BACTERIAL SURVIVAL IN SYSTEMS OF LOW MOISTURE CONTENT

PART V. COMPARISON OF TESTS FOR STERILITY OF OILS

BY KENNETH BULLOCK and N. H. BOOTH

From the Department of Pharmacy of the University of Manchester

Received July 13, 1953

INTRODUCTION

THE degree of bacterial contamination of pharmaceutical preparations may be investigated by means of two types of test:—(1) The *in vivo* type of test, in which the preparations are injected into animals and the occurrence of any symptoms noted. This type of test detects pathogenic organisms but cannot prove sterility since non-pathogens will not be detected. (2) The *in vitro* type of test, in which the preparations are mixed with suitable nutrient media and the resultant growth or absence of growth noted. This type of test detects "common saprophytic contaminants, and the pyogenic cocci and spore-bearing bacteria pathogenic to man," but would fail to detect some of the more delicate pathogens.

It will be seen, therefore, that there can never be absolute proof of the sterility of a bulk of material. Firstly, the tests used will only detect certain types of organisms, and secondly it is, in the nature of things, only possible to test a fraction of the material because the bulk must be used although, on account of the thorough mixing involved in manufacture, it can perhaps be assumed that any contamination is evenly distributed throughout the bulk.

It is contamination of the type detected by *in vivo* tests which it is most important to avoid in pharmaceutical preparations but such tests are expensive, difficult to carry out, and, unless large numbers of animals are used, the results are inexact. The "Tests for Sterility" of the pharmacist are usually tests of the *in vitro* type. This is justified only because, under the special conditions of manufacture, contamination with robust saprophytes, cocci and spores is more likely than contamination with delicate pathogens. It is for the same reasons that it is not illogical to evaluate the quality of "Tests for Sterility" by determining their ability to detect slight contamination with such organisms as *Bacterium coli*, the spores of *Bacillus subtilis*, *Streptococcus fæcalis*, etc. Sterility is an absolute term allowing of no comparative degrees, it involves the complete absence of living organisms. It should be clearly understood that, on the other hand, "Tests for Sterility," in conjunction with a statistical analysis of the results obtained, cannot do more than give a probability of the absence of more than a certain degree of contamination. This is of course quite sufficient. Since the B.P. test and other such tests are in fact *tests for contamination*, which effectively limit but do not completely exclude the possibility of contamination in the bulk of the material, it might be better to replace the phrase "complies with the tests for sterility" by the more accurate phrase "does not respond to the tests for contamination."

"Testing for Sterility" in oily preparations presents difficulties of its own. As previously shown in this Department[1], while spores remain viable in oils for long periods of time they do not germinate and do not multiply; vegetative organisms do not multiply and tend to die off. To detect bacteria in oils by an *in vitro* "Test for Sterility" it is therefore necessary first to cause the bacteria to be transferred from the oil phase to the aqueous phase of the nutrient medium.

It has sometimes been urged that a particular "Test for Sterility" must be satisfactory because it has been used for many years to control, on a large scale, a variety of preparations for parenteral use which have proved to be satisfactory in practice. All that such experience proves is that material failing to give rise to growth in *in vitro* tests also fails to give rise to infection when used parenterally. It indicates nothing about the ability of the test to detect minimal contamination of a type or degree which will not give rise to infection. Indeed it might well be that in a well managed works, even after many years use, the test might never have been tried on contaminated samples. The difficulty is not overcome by introducing a little contaminated dust into the preparations and then showing that the test indicates contamination.

To examine a "Test for Sterility" of oils it is first necessary to prepare samples of oils containing micro-organisms, preferably of known species, evenly distributed, in very low, but approximately known, concentration. Suitable quantities of such oils can then be examined by the particular test and the results submitted to statistical examination to see whether the proportion of samples showing growth, to those showing absence of growth, is consistent with the known degree of contamination of the oil. It is important that the concentration of the organisms in the oil should be known, at least approximately, because it might well be that only a fraction of the organisms present pass from the oil phase to the aqueous phase in a particular test. Under such conditions, with an oil containing on an average one or more organisms per sample quantity, some, or even all, of the particular samples might fail to give rise to growth.

For the above reasons the work described in this paper is divided into the following sections—(1) A description of the materials and methods used including a description of the three "Tests for Sterility" examined, namely, the test of the British Pharmacopœia, the Filtration Test of Davies and Fishburn[2], and a modified limiting viable count test described in this paper. (2) An account of the preparation of oils lightly contaminated with spores of *B. subtilis* suitable for use in evaluating "Tests for Sterility." (3) The results obtained by submitting the prepared oils to the three "Tests for Sterility" and a statistical examination of the results.

Materials and Methods

The *test organisms* used were the spores, prepared as previously described[3], of *B. subtilis*, Marburg, National Collection of Type Cultures No. 3610.

The *oil* used was arachis oil B.P.

The *peptone* used was Evans bacteriological peptone.

Solutions of peptone powder were made with sterile glass-distilled water, which was also used for diluting them; a separate investigation[3] having shown that glass-distilled water was not likely to be harmful to spores of *B. subtilis* during the relatively short time of exposure in the dilutions.

The *oil solvent* used to remove the oil from suspensions in oil was commercial light petroleum b.pt. 40° to 60° C. once redistilled and passed through a Seitz filter.

The *media* used were nutrient agar, prepared as previously described[3], and broth containing 2 per cent. of peptone, 1 per cent. of Hepamino and 0·5 per cent. of sodium chloride.

All *incubations* were carried out at 37° C.

Since one type of ærobic test organism only was used in the work described in this paper only those portions of the B.P. "Tests for Sterility" relative to ærobes are considered. Any conclusions expressed about the three "Tests for Sterility" considered, refer only to those tests as applied to ærobes.

The B.P. "Test for Sterility." In the test as described in the B.P. 1948 there was no reference to oily injections. By inference, the oily material was to be added to an unspecified quantity of medium (provided that the ratio of medium to oil was sufficient to annul the effects of any antiseptic present). No mention was made of the extent of the oil-water interface nor of intermixture of the two phases by shaking. These latter factors influence not only the transfer of the organisms from the oil phase to the aqueous phase but also the availability of oxygen since oxygen does not easily diffuse through an oil layer.

The B.P. 1953 remedied these omissions to some extent by stating "When oily solutions or suspensions are being tested, they are distributed as uniformly as practicable throughout the media, and the medium is shaken at intervals during cultivation." The importance of the extent of the oil-water interface and the effect of shaking had been investigated in experiments carried out prior to the publication of the B.P. 1953.

Quantities of 1 ml. of an oil containing approximately 4 spores in 10 g. were introduced into 10 ml. quantities of broth, (*a*) in bottles of 2 cm. internal diameter, so that the oil formed a visible continuous layer on the broth, and (*b*) in jars of 6·5 cm. internal diameter so that the oil was insufficient to form a visible continuous layer on the broth. Of the 60 samples in each type of container, 30 were shaken daily (50 up and down movements on one occasion each day) and 30 were not shaken, all for a period of 5 days incubation. During a further 5 day period of incubation all the containers were shaken daily. Growth or its absence was recorded at 5 and 10 days, the results being shown in Table I. A statistical analysis of the results is shown in Table II from which it can be concluded that using a 5 day incubation period the test is satisfactory using wide jars, whether shaken or not, and in narrow bottles, if these are shaken. It is not satisfactory to use the narrow bottles if they are not shaken. Thus the B.P. 1953 test is satisfactory in this respect. In further work described in this paper 2 cm. internal diameter bottles shaken daily were used. A

sample was reported as negative if there was absence of growth and positive if growth occurred.

The Filtration "Test for Sterility" of Davies and Fishburn. The test was carried out exactly as described by the authors[2] except that the light petroleum had a boiling point of 40° to 60° C. instead of 80° to 100° C.[4]

A sample was reported as negative if there was absence of growth and positive if growth occurred.

TABLE I

RESULTS OF B.P. TESTS ON OIL ST3

Type of container	After 5 days at 37° C.	After a further 5 days at 37° C. all samples shaken daily
Narrow bottles shaken daily	15	16
Wide jars shaken daily ..	17	17
Narrow bottles not shaken ..	1	8
Wide jars not shaken ..	12	15

Figures give the number of 1 ml. samples out of 30 showing growth.

The Limiting Viable Count Technique as such and modified as a "Test for Sterility." The most desirable "Test for Sterility" for oils would incorporate a viable count technique so that if the oil were not sterile, not only would this fact be shown, but also the degree of contamination would be indicated. A technique satisfactory for use in carrying out viable counts on heavily contaminated oils has already been described[4].

TABLE II

STATISTICAL ANALYSIS OF THE RESULTS GIVEN IN TABLE I

Period of incubation	Comparison	χ^2	P
5 days	Wide jars shaken compared with wide jars not shaken	1·67	0·1 to 0·2
	Narrow bottles shaken compared with narrow bottles not shaken	16·7	<0·001
	Wide jars shaken compared with narrow bottles shaken	0·268	0·5 to 0·7
	Wide jars shaken compared with narrow bottles	11·9	<0·001
Further 5 days all samples shaken daily	Narrow bottles not shaken during first five days' incubation compared with all other samples	6·43	0·01 to 0·02

P Is the probability of obtaining by chance as big a value of χ^2 as the one shown if there is no difference between the results compared.

Two difficulties were encountered in applying this technique to oils containing only a few spores per g. To appreciate the first difficulty it is necessary to bear in mind the fact that the oil samples examined either already contained some spray-dried peptone, or, for the reason described below, some sterile spray-dried peptone was mixed into the oil. The particles of a spray-dried powder have the form of whole or broken shells. Some of these hollow particles retain air and on centrifuging a suspension in oil and light petroleum a few of the particles, carrying with them adherent spores rise to the surface, instead of sinking to the bottom of the

liquid, and are thus lost on decantation. This error is relatively small for heavily contaminated oils but may be appreciable when only a few spores are present. Breaking down the air-retaining particles by trituration in a pestle and mortar was unsatisfactory because at the same time an appreciable number of spores were found to be killed (up to 50 per cent. in 15 minutes). This is in accordance with the findings of Curran and Evans[5] and King and Alexander[6].

The difficulty was finally overcome by milling the original powder with glass beads in a closed jar; a technique previously successfully used by Bullock, Keepe and Rawlins[3] for obtaining an even distribution of *Bact. lactis ærogenes* in spray-dried powders.

The second difficulty in performing viable counts on only slightly contaminated oils is that under natural conditions, i.e., unless the organisms have been introduced in a large bulk of powder, on mixing the oil with light petroleum and centrifuging, only a negligible deposit is obtained; far too small a quantity to use for a viable count after washing with more light petroleum. To overcome this difficulty the oil to be examined was always mixed with 5 per cent. of sterile, spray-dried, milled, peptone powder before diluting with solvent and centrifuging. This peptone carried the spores down with it and formed a workable deposit. The small but definite improvement in the recovery of spores from oil, resulting from the use of additional peptone powder is shown in Table III. The percentage recovery figures are discussed later.

TABLE III

PERCENTAGE RECOVERY OF SPORES FROM OIL

Treatment	Spores added per g. of oil	Spores recovered per g. of oil	Percentage recovery	t	P
No sterile peptone added to oil ..	3848	3480	90·4	3·808	<0·001
Unmilled sterile spray-dried peptone lightly triturated with the oil	3848	3620	94·1	2·752	0·001 to 0·01
Milled sterile spray-dried peptone shaken with the oil	3848	3740	97·2	1·290	0·2 to 0·3
Milled sterile spray-dried peptone lightly triturated with the oil ..	3848	3860	100·3	0·246	0·8 to 0·9

P is the probability of obtaining by chance as big a value of t as the one shown if there is no difference between the results compared.

Incorporating these two modifications, the limiting viable count technique was carried out according to the following directions.

Accurately weigh about 5 g. of oil into which exactly 5 per cent. of sterile, milled peptone has been introduced by light trituration. Add 5 ml. of light petroleum, mix, centrifuge and discard the clear supernatant liquid; repeat this process once. Remove the last traces of solvent from the deposit under reduced pressure. Take up the deposit in water in the exact proportion of 10 ml. of water for every 5 g. of oil sampled and perform a roll-tube count on the solution using 5 quantities, each of 1 ml., of the aqueous suspension to give quintuplicate tubing.

The total number of colonies in the 5 tubes gives the number

of spores present in 2·5 g. of the oil sampled. So far this constitutes the limiting viable count technique. To compare this test with the other two "Tests for Sterility," a sample was reported as negative if all 5 roll-tubes showed no colonies and as positive if one or more colonies occurred in one or more tubes. This constitutes the modified limiting viable count technique "Test for Sterility."

If it were at any time considered that the limiting viable count technique should be recommended as a simple "Test for Sterility" and not as a method of obtaining a viable count to assess other "Tests for Sterility," it would be advisable to take up in broth the whole washed residue centrifuged out of the oil and light petroleum mixture and to record absence of growth as negative and growth as positive as is usual in "Tests for Sterility."

The Preparation of Oils Suitable for Use in Evaluating "Tests for Sterility"

3 procedures for the preparation of the oils contaminated with spores of *B. subtilis* were available—

(1) Contamination of the oil with peptone powder obtained by spray-drying a solution of peptone containing only a small number of spores per g. of peptone. This procedure was discarded because it involved submitting a relatively small sample of spores to the drying process and might therefore result in the use of a selected sample.

(2) Contamination of the oil with a lightly contaminated peptone powder obtained by serial dilution of a heavily contaminated spray-dried powder with sterile spray-dried peptone. This procedure was tried and found to be satisfactory provided that the dilutions were mixed thoroughly by milling as previously described[3]. The method was, however, not adopted because the long milling periods required were time consuming.

(3) Contamination of the oil with a quantity of heavily contaminated, spray-dried, milled, peptone powder and serial dilution of the mixture with sterile oil. This method was found to be satisfactory, and was adopted.

2 spray-dried peptone powders containing spores of *B. subtilis* were used in the preparation of the oils. The first powder (P1) contained approximately 3200 spores per g. It was mixed with sterile oil to give a contaminated oil (Oil P1) containing approximately 260 spores per g. of oil. A portion of Oil P1 was diluted with a further quantity of sterile oil to give an "Oil for Sterility Test" (Oil ST1) containing approximately 2 spores per g. of oil.

The second powder (P2) contained approximately 96,000 spores per g. and was mixed with sterile oil to give Oil P2 containing approximately 1700 spores per g. This latter was again diluted with sterile oil to obtain Oil P2′ containing approximately 20 spores per g.

Two quantities of Oil P2′ were diluted separately with sterile oil to give two "Oils for Sterility Test," Oil ST2 and Oil ST3 both containing approximately 4 spores in 10 g. of oil.

The next problem was to form an estimate of the number of spores per

g. in the oil, and to show whether that number could satisfactorily be recovered by the limiting viable count technique. The estimate was made by carrying out a viable count on the original powder when, knowing the proportion of powder added to the oil, the number of spores per g. of the latter was calculated. A limiting viable count technique on the oil was then performed and the recovery calculated as a percentage. These were the methods used to obtain the percentage recovery figures in Table IV.

TABLE IV

RECOVERY OF SPORES FROM THE OILS BY THE LIMITING VIABLE COUNT TECHNIQUE

Material examined	Number of spores added per 10 g. of oil	Number of spores recovered per 10 g. of oil	Percentage recovery	t	P
Powder PI		32020			
Oil PI	2595	2605	100·4	0·272	0·7 to 0·8
Oil STI	19·98	20·48	102·5	0·205	0·8 to 0·9
Powder 2		962000			
Oil P2 ..	17590	17410	98·98	0·461	0·6 to 0·7
Oil P2′ ..	207	204	98·54	0·385	0·6 to 0·7
Oil ST2 ..	4·101	4·160	101·5	0·0967	>0·9
Oil ST3 ..	4·028	4·0	99·31	0·064	>0·9

P is the probability of obtaining by chance as big a value of t as the one shown if there is no difference betweeen th results compared.

This treatment is inexact because the counts per g. for both powder and oil are means derived by the use of dilution factors from the mean counts of batches of roll-tubes. In the above elementary treatment no indication is given of the possible errors of these mean counts of batches of roll-tubes so no definite degree of significance can be given to the figures representing the percentage recovery. These defects can be remedied by the following statistical treatment.

Let $\bar{x}$ be the mean roll-tube count of the contaminated oil, $\bar{y}$ be the mean roll-tube count of the powder used to contaminate the oil. It must be emphasised that $\bar{x}$ and $\bar{y}$ are the means of the counts of the number of colonies in roll-tubes, the roll-tubes having been obtained by suitable serial dilutions of solutions of either the contaminating powder or the powder recovered from a sample of oil.

The extent of these serial dilutions is taken into account by means of a constant r which incorporates three factors. (1) A factor representing the ratio of the volume of water used to reconstitute the sample of contaminating powder to the weight of this powder sample; (2) a factor representing the ratio of the weight of oil contaminated to the weight of powder used to contaminate it; (3) a factor representing the ratio of the volume of water used to reconstitute the powder recovered from the sample of oil, to the weight of this oil sample.

If the recovery is satisfactory we have

$$\bar{y} = r\bar{x}$$
$$\therefore r\bar{x} - \bar{y} = 0$$

To ascertain if $r\bar{x} - \bar{y}$ differed significantly from zero, use was made of the expression

$$\frac{rx - \bar{y}}{\sqrt{\left[\frac{r^2}{n_1}\left\{\frac{\Sigma(x-\bar{x})^2}{n_1-1}\right\} + \frac{1}{n_2}\left\{\frac{\Sigma(y-\bar{y})^2}{n_2-1}\right\}\right]}}$$

in which the symbols have their usual meaning. If n_1 and n_2 are large, the significance of this expression can be assessed by reference to the table of the t distribution with ∞ degrees of freedom. The results obtained by this procedure are shown in columns 5 and 6, Table IV.

If an oil is to be used to assess the effectiveness of "Tests for Sterility" it is essential that there should be evidence that the organisms in it are evenly distributed. Such evidence was obtained by submitting the results obtained from a number of samples subjected to the limiting viable count technique to one of four different mathematical treatments according to the degree of contamination of the oil as follows. (1) If the oil contained 20 or more spores per g. an analysis of variance was carried out as previously described[3]. (2) With oils containing between 4 and 20 spores per g. the transformation $y = \sqrt{x + 0{\cdot}375}$, where x represented an individual roll-tube count, was used prior to carrying out an analysis of variance on the y values in the usual way. (3) If the oil contained between 8 and 40 spores in 10 g. a similar transformation $y = \sqrt{T + 0{\cdot}375}$ was used where T represents the sum of the counts of the five roll-tubes obtained by the quintuplicate tubings from one sample of oil. The mean square was compared with the theoretical value of 0·25 to obtain a variance ratio and probability value. (4) If the oil contained less than 8 spores in 10 g. yet another treatment was necessary.

Quintuplicate counts were made on 50 samples of oil. For the 5 tubes representing each sample two figures were recorded—the number of tubes free from colonies, and the number of tubes containing one or more colonies. A frequency table was constructed from which a value of χ^2 could be calculated according to the usual formula. Provided the number of samples is large, χ^2 can be taken to have a normal distribution, whose mean and variance can be calculated.

The value of the ratio—

$$\frac{\chi^2 - \text{mean}}{\sqrt{\text{variance}}}$$

was therefore referred to the Normal Distribution Table to obtain a normal deviate and probability value.

The data in Tables IV and V shows that Oils ST1, ST2 and ST3 contain the spores in even distribution and that approximately 100 per cent. of the spores can be recovered by the limiting viability count technique. The oils are thus suitable for use in assessing the efficiency of "Tests for Sterility."

It should be mentioned that the probability values for Oils ST2 and ST3 given in the last column of Table V are slightly lower than the normally accepted 5 per cent. level but this was not considered to be unsatisfactory

in view of the extremely low counts of these oils and the fact that the approximations made in the calculation might emphasise any tendency to give a low value to P.

Comparison and Evaluation of the "Tests for Sterility"

Two series of tests were carried out. In the first series Oil ST1 containing approximately 2 spores per g. was used as the test oil. 50 approximately 5 g. samples of this oil were examined by the modified limiting viable count technique. Since the spores recovered from each sample of oil were suspended in 10 ml. of water and 5 × 1 ml. quantities of this were roll-tubed the total number of colonies counted in the 5 tubes corresponded to the number of spores in 2·5 g. of oil. All 50 samples gave one or more colonies in one or more of the 5 roll-tubes, i.e. 50 positives out of 50 samples were obtained. 50 approximately 1 ml. samples of Oil ST1 examined by the B.P. test all gave rise to growth in the broth. Similarly 50 approximately 5 ml. samples submitted to the Filtration Test all gave rise to growth in the broth. It can thus be concluded that all 3 "Tests for Sterility" can be relied on to show contamination in oils containing 2 or more spores per g. of oil.

TABLE V

Evenness of distribution of spores in the oils

Material examined	Method of calculation of variance ratio (see text)	Variance ratio	P
Powder P1	1	2·077	0·5 to 0·1
Oil P1	1	1·174	>0·2
Oil ST1	3	1·308	0·1 to 0·2
Powder P2	1	2·525	0·05 to 0·1
Oil P2	1	1·142	>0·2
Oil P2′	1	1·584	0·1 to 0·2
Oil ST2	4	*2·262	0·024
Oil ST3	4	*2·098	0·036

* Normal deviate.

P is the probability of obtaining by chance as big a value of variance ratio as shown if the spores are evenly distributed in the oils.

The second series of tests was more exacting since the test oil was Oil ST2 containing approximately 4 spores in 10 g. Considering a positive result to be growth in the broth in the case of the B.P. and Filtration tests and one or more colonies in one or more tubes of the limiting viable count technique test, the results are given in Table VI.

TABLE VI

Results of submitting 50 samples of oil ST2 to each of the 3 tests

Modified limiting viable count technique	Filtration test	B.P. test (1 ml. samples)	B.P. test (5 ml. samples)
42	31	25	43
(41·6)	(30·2)	(25·0)	(43·0)

Figures give the number of samples out of 50 showing growth.

The B.P. test is carried out on 1 ml. samples of oil. In order to make the results more comparable with the results of the other two tests the B.P. test was repeated using 5 ml. samples of oil.

The results for the three possible pairs of the three "Tests for Sterility"

have been compared by obtaining the corresponding values of χ^2 from a 2×2 contingency table. These values are given in Table VII, together with the figures representing the probability of obtaining by chance as large a value of χ^2 assuming that there was no difference between the two tests compared. The results obtained by the Filtration Test differ significantly from those obtained by the other two tests which themselves give concordant results.

TABLE VII

STATISTICAL ANALYSIS OF THE RESULTS GIVEN IN TABLE VI

Comparison	χ^2	P
Modified limiting viable count technique compared with the filtration test	6·42	0·01 to 0·02
Modified limiting viable count technique compared with the B.P. test (5 ml. samples)	0·142	0·5 to 0·7
Filtration test compared with the B.P. test (5 ml. samples)	8·352	0·001 to 0·01

P is the probability of obtaining by chance as big a value of χ^2 as the one shown if there is no difference between the results compared.

In all work with "Tests for Sterility" it is assumed that all manipulations will be carried out aseptically. In practice a low proportion of accidental contaminations does occur. Whenever in the above experiments a sample of an "Oil for Sterility Test" was examined an identical test was carried out on a sample of sterile oil. In these controls for the B.P. test no accidental contamination occurred. The proportion of controls accidentally contaminated was 4·0 per cent. for the Filtration Test and 4·7 per cent. for the modified limiting viable count technique. It is to be expected that these figures will vary with the worker and with the laboratory but they should always be low. With a skilled worker accidental contamination does not appreciably alter the results obtained by the 3 "Tests for Sterility." This can be seen by comparing in Table VI the figures not in brackets which were obtained by not allowing for accidental contamination with those in brackets which were obtained from the same results but making an allowance for accidental contamination.

DISCUSSION

Of the "Tests for Sterility" of oils examined, two ensure the transfer of the organisms from the oil phase to the aqueous phase of the nutrient media by dissolving out the oil in a volatile solvent and removing and washing mechanically the organisms by filtration or by centrifugal force before taking up in water or broth. In the B.P. "Test for Sterility" simple shaking of the oil and broth is relied upon to effect the transfer. At first sight this latter method does not appear to be very effective and indeed it is possible that the effectiveness may vary with the nature of the organism, particularly it may vary according as whether the organism is, or is not, acid-fast. That the B.P. test is satisfactory in this respect for the spores of *B. subtilis* is indicated even in the preliminary results shown in Table I and analysed in Table II. Provided that the containers are shaken daily for 5 days it was found that for an oil containing approximately 4 spores in 10 g. about half the 1 ml. samples examined showed growth in the broth.

This preliminary work could not be accepted as proof of the reliability of the B.P. test however, because it did not take into account the limits of error involved in the estimate of approximately 4 spores per 10 g. of oil. Further, to use the B.P. test in this way to confirm a count amounts to "counting by dilution to extinction," a method involving considerable uncertainty[7].

3 oils suitable for use in assessing the sensitivity of "Tests for Sterility" of oils, Oils ST1, ST2 and ST3 were prepared by introducing into sterile oil a known proportion of a contaminated powder. In this way a calculated figure could be obtained for the number of spores per g. of oil. A modification of the viable count technique for oils as previously described[4] was then used to obtain an experimental figure for the number of spores per g. of oil. An inspection of the agreement between the calculated and experimental figures given in Table IV indicated the reliability of the modified technique which, to avoid confusion has been called "the limiting viable count technique." This indication was confirmed by a statistical analysis of the figures, the results of which are given in the last two columns of Table IV. It is clear that the probability values indicate that there is no significant difference between the calculated and experimental figures for the number of spores per g. of the oils examined. It was shown that the spores in the oils were evenly distributed by a different mathematical treatment (Table V) of the same experimental results. It can be seen from the probability values that the spores are evenly distributed in the oils. At this point it had been established that the limiting viable count technique was reliable and that the spores in the S.T. oils were evenly distributed in approximately known concentrations.

The Filtration and B.P. "Tests for Sterility" were then critically examined in two ways. (1) By ascertaining by inspection whether the results obtained by them were consistent with the known concentrations of the spores in the oils; (2) by comparing statistically the results obtained by them with the results obtained by using the limiting viable count technique as a "Test for Sterility," i.e., by recording only growth or no growth from each sample instead of recording the number of colonies obtained from each sample.

It was first shown that with an oil containing 2 spores per g. all 3 tests consistently indicated contamination in all samples.

As shown in Table VI the B.P. test gave 25 positives out of 50 $\times$ 1 ml. samples of an oil containing approximately 4 spores in 10 g. Such a result would give rise to considerable confidence in the B.P. test. According to the other results quoted in Table VI the most sensitive "Test for Sterility" was the modified limiting viable count technique since with 50 samples 42 positives were recorded. Although 5 g. samples were taken for this test it will be recalled that only half of the powder recovered from the oil in each case was tubed and examined for growth; thus the actual results correspond to 2·5 g. samples of the oil, which on the average could be expected to contain only one spore each. The B.P. test came next with 43 positives out of 50 $\times$ 5 ml. samples, each sample liable to contain on an average 2 spores. The Filtration Test gave a lower recovery of 31

positives out of 50 × 5 ml. samples. These results indicate that all 3 tests as applied to oils are capable of giving a positive result with a sample of oil containing only one spore.

The above conclusions, drawn from a simple inspection of the figures are supported by the statistical analysis presented in Table VII. It is here shown that the limiting viable count technique relating to 2·5 g. samples of Oil ST3 gave similar results to the B.P. test using 5 ml. samples while the results obtained by the Filtration Test were significantly different but only slightly less satisfactory.

It should be pointed out that these conclusions by no means show that the Filtration Test is not satisfactory although in our hands it has proved to be somewhat less sensitive than the two other tests in the absence of antiseptics. One of the claims made for the Filtration Test was that in it oil soluble antiseptics could be extracted from the organisms rendering them capable of multiplying in broth when otherwise they would not have multiplied even in a suitable nutrient medium or, presumably, if introduced into animal tissues.

In the experiments reported in this paper only the spores of *B. subtilis* have been used as test organisms. It should be remembered in this respect that there is evidence that vegetative bacteria tend to die off in oils and other systems of low moisture content[1]. Work is indeed at present in hand using *Str. fæcalis* as test organism and it is hoped later to use anærobic bacteria and spores. Meanwhile it was thought that the results obtained so far were of sufficient interest to warrant publication.

Summary

1. The preparation of oils containing as few as 4 spores in 10 g. in even distribution has been described. Such oils are suitable for use in evaluating "Tests for Sterility."

2. A limiting viable count technique has been elaborated by means of which the number of spores in such oils can be counted with reasonable accuracy.

3. The limiting viable count technique was then modified so as to serve as a "Test for Sterility" of oil. This and the B.P. "Test for Sterility" as applied to oils and the Filtration Test of Davies and Fishburn have been compared and evaluated. Although all 3 tests were found to be reasonably satisfactory the above order was that of decreasing sensitivity.

It is a pleasure to express our thanks to Mr. A. M. Walker, B.A., for suggestions and advice concerning the statistical treatment of the results reported in this paper.

References

1. Bullock and Keepe, *J. Pharm. Pharmacol.*, 1951, **3**, 717.
2. Davies and Fishburn, *ibid.*, 1946, **3**, 365.
3. Bullock, Keepe and Rawlins, *ibid.*, 1949, **1**, 878.
4. Bullock and Keepe, *ibid.*, 1951, **3**, 700.
5. Curran and Evans, *J. Bact.*, 1942, **43**, 125.
6. King and Alexander, *J. gen. Microb.*, 1948, **2**, 315.
7. Porter, *Bacterial Chemistry and Physiology*, 3rd Ed., Wiley, New York, 1947, p. 97. (14 References).

DISCUSSION

The paper was presented by MR. N. H. BOOTH.

MR. E. ADAMS (Plymouth) suggested the use of a tube of 8 mm. internal diameter by 25 mm. long, open at both ends, half immersed inside one of the bottles. He considered that perhaps the insertion of such a tube, into which the oil was transferred, brought the oil into contact with the broth and allowed the organisms to diffuse downwards and into the surrounding medium which was in contact with the air, thus obviating the necessity for shaking both the narrow bottles and the wide jars.

MR. G. SYKES (Nottingham) said there was little doubt that the B.P. method of shaking the mixture of oil and broth daily was a great improvement over leaving it to stand for 5 days. In connection with work which he had done on filtration of oils, an endeavour had been made to estimate the number of organisms in the oils, and one of the first observations made was that vegetative organisms died out very rapidly. An attempt to recover bacterial spores in a similar way was not quite so successful as that of Dr. Bullock and his co-workers. It was suspected that organisms were being lost somehow, and having made the suspension of organisms in the oil, shaking them once with water or with broth gave only a partial recovery—something of the order of 40 to 60 per cent.—of the anticipated number of organisms. Similarly, in an aqueous suspension of bacterial spores, when shaken with oil the organisms disappeared from the aqueous phase. In terms of the filtration test, he was less fortunate in the choice of organisms than Dr. Bullock and his team in that the treatment of the organisms with light petroleum always gave a significant kill. In order to avoid the use of the word "sterile" on labels, a more true statement of scientific fact would be to say "sterilised" or "Passed the test for sterility."

DR. G. E. FOSTER (Dartford) said that although the paper was a valuable contribution, it could only be regarded as a beginning, because in carrying out tests for sterility a great number of factors were involved. From experience he appreciated that the composition of the culture medium was important.

MR. A. ROYCE (Nottingham) said that he had some experience in testing oils for sterility. He agreed that narrow tubes were not very good and that the wide tube gave much better results. A sloppy agar (0·2 to 0·25 per cent.) was used, and it was found that the recoveries from a lightly contaminated sample of oil were improved by as much as 50 per cent. by shaking with this medium instead of plain broth. It would be interesting to know whether the authors had tried varying the culture medium.

MR. J. W. LIGHTBOWN (Mill Hill) expressed the view that the method of contaminating the oils should be carefully examined. The oil was being contaminated, not with bacterial cells or spores, but with spray dried peptone particles in which the organisms were embedded. The size of the spray dried particles (something of the order of 20 to 50 μ) was larger than that of a spore. Also the surface of the particles must be quite different from that of the spore. Those two factors would

have an influence in assessing the value of the B.P. test. In the results described where the B.P. test was carried out with a continuous layer of oil over the surface of a tube, without shaking, no growth was obtained. It was likely that the spores had gone into the broth. That would be worth investigating using an oil with a fairly high concentration of peptone and examining microscopically. It was quite likely that growth was obtained on shaking because of aeration. Another factor which should be considered in the B.P. test was the presence of unsaturated fatty acids. Those were markedly toxic in small concentrations to some organisms. Obviously the strain of organism being used did not appear to be sensitive, but a number of strains of sporing ærobic organisms would not germinate in the presence of small traces of these acids. The claim that trituration caused 50 per cent. mortality of spores was interesting. Was it trituration of the spray dried powder with oil or trituration of the spray dried powder alone? It was difficult to believe that conditions were much less drastic than shaking with glass beads or particles of sand. He wondered why the authors did not use another technique of preparing the contaminated oils, that is, spray drying spores in some material which would be soluble in oil so that it would dissolve and leave the spores completely free.

MR. G. SYKES (Nottingham) raised the further point that peptone, being hygroscopic, might offer encouragement to the organisms to pass from the oily phase into the water phase.

DR. K. BULLOCK referring to the difference between his results and those obtained by Mr. Sykes, said that in the work done by Mr. Sykes there was some moisture present whereas in his own work there was not. He had been able to show in another connection that, in the absence of moisture, enzyme systems in general were much more resistant to all toxic agents which he had tried, particularly volatile solvents. In his view the difference in results might be due to the amount of moisture present.

MR. N. H. BOOTH, in reply, said that Mr. Adams's suggestion of putting an open tube into the bottle of broth was interesting, but he doubted whether it would work. With such a narrow tube there would probably be a smaller interface between the oil and broth, and better results could in those circumstances hardly be expected. With regard to the killing of organisms with solvent, he had made extensive tests with various solvents using *Bacillus subtilis* as the test organism and had found that light petroleum (b.pt. 40 to 60° C.) was quite satisfactory and did not give any kill. Previous work substantiated that vegetative organisms died off very rapidly in oils. He agreed that the culture medium used for the test had an effect on the recovery. Oil-soluble material to aid contamination of the oil had already been tried using stearin, and the use of such material had been considered. The trituration which resulted in 50 per cent. kill was of powders without oil present and carried out with a pestle and mortar. With regard to the relationship between peptone particles and spore size, milled powder had been used for contamination and the particles of peptone had been broken up as much as possible without actually killing any of the spores present. Therefore the particles

of peptone present in the oils were very much smaller than 20 to 50 μ. The point concerning aeration in the B.P. was quite valid. A sloppy agar medium was used for shaking out oils and found to be quite satisfactory.