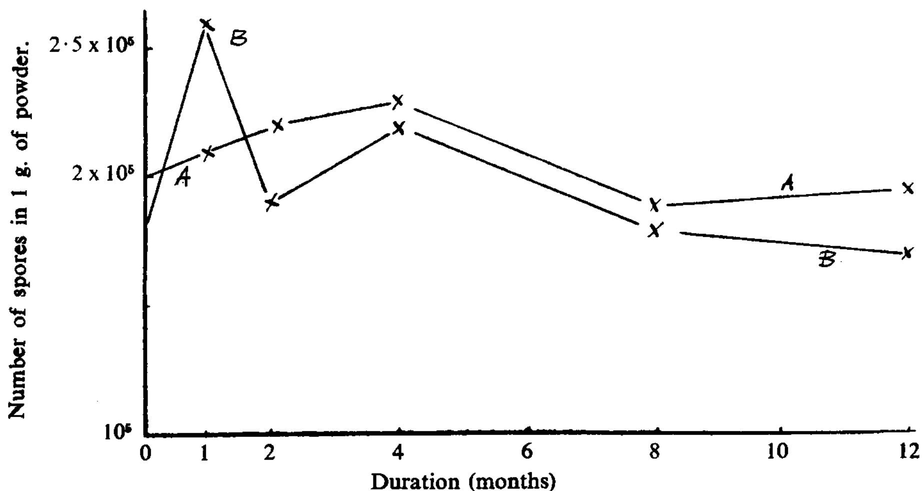

FIG. 1A. Survival of *Bacillus subtilis* spores, at different humidities, in arachis oil.
B. Test. phosphorous pentoxide.
A. Control powder.
× Experimental determination.

Graphs C and D of Figure 1B and the pairs of graphs in Figure 6 show the effects of varying the lipid vehicle, but using in all cases a peptone powder dried over anhydrous calcium chloride. Arachis oil, ethyl

oleate, cod-liver oil, and oil of theobroma were used. Cod-liver oil was included on account of its reputed antiseptic properties.

The pairs of graphs in Figures 4 and 5 illustrate the small effects produced by the presence of substances commonly used under other conditions as bactericides: 1 per cent. of chlorophyll in arachis oil, 2 per cent. of chlorocresol in liquid paraffin, and iodatol were employed.

Chlorocresol is a bactericide having wide applications in pharmacy;

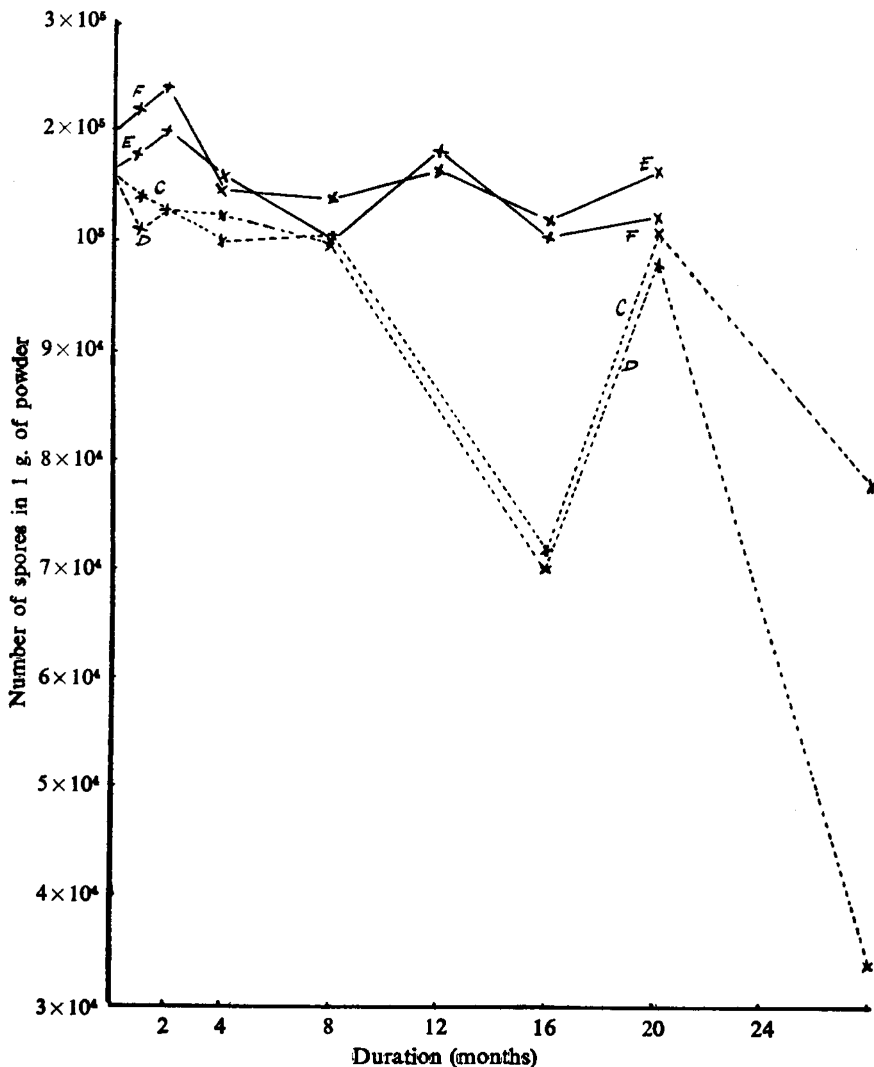

FIG. 1B. Survival of *Bacillus subtilis* spores, at different humidities, in arachis oil.
D. Test. anhydrous calcium chloride. C. Control powder.
F. Test. hydrated calcium chloride. E. Control powder.
× Experimental determination.

it is also a phenol. It has been suggested in the past that phenols depend upon the presence of moisture for their activity. For these reasons a further group of experiments were undertaken using a 0·2 per cent. or 2 per cent. solution of chlorocresol B.P. in arachis oil as suspending fluid and spores of *B. subtilis* in peptone as the infective agent. The pairs of graphs in Figures 2A and 2B show the effects of the 0·2 per

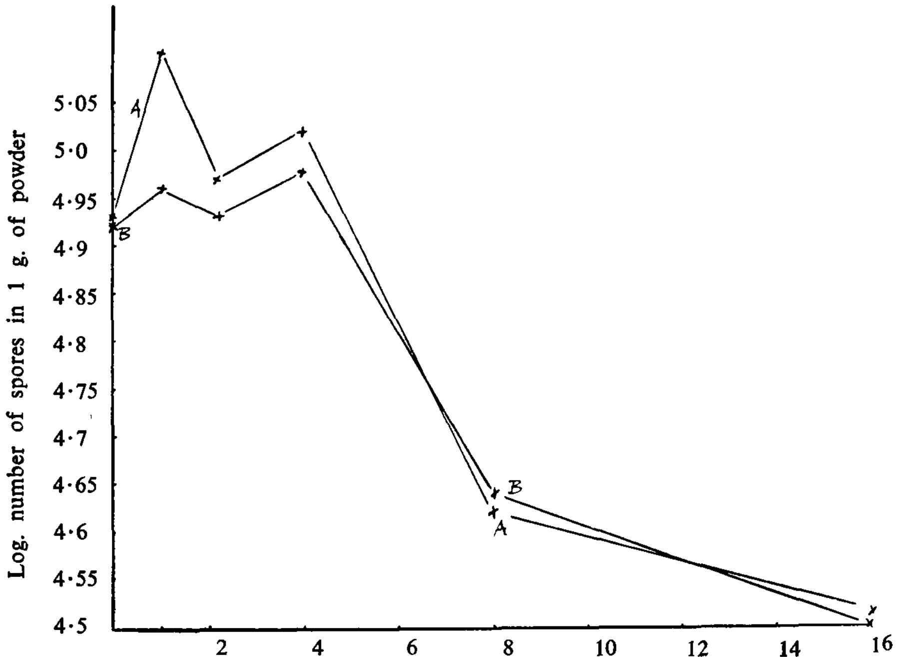

FIG. 2A. Survival of *Bacillus subtilis* spores, at different humidities, in solutions of chlorocresol B.P. in arachis oil.

B. Test. phosphorus pentoxide.
A. Control powder.
× Experimental determination.

cent. chlorocresol B.P. solution in arachis oil combined with powders of varying moisture content, while the graphs of Figure 3 represent the results obtained by a repetition of this work using a 2 per cent. solution of chlorocresol B.P. in arachis oil.

In the final group of experiments using spores of *B. subtilis* a stearin powder was employed as the infecting agent. It will be remembered that in such experiments an almost clear oily suspension of the spores is obtained in which the latter are practically free from moisture and free from a coating of nutrient media. Arachis oil, arachis oil containing 2 per cent. of chlorocresol B.P. and liquid paraffin containing 2 per cent. of chlorocresol B.P. were used as the oily media in these experiments, the results being shown in Figure 7.

To investigate the effects of oils and oily solutions on vegetative cells,

a peptone powder containing *S. fæcalis* brought to equilibrium over anhydrous calcium chloride was in all cases used as the infecting agent. Figure 8 shows the effects on the viability of the cells produced by storing a suspension of such a powder in arachis oil, liquid paraffin, ethyl oleate, and oil of theobroma.

The pairs of graphs in Figures 9 and 10 show the effects of adding 0·2 per cent. or 2 per cent. of chlorocresol to the arachis oil or 2 per cent. of chlorocresol to the liquid paraffin.

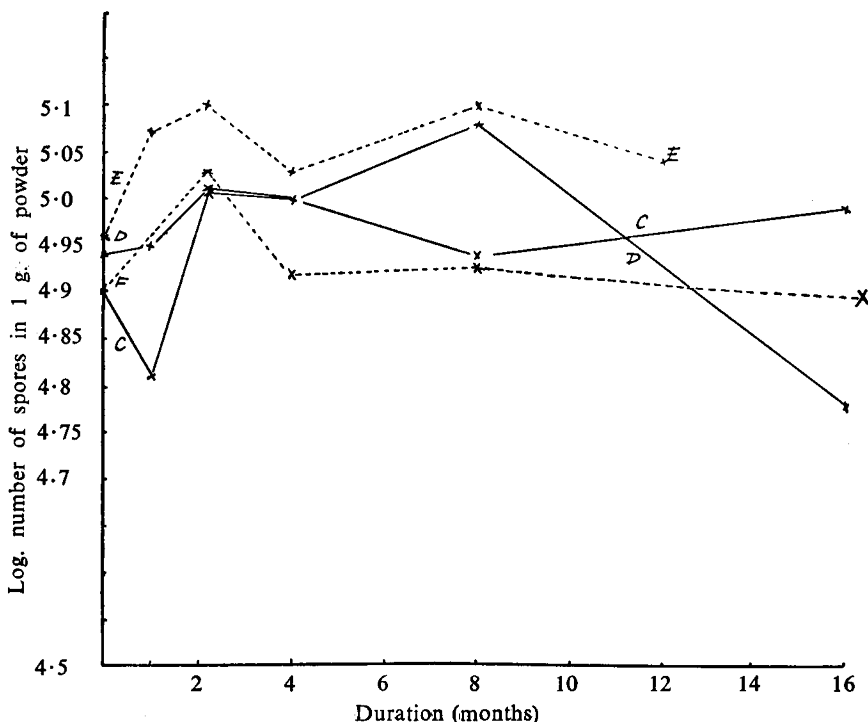

FIG. 2B. Survival of *Bacillus subtilis* spores, at different humidities, in 0·2 per cent. solution of chlorocresol B.P. in arachis oil.
D. Test. anhydrous calcium chloride. C. Control powder.
F. Test. hydrated calcium chloride. E. Control powder.
× Experimental determination.

DISCUSSION

When the above results are considered, three striking facts emerge: (i) the capacity of spores of *B. subtilis* to remain viable in oils and fats for long periods; (ii) the marked resistance of vegetative cells in the dry state to the effects of storage in oily media; (iii) the comparatively small bactericidal effects of substances dissolved in the oily media, even though such substances may be noted for their activity under other circumstances. Large numbers of organisms, especially vegetative cells, die

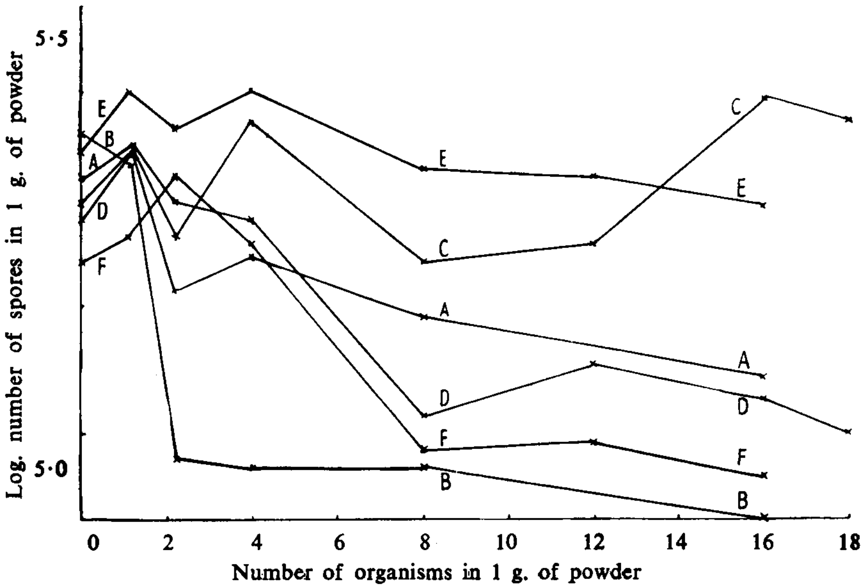

FIG. 3. Survival of *Bacillus subtilis* spores at different humidities in 2 per cent. solutions of chlorocresol B.P. in arachis oil.
B. Test. phosphorus pentoxide. A. Control powder.
D. Test. anhydrous calcium chloride. C. Control powder.
F. Test. hydrated calcium chloride. E. Control powder.
× Experimental determination.

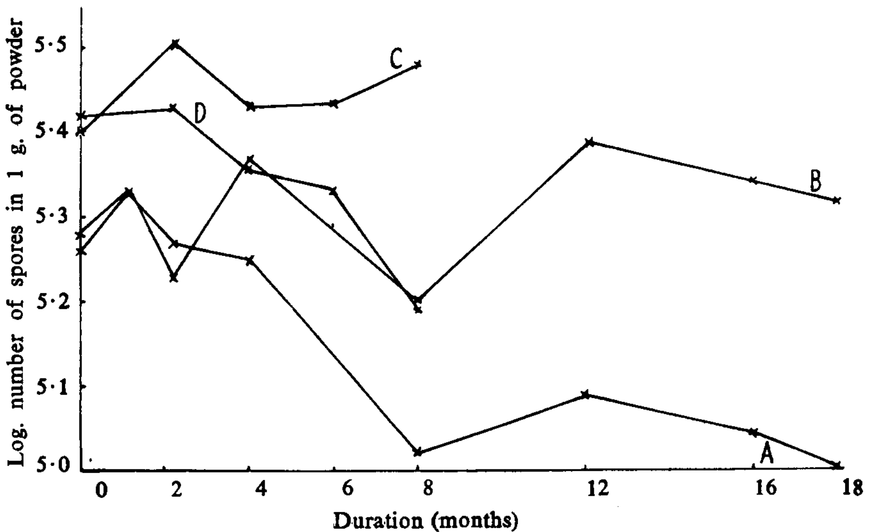

FIG. 4. Survival of *Bacillus subtilis* spores, stored at the same humidity, in 2 per cent. solutions of chlorcresol B.P. in various oils.
A. Test. Arachis oil + 2 per cent. chlorcresol B.P. B. Control powder.
D. Test. Liquid paraffin + 2 per cent. chlorcresol B.P. C. Control powder.
× Experimental determination.

off rapidly in the oily media, but as they do the same apparently in the dry powders, their destruction is not a function of the oil.

The results show quite clearly that oils are not reasonably self-sterilising. Large numbers of even vegetative cells will survive 6 months' storage in arachis oil. (Figure 8). The oils have so little effect on the spores and vegetative cells that it will be necessary to reduce the errors of the technique used up to the present in this work and to increase the number of experiments and so to devise the latter that the results will be more amenable to the statistical treatment required before it can be conclusively stated that the oils have or have not any definite action on the viability of the organisms suspended in them.

From Figures 1A and B, 2A and B and 3 it can be seen that neither the type of oil or fat nor the moisture content of the suspended powder has any effect on the survival of the spores of *B. subtilis*. Similar conclusions apply to *S. fæcalis* except that it appears that ethyl oleate may be destructive to *S. fæcalis*.

Substances at concentrations known to be bactericidal under other circumstances have surprisingly little activity in oily media, even when the moisture content of the suspended powder is raised to 6·8 per cent. (Figures 2A and B, 3 and 4). The results obtained with chlorocresol deserve special mention. It is impossible to say conclusively that 0·2 per cent. or 2 per cent. of this substance in oily media has any bactericidal effect on the spores of *B. subtilis* in over 12 months or on *S. fæcalis* in over 6 months. On the other hand, it is quite certain that oil containing 2 per cent. of chlorocresol may after 16 months' storage contain over 10,000 spores per g. or after 6 month' storage it may contain over 100 viable *S. fæcalis* organisms per g.

From Figures 4 and 5 it would appear that some bactericidal activity might be attributed to iodine as iodatol and to 1 per cent. of chlorophyll in olive oil. This is, however, somewhat straining the customary use of the word " bactericidal." This word is not usually applied to substances which require 2 to 10 months before they begin to show a slight effect, and even then leave more than 100,000 organisms per g. viable. In other words, it is doubtful whether oils, and oily solutions have any adverse effect upon organisms suspended in them, but it has been conclusively shown that large numbers of vegetative cells and spores can remain viable on storage in such conditions for over 6 months.

It should be clearly understood that the work described above does not prove that bactericides in oily solutions are useless. The organisms are viable in the sense that if the oily antiseptic is washed away *without the addition of moisture*, i.e., by a dry volatile solvent they are capable of multiplying to form a colony. If the oily solution is injected it is difficult to see how the oil could possibly be removed from the organism without access of aqueous solutions. i.e., solutions of lipase. In these aqueous solutions the bactericide would presumably dissolve and might

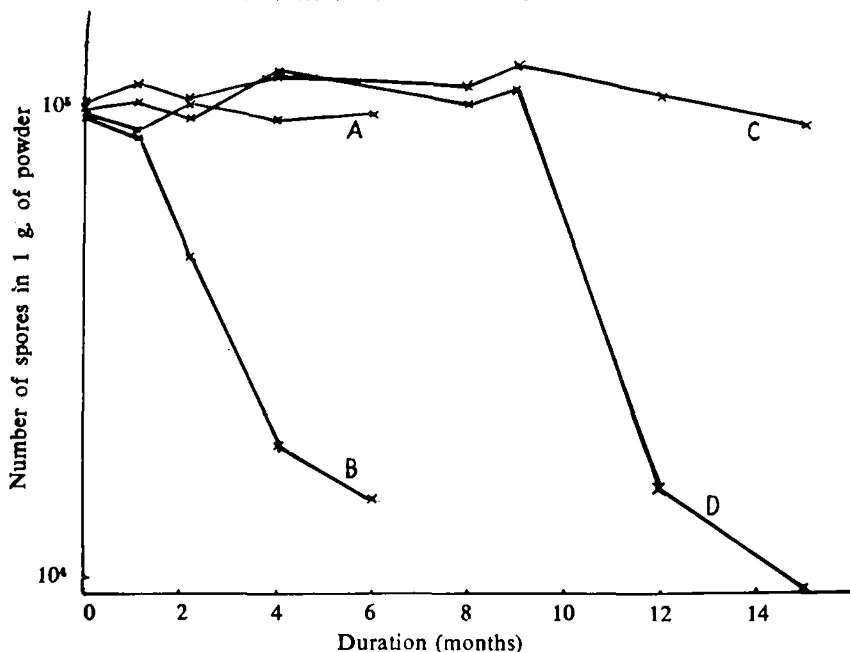

FIG. 5. Survival of *Bacillus subtilis* spores, stored at the same humidity, in various oils.
B. Test. Arachis oil + 1 per cent. chlorophyll. A. Control powder.
D. Test. Iodatol. C. Control powder.
× Experimental determination

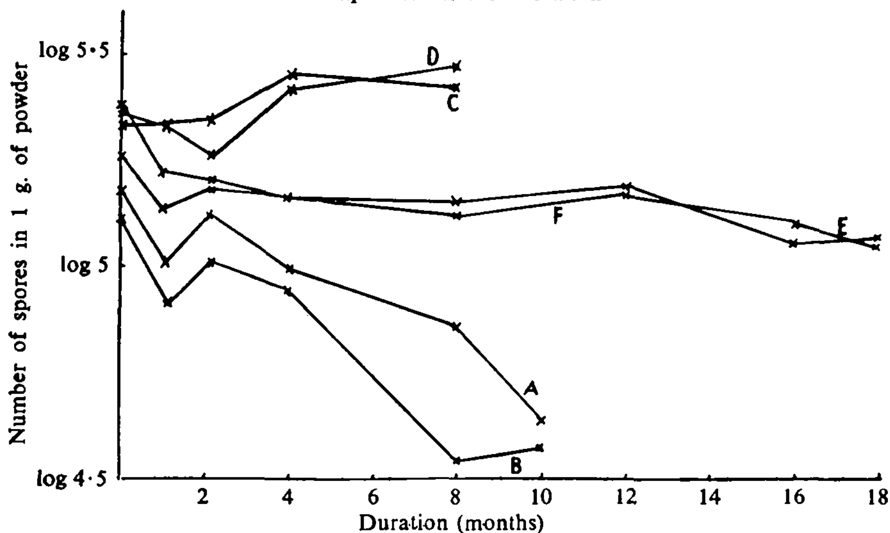

FIG. 6. Survival of *Bacillus subtilis* spores, stored at the same humidity, in various oils.
B. Test. ethyl oleate. A. Control powder.
D. Test. Oil of theobroma. C. Control powder.
F. Test. Cod-liver oil. E. Control powder.
× Experimental determination

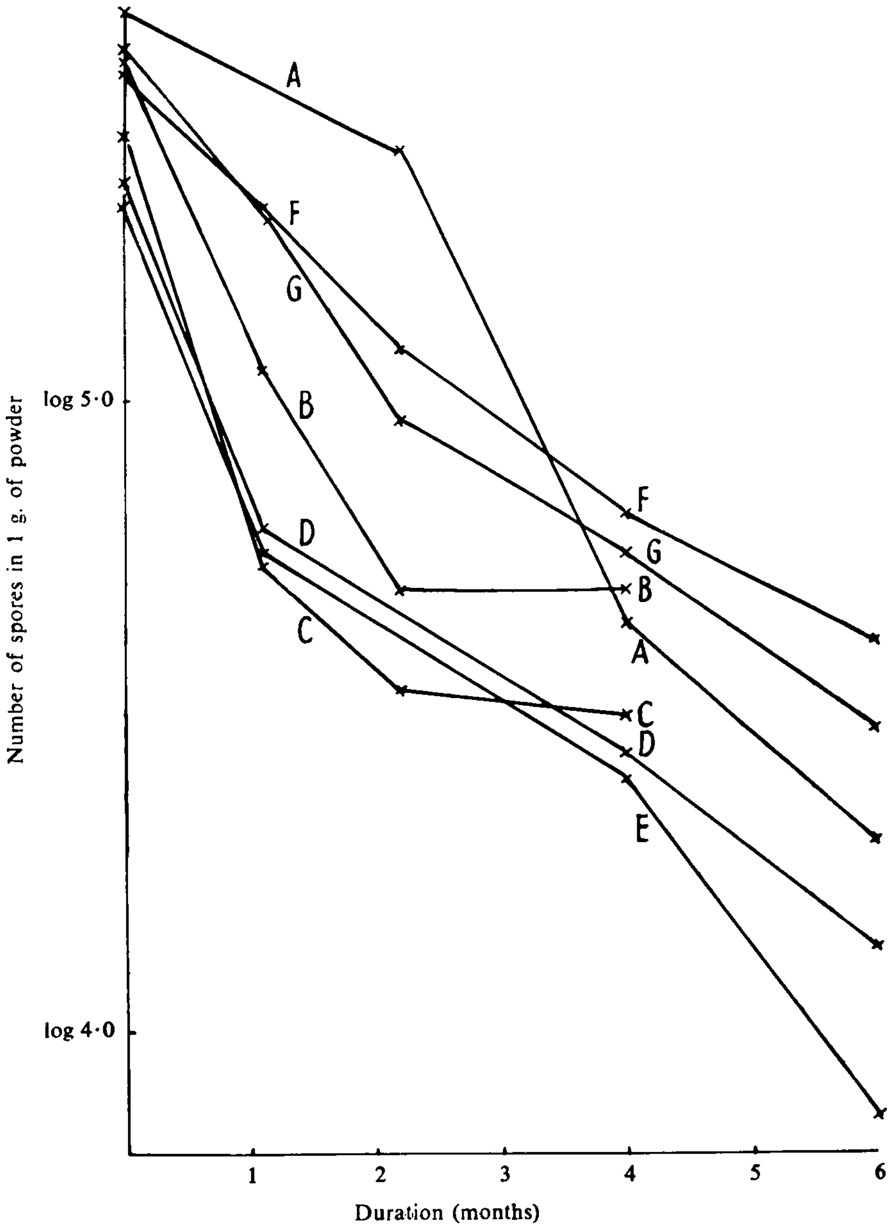

FIG. 7. Survival of *Bacillus subtilis* spores in spray-dried stearin powders dissolved in various oils, with or without the addition of 2 per cent. chlorcresol B.P.
A. Control powder.
C. Test. Arachis oil. B. Control powder.
E. Test. Arachis oil + 2 per cent. chlorocresol B.P. D. Control powder.
G. Test. Liquid paraffin + 2 per cent. chlorocresol B.P. F. Control powder.
× Experimental determination.

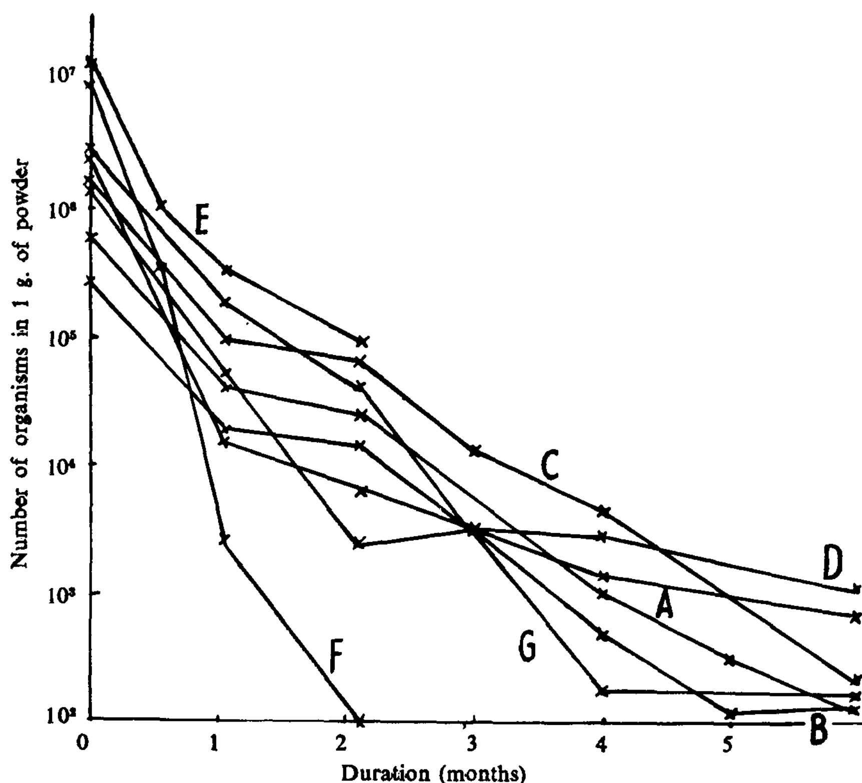

FIG. 8. Survival of the cells of *Streptococcus fæcalis* in various oils.
B. Test. Arachis oil. A. Control powder.
D. Test. Liquid paraffin. C. Control powder.
F. Test. Ethyl oleate. E. Control powder.
H. Test. Oil of Theobroma. G. Control powder.
× Experimental determination.

well kill the spore or bacterial cell on its liberation from the oily solution. To decide this point it would be necessary to repeat the type of experiments carried out by Coulthard, Chantrill and Croshaw[2] using the oily suspensions of spores and cells described above.

A further, somewhat anticipated, but nevertheless important, conclusion, has emerged from this work. There has been no evidence at any time that the viable count of *B. subtilis* or *S. fæcalis* has increased in the oily suspensions, all apparent increases being within the limits of experimental error, as can be seen from the graphs. Further, on testing the recovered spores from time to time they have always been found to retain their resistance to moist heat at 80°C. for 5 minutes, indicating that there has been no sign of germination of the spores in the oily media. Thus, while bacteria and spores may persist in oily media they do not multiply.

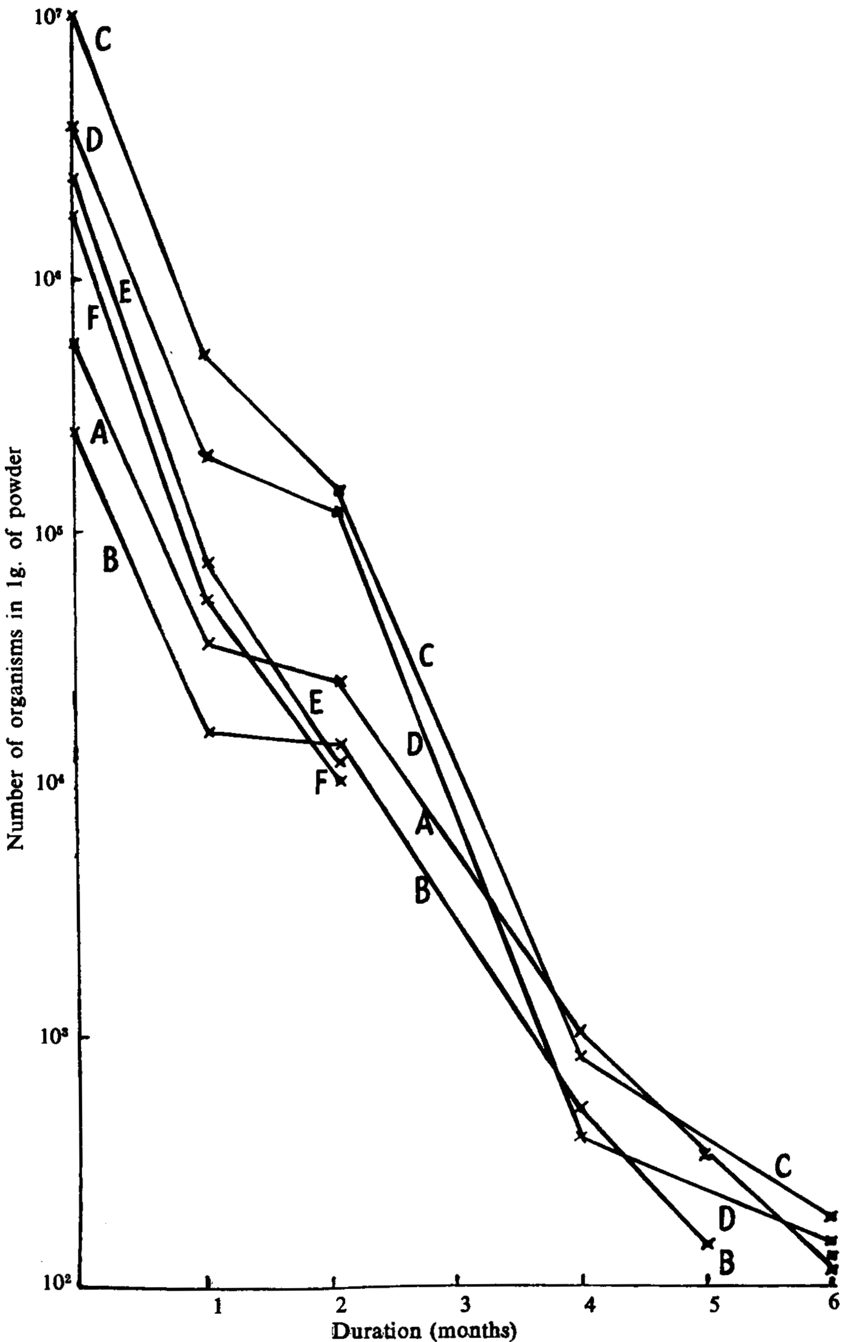

FIG. 9. Survival of the cells of *Streptococcus fæcalis* in oily solutions of chlorcresol B.P.
B. Test. Arachis oil. A. Control powder.
D. Test. Arachis oil + 0·2 per cent. chlorcresol B.P. C. Control powder.
F. Test. Arachis oil + 2 per cent. chlorcresol B.P. E. Control powder.
× Experimental determination.

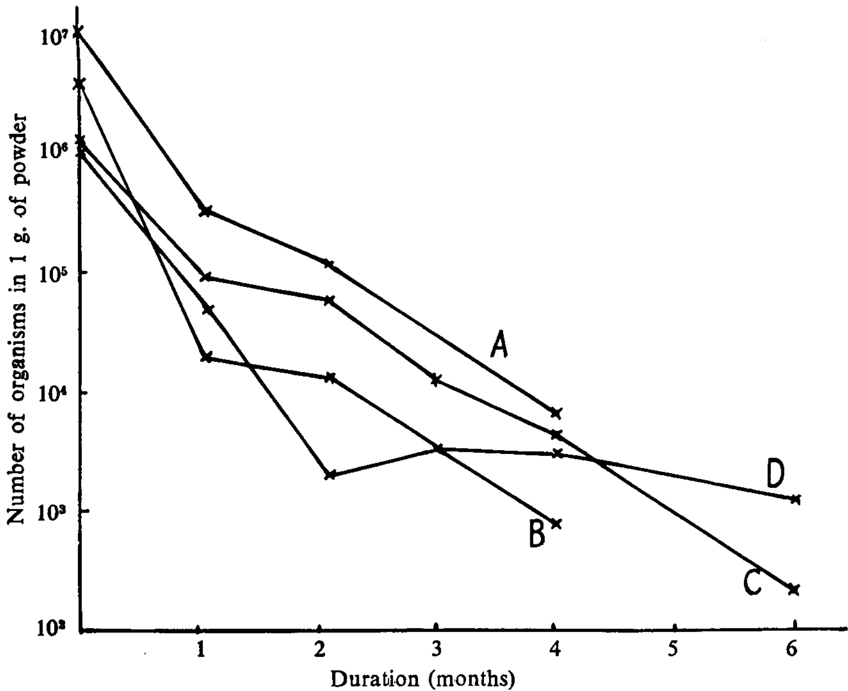

FIG. 10. Survival of the cells of *Streptococcus fæcalis* in liquid paraffin with or without the addition of chlorcresol.

B. Test. Liquid paraffin + 2 per cent. chlorocresol. A. Control powder.

D. Test. Liquid paraffin. C. Control powder.

× Experimental determination.

SUMMARY

1. Spores of *B. subtilis* have been shown to remain viable in oils and fats and liquid paraffin for over 2 years. This is true even when the spores are free from nutritive media and dried with acetone.

2. *S. fæcalis* has been shown to remain viable in oils, fats, and liquid paraffin for over 6 months.

3. The death rate of the organisms suspended in the oils and fats is comparable to that of the organisms in the control powder. Indeed from the present experiments it is not possible to conclude that the oils exert any specific effect, other than acting as a medium preserving the low moisture content.

4. There is little difference in the effects of the various oils so far examined, but there is some indication that ethyl oleate accelerates the death rate of *S. fæcalis*.

5. No indication has been obtained of the multiplication of *S. fæcalis* or the germination of spores of *B. subtilis* in oily media.

6. No bactericide so far examined has exerted any marked action on the suspended organisms.

7. While the two organisms used in this work are not noted for their pathogenicity the presumption is raised that other spores and bacteria might be expected to possess similar resistance. The bearing of these conclusions in the dispensing of oily solutions for injection is discussed.

REFERENCES

1. Sykes and Royce, *J. Pharm. Pharmacol.*, 1950, **2**, 639.
2. Coulthard, Chantrill and Croshaw, *J. Pharm. Pharmacol.*, 1951, **3**, 215.
3. Hammer and Long, *Bact. Rev.*, 1941, 337.
4. Williams and Feiger, *J. biol. Chem.*, No. 166, 335.
5. Foster and Wynne, *J. Bact.*, 1948, **554**, 484.
6. Crimm and Martos, *J. Thoracic Surg.*, 1945, 265.
7. Tausson, *Meftyanac. Khos.*, 1928, 220.
8. MacMaster, *J. Infec. Dis.*, 1919, No. 24, p. 378.
9. Fairhall and Bates, *J. Bact.*, 1920, **5**, 49.
10. Bullock, Keepe and Rawlins, *J. Pharm. Pharmacol.*, 1949, **1**, 878.
11. Bullock and Keepe, *ibid.*, 1951, **3**, 700.

DISCUSSION

DR. BULLOCK presented the two papers.

The CHAIRMAN said that those who were intimately concerned with the preparation of parenteral injections realised that the B.P. had not been specific as to the bacteriostatic which should be put into oily injections to be dispensed in multiple dose containers, but the information was not available.

A point of interest in the paper was that bacteria could remain viable in an oily medium: this was a step towards solving the problem of arranging experimental conditions comparable with the contamination of an oily injection by dust. The reference to ethyl oleate was interesting because of the possibility that peroxide formation with age gave increased killing action. The lack of resistance of vegetative organisms to spray drying was also of interest as it had been noted that *B. pyocyaneus* on freeze-drying succumbed very quickly. It had been a useful organism for testing the bactericidal action of phenolic substances.

DR. H. DAVIS (London) said that Dr. Bullock had shown that the oils themselves appeared to be bacteriostatic; but the B.P. laid down that a bacteriostatic should be added. This raised the question as to what is meant by the terms bactericide and bacteriostatic. Pentachlorophenol might possibly be a suitable bacteriostatic for oil, but it would first be necessary to make sure that it was harmless to the tissues. It would be interesting to know whether the authors encountered any spreading of growth on the surface when using *B. subtilis* in the roll tube technique. If so, how did they prevent it, and for how long were the tubes incubated?

DR. N. EVERS (London) suggested, in view of the Chairman's remarks, the possibility of using a synthetic peroxide similar to those which occurred naturally in fats and oils.

DR. F. HARTLEY (London) suggested that the ultimate question would be not whether bacteria survived in the oil, but whether they retained their pathogenicity, and to what extent other characteristics were changed.

MR. T. D. WHITTETT (London) asked the authors whether they had considered the use of sorbitan derivatives such as Tween 80 to solubilise oil prior to carrying out tests for sterility. These substances were not bacteriostatic and were employed as constituents of culture media.

DR. A. H. COOK (London) drew attention to the footnote to Table III stating that it was difficult to count colonies after 24 hours' incubation, whereas in the Table itself a much smaller variation was shown for the period of 24 hours than for any other time. That appeared to be contradictory—had the authors any explanation to offer? Table X recorded counts on spray-dried powder containing *S. fæcalis* dried at 180°C., a temperature already stated to produce a mortality of 98 per cent. It would be useful to know whether the authors used the same powder for the remainder of their experiments; if so, they would be picking out a resistant organism. It would be of interest to know whether the resistance of the 2 per cent. of survivors was any different from that of the organisms of the original suspension.

MR. C. E. COULTHARD (Nottingham) agreed as to the considerable resistance of *S. fæcalis*. He had recently discovered a strain of *Cl. septicum* which was highly virulent and at the same time resistant to drying. A number of tests had been carried out by drying it in ampoules and subsequently filling them in the normal way with various pharmaceutical products and noting survival times. In that way the following had been examined: arachis oil, arachis oil and cresol, arachis oil and phenol, liquid paraffin, liquid paraffin and cresol, liquid paraffin and phenol. In those solutions it had been found that the organisms retained their virulence for at least 35 weeks.

DR. G. FOSTER (Dartford) referred to the use of anæsthetic ether to precipitate bacteria from oily injections, and asked whether Dr. Bullock had carried out any experiments with ether containing peroxide. If so, what were the results of such experiments?

DR. BULLOCK, in reply, said it was gratifying to note that others were interested in the correct definition of such terms as "bactericide" and "bacteriostatic." The authors' object had been to study the effect on bacteria, and they had not been concerned with the effect of injecting infected oily preparations into the animal body. Peroxide formation and the use of *B. pyocyaneus* were questions which had yet to be solved. He thanked Dr. Davis for his suggestion that pentachlorophenol might be used as a bacteriostatic, and added that copper salts of lower fatty acids had also been suggested.

Roll tube counts had been a problem, but Mr. Rawlins had carried out extensive tests which showed that repeatable accurate counts could be obtained. He agreed that the work required extending by the use of pathogens, but there was the difficulty of spray drying such organisms

due to the obvious dangers involved with a large apparatus. An interesting feature was the general agreement between the authors' *in vitro* results and the *in vivo* results obtained by Mr. Coulthard. Work on sterility tests was proceeding, but the difficulty was still that the type and extent of the infection was unknown. The ideal was to obtain approximately 1 organism in 10 ml. of oil and use that to investigate the various proposed tests. Organisms spray-dried at 180°C. were not used in subsequent experiments as the organisms would not have been evenly distributed throughout the product. In any case the temperature was that of the air inlet and the organisms would not have been exposed to a temperature higher than 60°C. Anæsthetic ether B.P. was used throughout the experiments.