THE REMOVAL OF BACTERIA FROM OILS BY FILTRATION

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ATTEMPTS to influence the rate of diffusion of drugs given by injection have directed attention to the importance of the vehicle and as a result of this an oily basis is frequently used for injections of thermolabile drugs. The sterilisation of these injections requires some consideration in view of opinions expressed that the method of sterile filtration so generally applicable to aqueous solutions is unsatisfactory with oils. Indeed, the Pharmacopœia of the United States (XIII, pp. 696-697) states: "Liquid petrolatum and other oils are not to be sterilised by this method as they may increase the permeability of the filter to bacteria." It then goes on to explain that the process of filtration is not simply a matter of relative sizes of pores of the filter and of particles to be filtered, but involves many other factors.

As early as 1926, Holman[1] showed that by pre-treatment with a mixture of petroleum and paraffin oil, candle filters could be rendered much more permeable to the passage of certain bacteria than before treatment or even after the removal of the oil. He did not give the porosity of his candles, but it would appear that they were not of sufficiently fine porosity to yield sterile filtrates of aqueous solutions. The observation on increased permeabilities was later extended by Kramer[2], by Varney and Bronfenbrenner[3], and by others, to vehicles other than oils. The generally accepted explanation of this phenomenon is that fine-pore filters depend for their efficiency not only on mechanical sieving but also on the relative electrical charges on the filter and on the particles in the liquid being filtered. Thus, coating a filter with oil may be considered to insulate its charge and thereby reduce its efficiency.

In contrast, Hurni[4] believed that the only method of sterilising oil, other than by dry heat, was filtration through a Berkefeld candle under reduced pressure at a temperature of 80° to 90°C. He used bacterial spores as the test organisms and presumably employed the elevated temperature to assist in the rate of filtration. He found that these same organisms suspended in oil would pass through a Seitz-type filter pad under the comparatively high filtration pressure of 1½ atmospheres, and concluded that Seitz and other asbestos-type filters are useless for the sterilisation of oils. He gave no information on the efficiency of Seitz filters under reduced pressure. Berry[5] considered that the pressure at which a filtration was carried out materially affected the efficiency of the filter, thus the contrast in response of the candle filter and filter pad reported by Hurni may have been due to the different conditions under which his experiments were carried out.

Berry[6] also stated in a paper on bacterial filtration that a Seitz filter depended for its efficiency on the swelling of the fibres of the pad and that where such swelling, caused by inbibition of the solvent being filtered, did not take place, the effectiveness of the filter was seriously impaired. Davies and Fishburn[7], on the other hand, describing their filter pad technique for sterility tests, stated that oils could be tested satisfactorily by this method, thus implying the complete retention by the filter pad of any organisms present in the oils.

In the past few years we have successfully handled some hundreds of batches of oily solutions. Each batch has been filtered under reduced pressure at normal temperatures through sterilised and dry Ford Sterimats (S.B. grade), and tested for sterility by approved methods at both the bulk and final container stages. No batch has yet been failed and only very occasionally has a repeat test been found necessary. Our success may have been due to the comparative freedom of oils from bacterial contamination (as suggested by Hurni[4]), and to the recognised difficulties in detecting organisms in small numbers in water-immiscible liquids, nevertheless, it gives substantial support to the practical value of bacterial filters in the sterilisation of oils. In view of the statements quoted above, it seemed desirable to re-investigate this whole matter, and the following paper describes some of the results of this work.

Practical Considerations.—Coulthard and Chantrill[8] and Coulthard and Croshaw[9], in private communications, have shown that hæmolytic streptococci protected with blood, and also tubercle bacilli, will remain infective in oils for periods up to several weeks, and it is well known that dry bacterial spores will also survive for prolonged periods in oils. We considered it desirable, however, to carry out our experiments with very small, unprotected, vegetative organisms, and chose, therefore, for most of the work *Bacterium prodigiosum* either on nutrient agar slopes or in broth culture. A number of preliminary tests showed that these relatively unprotected cells became non-viable in the space of a few hours when suspended in the oils commonly used as vehicles for injections, and so adequate controls were included in every group of experiments to ascertain the rate of survival during the filtration period. In some tests a soil-dust-spore mixture was substituted. Both candle filters and asbestos pad filters were tested under the different conditions outlined below.

Experimental Technique

Liquid paraffin, arachis oil and ethyl oleate, all of B.P. quality, were used in the experiments, and 18 to 24-hour cultures on agar slopes or in broth of *Bact. prodigiosum* provided the source of this test organism. It was not found possible to prepare a completely dry suspension in oil of sufficiently high viability for the purpose required, and so the following method was adopted. Small agar slope cultures were sludged with a few ml. of the oil, taking care not to break the surface of the agar, and this suspension was diluted with a large volume of sterile oil—usually 1 to 2 l.—giving a final concentrated bacterial suspension of very low moisture

content. That these organisms were virtually unprotected receives considerable support when their very high death-rate is compared with the comparative stability of blood-protected organisms such as those used by Coulthard and Chantrill[8].

Counts were made on the oil suspensions by preparing tenfold serial dilutions in the same oil (using a fresh sterile pipette at each stage) and testing each dilution for the presence of viable bacteria. This was done by a method similar to that given in the U.S.P., XIII, p. 691:—1 ml. of the sample was inoculated into 50 ml. of sterile nutrient broth in a screw-capped bottle and the mixture shaken vigorously. It was incubated at 37°C., shaking daily, and at the end of the incubation period of 5 days subcultures were made to confirm the presence or absence of viable bacteria. Counts were made in this manner just before the commencement of the filtration period and immediately on completion of the filtration, usually some 6 to 8 hours later, the latter sample being tested simultaneously with the filtrate. This precaution was taken to confirm that viable organisms were still present in the oil and to counteract as far as possible any false-negative results due to spontaneous death of bacteria in the filtrate. All the counts recorded indicate the weakest dilution showing a positive response on subculture, for example, a recorded bacterial count of 1,000 per ml. indicates growth in the 1 in 1,000 dilution but not in the 1 in 10,000 dilution.

All filtrates were examined by a similar shaken broth technique except that much larger samples, up to 50 ml. (in 5 ml. portions) were employed with correspondingly larger volumes of broth.

In experiments not using *Bact. prodigiosum*, 5 g. of a dry soil-dust-spore mixture was shaken with 1 l. of the oil, and roughly filtered to remove coarse particles. The oily suspension was then used in the manner described above.

Candle Filters.—It is our experience, based on work we hope to publish in the near future, that candles of an estimated maximum pore size of about 2·4 microns, corresponding to a "bubble-pressure test" in water of about 18 lb. per sq. in, must be used to obtain a sterile filtrate from a nutrient solution over a sustained filtration period. We, therefore, chose for this series of experiments Doulton and Berkefeld candles of bubble pressure to 18 to 22 lb./sq. in. We also included a few tests with candles of greater pore size with bubble pressures of approximately 10 lb./sq. in. The bacterial suspensions in oils were prepared as already described and the filtrations were carried out under reduced pressures. The results of the experiments are given in Table I.

It is obvious that the rate of filtration of the relatively viscous oils through candles of such porosity is very low, too low in fact to be of practical value on the large scale. The results do show, however, that candles which are reliable in giving sterile aqueous filtrates will sterilise even highly contaminated oils. A candle of greater pore size, and, therefore, not suitable for sterile filtration of aqueous solutions, was also found

unsuitable for removing small organisms such as *Bact. prodigiosum* but was apparently effective in removing the larger sized bacterial spores.

Ford Sterimat Filters.—Single 14 cm. Ford Sterimats (S.B. and F.C. grades) were sterilised and used dry for these experiments. Filtrations were carried out at reduced pressures (Table II) and also at positive

TABLE I

FILTRATION OF OILS THROUGH CANDLES AT REDUCED PRESSURES

Type of candle	Test organism	Oil	Period of filtration (hours)	Volume filtered (litres)	Count/ml. of oil during experiment		Tests on filtrate	
					Start	Finish	Samples Tested	Number Sterile
18 to 22 lb. bubble pressure	*Bact. prodigiosum*	Arachis oil	6·5	1	1000	100	10×5 ml.	All
		Liquid paraffin	24	1	1000	100	,,	,,
		Ethyl oleate	6·5	2·5	100	10	,,	,,
18 to 22 lb bubble pressure	Soil-dust-spores	Arachis oil	6·5	0·5	1000	1000	10×5 ml.	All
		Liquid paraffin	24	1	1000	100	,,	,,
		Ethyl oleate	6·5	3	1000	100	,,	,,
10 lb. bubble pressure	*Bact. prodigiosum*	Arachis oil	6	1·5	1000	100	10×5 ml.	4×5 ml.
	Soil-dust-spores	,, ,,	6·5	3·5	1000	1000	,,	All

TABLE II

FILTRATION OF OILS THROUGH FORD STERIMATS AT REDUCED PRESSURES

Filter grade	Test organism	Oil	Period of filtration (hours)	Volume filtered (litres)	Count/ml. of oil during experiment		Tests on filtrate	
					Start	Finish	Samples tested	Number sterile
S.B.	*Bact. prodigiosum*	Arachis oil	4	1·5	100,000	1000	10×5 ml.	All
		Liquid paraffin	7	2	10,000	1000	,,	,,
S.B.	Soil-dust-spores	Arachis oil	7	1	1000	1000	10×5 ml.	All
		Liquid paraffin	7	1	1000	1000	,,	,,
S.B.	*Bact. prodigiosum* in broth culture*	Arachis oil	4	1	100,000	100,000	10×5 ml.	All
		Liquid paraffin	6·5	0·5	100,000	100,000	,,	,,
F.C. ...	*Bact. prodigiosum*	Arachis oil	0·5	1	100	100	3×5 ml.	2×5 ml.

* Prepared by shaking 1 per cent. of a broth culture of *Bact. prodigiosum* into a temporary emulsion in the oil.

pressures of 25 lb./sq. in. (Table III); the latter in an attempt to reproduce the finding of Hurni (*v.s.*). The rate of filtration was greater than with candle filters and, here again, all experiments using the sterilising grade of filter (S.B.) were completely successful, and there appeared

TABLE III

FILTRATION OF OILS THROUGH FORD STERIMATS UNDER POSITIVE PRESSURE

Test organism	Oil	Period of filtration (hours)	Volume filtered (litres)	Count/ml. of oil during experiment		Tests on filtrate	
				Start	Finish	Samples tested	Number sterile
Bact. prodigiosum ...	Arachis oil	4	2·5	10,000	10,000	10 × 5 ml.	All
	Liquid paraffin ...	3·5	3	1000	1000	,,	,,
	Ethyl oleate	2	10	100	10	,,	,,
Soil-dust-spores ...	Arachis oil	3·5	2·5	1000	1000	10 × 5 ml.	All
	Liquid paraffin ...	4	2·5	1000	100	,,	,,
	Ethyl oleate	2·75	18	1000	100	,,	,,
Bact. prodigiosum in broth culture*	Liquid paraffin ...	4	3	1000	1000	10 × 5 ml.	All
	Ethyl oleate	2	10	100	10	,,	,,

All filtrations using S.B. grade pads, and under an applied pressure of 25 lb./sq. in.
* Prepared by shaking 1 per cent. of a broth culture of *Bact. prodogiosum* into a temporary emulsion in the oil.

to be no difference between filtrations carried out under reduced pressure or at the positive pressure employed, although, of course, the rate of filtration was materially affected.

The F.C. grade filter, only intended for use as a clarifying filter, failed, as anticipated, to hold back bacteria in oily suspension.

ADDITIONAL TESTS

A small number of experiments, involving animal inoculations as well as *in vitro* tests, have been carried out by Coulthard, Chantrill and Royce[10] and the results will be reported in detail elsewhere. Briefly, 25 ml. amounts of heat-sterilised liquid paraffin were inoculated with *Streptococcus hæmolyticus* (*Richards strain*) by adding small amounts of blood taken from a mouse fatally infected with the organism. In one instance 2 drops of blood were added giving a theoretical count of 28,000 viable cells per ml. of oil, and in another 0·1 ml. of blood yielding theoretically 3,000 cells per ml. of oil. Part of the infected oil was filtered under reduced pressure through a 6 cm. Ford Sterimat (S.B.). Filtered and unfiltered portions of the oils were injected intraperitoneally into groups of about 15 mice. In each case, all the mice injected with the filtered oil survived the test period of 5 days, and all those injected with the unfiltered oil died within 48 hours. At the same time, counts were made on the oils as outlined in an earlier section but using 10 per cent. serum broth as the cultage medium. No bacteria were found in 0·6 ml. of the filtered oil, but the unfiltered oil had streptococci in the 1 in 1,000 dilution and not in the 1 in 10,000 dilution.

Breakdown Filtrations.—It has already been shown in the foregoing experiments that filters which are effective in removing bacteria from aqueous media are also effective in removing them from oily vehicles. In an attempt to find the conditions under which filters cease to be effec-

tive, as suggested by Holman[1] and others, a number of experiments have been carried out filtering oil-broth mixtures and also oil and aqueous suspensions alternately.

The oil-broth mixtures were made by shaking 10 ml. of an 18 to 24-hour broth culture of *Bact. prodigiosum* into a temporary emulsion in 1 l. of oil (giving a 1 per cent. water content). Filtrations were through Sterimats (S.B.) at either reduced pressures or under positive pressures, the oil suspension being shaken at intervals. Five such experiments were carried out, two with arachis oil, two with liquid paraffin and one with ethyl oleate. Although high initial counts of well over 100,000 viable cells per ml. of oil were found in some cases, the filtrates were all sterile, that is, no organisms were detected in 50 ml. of oil. Thus, the presence of small amounts of water admixed with the oil did not affect the efficiency of the filtration.

When 500 ml. of a diluted broth culture of *Bact. prodigiosum* (1 ml. of culture in 5 l. of saline solution) was first put through a Sterimat followed by 250 ml. of arachis oil and then a further 250 ml. of aqueous culture the whole of each broth fraction and the oil were found to be sterile. On the other hand, when a dry pad was saturated with oil and then an aqueous suspension of the test organism forced through it, the aqueous fraction was heavily contaminated. In one experiment the count was between 10 and 100 organisms/ml. and in another over 1,000 organisms/ml. (Table IV.)

In contrast, candle filters of 18 to 22 lb. "bubble pressure," which had previously been impregnated with arachis oil, liquid paraffin, or ethyl oleate, were completely successful in preventing the passage of bacteria in aqueous suspension even though the filtrations were continued for periods of 6 to 7 hours. (Table IV.)

TABLE IV

FILTRATION OF ALTERNATE OIL AND AQUEOUS SUSPENSIONS

Filter	Oil	Successive fractions filtered			Results
		1st	2nd	3rd	
Sterimat S.B. ...	Arachis oil	Saline + *Bact. prodigiosum*	Oil + soil-dust-spores	Saline + *Bact. prodigiosum*	All fractions sterile
,, ,,	,,	Oil	Saline + *Bact. prodigiosum*		Aqueous fraction contaminated
Candle 18 to 22 lb. bubble pressure	Arachis oil	Oil	Saline + *Bact. prodigiosum*		Aqueous fraction sterile
	Liquid paraffin	,,	,,		,,
	Ethyl oleate	,,	,,		,,
Candle 10 lb. bubble pressure	Arachis oil	Oil	Saline + *Bact. prodigiosum*		Aqueous fraction contaminated.

Discussion

The time factor is probably very important in sterile filtration and it may well be that filters, satisfactory when the period of filtration is brief, will break down when used over longer periods. This is, of course, particularly likely to occur with nutrient solutions. Asbestos pad filters of the type Ford Sterimat (S.B. grade) are quite satisfactory for periods of a few hours. In the case of candle filters, a grade giving a response of 18 lb./sq. in. in the " bubble pressure" test in water (equivalent to an estimated maximum pore size of 2·4 microns) may be necessary for filtrations sustained during several hours, whereas a grade of about 12 lb./sq. in. response may be satisfactory for short period filtrations. From the experiments reported here, using a variety of oily vehicles and of bacteria, it appears that these same standards might safely be applied to the sterile filtration of oils.

We have no evidence to show that filters of sufficiently good quality are normally less efficient in dealing with oily than with aqueous preparations. Neither have we any evidence to support the claim put forward by Hurni that pressure filtration through asbestos filters is unreliable. We have indicated, however, that coarser grade filters, the Sterimat F.C. grade or larger pore filter candles, which are not claimed to hold back all bacteria, do not yield sterile filtrates.

Filters have been caused to break down by somewhat artificial means, for example, by alternatively filtering oil and water. This applied to Sterimats only and not to candle filters as claimed by Holman. The difference was most probably due to Holman's use of a coarser grade candle, since he did not claim in the the first place to obtain a completely sterile filtrate even with an aqueous solution. This finding gives support to the electrostatic theory of filtration, but it also shows that the phenomenon is manifest over only a limited range of porosity and can be offset by the probably more reliable mechanical process of filtration with filters of sufficiently fine pore size. The fact that the efficiency of some filters can be reduced by coating with oil has little or no practical application with Seitz-type pads, which are used only once, but it may be of importance in dealing with candle filters. These should be washed free of oily vehicle before being re-used for aqueous solutions.

Summary

(1) Oils deliberately contaminated with *Bact. prodigiosum*, hæmolytic streptococci or bacterial spores have been successfully sterilised by filtration through Ford Sterimats or candle filters. Large scale batches of oily injections have also been handled with equal success over many years.

(2) It is important that the right grade of filter be used. Filters of grades suitable for the sterilisation of aqueous solutions are also suitable for handling oily vehicles.

(3) Sterimats have been caused to break down by first " insulating " the pads with oil and then filtering an aqueous suspension of organisms.

They are, however, satisfactory with oils containing only a small amount of water, or if a previously wetted pad is used.

(4) The breakdown effect has not been demonstrated with candles of sufficiently fine pore size.

It is a pleasure to record our thanks to Mr. C. Bowler for his able technical assistance. We are also indebted to Mr. C. E. Coulthard for his interest and criticisms, and to the Directors of Boots Pure Drug Co., Ltd., for permission to publish this work.

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DISCUSSION

An abstract of the paper was read by Mr. Sykes.

PROFESSOR H. BERRY (London) thought that there was difficulty in proving the results in work of this kind. The forcing of oil through a filter with a dispersed aqueous suspension of bacteria was not to be trusted, because of the difficulty that water has in passing through an oil-wetted filter. Normally oils were infected with air-dried organisms and it was therefore preferable to obtain a test organism which could be deprived of surface moisture and remain viable. Also the organism should be of the size of, say, *Bact. prodigiosum*, 0·75 mμ or it was no real test of the filter. He had tried to dry organisms with acetone but *Bact. prodigiosum*, *Bact. coli* and staphylococci died very rapidly. *B. subtilis*, particularly the spore form, would survive for long periods in acetone but was not a suitable organism with which to test the filter. He accepted the authors' results, but their negative character reduced their significance. He would like to know the size of the dust particles used and if the authors could confirm any difference in the behaviour of the Seitz pad when dry, wetted with water, and when wetted with oil. Had they observed a critical pore value for a filter candle which would pass either aqueous-wetted bacteria or bacteria wetted with oil ?

MISS V. W. BURRELL (Pinner) said that she had successfully used spores of *B. subtilis* and, for vegetative organisms, *Bact. coli* in similar experiments. Sterilisation had been effected by Ford sterimats of G.S. and S.B. grades. Suspensions of suitably dry organisms were obtained by taking a small portion of an agar slope culture and rubbing it down in a mortar. The experiments were all carried out under vacuum, and air was eventually allowed to pass in after being dried with sulphuric

acid. Out of well over a 100 heavily contaminated experimental batches, only two were found not to be sterile, possibly due to breakdown of the filter.

DR. K. BULLOCK (Manchester) asked for details of the authors' sludging process. When he had tried it he obtained an emulsion presumably of clumps of bacteria in a water phase in oil. It would not surprise him if they were filtered off by an oil-saturated filter. Did the authors check whether there was any clumping or whether the organisms were evenly distributed, and had they tested the oil for sterility before filtration as well as afterwards?

MR. R. LEVIN (Leeds) said that he had subcultured previously contaminated oils after incubating them for 6 weeks. Olive oil, with and without a fungstatic, showed growth, but samples of ethyl oleate without a fungistat did not. Had the authors any similar experiences and had they found similar properties for ethyl oleate?

MR. G. SYKES, replying to the discussion, said that the making of a bacterial suspension from an agar slope was the nearest they could get to the ideal. Time, porosity, thickness of the filter bed and the nature of the solution were all important factors in the filtration of bacteria. They had not tested production batches of oily preparations before filtration as the survival of bacteria in oils was very low and it was difficult to test oils for the presence of so few bacteria. It was possible that self-sterilisation occasionally occurred.

MR. A. ROYCE said that they, too, had found that certain organisms did not survive treatment with acetone. Their soil dust preparations were ground down in a mortar and were fairly fine. After initial trituration with oil, the mixture was filtered through gauze or thin cotton wool and no large particles of dust were visible in the final preparation. However, they could not guarantee that they had a suspension of single organisms only. A considerable number of organisms in an aqueous phase passed through an oil-saturated pad, but an oil filtrate from a water-wetted pad was sterile. Subsequent aqueous fractions filtered through the now oil-coated water-wetted pad gave filtrates which were also sterile. Work had not yet been completed on critical pore values, nor had G.S. grade pads been tested, and no attempts had been made to achieve perfect dryness in the system. The sludging technique consisted of adding a few drops of oil to an agar slope, mixing and then triturating the concentrate in a mortar with a large quantity of oil. Although a high dilution was used and there were no visible clumps they had not done any experiments to prove the absence of clumping. Ethyl oleate was markedly hostile to vegetative organisms, but the authors did not agree that it was self-sterilising.