# Membrane Filter Technique for Testing the Bactericidal Activity of Iodophors

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**A method has been devised for determining the contact killing time of a water-soluble antiseptic or disinfectant agent using a membrane filter as the test organism carrier. The method is useful in comparing the bactericidal activity of different concentrations of an agent since the number of organisms killed following a specific contact time can readily be determined on a percentage basis compared to that of a control. The problems of bacterial clumping, "wild plusses," and carry-over bacteriostasis are eliminated. A suspension of the test organism was filtered through a membrane filter, a solution of the agent was added, was allowed to remain in contact with the test organism for a specific time, and was filtered. The treated membrane was washed with a neutralizer, was rinsed, and was incubated on nutrient agar. The number of surviving organisms, if any, was determined by colony count. Solutions of povidone-iodine as well as commercial products containing it were tested against *Staphylococcus aureus*, FDA 209, ATCC 6538. Solutions containing a 1:15,000 concentration of available iodine gave a kill of 99.91 percent following a 1-min. contact.**

A REVIEW of the history and descriptions of the methods currently used to test the bactericidal activity of antiseptics, disinfectants, and sanitizers has been published by Lawrence and Block (1). The Association of Official Agricultural Chemists recognizes two methods for testing disinfectants: the AOAC phenol coefficient method (2), originally devised in 1929, and the AOAC use-dilution method (3), introduced in 1953. Both of these methods measure the ability of an agent to elicit a 100% kill of the test organism. In 1961 Ortenzio and Stuart (4) introduced a modification of the use-dilution method where the percentage kill of various dilutions of test organism could be determined and where a result within 95% confidence limits is claimed. Two other methods which depend upon percentage kill and which are employed mostly for determining the effective dilutions of sanitizing agents are the Chambers modification of the Weber and Black method (5) and the modified Cade and Halvorsen method (6). All of the methods for evaluating bactericidal agents in use today show certain disadvantages and defects which are well known to workers in this field. This is evident from the many modifications in technique which have been made from time to time. In the seed tube to broth culture transfer techniques, the phenomenon of "wild plusses" is of frequent occurrence. In such instances, particularly with the quaternary ammonium compounds it often happens that one subculture will show growth while another one made from a less concentrated disinfectant solution or one made from the same solution after a shorter contact time will show no growth. Bacterial clumping and adherence to the sides of the seed tube have been suggested as reasons for these inconsistencies.

The problem of carry-over due to the bacteriostatic action of the test agent when transferred to subculture tubes must also be contended with. High activity substances such as quaternary ammonium compounds, mercurials, and iodophors must be neutralized by adding inactivators to the subculture medium. One can never be sure about the effect of neutralizers unless additional time-consuming control measures are taken. These steps involve making sure that the inactivator completely neutralizes the agent and at the same time does not itself inhibit bacterial growth. Kayser and van der Ploeg (7) have shown that sodium thiosulfate (0.25%) in an agar medium will completely inhibit the growth of some strains of *S. aureus*. They suggest that its use be avoided in growth media designed to neutralize iodine in disinfection efficiency tests. When stainless steel cylinders of the specifications described in the official use-dilution method (1) are used as carriers, the problem usually can be solved only by making additional transfers to secondary subculture tubes.

In an attempt to overcome some of these difficulties, a membrane filter technique was devised. It can be used to test any water-soluble agent which can be removed from a cellulose ester-type of membrane filter by washing with water or which can be inactivated rapidly by an appropriate neutralizer or both. It simplifies the determination of percentage kill, if this determination is sought after, eliminates the phenomenon of "wild plusses," and avoids the carry-over effect. The test agent selected was polyvinylpyrrolidone-iodine[1] (PVP-I) (povidone-iodine), and commercially available products containing it.

## EXPERIMENTAL MATERIALS

**Test Organism**—*Staphylococcus aureus* FDA 209, ATCC No. 6538. Maintain stock cultures on nutrient agar slants by monthly subcultures. Incubate new stock transfer 2 days at 37°; then store at 2–5°. From the stock culture inoculate a tube of brain heart infusion broth (BBL) and make at least 4 consecutive daily transfers incubating at 37°, before using culture for testing. Use 22–26 hr. culture of organism growth in BHI at 37° in test. Mix well by high speed agitation on a Vortex Genie (Scientific Industries, Inc., Springfield, Mass.) for 1 min. before using.

[1] Betadine, Purdue Frederick Co., Yonkers, N. Y.

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**Culture Media**—Brain heart infusion broth (BBL) for daily transfers of test organism and Trypticase Soy agar (BBL) for colony counts.

**Inactivator (Neutralizer)**—Dissolve 0.5 g. of sodium thiosulfate in 100 ml. of distilled water and sterilize by autoclaving.

**Filtering Apparatus**—Millipore membrane filter holder (XX1004700) used with a filter membrane (DAWG 04700). Assemble with a liter flask and sterilize with steam under pressure. Plastic Petri dishes (60 × 20 mm.).

**Transfer Loop**—Make a 4-mm. i.d. single loop at end of 5–7.5 cm. (2–3 in.) Pt or Pt alloy wire No. 23 B and S gauge. Fit other end in suitable holder.

## PROCEDURE

**Dilutions of PVP-Iodine**—Prepare an aqueous stock solution of the PVP-iodine material (powder or solution) in sterile distilled $H_2O$ such as to represent a 1:1,000 concentration of available iodine. Set up a series of 10 sterile culture tubes in a rack and prepare 5-ml. quantities of each of the 10 dilutions shown in Table I.

**Preparation of Test Organism**—Transfer a loopful of a 22–26 hr. old broth culture of *S. aureus* to 100 ml. of BHI broth. Mix well. This suspension (contact suspension), containing between $2 \times 10^4$ and $4 \times 10^4$ organisms/ml., is used for testing the activity of the PVP-iodine dilutions. Dilute the contact suspension in BHI (1:100) and mix well. This is used as the organism numbers control suspension and also to determine the effectiveness of the inactivator.

**Organism Numbers and Inactivator Controls**—Assemble a sterile filtering apparatus with membrane. Place 5 ml. of sterile $H_2O$ in the funnel, add 2 ml. of organism numbers control suspension, swirl to mix, and filter by suction so as to form a layer of bacteria evenly spread over the membrane surface. Prepare five such membranes and place on Trypticase Soy agar. Incubate at 37° for 24 hr. Count the colonies on each plate, obtain the average, multiply by 100, and use this figure in calculating the percentage kill for the PVP-iodine dilutions. Filter 5 ml. of the most concentrated solution of PVP-iodine through a sterile membrane. Neutralize the iodine remaining on the membrane by overlaying with 10 ml. of inactivator. Filter after a few seconds of contact. Rinse immediately with 20–25 ml. of sterile $H_2O$. Impregnate the membrane by filtering 2 ml. of organism numbers control suspension. This is the "wash" control. Incubate at 37° for 24 hr. and compare the colony count with the average of the five controls. There should be no significant difference in colony counts showing that the iodine had been inactivated and the $Na_2S_2O_3$ had been removed.

Table I—Percentage Kill of PVP-I Solutions of Varying Concentrations of Available Iodine against *S. aureus* after a 1-min. Contact

| Avail. I | Colony Count | % Kill |
|---|---|---|
| 1:1,000 | 0 | 100 |
| 1:2,000 | 0 | 100 |
| 1:4,000 | 1 | 99.99 |
| 1:6,000 | 12 | 99.95 |
| 1:8,000 | 20 | 99.93 |
| 1:10,000 | 24 | 99.92 |
| 1:15,000 | 32 | 99.91 |
| 1:20,000 | 262 | 99.23 |
| 1:25,000 | 335 | 99.01 |
| 1:30,000 | 4,180 | 87.07 |
| Control | $3.39 \times 10^4$ | |

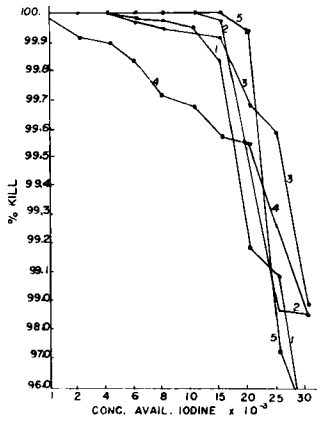


*Fig. 1—Percentage kill of PVP-I products against S. aureus after 1-min. contact. Key: 1, mouthwash; 2, solution; 3, surg. scrub; 4, shampoo; 5, douche.*

## OPERATING TECHNIQUE

Prepare an organism impregnated membrane for each PVP-iodine solution to be tested using 2 ml. of contact suspension for each membrane. The membranes can be prepared, dried, and stored aseptically at 2–5° for several days prior to the test, if desired. When prepared extemporaneously, pack the contact suspension in an ice bath when not in use to inhibit bacterial growth. Remove any vacuum in the filtering flask and overlay an impregnated membrane with 5 ml. of each dilution of test agent. After exactly 60 sec. of contact, pour 10 ml. of inactivator into the funnel and filter rapidly. Immediately flush with 20–25 ml. of sterile $H_2O$. A contact period other than 60 sec. may be used if desired. Place the treated membranes on Trypticase Soy agar contained in 60-mm. Petri dishes and incubate at 37° for 24 hr. Count the number of colonies, if any, and determine the percentage reduction over the numbers control. The same filtering apparatus can be used for each membrane providing the platform and funnel are well rinsed between each change of membranes.

## RESULTS AND DISCUSSION

Using this method, aqueous solutions of PVP-iodine containing the amounts of available iodine shown in Table I were prepared and tested. The percentage kill for each dilution is shown and the killing power can easily be assessed. Similarly five commercial products were tested and their activities were compared. The results are shown in Fig. 1.

The membrane filter technique seems to present certain features which are an improvement over the AOAC methods of testing for bactericidal activity, particularly for soluble antiseptics and disinfectants.

1. It eliminates the questionable pour plate technique for determining viable cell counts. Every organism contacted by the agent is cultured for viability not just an aliquot portion. Therefore,

[2] Betadine products, Purdue Frederick Co., Yonkers, N. Y.

there can be no "skipping" or "wild plusses," a phenomenon often seen when aliquot amounts are subcultured.

2. When a diluted bacterial suspension is filtered through a membrane, the organisms form an even, thin layer over its surface and they cannot be removed by washing as from stainless steel carriers. This distribution more closely resembles the spread of bacteria on human skin, mucosa, and contaminated surfaces. The problem of cell clumping which often occurs with bacteria suspended in solutions of quaternary ammonium compounds is eliminated completely.

3. There is no possibility of any carry-over of PVP-iodine activity, since available iodine is neutralized and thoroughly rinsed from the organism prior to culturing. The effectiveness of the neutralization can easily be determined without delay. Since the neutralizer is also removed by washing, the possibility of any interference with the growth of the test organism is eliminated.

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*Keyphrases*

Iodophors—bactericidal activity testing
Membrane filter technique—bactericidal activity test
Bactericidal activity—povidone-iodine solutions

# Communications

## Mechanism of Flow Improvement by the Addition of Fine Particles to Bulk Solids

*Sir:*

The flowability of a bulk particulate solid may be improved by the addition of a small proportion of fine particles. These fine particles, or glidants, may be chemically similar or dissimilar to the material with which they are admixed (1). In the former case the term "fines" may be used to describe the glidant particles.

Gold *et al.* (2) suggest that fine particles may act as glidants by filling the void spaces between particles, in addition to reducing interparticulate cohesive forces and reducing surface rugosity (3).

Now it can be shown that the voidage of a random array of spheres is 0.38 and 0.26 for a close packed array (4). Provided the size ratio of fine:coarse particles is less than 0.4 → 0.6, fine particles percolate into the interstitial void spaces. Thus, increasing the percentage of fines in a granulation should increase the bulk density until the void spaces are full.

Figure 1 is representative of a number of binary mixtures of magnesia and shows that the bulk density [determined by the British Standard Method (5)] increases as the intergranular voids are filled and then decreases due to bed expansion by the fine component.

If the flowing bulk solid is considered as a continuum, then it should follow that the higher the bulk density the greater the mass flow rate since more material can flow through the orifice due to a closer packing.

The flowability of the binaries outlined in Fig. 1 have been determined (3) and it can be seen

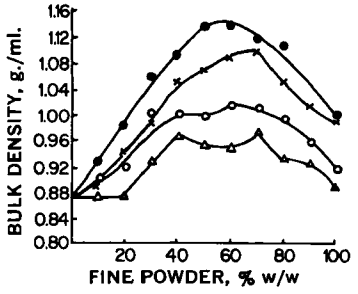


*Fig. 1—The changes in bulk density in binary mixtures consisting of coarse granules (0.0561 cm.) with increasing concentrations of fine powders. Arithmetic mean diameter of fine powders. Key: △, 0.0158 cm.; ○, 0.0090 cm.; ×, 0.0059 cm.; ●, 0.0048 cm.*