STERILIZATION OF AIR BY HEAT

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(With 5 Figures in the Text)

INTRODUCTION

The need for the production and supply in adequate quantities of sterile air in many microbiological processes is evident and several workers have considered the problem. In particular Stark & Pohler (1950) have summarized the following criteria for an ideal sterile air system as the ultimate aim for future work:

(1) Complete elimination of all viable micro-organisms.
(2) A high degree of reliability.
(3) Ease and simplicity of operation.
(4) Minimum capital and operating costs.

Sykes & Carter (1954) have reviewed several methods of air sterilization and concluded that scrubbing methods, using chemical disinfectants, are unsatisfactory owing to the danger of carry-over into the culture medium. They state that heat is too expensive, and that ultraviolet light and electrostatic precipitation are inefficient. They have shown that granular and fibrous filter materials can be used, and that slag wool is the best of all. While this assessment is no doubt true for an industrial process, we believe that on the criteria quoted above, sterilization by heat has the greater merit except over capital and operating costs, and that in experimental work up to pilot scale its advantages of greater reliability and efficiency are overwhelming.

The disadvantages of filters are well known. In the first place great care must be taken in packing the filter-bed. Secondly, the bed is liable to disruption with consequent loss of efficiency during steam sterilization, or during operation if the equipment is subjected to rapidly changing air velocities. Thirdly, unless precautions are taken to ensure that the air to be filtered is not fully saturated with water, the passage of infected droplets of water is another serious hazard. Lastly, it is necessary to ensure that the compressors supplying the air are kept in sound mechanical condition so that the filter-bed does not become clogged with oil droplets. On the other hand, the operation of a heat sterilizer requires control of two process variables only: the temperature of the air in the exposure chamber, and the air rate.

Under the best laboratory conditions the minimum penetration reported by Thomas (1952) and Henderson (1952) was one particle in 10,000 for 1μ particles of methylene blue. In the work to be described we have shown that, by using heat sterilization, penetration can be reduced to one particle in 470 million.

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Current opinions on testing the sterility of air are well represented by the views of Parker & Cherry (1950) who state:

'...When dealing with large quantities of air, it is difficult to assess the efficiency of the filtration system. If the system is operating at nearly 100 % the size of samples which can be taken by slit samplers is very small when compared with the whole, and the chances of trapping the odd organism which may be passing are proportionately small. Alternatively, the use of a vessel as a test tank containing agitated sterile medium, through which a much larger sample of the air passing through the filtration system can be taken and tested, only distinguishes between sterile and non-sterile air and does not give any measure of the efficiency of filtration.

The fact that the system can be operated on the large scale to give long series of batches without contamination is perhaps the best criterion that can be applied and when this operates as in our experience it does, the system can be said to be giving sterile air....'

This view has not been universally adopted and work on testing the sterility of air has been pursued and described by Bourdillon, Lidwell & Lovelock (1948), Terjersen & Cherry (1947), and by Decker, Citek, Harsted, Gross & Piper (1954). By heating grossly contaminated air in a furnace Bourdillon *et al.* concluded that the survival rate of spores was not more than 1 in 4000. Terjersen & Cherry, who sterilized by filtration, quoted survivals of not greater than 1 in 270,000; whilst Decker *et al.* claimed values of not more than one in a million for a proprietary heat sterilizer which they tested.

In the work described in the present paper we have shown that a heat sterilizer, with an estimated minimum exposure time of 0·14 sec. at 300° C., gives a spore penetration of not more than one in 470 million at the most conservative estimate. The sensitivity of the test has been improved by increasing the dosage of spores and the sample size, by the substitution of fibrous filters for the slit sampler used by other workers, and by the adoption of scrupulous aseptic techniques.

The test method adopted, a 140 l./min. air sterilizer, and a larger version with a capacity of 1700 l./min. are described below.

EXPERIMENTAL

Test method

Use of micro-filter. The slit sampler was developed for quantitative use in air hygiene studies to detect minimum concentrations of air-borne bacteria, but it is not an ideal instrument for testing absolute sterility. It has the following disadvantages, from which the sieve sampling method used by Decker *et al.* also suffers:

(1) For whatever purpose used, the chance of contamination by air-borne organisms in the surrounding atmosphere is high. This introduces the complication of requiring a distinction to be made between the extraneous organisms and the tracer organism in the infected air.

(2) It is not easy to disinfect the sampler after gross contamination.

With the micro-filter described by Henderson (1952) these disadvantages are minimized. The contrast in the degree of chance contamination between the various methods is shown in Table 1, which gives the proportion found contaminated in

control samples, subjected to all manipulations except the passage of the air to be examined; these samples will subsequently be referred to as *handling controls*.

Table 1. *Fortuitous contamination*

Sampling method	Total no. of samples	No. of contaminated samples	% contamination
Slit sampler*	25	17	68
Sieve sampler†	72	13	18
Micro-filter‡	427	10	2·3

* Terjersen & Cherry (1947).
† Decker, Citek, Harsted, Gross & Piper (1954).
‡ Present study.

The collection efficiency (as defined by Bourdillon *et al.* 1948, p. 21), of a micro-filter is 98·6 % compared with 92–97 % for a slit sampler; and its pressure drop is 12 in. of water at an air flow of 14 l./min. It can be coupled direct to the pipe-line to be sampled, and provided this is at a sufficient pressure there is no need for the complications of the aspiration device which forms part of the slit sampler.

Construction of a micro-filter. When a few are required, they can be machined out of brass or aluminium but in quantity are best made from a moulded *PF* plastic (Fig. 1). The filter-bed is $\frac{1}{2}$ in. in diameter, and $\frac{5}{8}$ in. long. It is packed with 0·16 g. of filter material consisting of a carded mixture of merino wool noils and blue asbestos in the proportion of 88 parts by weight of wool to 12 parts by weight of asbestos, which may be obtained from the Cape Asbestos Co., Barking, Essex.

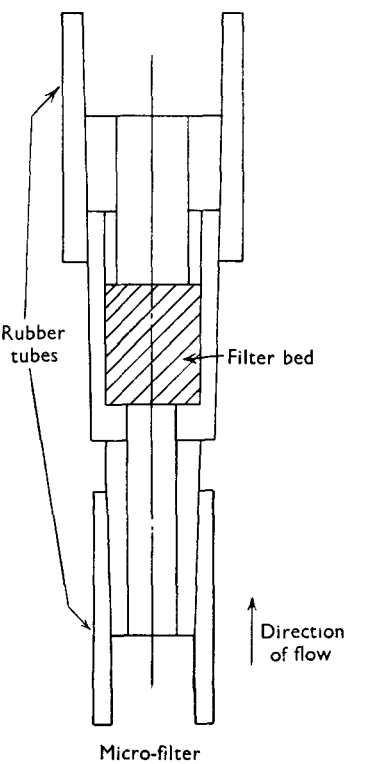

Fig. 1

Sampler efficiency. As Terjersen & Cherry (1947) have pointed out, since identical samplers are used both for the infected and treated air, the absolute value of the sampling efficiency is not of direct importance for the determination of the degree of penetration occurring in the sterilizer. It is, nevertheless, of some interest to know this figure as it furnishes a measure of the sensitivity. Collection efficiency is determined by passing an aerosol of suitable particle size through a pair of filters in series. The number of particles collected on each filter is determined by methods to be described later, and the collection efficiency is expressed as

$$\frac{N_1 - N_2}{N_1} \times 100\,\%,$$

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where N_1 and N_2 represent the number of particles found in the first and second filters respectively. As collection efficiency approaches 100 %, the confusion that results in considering a value consisting of a succession of nines can be resolved by expressing efficiency as penetration (N_2/N_1) written in the form 'One in N_1/N_2'.

Results obtained from a slit sampler are interpreted by assuming that every colony on the agar plate (after allowing for those introduced fortuitously) represents an infected particle originally present in the air stream being sampled. It is thus a quantitative tool. In our use of the micro-filter we have used a qualitative method to show whether or not infection is present. It is therefore essential to show that the complete method (i.e. filtration of the air followed by culture of the filter material) will detect a minimum number of organisms.

The sampling point. The loss of particles during collection is discussed by Bourdillon *et al.* (1948, p. 27). They show that this is negligible for particles of less than 10 μ diameter and is minimized by using short, straight and narrow-bore pipes. In our work iso-kinetic sampling was not adopted, but the length of connexion between the sample point and the filter was reduced to a straight piece of 0·635 in. inside diameter pipe ($\frac{1}{2}$ in. British Standard Pipe), 12 in. in length.

Steam sterilization of equipment

A typical arrangement used for the 140 l./min. sterilizer is shown in Fig. 2. Air is supplied through valve 5 to the base of the heater. A Collison spray (Henderson, 1952) injects a cloud of organisms into the ingoing air which after treatment in the heater passes to atmosphere at valve 3. A portion of the treated air stream is sampled at valve 2. The filter attached to the exit of valve 3 prevents contamination of the laboratory air should the heater fail to sterilize.

Sterilization follows accepted principles. With the heater switched off and the spray bottle disconnected, steam from a supply at 20–30 lb./in.^{2} gauge pressure is introduced through valve 1 and purged through valves 2–4, these being adjusted to maintain the internal pressure of the system at a minimum of 20 lb./in.^{2} gauge pressure. The outlets of valves 2 and 3 are fitted with caps (Fig. 3). After a suitable time of 1–4 hr. the heater is switched on and, when it reaches a steady maximum temperature, the valves 2, 3, 4 and 1 are shut in this order (including the valves on the cap in the case of valves 2 and 3 which are shut before the main valve). Compressed air is then introduced at valve 5 to give a positive pressure of 5–10 lb./in.^{2} in the system and to prevent the ingress of contaminated atmospheric air. Care is taken to charge the air slowly so as not to exceed the sterilizing capacity of the heater. In practice this amounts to admitting air at a predetermined rate and gauging flow by the rate of increase of the internal pressure. Ideally there should be some form of flowmeter between valve 5 and the heater inlet. A merino wool-asbestos filter, having a filter-bed $\frac{1}{2}$ in. thick and $5\frac{3}{4}$ in. in diameter and containing 49 g. of filter material, is steam sterilized in an autoclave and fitted aseptically in place of the cap on valve 3. A suitable flowmeter is fitted to the filter exit, arranged to purge the delivery of air outside the building or, if not expedient, at a point remote from the sampling chamber. The air flow is controlled at valve 5

with valve 3 wide open, or vice versa depending on whether the system is to be operated at near-atmospheric or at the pressure of the air main.

Spore injection. A distilled-water suspension of *Bacillus subtilis* spores (Porton 'U' strain) is prepared by heating to 60° C. for 30 min. and is then diluted to a

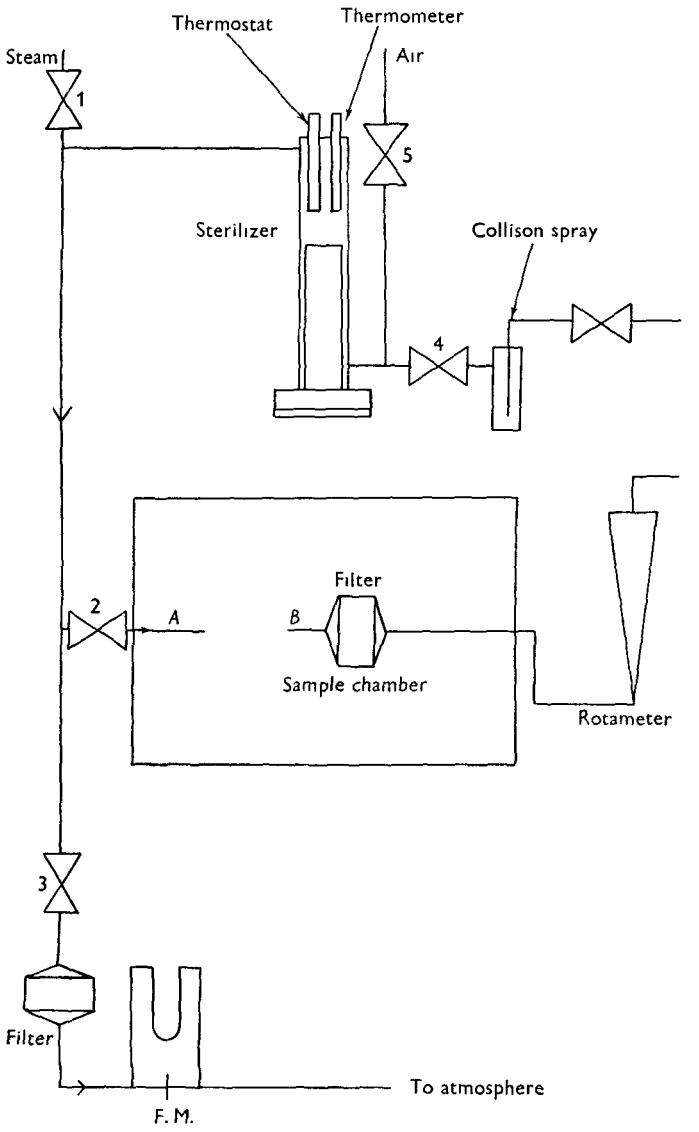

Fig. 2

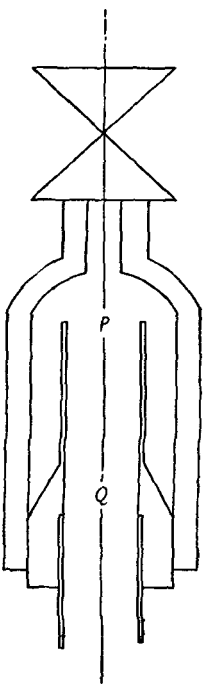

Fig. 3. Connection made aseptically to portion *PQ*.

concentration of 1×10^9 viable cells/ml. This is dispersed from the Collison type spray, fitted in a stainless steel bottle (net capacity 70 ml. suspension) which is coupled to valve 4, with a pressure gauge, control valve and air supply on the upstream side.

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Handling, connexion and operation of the spray bottle is carried out by an operator who takes no part in sample taking. For an aperture setting of 0·008 in. the differential air pressure across the spray is adjusted to 30 lb./in.^{2} which on opening valve 4 gives an injection rate of 40 ml./hr. of suspension, allowing about 90 min. operation for each charge.

Sample taking. This is performed in a glass or Perspex-fronted chamber enclosing the parts shown in Fig. 2. Before sampling, the chamber is washed down with diluted sodium hypochlorite solution (e.g. 10 %, v/v, of Chloros in water). All subsequent operations are performed by a gowned operator who, as described, has not taken part in spore injection. Before operations the hands and forearms must be washed, and a disinfected gown fitted. During the sampling period the operator must not handle or touch extraneous equipment. A freshly sterilized $5\frac{3}{4}$ in. diameter filter is placed in the chamber and connected to the rotameter. The protective cover on the glass tube *B* which forms the filter inlet, and the cap on the pipe at *A* adjacent to valve 2, are removed. A sterile rubber connector and glass tube are aseptically coupled at *A*.

A sampling filter is connected and disconnected as follows: a rack containing a supply of sterile filters enclosed in cotton-wool plugged tubes is placed near the chamber. A tube is conveyed to the Bunsen burner in the chamber and, using aseptic precautions, the filter is removed and the tube, with the plug re-inserted, returned to the rack. After flaming the exposed glass tubes (*A* and *B*) the sterile filter is connected between them, with the female end of the filter pointing upstream. On disconnexion, the filter is flamed at both ends before insertion (female end upwards) into its original tube which has been returned to the chamber for this operation. It is stressed that in order to minimize chance infection this rigorous procedure is necessary.

A handling control sample is taken by connecting and disconnecting a filter, without the passage of air. For an air sample, the rate of flow is adjusted at valve 2 to give 14 l./min. for a period of 5 min., giving a sample size of 70 l.

Spore concentration. Deposition of spores through impact effects occurs between the injection point and the sample point. To determine the maximum concentration recoverable at the sample point injections are made with the heater at room temperature. Samples are taken as previously described and the filter wads are removed from the filters and placed in 1 oz. screw-capped bottles containing 10 ml. sterile distilled water and 0·05 % Manucol, as well as a few glass beads. The bottles are shaken until the wool disintegrates, and a spore count is done on the supernate. This method has been found to give satisfactory extraction. At the conclusion of such tests the sampling chamber is decontaminated.

Sterility of filters. This is evaluated on the results of three tests:

(1) The first, called a *filter control*, is carried out on a series of sterilized filters, the wads from which are ejected into sterile broth, which is then examined for growth after 5 days incubation. This determines the efficiency of filter sterilization and the transfer of filters into sterile broth.

(2) The second, called a *handling control*, is performed on another series of filters which are connected and disconnected at the sampling point and then treated as in

(1) above. This, in conjunction with the first test, shows the hazards arising in the sampling chamber.

(3) The third, called the *infected air sample*, includes, as well as the foregoing risks, measurement of the penetration through the heat sterilizer.

All filters are examined in the same way and at the same time. The room in which the subsequent operations are carried out is prepared by closing windows and doors and washing down surfaces with diluted Chloros. Fifteen minutes after preparation, the gowned operator, observing aseptic practice, transfers each filter wad into 25 ml. of sterile tryptic meat broth (such as that of Cole and Onslow) contained in a $7 \times 1\frac{1}{2}$ in. diameter cotton-wool plugged tube. The tubes are then incubated at 37° C. for 5 days. When growth is detected the culture is examined to determine whether the organism is *Bacillus subtilis* or otherwise.

An air sterilizer (140 *l./min. capacity*)

This is shown in Fig. 4. It was adapted from a heater intended to sterilize air vented into tanks of sterile broth and is constructed from stainless steel to B.S.S. En. 58B. The heater element is wound on a porcelain former, a suitable type of which can be obtained from Messrs Ferranti Ltd. The thermostat is set to operate at 310° C., and controls the air temperature to $\pm 25°$ C.

A precise value for exposure time is difficult to give since it is made up of three parts. First, heating-up in the zone AB; secondly, a period of relatively constant temperature in the portion BC; and finally, an unmeasured period before the air has cooled to a temperature at which organisms are unaffected by heat. This first value we have called heating-up time, t_1, and the second, exposure time t_2. Both have been calculated in the same way to give an estimate of the *actual* time.

$$\text{Time} = \frac{V \times P \times 60 \times 293}{F \times (T + 273) \times 14{\cdot}7} \text{ sec.},$$

where V = volume (l.) of heating-up portion (V_1), or that of exposure chamber (V_2); P = air pressure in lb./in.^{2} absolute; F = air flow in l./min. measured at 20° C. and 1 atm.; T = air temperature in ° C. For heating-up time, the air temperature is taken as the average of the inlet air temperature (generally 20° C.) and the exposure chamber mean temperature. Since this work was undertaken primarily to obtain design data, exact knowledge of t_2 was the principal requirement. For this unit V_1 is 0·276 l. and V_2 is 0·66 l. At an air rate of 140 l./min. and exposure chamber temperature of 300° C., t_1 is 0·08 sec. and t_2 is 0·14 sec.

An air sterilizer (1700 *l./min. capacity*)

This consists of banks of heaters similar to the one in the unit described above, and is in regular use in this laboratory. It has now completed 1700 days operation without any failure, and in that time has required very little maintenance. The heating elements fitted originally are still in use.

The heaters (Fig. 5) consist of a series of annular chambers made from two tubes. The heater is inside the inner tube and the air passes through the annulus. The total

length of tube is 38 ft., which is equivalent to a heating surface of 22·5 ft.^{2}, and the material of construction is stainless steel to B.S.S. En. 58 B. Depending on the air rate being used, temperature is controlled, slightly in excess of that required, by

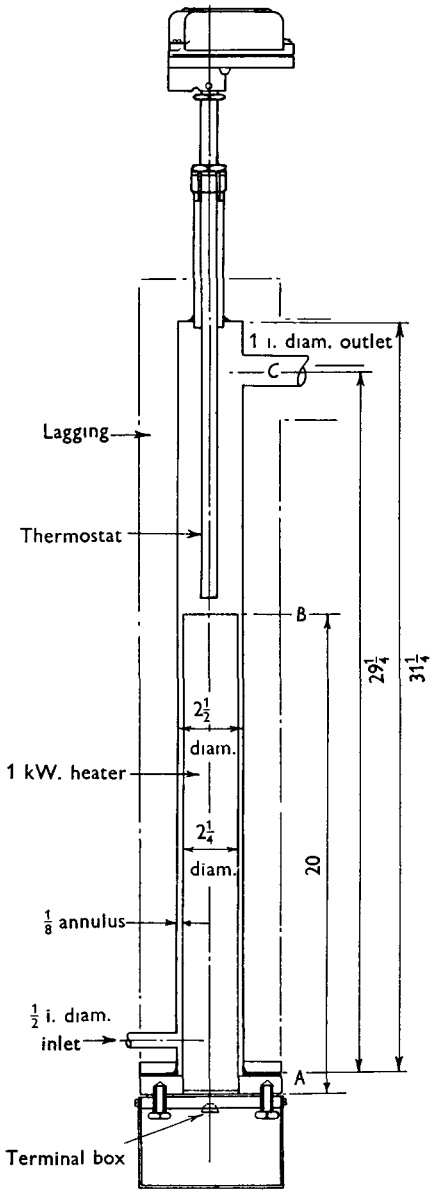

Fig. 4. Dimensions in inches.

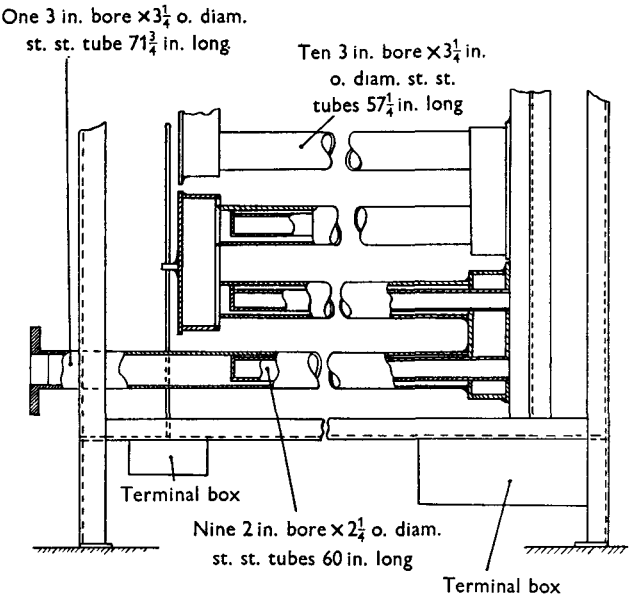

Fig. 5

manual adjustment of the heater current. A thermostat system is then brought into operation which controls automatically at the desired temperature. The thermostat detector elements are in the exposure chamber which consists of two 5 ft. (approximately) lengths of 3 in. bore pipe. The volume of the heating-up portion (V_1) is

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35·3 l. and that of the exposure chamber (V_2) is 16·8 l. At an air rate of 1700 l./min., operating pressure of 60 lb./in.^{2} (gauge) and exposure chamber temperature of 300° C., t_1 is 4·3 sec. and t_2 is 1·6 sec.

RESULTS

Validity of test method

The collection efficiency of sterilized filters was determined by the methylene blue penetration test using 1 μ particles as described by Thomas (1952). This gave a value of 98·6 %. When the spore concentration of the infected air sample was determined, the result was confirmed by placing a second filter in series with the first and examining each filter separately. Henderson (1952) reported collection efficiencies of 99·99 %, but this was for a resin-impregnated wool which deteriorates on sterilizing in steam. We have used in the present work a plain merino wool-asbestos mixture (Thomas, 1952) which has a satisfactory performance after sterilizing.

Table 2. *Comparison of slit sampler and micro-filter*

Slit sampler			Micro-filter		
Infected air samples	Observed	Expected	Infected air samples	Observed	Expected
Plates showing:			Tubes showing:		
nil organism	26	26·3	no growth	31	31·4
1 organism	11	11·3	growth	17	16·6
2 organisms	3	2·4			
Control test: no. of samples sterile, 76 no. of samples not sterile, 4			Control test: no. of samples sterile, 50 no. of samples not sterile, 0		

The sensitivity of the method, that is the ability to detect a minimum number of organisms, was measured by comparison with a slit sampler. Air containing on an average one spore in 200 l. was examined simultaneously by the two methods. The size of each air sample was 84 l., and the results, including controls to test for chance contamination, are given in Table 2.

Those from the slit sampler were shown to fit a Poisson distribution and it was therefore possible to predict the expected number of non-sterile micro-filters. The observed number of filters producing growth was in excellent agreement with the expected number, and since from the slit sampler eleven air samples each contained one organism a proportion of the seventeen growths from the filters must have originated from single organisms. Hence the conclusion is drawn that the micro-filter will detect single organisms.

A 140 l./min. air sterilizer

Performance. The results for this unit are given in Table 4.

In the assessment of performance, Bourdillon *et al.* (1948) and Terjersen & Cherry (1947) have argued that when no penetration was detected one spore might have been present, and based their calculations on this value.

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Such assumption needs qualification because this is an example of sampling where the number of spores observed, p, fits a Poisson distribution. The probability P that these p spores could not have come by chance from a population of mean value greater than m is given by

$$P = 1 - \sum_{x=0}^{x=p} e^{-m} \frac{m^x}{x!}.$$

Table 3 gives the possible upper limit for m at a probability of $P = 0{\cdot}95$.

In selecting 1 as the upper limit, when they failed to detect anything, the above named workers have in fact assumed a probability of $P = 0{\cdot}63$. It would have been more reasonable to assume $P = 0{\cdot}95$ when the value of m would have been increased to 3 (Table 3).

Table 3. *Values of m at* $P = 0{\cdot}95$

No. of spores observed	0	1	2	3	4	5
Possible upper limit of m	3·00	4·74	6·30	7·75	9·15	10·51

From Table 4 it will be seen that the exit air is grossly contaminated when operating at atmospheric pressure at an air rate of 168 l./min. The effective capacity at this pressure is 140 l./min. Let us consider the results obtained at this air rate (Expts. 27–31) where fifty-eight infected air samples (each of 70 l. volume) showed no contamination. The spore concentration at this air rate was shown to be $2{\cdot}6 \times 10^5$ particles/l., the mean of eighteen observations. This shows that approximately 95 % of the organisms originally added at the spray bottle are lost by impact or other effects. Since penetration, D, is given by

$$D = \frac{\text{Possible upper limit of } m \text{ (Table 3)}}{\text{No. of samples} \times \text{sample volume} \times \text{spore concentration}}.$$

Then

$$D = \frac{3}{58 \times 70 \times 2{\cdot}6 \times 10^5}$$

$$= 1/352 \text{ million}.$$

If we pool all the results where sterility was achieved, we find that out of 238 samples of infected air four were contaminated. On the basis of the sensitivity tests described previously, it is reasonable to assume that each of these instances of contamination was due to single organisms. Thus four contaminating organisms were detected in 238 samples of infected air. It will be noticed that in 237 handling samples there were six cases of contamination. The four organisms might therefore have arisen through faulty handling. If in spite of this it is assumed that they all arose as the result of penetration (a probability of less than 0·03) then by consulting Table 3 an upper limit of 9·15 organisms is obtained. Calculating as before and using the same value of spore concentration (a low figure in this case) the degree of penetration is now found to be one in 470 million, which is a very conservative estimate. At 60 lb./in.^{2} operating pressure the effective capacity is 168 l./min. The estimate of penetration in this instance is one in 364 million.

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Table 4. *Sterility tests*, 140 *l./min. sterilizer*

Expt. no.	Temperature range (° C.) Minimum	Temperature range (° C.) Maximum	Air flow l./min. referred to 20° C. and 1 atm.	Time (sec.) Heating-up t_1	Time (sec.) Exposure t_2	Sterility tests: Filter control No. of samples	Filter control No. infected Total	Filter control No. infected No. due to *B. subtilis*	Handling control No. of samples	Handling control No. infected Total	Handling control No. infected No. due to *B. subtilis*	Infected air No. of samples	Infected air No. infected Total	Infected air No. infected No. due to *B. subtilis*
Heater operating at atmospheric pressure														
15–18	319	330	56	0·20	0·35	56	2	1	60	4	3	60	3	3
19–23	311	320	84	0·13	0·24	53	2	2	60	3	3	60	1	0
24–26	286	320	112	0·10	0·18	56	1	1	60	0	0	60	1	1
27–31	275	325	140	0·08	0·14	60	2	2	57	0	0	58	0	0
32–33	269	296	168	0·07	0·12	30	2	2	30	0	0	30	25	25
Heater operating at 60 lb./in.² gauge air pressure														
37–39	240	320	168	0·35	0·64	43	0	0	60	2	1	60	0	0
40–41	228	278	196	0·31	0·58	50	0	0	60	0	0	58	26	25
42–43	202	262	225	0·24	0·45	31	0	0	40	1	1	40	38	38

An air sterilizer (1700 *l./min. capacity*)

Performance. Using the same test method this heater has been shown to have a similar efficiency to that of the smaller unit already described. The results are given in Table 5.

Table 5. *Sterility tests*, 1700 *l./min. sterilizer*

Air rate, l./min. referred to 20° C. and 1 atm.	Time (sec.) Heating-up t_1	Time (sec.) Exposure t_2	Temp. (° C.)	Result
	Operating pressure, 30 lb./in.² (gauge)			
280	16	5·6	300	Sterile
280	17	6·0	260	Sterile
280	17	6·5	220	Sterile
280	18	7·1	180	Contaminated
	Operating pressure, 60 lb./in.² (gauge)			
1700	4·3	1·6	300	Sterile
1700	4·5	1·7	260	Sterile
1700	4·7	1·8	220	Sterile
1700	5·0	2·0	180	Contaminated

Although a temperature of 220° C. produces sterile air, 300° C. has been selected as the normal operating temperature. As an independent check, the unit was tested by infecting the inlet air to give 10,000 spores per litre at the air inlet to a culture vessel when the unit was operating at room temperature. Infected air treated at 300° C. was then passed for 5 hr. into a culture vessel containing 100 l. of a peptone medium. No growth was observed. In this instance it was calculated that the penetration, if occurring, was less than one in five million.

It will be noted that in the smaller unit the limiting exposure time at 300° C. below which sterility is not affected is given as 0·14 sec. In the larger unit at 300° C. the estimate of exposure time is 1·6 sec. This is not a limiting value, but is the lowest at which a test could be made owing to the inadequacy of the air supply and electric heater capacity. In this instance we had to be content with determining the limiting temperature for a given air rate and pressure. Reducing pressure to decrease exposure time was not done, as the immediate object of the work at that time was to determine the maximum possible output of sterile air which, as it happened, was limited by compressor capacity.

SUMMARY

Equipment for the sterilization of air by heat with capacities of 140 l./min., and 1700 l./min. respectively is described. An improved method for testing sterility is given.

It was at Dr Henderson's instigation that the micro-filter was tested in this application. The larger size air sterilizer was designed and installed in this laboratory by Mr F. H. Conway of Ministry of Supply, C.D.E.E., Porton. We are indebted to Mr S. Peto for the statistical treatment of results.

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