

CHAPTER 45

Determination of Membrane Filter Porosity by Microbiological Methods*

B. G. ROGERS** AND H. W. ROSSMOORE

Department of Biology, Wayne State University, Detroit, Michigan

Porosity of membrane filters has previously been determined by physical methods and validated microbiologically through fixed-load, total sterility tests. Observations of bleed-through from heavy bacterial suspensions equal to or greater than 10^9 organisms in 100 ml and total retention at lower loads equal to or less than 10^8 organisms in 100 ml led to an investigation of some of the parameters responsible for membrane filter retention efficiency. A test method employing positive pressure filtration of serially diluted suspensions was used to determine or validate (from physical data) the membrane porosity. The number of organisms passing through were estimated according to the MPN procedure in *Standard Methods for the Examination of Water and Wastewater*, 12th edition. Pore size was based upon the mean diameter of the organisms retained 100% at total loads of approximately 10^9 organisms per 100 ml and in which increments from this value produced bleed-through. This bleed-through was found to be directly proportional to the input of the above previously mentioned sizing load. Sizing of four membranes, from 0.22–0.80 μ , was accomplished with species of Eubacteria. The results coincided with published physical data for cellulose ester membranes and proved definitive for glass fiber membranes for which physical measurements have proved inconsistent.

Filtration membranes are currently produced in a variety of pore sizes ranging from 10 nm–8 μ . Cellulose esters, produced in 150- μ thick sheets, are the predominant construction material. The porosity of such membranes is routinely derived from physical tests such as the bubble-point (Sauer, 1961), mercury intrusion (Ritter and Drake, 1945), pore-size distribution (Erbe, 1933), or particle passage methods (ASTM, 1965). In addition, membranes destined for use in sterility testing or sterile filtration are tested for their retention of bacteria (ASTM, 1965; Bowman et al., 1967; Singer and Kinbauer, 1968).

Presently, bacterial retention tests are in a secondary, supportive role as quality control checks on physically determined porosity. In our opinion, the lack of uniform methods and evaluation procedures has resulted in confusion and in a nebulous concept of the value and application of retention tests. In 1967, Bowman presented the first uniform test procedures for the quality control evaluation of 0.22 μ cellulose ester membranes. These procedures helped to relate retention performance to field use.

The need for improved microbiological evaluation methods has been emphasized by inconsistencies between physically determined porosity and field performance. Singer and Kinbauer (1968), for instance, were unable to correlate bubble point with 100% retention, and they also point to mercury intrusion data as yielding erroneously small porosity determinations. Such inconsistencies also occur in the development of new filtration media from materials other than cellulose esters. In determining the porosity

* Contribution No. 258, Dept. of Biology.

** Partial fulfillment of the M.S. in Biology.

of these new media, the use of physical tests which were developed for cellulose ester membranes often give misleading results. Such was the case with prototype glass fiber media with which we initiated these studies. The test methods developed differed from other membrane retention tests. We were able to directly measure biological pore size. A unique feature requires a limited amount of bacterial penetration of the membrane as the organism size approaches pore size. This necessitated the filtration of numerous species of overlapping mean diameters approaching and surpassing the presumed pore size.

Initially, we examined 0.22- μ and 0.45- μ cellulose ester membranes to determine the relationship between total cell loads, cell size, and effluent bleed-through in a medium with well established physically determined pore size. Each series of tests involved the filtration of five bacterial suspensions ranging from 10^9 - 10^{10} cells in 100 ml.

We then selected two prototype glass fiber filters (designated as S and R) to demonstrate the use of microbiological porosity measurements for media with which physical data had not been consistent with biological results. In these studies we increased the load range to 10^6 - 10^{10} cells in 100 ml.

PROCEDURE

We used a five-place filter manifold operated at 15 psi with sterile filtered air. Each unit had a 300-ml glass reservoir and an aseptically vented 500-ml collection flask and was autoclaved before final assembly, at which time the appropriate 47-mm membrane was inserted. We determined filtration leaks by postincubation inspection of the membrane. Filtrates were recovered aseptically and evaluated by the multiple tube most probable number (MPN) method, in which five tubes each of Trypticase soy broth (TSB-BBL) plus 0.1% agar were inoculated respectively with 10 ml, 1 ml, and 0.1 ml of the 100 ml effluent. Incubation was at 32 C for a minimum of 5 days before recording negative results. Codes for delivering MPN values were taken from the Swaroop tables in the "Standard Methods for the Examination of Water and Waste Water (1965)." We calculated efficiencies of retention as follows:

$$\% \text{ retained} = \frac{\text{influent no. cells} - \text{effluent no. cells}}{\text{influent no. cells}} \times 100$$

In order to bracket the presumed filter sizes adequately, we selected seven bacterial species for filtration. Details regarding ATCC No., sizes, and confirmatory indicator features are given in Table 1.

The medium and method of growth used was based on the need for an end result with uniform and repeatable cell size. All cultures were grown as agar flats in 6-oz prescription ovals on Antibiotic Medium #1 (Difco) for *Serratia marcescens* and on Trypticase soy agar (TSA-BBL) for the others. The former species was incubated at 25 C while the remainder were held at 32 C. In all cases, the flats were inoculated with 1 ml of a 1:100 TSB culture. After a 24-hr incubation, cells were harvested and washed twice and resuspended in 40 ml of distilled water. This cell suspension was agitated with 6 g of 0.2 mm glass beads for ½ to 1 hour to break up clusters and chains, decanted, diluted to 80 ml and coarse filtered through 70-100 mm sintered glass. This technique routinely yielded approximately 10^9 Gram negative cells/ml and 10^8 Gram positive cells/ml.

A most important aspect of our procedure is accurate, rational sizing. The size of cells not only varies with the physiological state but also with the method of preparing the cells for viewing. After viewing stained and phase-contrast cells, we decided

TABLE 1. *Characteristics of species used in porosity determinations^a*

| Organism & ATCC No. | Diameter Range | Mean Diameter & s.d. ^b | Length Range | Length Mean | Indicator Feature |
|--|-------------------|--------------------------------------|-----------------|----------------|--|
| <i>Bacillus cereus</i> 14579 | 0.91-1.1 | 1.0 (.03) | 2.0-4.0 | 3.0 | Morphology |
| <i>Bacillus subtilis</i> 9858 | 0.69-0.81 | 0.75(.02) | 1.8-3.0 | 2.4 | Morphology |
| <i>Escherichia coli</i> 11229 | 0.58-0.70 | 0.64(.02) | 1.0-2.0 | 1.5 | Growth in 2% brilliant green bile |
| <i>Pseudomonas aeruginosa</i> 10145 | 0.49-0.63 | 0.56(.02) | 1.0-2.0 | 1.5 | Blue-green pigment |
| <i>Pseudomonas</i> spp 19146 | 0.26-0.35 | 0.30(.02) | 0.8-1.0 | 0.9 | Morphology |
| <i>Serratia marcescens</i> 14756 | 0.44-0.56 | 0.50(.02) | 0.8-1.5 | 1.2 | Red pigment |
| <i>Staphylococcus aureus</i> 6538 | 0.76-0.94 | 0.85(.03) | — | — | Morphology and golden- yellow pigment |

^a Measurement in microns.^b Standard deviation from the mean.

that background treatment with nigrosin produced the least distortion. Size distribution (Table 1) is each based on 500 individual measurements performed on five separate cultures. Nigrosin negative stain was done routinely prior to each filtration to check proper size distribution. All measurements were made at 1000 \times with an ocular micrometer used in conjunction with an American Optical Company Series 10 microscope.

RESULTS AND DISCUSSION

In each porosity determination, asymptotic retention efficiencies are interpreted as indicating minimal difference between bacterial diameter and filter pore size. The size range, therefore, of organisms penetrating the membrane at asymptotic retention efficiencies can be taken as a measure of the filter's maximum porosity. Since bacteria occur in a range of sizes distributed about a mean, asymptotic retention efficiencies signify bleed-through of organisms whose diameter range is less than their mean diameter. One can further limit the range of diameter sizes which are penetrating the membrane by testing the filter with an overlapping size distribution of another species of bacteria. Total retention of the bracketing organism indicates those size ranges which are not great enough to assure that filter porosity is less than the mean bacterial diameter. Such instances occur frequently in porosity determinations of prototype media.

From preliminary investigations, the highest retention efficiencies occurred at total loads in 100 ml of 10^9 organisms when the mean bacterial diameter was approximately 0.05μ greater than the mean filter porosity. Under these conditions, retention efficiencies were 99.9999999% (Table 2). Slight deviations from this value are con-

TABLE 2. Relationship between retention efficiency and total bacterial load in determining cellulose ester membrane pore size^a

| Load of Viable Cells in 100 ml | Viable Cells Recovered in 100 ml Filtrates ^b | Retention Efficiencies ^b | Filtration Time for 100 ml |
|--------------------------------|---|-------------------------------------|----------------------------|
| <i>Serratia marcescens</i> | | | |
| 3.2×10^{10} | 3300 (from plate count) | 99.999988% | 76 min. |
| 2.5×10^{10} | 542 | 99.9999978% | 62 min. |
| 2.4×10^{10} | 542 | 99.9999977% | 49 min. |
| 1.9×10^{10} | 49 | 99.9999974% | 39 min. |
| 1.6×10^{10} | 13 | 99.99999919% | 23 min. |
| 1.3×10^{10} | 5 | 99.99999962% | 18 min. |
| 8.0×10^9 | 2 | 99.99999975% | 4 min. |
| 6.3×10^9 | 0 | 100.0% | 4 min. |
| 3.2×10^9 | 0 | 100.0% | 18 sec. |
| 2.5×10^9 | 0 | 100.0% | 18 sec. |
| <i>Pseudomonas aeruginosa</i> | | | |
| 3.0×10^{10} | 0 | 100.0% | — |
| 5.0×10^9 | 0 | 100.0% | — |

^a A membrane manufactured by the Millipore Corp., Bedford, Massachusetts. Porosity physically rated as 0.45 ± 0.02 microns.

^b As evaluated according to the Most Probable Number method in *Standard Methods for the Examination of Water and Waste Water*, 12th edition.

^c Calculated according to the following formula:

$$\text{Retention Efficiency} = \frac{\text{Load of viable cells in 100 ml} - \text{Viable cells recovered in 100 ml filtrates}}{\text{Load of viable cells in 100 ml}} \times 100$$

sistent with the magnitude of the standard deviation from the mean diameter and with its postfission arrangement. Clusters, for instance, tend to increase retention efficiency, whereas increased standard deviation from mean diameter tends to decrease retention efficiency. One-hundred per cent retention is generally produced when the disparity between mean filter porosity and mean bacterial diameter is equal to or greater than 0.10μ .

The data used for the determination of the porosity of the 0.45μ cellulose ester membrane are presented in Table 2. The high retention efficiencies for *S. marcescens* are interpreted as meaning that the filter is definitely within the 0.44 - 0.56μ range. High load bracketing filtrations with *Pseudomonas aeruginosa* revealed the membrane incapable of passing $0.49\text{-}\mu$ size variants. Therefore, the 0.44 - $0.45\text{-}\mu$ portion of the diameter size distribution for *S. marcescens* was judged to be the porosity of the filter. Published physical data for this membrane is $0.45 \pm 0.02 \mu$. Porosity determinations for $0.22\text{-}\mu$ cellulose filters were inconclusive in that loads equal to or greater than 10^9 organisms in 100 ml produced confirmed bleed-through amounting to only one organism in 100 ml filtrate in two instances out of five replicates. The mean disparity in size between the $0.22\text{-}\mu$ filter and *Pseudomonas* spp. 19146 ($= 0.08 \mu$) is thought to be responsible for the high degree of retention. Currently, we are investigating smaller organisms in the genus *Xanthomonas* for use with $0.22\text{-}\mu$ media.

Porosity determinations for prototype glass fiber media designated S and R are presented in Tables 3 and 4. Physical measurements reported to us by the manufacturer indicated S and R to have a pore size of 0.32μ and 0.65μ , respectively. We evaluated S media with three organisms (Table 3). Retention data with *Escherichia coli* suggest that the pore size may be critically close to mean cell diameter and absolute retention of *Bacillus subtilis*, only 0.1μ larger, served to reinforce that conclusion and also to place an upper limit on media pore size. Although we are basing pore size of S media on *E. coli* retention efficiency, it should be noted that this efficiency is far lower than that given for *S. marcescens* (Table 2). Previous observations suggest that the lower retention efficiencies of *E. coli* with S media are probably a function of the type of media rather than one of species anomaly, although differences in size distribution should not be ruled out.

Staphylococcus aureus and *Bacillus cereus* were used to determine R porosity (Table 4). High retention efficiencies at 10^9 cell loads indicated minimal organism and

TABLE 3. Estimation of pore size for S-type glass fiber filters using retention efficiencies

| Organism | Size (μ) | Maximum Retention Efficiency | Estimated Pore Size (μ) |
|-------------------------------|----------------|------------------------------|-------------------------------|
| <i>Pseudomonas aeruginosa</i> | 0.49-0.63 | 99.99982% | 0.65-0.69 |
| <i>Escherichia coli</i> | 0.58-0.70 | 99.99998% | |
| <i>Bacillus subtilis</i> | 0.69-0.81 | 100.0% | |

TABLE 4. Estimation of pore size for R-type glass fiber filters using retention efficiencies

| Organism | Size (μ) | Maximum Retention Efficiency | Estimated Pore Size (μ) |
|------------------------------|----------------|------------------------------|-------------------------------|
| <i>Staphylococcus aureus</i> | 0.76-0.94 | 99.999996% | 0.76-0.85 |
| <i>Bacillus cereus</i> | 0.91-1.10 | 100.0% | |

porosity size differences. The filter was tested by a bracketing filtration with *B. cereus* as a confirmation of the filter's maximum porosity. The filter was assessed at 0.76–0.85 μ . Part of the high retention efficiency for *S. aureus* (Table 4) may arise from postfission clustering. Agitation times with glass beads in the preparation of the *S. aureus* filtration suspension were twice as long for this reason. Filtration with the 70–100 μ sintered glass filter also helps to remove clusters. Examinations of cultures produced a great many free cocci and significantly reduced the number and size of chains and clusters.

From the examination of the porosity determinations, it is interesting to note that there is a certain minimum threshold load (breakpoint load) required before bleed-through becomes apparent. Higher load increments from this breakpoint produce bleed-through directly proportional to load. Concomitant with this phenomenon are the gradual reductions in retention efficiencies and increase in filtration time (Table 2). The explanation of the breakpoint load can only be surmised at this time. Higher loads certainly present a greater concentration of minimal-sized variants, and increased filtration times afford a longer migration time for organisms to penetrate the filter. It can be further postulated that the breakpoint is in part also a result of a literal saturation of the pore structure with bacteria. This saturation could effect a buildup of pressure, causing bleed-through by a pushing action. Because of the extremely small numbers of bacteria recovered in filtrates where high retention efficiencies are apparent, one is tempted to include the possibility of filterable stages of bacteria being responsible for at least part of the bleed-through. Gan's (1965) studies on the dialyzability of bacteria and the observation of Herold et al. (1967) of penetration of 0.22- μ and 0.45- μ cellulose ester membranes by *S. aureus* indicate, however, that a great deal more time is needed for this sort of penetration than is afforded by our test method. In Gan's dialysis studies, bacteria generally took approximately 5 days to penetrate a dialysis membrane and in studies of Herold et al. *S. aureus* penetrated 0.22- μ and 0.45- μ media in 8 to 50 hr. They ascribe penetration to diapedesis, formation of protoplasts, or the formation of minimal reproductive units.

Probably the most significant finding of this investigation is the great importance of the effect of load on retention efficiency. Bacterial size distribution is important also, but it is apparent that relatively significant disparities in size do not affect retention efficiencies unless one filters appreciably high loads of organisms. These factors must be considered therefore in the design of any microbiological regime for the evaluation of membrane porosity or sterility test applications.

In sterility test procedures the relatively low loads of less than 10^5 bacteria are entirely acceptable. As in Bowman's method (Bowman, 1967), such tests need only to recover low loads routinely encountered in serum, plasma, antibiotics, or water analysis work. Such tests, although excellent indicators for low loads, are poor indicators of cell concentration more than 10^7 and only gross indicators of pore size.

Porosity evaluation for membranes used in sterile filtration applications should have more exacting requirements. These are met when both the size of the bacterium and the load are so selected as to yield asymptotic retention efficiencies, i.e., approaching 100%. Under such conditions, bleed-through assures the close proximity of bacterial dimensions to maximal porosity, and total retention of loads one log under the breakpoint load (ca. 10^8 cells) attests to the high load retention qualities needed for sterile filtration. Higher loads may be incorporated in the serial MPN filtration procedure to evaluate membrane performance at increasing loads. Such loads are not thought to be necessary or significant for the determination of porosity. Extremely high loads (above 10^{10}) are not representative of the conditions of actual use. They also introduce the

problems of extremely long filtration times and perhaps the effect of a squeezing action upon the bacteria.

LITERATURE CITED

- ASTM Proposed Tentative Test Method. Test for determination of characteristics of membrane filters for use in aerospace liquid, June 1965.
- Bowman, F. W., M. P. Calhoun, and M. White, 1967. Microbiological methods for quality control of membrane filters. *J. Pharm. Sci.*, **56**: 222-225.
- Erbe, F. 1933. Die Bestimmung der Porenverteilung nach ihrer Grösse in Filtern und Ultrafiltern. *Kolloid-Zeitschrift.*, **63**: 277-285.
- Gan, H. K. 1965. Dialysis studies. Experiments dealing with dialyzability of bacteria. *J. Hyg. Epidemiol. Microbiol. Immunol.*, **7**: 422-435.
- Herold, D. J., J. S. Schultz, and P. Gerhardt, 1967. Differential dialysis culture for separation and concentration of a macromolecular product. *Appl. Microbiol.*, **15**: 1192-1197.
- Ritter, H. L., and L. C. Drake, 1945. Pore-size distribution in porous materials: I. pressure porosimeter and determinations of complete macro-pore-size distributions. *Ind. Eng. Chem., Anal. Ed.*, **17**: 782.
- Sauer, T. C. 1961. Filter pore size-bubble test. Technical Progress Series—*Fuel filter test methods*. Society of Automotive Engineers. **1**: 25-29.
- Singer, S., and E. Kinbauer, 1968. Quality control of bacterial removal filters. *Proceedings from the American Association for Contamination Control*. pp. 46-49.
- Standard Methods for the Examination of Water and Wastewater*. 12th ed., New York. American Public Health Association, Inc.