# Microbiological Methods for Quality Control of Membrane Filters

By FRANCES W. BOWMAN, MIRIAM P. CALHOUN, and MACK WHITE

**Microbiological methods are presented for quality control of membrane filters of 0.45 and 0.22 $\mu$ porosity.**

ALTHOUGH MEMBRANE filters have been produced for more than 100 years, they were not widely used in the United States until after World War II. According to a report by Goetz (1), the Germans made dramatic use of them during the war for preventing epidemics that could have been caused by contaminated water supplies. Using membrane filters, they developed a rapid test to determine the safety of public water supplies after bombings.

The technology for producing membrane filters was later brought to the U. S. where, under the sponsorship of the U. S. Department of the Army, extensive research was conducted on their production. This research resulted in practical procedures for commercial production. The filters were produced from cellulose esters in sheets approximately 150 $\mu$ thick. The pore sizes were predetermined and controlled in the manufacturing process.

At the present time, 12 distinct pore sizes or classes ranging from 8 $\mu$ (class 1) downward in pore size to 10 m$\mu$ (class 12) are commercially available. According to the reports by the manufacturers, some of the membranes are composed of pure cellulose acetate, whereas others contain a mixture of cellulose acetate and cellulose nitrate. Due to the demand for these membranes, an Interim Federal Specification (2) was published for the purpose of securing bids for the Defense Supply Agency. This federal specification includes methods for evaluating the quality of each class of membrane.

In the past decade, scientists have found innumerable applications for the membrane filters as an analytical tool. The filters have been widely used in the sampling and measurement of airborne particulates, microbiological analysis of water and foods, clinical diagnosis, sterile filtration, beer manufacturing, and sterility testing.

Holdowsky (3) first suggested the use of bacterial retentive membranes in sterility testing of antibiotics to provide a means of removing the antibiotic from the test medium. Using the membrane filtration technique, Bowman (4) developed procedures for soluble antibiotics, and the Antibiotic Regulations were subsequently amended to include the membrane filtration sterility test. For this test, the Antibiotic Regulations state that the bacterial membrane filter should have a nominal porosity of 0.45 $\pm$ 0.02 $\mu$, a diameter of approximately 47 mm., and a flow rate of 55 to 75 ml. of distilled water passing each sq. cm. of filter area per min., with a differential pressure of 70 cm. of mercury at 25°. Bacterial retentive membranes that fulfill these specifications are available from a number of suppliers. Most manufacturers conduct laboratory quality control tests. In addition to these tests, it is desirable that laboratories conducting sterility tests assess the quality of the membranes to be used. Therefore, a reliable standard method for evaluating the bacterial retentive membranes was sought.

Among the methods currently used for determining pore size are Sauer's pore-size bubble test (5), Erbe's pore-size distribution determination (6), Ritter's mercury intrusion measurement of pore radius (7), and particle passage methods (8). The latter are the most commonly used, and are the basis of the current pore-size rating system. These tests tell whether the filter will retain particles larger than the nominal pore size. For large pores, colored or radioactive particles are used; for microbiological filters, bacteria are used.

The particle passage method provides a practical method for determining the quality of class 7 (0.45-$\mu$) membranes used for sterility testing. Special equipment such as that used for the mercury intrusion and other methods is not needed. Most of the equipment and materials required for this test are found in any laboratory performing membrane filtration sterility tests. For this reason, a passage test using *Serratia marcescens* ATCC 14756 has been developed for accepting the membranes used for the sterility testing of antibiotics under the certification program. The acceptance test employs a general inspection level I, double sampling plan, MIL-STD-105, at a 1% acceptable quality level. For example, if a lot (batch) of 1201 to 3200 membrane filters is to be sampled for tests, 32 filters

Received October 27, 1966, from the Division of Antibiotics and Insulin Certification, Bureau of Science, Food and Drug Administration, U. S. Department of Health, Education and Welfare, Washington, D. C. 20204.
Accepted for publication November 10, 1966.

will be selected randomly from the lot and tested for retention of *S. marcescens*. If none is found defective, the batch is acceptable. If one is defective, a second test is performed on an additional 32 filters. If two or more are found defective in either the first or the first and the second test, the lot is rejected. The double sampling plan allows acceptance of acceptable quality filters with minimum testing of one sample; the second sample allows for the probability occurrence of one defective membrane in a sample of 64 filters having an average quality level of 1% defective.

## ACCEPTANCE TEST PROCEDURE FOR CLASS 7 MEMBRANES

**Bacterial Culture.**—Prepare a suspension of *S. marcescens* ATCC 14756 as follows. Transfer the organism to a Roux bottle containing 300 ml. of U.S.P. peptone–casein agar, incubate at 25° for 24 hr., and harvest the growth with 50 ml. of sterile 0.1% (w/v) peptone diluting fluid and sterile glass beads. Perform a viable cell count on the suspension and determine the dilution that will yield between 50,000 and 100,000 cells/ml.

**Apparatus.**—Assemble 32 filtration units as shown in Fig. 1. Each unit consists of a 1-L. side-arm Pyrex suction flask (*1*) fitted with a No. 8 rubber stopper which has a hole in the center for the support of a 300-ml. Pyrex filter funnel. The fritted-glass base which supports the membrane is held to the 300-ml. funnel with a spring action aluminum clamp. One end of a 2-in. long piece of vacuum tubing ($^1/_4$-in. bore and $^3/_{16}$-in. wall) is attached to the side-arm of the 1-L. flask. At the other end (*2*) a female adapter, Luer-Lok, is inserted to hold a Swinny hypodermic adapter (*3*) containing a 13-mm. diameter microfiber glass air filter. A male adapter, Luer-Lok (*4*), attached to the Swinny adapter is inserted in additional tubing which is connected to the vacuum source. The top of the side-arm flask and the base of the funnel are covered with aluminum foil. The top of the funnel is closed by a rubber stopper pierced with an 18-gauge hypodermic needle attached to a Swinny adapter as described above. After the entire unit is autoclaved for 1 hr. at 121°, a sterile class 7 membrane is aseptically placed on the fritted-glass base of the funnel with flat bladed forceps to avoid damaging the membrane.

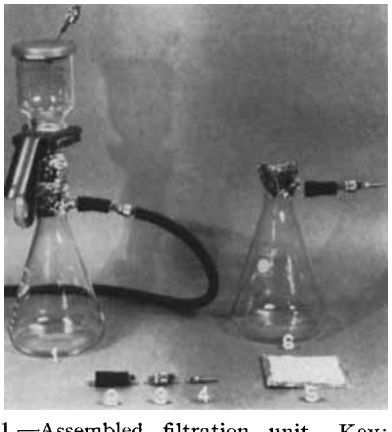

Fig. 1.—Assembled filtration unit. Key: *1*, Pyrex suction flask for filtration; *2*, female adapter; *3*, Swinny adapter with air filter; *4*, male adapter; *5*, gauze square; *6*. Pyrex suction flask for incubation.

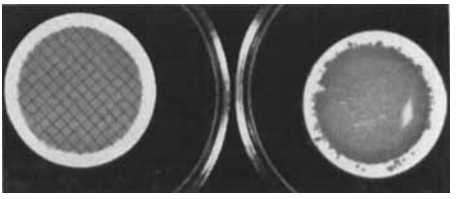

Fig. 2.—Membrane with satisfactory growth of *S. marcescens* (left) and unsatisfactory growth (right) after incubation in acceptance test.

**Test Procedure.**—Lift the stopper of each of the 32 filter funnels prepared as described above and carefully pour in 200 ml. of U.S.P. peptone broth that has been seeded with 1 ml. of the *S. marcescens* suspension appropriately diluted as previously described. Replace the stopper firmly on the funnel and apply negative pressure to start the filtration. When the filtration has been completed, stop the vacuum and allow air to be drawn rapidly into the flask through the air filter inserted in the rubber tubing. With the funnel still attached, remove the rubber stopper from the suction flask and cover the top of the flask containing the filtrate with a presterilized gauze square approximately 11.5 × 11.5 cm. (*5*), saturated with 5% phenol. Remove each membrane aseptically and place it on the surface of peptone–casein agar in a sterile Petri dish (20 × 100 mm.). Incubate the 32 agar Petri dishes containing the membranes and the 32 side-arm flasks (*6*) at 32°. Observe the growth on the membranes after 24 and 48 hr., and examine the filtrates in the flask after 5 days' incubation. No growth of organisms in the filtrates proves complete retention of the organisms by the membranes and indicates their suitability. As shown by the membrane on the left in Fig. 2, growth on the plates should be confluent and contained on that part of the membrane inside the filtering area. Growth of the organisms on the edge of the membrane, as shown on the right in Fig. 2, indicates faulty technique and may produce growth in the filtrate. Side leakage can occur if the membrane is not perfectly sealed in the unit by the clamp. Therefore, extreme care should be taken when assembling the units to avoid leakage.

**Evaluation of Results.**—If no growth is detected in any of the 32 filtrates after 5 days and the growth of *S. marcescens* on the membranes is confluent, the batch of membranes is acceptable for sterility testing. If two or more filtrates show growth, the batch is rejected. However, if there is growth at the edge of a filter and the related broth exhibits growth, the test is judged invalid, and it is repeated in full. The results of tests judged invalid are not considered a part of the supplier's quality performance record.

If one filtrate shows growth, a repeat test is performed on 32 additional membranes. If no growth

TABLE I.—RESULTS OF ACCEPTANCE TEST ON CLASS 7 MEMBRANES[a]

| Mfgr. | Batch | Filtrates—First Test | Filtrates—Second Test | Membranes—First Test | Membranes—Second Test |
|---|---|---|---|---|---|
| *A* | 1 | 0 | NA | Satisfac. | NA |
| | 2 | 0 | NA | Satisfac. | NA |
| | 3 | 0 | NA | Satisfac. | NA |
| *B* | 1 | 0 | NA | Satisfac. | NA |
| | 2 | 1/32 | 0 | Satisfac. | Satisfac. |
| | 3 | 0 | NA | Satisfac. | NA |
| *C* | 1 | 0 | NA | Satisfac. | NA |
| | 2 | 0 | NA | Satisfac. | NA |
| | 3 | 0 | NA | Satisfac. | NA |

[a] 0, no growth; NA, not applicable; 1/32, one filtrate with growth out of 32 tested; satisfactory, confluent growth confined to the filtering area.

is detected in any of the additional 32 filtrates after 5 days at 32°, the batch of membranes is acceptable for sterility testing. If growth is detected in one or more of the filtrates, the batch is rejected.

**Experimental.**—In order to evaluate some of the commercially-produced class 7 membrane filters, membranes were obtained from three different manufacturers. An acceptance test, as described previously, was performed on three batches from each manufacturer. The manufacturers were identified as *A*, *B*, and *C* for the evaluation studies. It can be concluded from the results shown in Table I that all membranes tested were acceptable for sterility testing. A retest was necessary in only one out of nine acceptance tests.

## DISCUSSION

Although class 7 (0.45-$\mu$) and class 9 (0.22-$\mu$) membranes are both used to sterilize liquids by filtration, the latter are always used with solutions containing sera, plasma, trypsin, or other enzymes where species of *Pseudomonas* of small size are known to occur. The class 7 filter, having larger pores, yields flow rates approximately 3 times those of the class 9 filters, but can only be used for sterile filtration where prior experience indicates that organisms smaller than 0.45 $\mu$ are not present in the solution to be filtered. For example, in the production of penicillinase (9) from *Bacillus cereus* ATCC 13061, it was found in this laboratory that the class 7 membrane was not adequate for sterilizing the enzyme. This membrane removed all of the *B. cereus* from the broth in which the enzyme was produced, but on several occasions the broth was also heavily contaminated with a small Gram-negative bacillus which the membrane did not filter out completely. However, in every case the class 9 membrane was able to retain all cells of this organism and yield a sterile penicillinase. The organism was identified as a species of *Pseudomonas* and was later used to develop an acceptance test for 0.22-$\mu$ filters. Its ability to pass through a class 7 is dependent on its small size (approximately 0.3 × 1.0 $\mu$). It has been successfully used in the quality control of the class 9 membranes for over 1 year and has been transferred approximately 200 times with no increase in its size. Since it proved to be so useful in testing class 9 membranes, the organism was deposited with the American Type Culture Collection where it was given the accession number ATCC 19146.

The fact that class 7 membranes (0.45 $\mu$) will not retain all microorganisms does not preclude their use in sterility testing. Investigational studies proved that the passage of these small *Pseudomonas* cells occurs only when the population size is relatively high. Therefore, the passage of a few cells is not significant, since the majority of the cells are retained on the membrane and their recovery in the sterility test is assured. For this reason, class 7 membranes, which provide a better flow rate than class 9 membranes, were selected for the antibiotic sterility tests.

As previously mentioned, class 9 membranes are used in this laboratory to sterilize penicillinase for use in testing insoluble penicillins, such as benzathine penicillin G. The sterile enzyme is aseptically added to sterile thioglycollate medium for use in the direct method sterility test. The class 9 membranes purchased for sterile filtration are tested by using the same sampling plan and the same equipment as that used in the acceptance test for class 7, except for changes in the organism and the procedure as described below.

## ACCEPTANCE TEST PROCEDURE FOR CLASS 9 MEMBRANES

Maintain *Pseudomonas* sp. ATCC 19146 on U.S.P. peptone–casein agar. Transfer the organism to a conical flask containing 50 ml. of trypticase 2% (w/v) which contains 0.6% sodium citrate, and incubate the flask at 25° until the viable cell count reaches approximately 1,000,000 cells/ml. Filter 50 ml. of the broth suspension through a class 7 membrane into a sterile flask. Transfer the membrane to the surface of a sterile peptone–casein agar in a 20 × 100-mm. Petri dish. Incubate the Petri dish at 32° and observe the growth after 24 and 48 hr. Transfer three 1-ml. aliquots of the filtrate that has passed through the 0.45-$\mu$ membrane to each of three tubes containing 10 ml. of thioglycollate or any other suitable liquid nutrient medium. Filter the remaining portion of the filtrate through a class 9 membrane into a second sterile receiving flask. Incubate the flask for 5 days at 32°. Transfer the class 9 membrane to the surface of sterile agar in a Petri dish (20 × 100 mm). After 24–48 hr., the class 7 membrane should show confluent growth and after 48–72 hr., the class 9 membrane should show individual colonies of the test organism. The three tubes of thioglycollate medium should show growth. The filtrate that passed through the class 9 membrane should not show growth after 5 days' incubation at 32°. The evaluation of the results are the same as for the class 7 membranes except that the test organism must pass through the class 7 membrane and be retained by the class 9 membrane in each of the 32 tests performed.

## SUMMARY

A microbiological method has been developed for performing an acceptance test for class 7 (0.45-$\mu$) membrane filters, used in sterility testing, and class 9 (0.22-$\mu$) membrane filters, used in sterile filtration. *S. marcescens* is used as the test organism for the

former and *Pseudomonas* sp. for the latter. The sampling plan of MIL-STD-105-D is used for the statistical evaluation of the membranes for acceptance or rejection.

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# Mannich Bases from 2-Phenylindolizines I

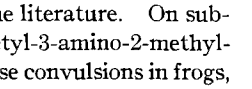
## 3-Alkyl-1-dialkylaminomethyl Derivatives

By WILLIAM B. HARRELL* and ROBERT F. DOERGE

**In a search for new therapeutic agents, a series of Mannich derivatives of 3-methyl-2-phenylindolizine and 3-ethyl-2-phenylindolizine has been prepared. The mechanism by which 2,3-disubstituted indolizines participate in the Mannich reaction has been examined and alternate $S_N1$ and $S_N2$ routes have been proposed. 1-Diethylaminomethyl-3-methyl-2-phenylindolizine has been tested and found to have central nervous system depressant activity.**

MANY PHYSIOLOGICALLY active compounds have the indole nucleus in their structure. The similarity between the indole and indolizine nuclei has prompted speculation that indolizine analogs of biologically important indoles could conceivably have potent physiological activity (1–3). It is this concept, coupled with the observation that only a few such investigations had been reported in this area, that encouraged the consideration of the indolizine nucleus for the present investigation.

Reports on the biological activity of indolizines are indeed very scarce in the literature. On subcutaneous injection 1-acetyl-3-amino-2-methylindolizine was shown to cause convulsions in frogs, mice, and rabbits (4). β-(1-Indolizinyl)-α-aminopropionic acid, the indolizine analog of the amino acid tryptophane, has been prepared (2) and shown to inhibit indole formation by an indole-accumulating mutant of *Salmonella typhimurium* (5). A series of arylindolizines was synthesized by Venturella for pharmacological testing (3, 6, 7), but to date no report on the activity of these compounds has appeared in the literature.

Having decided upon the indolizine structure, attention was focused on the various possibilities of chemical modification which might logically be expected to produce physiologically active compounds. Among the biologically active indoles, many are aminoalkyl derivatives such as serotonin, bufotenine, psilocin, reserpine, and lysergic acid diethylamide, all of which are known to have pronounced activity on the central nervous system. It was noted that in all of these compounds, the indole nitrogen and the extra-indole nitrogen are separated by four carbon atoms  , a spatial arrangement which might be a contributing factor in determining their activities. It was therefore reasoned that certain aminoalkylindolizines with the same carbon spread between the nitrogens might also possess activity on the central nervous system. After considering several possible chemical modifications, the Mannich reaction was chosen because the resulting dialkylaminomethyl substituent, when introduced at the C-1 position, would give the desired intramolecular spread. A comparison between bufotenine (I) and the Mannich bases (II) obtained by the reaction of 2,3-disubstituted indolizines with formaldehyde and dimethylamine illustrates this similarity.

Received July 20, 1966, from the School of Pharmacy, Oregon State University, Corvallis. 97331.
Accepted for publication October 11, 1966.
Abstracted from a thesis submitted by William B. Harrell to the Graduate School, Oregon State University, in partial fulfillment of Doctor of Philosophy degree requirements.
* Fellow of the American Foundation for Pharmaceutical Education, 1963–1964 and 1965–1966. National Science Foundation Science Faculty Fellow, 1964–1965. Present address: Department of Pharmaceutical Chemistry, School of Pharmacy, Texas Southern University, Houston.